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The effects of the red tide producing dinoflagellate, Karenia brevis, and associated brevetoxins on viability and sublethal stress responses in scleractinian coral: a potential regional stressor to coral reefs

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The effects of the red tide producing dinoflagellate, *Karenia brevis*, and associated brevetoxins on viability and sublethal stress responses in scleractinian coral: a potential regional stressor to coral reefs

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Abstract

Coral cover is in decline on a global scale, with increased mortality events being attributed to a number of global and regional stressors. While the impacts of global stressors (e.g. sea surface temperature rise, ocean acidification) are well documented, there is growing interest in identifying and understanding the impacts of regional stressors. The reason for this change in focus is that regional stressors can often work in combination, sometimes synergistically, with global stressors and that stressors on a regional scale tend to be more easily mitigated by management practices. One regional stressor that impacts a myriad of marine organisms in the southeastern United States is the annual red tide blooms produced by the dinoflagellate, *Karenia brevis*. Their impacts, along with the lipid soluble polyether neurotoxins they produce, termed brevetoxins, are well studied in economically important organisms, such as bivalves. However, little is known of their impacts on organisms that possess ecological importance, such as species of scleractinian coral. To address this gap in knowledge, a multifaceted study is discussed herein, which evaluated the effects of ecologically relevant concentrations of *K. brevis* and associated brevetoxins on different coral life history stages and coral species. The second chapter addresses the impacts of red tide on larval behavior, settlement and survival of the coral species *Porites astreoides*, as well as impacts of photochemical efficiency and oxidative stress within different coral species (*P. astreoides* larvae, *P. astreoides* adults, *Acropora cervicornis*, *Cladocora arbuscula*, and *Phyllangia americana*). The third chapter confers the use of broad-scale proteomic analysis to identify the cellular response of the non-model coral species, *P. astreoides*, following exposure to red tide. Coral larvae actively avoided both medium and high bloom conditions of *K. brevis* and

brevetoxins, while percent larval settlement and survival were impacted following exposure to high bloom concentrations of *K. brevis*. Photochemical efficiency of *in hospite Symbiodinium* was reduced following exposure to both *K. brevis* and brevetoxin in *P. astreoides* larvae, as well as exposure to *K. brevis* in *P. astreoides* adults, while being unimpacted in *A. cervicornis*. Compared to controls, high bloom conditions resulted in an increase in biomarkers of lipid peroxidation in *C. arbuscula*. This was also seen in *P. astreoides* larvae at 24 hours; however, this difference was indistinguishable following 48 hours. Surprisingly, no other biomarker of oxidative stress analyzed were impacted. Broad-scale proteomic analysis of *P. astreoides* following exposure to red tide conditions revealed variable changes in proteome expression depending on if the corals were exposed to *K. brevis* or brevetoxins. Exposure to brevetoxins resulted in differential expression of proteins related to DNA organization, chromatin formation and transcription expression; while exposure to *K. brevis* resulted in differential expression of proteins related to redox homeostasis, protein folding, energy metabolism, and production of reactive oxygen species. The results of this study demonstrate the potential for annual red tide blooms to act as a regional stressor on coral species. They highlight the ability of red tide conditions to negatively impact coral at multiple life history stages and that the extent of these effects may be species specific. They also provide further incite of coral's response to red tide exposure at the cellular level.

1.

Introduction

1.1 Scleractinian Corals

Under the phylum Cnidaria, the order Scleractinia represents coral species that produce a hard calcium carbonate skeleton, commonly referred to as the stony corals. These keystone species and the limestone structures they produce provide the foundation for one of the most biodiverse ecosystems in the world, creating critical habitat that sustains many important economic and ecologic systems. Globally, it has been estimated that there are 6,000-8,000 reef fish species that reside permanently on coral reefs, while many others utilize the reefs at a particular life stage (Lieske & Meyer, 2001). It is believed that coral reefs can harbor between 950,000 – 9 million marine species that have yet to be discovered, which could be potential sources for future medicines and commercially important materials (Reaka-Kudla, 1997). Coral reefs also support many industries, such as tourism, and commercial and recreational fishing. It is estimated that $375 billion of goods and services are acquired from coral communities each year (Costanza et al., 1997). Because of the services they provide, coral reef conservation and recovery must be considered a high-priority.

The success of coral reefs is largely dependent on the mutualistic relationship that corals share with *Symbiodinium.* These intracellular photosynthetic zooxanthellae provide corals with nutrients that are essential for their survival. Because of this, corals are typically limited to euphotic zones (<70m), with most species being found shallower than 30 m in depth (Kleypas, 1997; Lalli & Parsons, 1995). The breakdown of this mutualistic relationship results in coral bleaching. When stressed, corals will expel their symbionts which exposes their white calcium

carbonate skeletons, creating a “bleached” appearance. Over the past few decades there has been an unprecedented increase in reports of coral bleaching events on a global scale (Couch et al., 2017; Donner et al., 2005; Glynn, 1993; Hughes et al., 2017; Rodgers et al., 2015). Much of this is contributed either directly or indirectly to global sea surface temperature rise. This is because many coral species occupy habitat that is in their upper thermal tolerance where small increases in temperature, even over just a few weeks, could result in bleaching (Berkelmans & Willis, 1999; Glynn & Colgan, 1992; Glynn & D'croz, 1990; Jokiel & Coles, 1990). Another global stressor afflicting coral is ocean acidification, in which increased atmospheric CO_2 causes lower oceanic pH levels. The reduction in pH effectively impacts calcification rates, reducing a coral’s ability to produce its calcium carbonate skeleton (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007). Ocean acidification also induces coral bleaching, possibly as a result of the disruption of photoprotective mechanisms within the photosystems of the symbiont (Anthony et al., 2008). While global stressors have been accredited as the leading causes of coral bleaching, there is growing concern for identifying and understanding the impacts of local or regional stressors (e.g overfishing, disease, pollution). The rationale for this is due to the fact that: 1) management and intervention of regional stressors are more easily achieved; and 2) regional stressors can often work in combination, in some cases synergistically, with global stressors to yield bleaching events. Regional stressors can be large contributors to coral bleaching. This is evident in the fact that coral cover decline varies on a temporal basis but tends to be consistent on a regional scale (Gardener et al., 2003; Bruno & Selig, 2007). These stressors can be natural in origin (e.g. hurricanes, macroalgae growth, disease) or they can be anthropogenic (e.g. overfishing, pollution) (Hughes & Connell, 1999). The extent to which these stressors impact

coral is dependent on many factors, such as the frequency of the stressor, the duration that the stressor persists, the magnitude of the stressor, and if one or more stressors are impacting the coral simultaneously. In order to implement successful management practices, it is important to identify all the stressors that are affecting coral communities and assess the extent of their impacts. If no action is taken to protect and restore stressed reefs, recent projections predict global coral ecosystem collapse in just a few decades (Pandolfi et al., 2003)

1.2 Annual Red Tide Events in the Gulf of Mexico

In the Gulf of Mexico, seasonal red tide events are produced by the ichthyotoxic dinoflagellate algae, *Karenia brevis.* These blooms act as a regional stressor on an array of species throughout the Gulf and Atlantic coastline of the southeastern United States. Red tide blooms are not a recently discovered phenomenon. The presence of these blooms has been documented since the early sixteenth century with Spanish explorers having reported observations of water discoloration and fish kills in sailing log books, while reoccurring mass fish kills have been documented off the Gulf coast of Florida as early as 1844 (Feinstein et al., 1955; Martyr, 1912). These blooms typically occur in late summer to fall months, although blooms are capable of persisting year-round if conditions permit (Tester & Steidinger, 1997). Based on the periodicity of these events, it has been theorized that *K. brevis* has a seeded benthic cyst phase that lies dormant until conditions are right for bloom formation (Steidinger, 1975). Although a seeded phase has yet to be thoroughly investigated, *K. brevis* is ubiquitous throughout the Gulf waters year-round, typically occurring in concentrations of <1000 cells L^{-1}. However, when conditions are favorable, lethal blooms can form with densities of over 2.5 ×

10^6 cells L^{-1} (Flewelling 2008; Tester et al., 1993). On the west coast of Florida, algal blooms generally form off shore as a result of nutrient enhancement due to upwelling or biological sources. They are then carried to the coastline by cross-shelf transport mechanisms where the shallow, nutrient rich waters are optimal for further proliferation of the bloom. Following eddies and wind patterns, these blooms can then be further concentrated and migrate through the Florida Straight, where they can enter the Gulf Stream and impact the Atlantic coastline as far north as North Carolina (Tester & Steidinger, 1997).

Whether or not anthropogenic eutrophication is supporting growth and maintenance of these blooms near shorelines is still debated. Throughout the world there has been an increase in harmful algae bloom frequency, duration and density near coastlines and it is the general consensus that degraded water quality from nutrient loading is one of the main factors driving this change (Heisler et al., 2008). However, evidence for this in *K. brevis* blooms is lacking. *K. brevis* is capable of utilizing a wide variety of organic and inorganic sources of nitrogen and phosphorus (Baden & Mende, 1979; Killberg-Thoreson et al., 2014; Vargo & Howard-Shamblott, 1990). In addition, bloom maintenance and growth require many chelators and trace metals (Collier et al., 1969; Wilson, 1955; Wilson & Collier, 1955). Because of this, terrestrial source of estuaries, groundwater, rainfall and river discharge were originally believed to drive bloom formation. Odum et al. (1955) investigated the potential for ground water and three different estuaries as a source of nitrogen and phosphorus loading in southwest Florida. It was found that both the Tampa Bay and Peace River estuaries were capable of supplying enough N and P to explain large blooms in those areas; however, the Caloosahatchee River and ground water sources resulted in a reduction of nutrients by diluting coastal waters even though dense

blooms still occurred in those areas. Martin et al. (1971) investigated the potential of rainfall and river flow to increase iron concentrations but failed to find that these sources of iron were sufficient in explaining patterns in red tide blooms. Additionally, Hu et al. (2006) proposed that river flow could only partially provide the nitrogen needed to support documented blooms in west-central Florida. Although no one terrestrial source is capable of explaining observed bloom patterns, it is hard to ignore the potential of increased human activities, such as agriculture and reservoir draining, to contribute as nutrient sources directing bloom formation. Because of this it has been suggested that decades of released nutrients are being sequestered in the benthos of the West Florida Shelf, where upwelling events result in massive nutrient loadings and trigger a complex set of species interactions that lead to bloom formation (Brand & Compton, 2007; Walsh et al., 2009; Weisberg et al., 2014; Weisberg et al., 2016). Regardless of the cause, there is evidence that suggests red tide events, along with other harmful algae blooms, have been increasing over recent decades (Brand & Compton, 2007; Hallegraeff, 1993; Magaña et al., 2003; Smayda 1990; U.S. Commission on Ocean Policy 2004). In a study by Magaña et al. (2003) who investigated red tide events in historical records related to the western Gulf of Mexico, suggested that Texas has experiences an increase in bloom frequency in more recent years. In another study by Brand and Compton (2007), spatio-temporal analysis of bloom densities had shown that bloom concentrations have increased 13-18-fold since prior decades; while, because of wind and current patterns, the majority of these blooms occurred near shore, with a 20-fold increase 0-5 km from the shoreline (Brand & Compton, 2007).

The detrimental effects of *K. brevis* blooms are largely due to the neurotoxic polyethers they produced, known as brevetoxins (Brovedania et al., 2015). Two major structures of

brevetoxins have been identified, with many derivatives of each. The nomenclature of these compounds is based on the algae's former classification of *Ptychodiscus brevis* to give the acronym PbTx, followed by a number in the order of their discovery (Baden, 1989; Poli et al., 1986). Many naturally occurring and chemically-synthesized brevetoxin analogs have been reported; however, all derivatives are characterized by their polyether backbone structures as being one of two types. The chemical structures of type 1 and type 2 are represented by PbTx-1 and PbTx-2, respectively; while derivatives result from alterations of their functional groups (Fig. 1) (Baden, 1989). These lipid-soluble toxins interact with voltage gated sodium channels (VGSCs) resulting in an excitatory response to produce their neurotoxic effects (Atchison et al., 1986; Baden, 1989). Brevetoxins are believed to attach to site 5 of VGSCs, stabilizing their open configuration and disrupting the normal maintenance of action potentials (Furey et al., 2007; Poli et al., 1986). There are two main routes of exposure of brevetoxins, either through respiration or ingestion. Toxins in the water column can be carried to the sea surface through bubble mediated transport. Once at the surface, bursting bubbles and wave action then allow the brevetoxins to be aerosolized (Blanchard 1975; Pierce et al., 2003). The dangers of inhalation may be an acute threat, with aerosolized brevetoxins dissipating after a bloom collapse. However, the threat of ingesting brevetoxin-contaminated food can be long lasting. Brevetoxins are capable of bioaccumulating, typically in filter feeding organisms, and then can spread through the food-web via trophic transfer (Flewelling, 2008; Landsberg et al., 2009; Morris et al., 1991). Impacts of brevetoxins through both respiration and ingestion have been well documented in an array of species, including those at higher trophic levels, such as fish, marine mammals, and humans. In Florida, large bloom formations near coastal communities

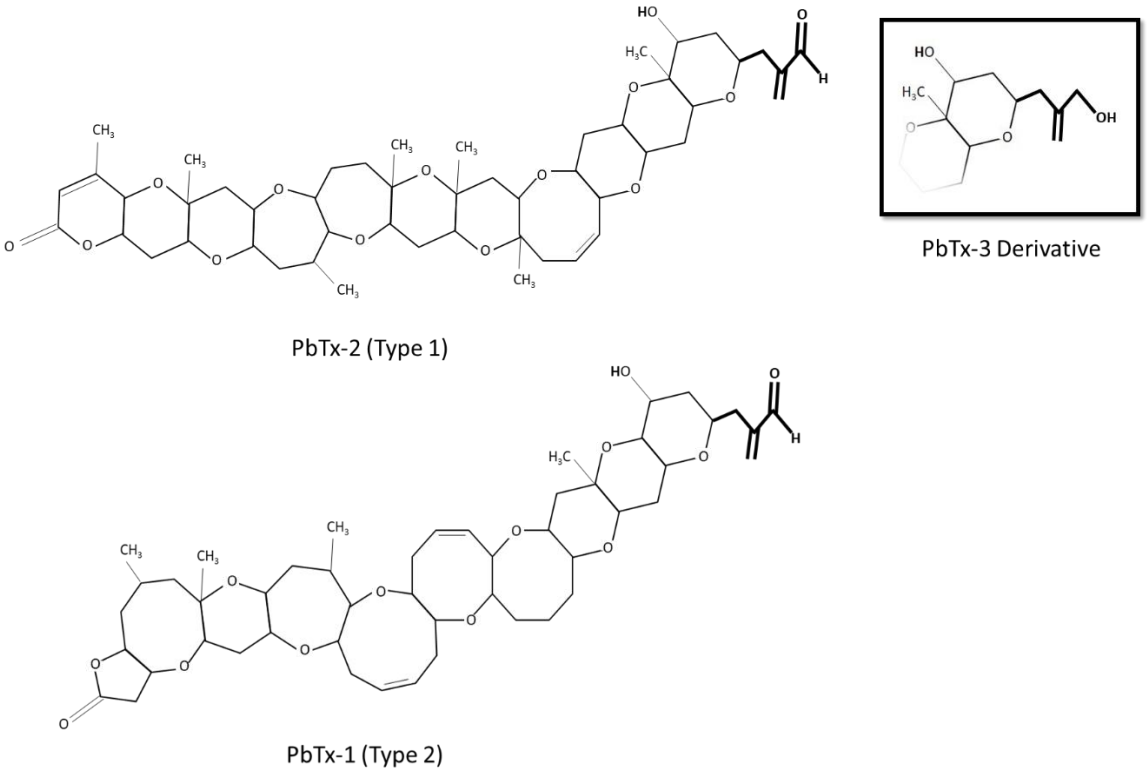

Figure 1 Brevetoxin type 1 and type 2 chemical structures (PbTx-2 and PbTx-1, respectively) with the PbTx-3 derivative of type 1. Functional groups are indicated in bold. Natural and synthetic derivatives of each brevetoxin type retain the polyether back bone and differ by their functional group. PbTx-3 is a naturally occurring derivative of the aldehyde reduced form of PbTx-2 (adapted from Baden, 1989)

typically coincide with increased hospital reports of airway related illnesses, such as pneumonia, bronchitis, and asthma (Backer et al., 2003; Kirkpatrick et al., 2006). It was estimated that in Sarasota alone, the capitalized cost of emergency room admission due to *K. brevis* related airway illnesses could be between $0.5 million to $4 million (Hoagland et al., 2009). However, this is probably an underestimation of the total impact since it does not take into consideration the cost to treat these illnesses, nor does it take into consideration the cost of exposure by ingestion. In humans, ingestion typically occurs from consumption of contaminated shellfish, which results in a condition known as neurotoxic shell fish poisoning. One discernable event occurred in the fall of 1987. In North Carolina, 48 cases of neurotoxic shell fish poisoning were reported from individuals eating shellfish harvested from an area that encountered a dense bloom (Morris et al., 1991). That same bloom in North Carolina was believed to be the cause of an eleven-month dolphin stranding event throughout the southeastern United States. This resulted in the death of 740 dolphins, where necropsies revealed ingestion of brevetoxin contaminated fish (Geraci, 1989). In 1996, off the southwest coast of Florida, a die-off of 149 manatees over a two-month period closely coincided with a significant red tide event, resulting in a depletion of nearly 5% of the population. It was later discovered that the impacted manatees showed signs of pulmonary damage as well as ingestion of brevetoxin contaminated seagrass (Bossart et al., 1998). Although mass fish kill events have been reported in the west coast of Florida since the early eighteenth century, it wasn't until 1948 that these events were recognized to cooccur from dense blooms of *K. brevis* (Davis, 1948). In addition to ingestion of contaminated food, fish can also be exposed to dissolved brevetoxins in the water column through absorption across their gill membranes

(Landsberg et al., 2009). Red tide has been known to impact many different species of invertebrates as well, such as sea urchins and sponges (Landsberg, 2002). One class of invertebrates whose sensitivity to brevetoxins has been well studied, particularly at early life stages, is Bivalvia. Due to their economic value, exhaustive efforts have been taken to discern what kind of effects *K. brevis* and brevetoxins have on these species. Leverone et al. (2006) demonstrated that exposure of three-day old and seven-day old larvae of *Mercenaria mercenaria*, *Crassostrea virginica* and *Argopecten irradiant* to *K. brevis* resulted in reduced survivorship in both age classes for all three species. Rolton et al. (2014) showed that short term exposure of *K. brevis* to the embryonic stages of *C. virginica* and *M. mercenaria* is not only lethal but can cause sublethal effects as well, including developmental abnormalities and reduction of larval size. Other studies suggest that exposure of gametes of *C. virginica* to *K. brevis* can result in reduced gamete quality and a decrease in fertilization success (Rolton et al. 2015). Currently, there is an alarming deficit in our understanding of the impact of *K. brevis* on coral species. Early life history stages should be of particular concern since any impact on the gamete or larval phases can negatively influence recruitment success of coral and cause a reduction in the genetic variability in the population. Since concentrations of red tide are increasing, and the majority of *K. brevis* blooms are occurring near shore where corals are most likely found, interactions between *K. brevis* and coral are inevitable. Hence, it is important to assess the impact of *K. brevis* as a potential regional stressor on coral species throughout the southeastern United States.

Some evidence exists that suggests red tide can act as a potential stress. The preliminary work of Ross et al. (2010) found that a 20-hour exposure of larvae of the coral species *Porites*

astreoides to ecologically relevant concentrations of *K. brevis* resulted in a decrease in respiratory demand and a simultaneous increase in selected biomarkers of oxidative stress. These findings prompted this current study which was aimed at further elucidating the potential impacts of *K. brevis* on different coral species and life history stages. There are many different factors that can influence the potential impacts of these blooms on corals, such as which life history stage is being challenged, the ability for certain species to be resistant or susceptible to exposure, and if behavioral or cellular changes ensue. Since our understanding of the potential effects are so limited, a broad, multifaceted approach was used to elucidate impacts on coral. Therefore, the goal of this study was to address the following questions.

1) Does exposure to live *K. brevis* or purified brevetoxins result in behavioral changes in coral larvae?
2) Does exposure to live *K. brevis* or purified brevetoxins impact recruitment success of larvae?
3) Does exposure to live *K. brevis* or purified brevetoxins impact photochemical efficiency of symbionts? If so, does this vary among species of coral, as well as at different life history stages?
4) Does exposure to live *K. brevis* or purified brevetoxins induce oxidative stress and can this stress be detected through the use of biomarkers?
5) Does exposure to live *K. brevis* or brevetoxins induce other sublethal stress responses that can be assessed through the use of quantitative proteomic analysis?

This thesis is composed of two major studies in order to answer these questions. The first study aimed to address the first four question. It incorporated techniques capable of identifying

larvae avoidance of *K. brevis* or brevetoxin contaminated seawater, quantified rates of settlement and survival in larvae, assessed induction of coral bleaching in different coral species and at different life history stages, and analyzed different biomarkers of cellular stress following exposure. The second study utilized a mass spectrometry-based proteomics approach (iTRAQ proteomic analysis) to determine what metabolic pathways of *P. astreoides* were impacted by *K. brevis* or brevetoxins.

2.

Behavioral and physiological responses of coral species to the red tide producing dinoflagellate, *Karenia brevis,* and associated brevetoxins

Abstract

Because of the global decline in coral coverage, it is important to identify all stressors that can potentially impact coral reef ecosystems. In the Gulf of Mexico, annual red tide events occur from the bloom formation of the neurotoxin producing dinoflagellate algae, *Karenia brevis*. These blooms have negative impacts on an array of species, from higher trophic-level organisms such as fish, dolphin and humans, to invertebrates such as bivalves. Since little is known of the effects of red tide on coral communities, it was the goal of this study to determine if *K. brevis* and their associated brevetoxins can act as a potential regional stressor on corals species. This was examined using a multifaceted approach to determine impacts on: larvae behavior, larvae settlement and survival, photochemical efficiency of symbionts, and induction of oxidative stress. An Atema choice flume was used to present larvae of the coral species *Porites astreoides* with a pairwise choice between contaminated and uncontaminated seawater. Also, larvae of *P. astreoides* and adult fragments of *P. astreoides*, *Acropora cervicornis*, *Cladocora arbuscula,* and *Phyllangia americana* were exposed to ecologically relevant concentrations of purified brevetoxins (0.018 µg mL^{-1} PbTx-2 and 0.0018 µg mL^{-1} PbTx-3) or *K. brevis* cells (2.5×10^6 cells L^{-1}). Following exposure, larval percent settlement and survival, maximum quantum yield and biomarkers of oxidative stress were analyzed. Larvae showed a significant preference for seawater devoid of brevetoxin and *K. brevis* cells down to

concentrations of 5×10^5 cells L^{-1}. Analysis of larval settlement demonstrated that red tide exposure impacted both larvae percent settlement and survival. Maximum quantum yield (F_v/F_m) was impacted in *P. astreoides* larvae and adults but not in adult fragments of *A. cervicornis*. Evidence of lipid peroxidation was detected for *P. astreoides* larvae following a 24-hour exposure and in *C. arbuscula* adult fragments following a 48 -hour exposure; however, no other biomarker of oxidative stress measured showed a significant increase. The results herein suggest that acute exposure to blooms of *K. brevis* and associated brevetoxins can act as a potential regional stressor to coral species, with larvae appearing to be particularly affected. Reallocation of larval energy to avoid blooms and impacts on larvae settlement and survival can have serious consequences on coral reef recruitment, recovery and resilience; while impacts on symbiont photochemical efficiency and induction of oxidative stress can result in coral bleaching and reduced coral cover. While this study focused on short term exposure to red tide, blooms have been reported to last year-round. This merits future investigation into prolonged exposure of red tide on coral species.

2.1 Introduction

2.1.1 Reef Building Coral

Coral populations have been declining on a worldwide basis due to the combined effects of global scale stressors acting in concert with regional or local scale pressure (Carpenter et al., 2008; Hughes et al., 2007). In the Florida Keys and the Gulf of Mexico, a number of global and local stressors have clearly been identified as contributing towards the overall coral cover decline in those areas (Causey et al., 2002). These stressors may have an impact during a

particular or all life history stages of coral. Of specific concern are impacts on the distribution, survival, and metamorphosis of the pelagic larval stage of coral, since this can negatively affect coral recruitment and have implications for the resistance and recovery of coral reef communities (Edmunds et al., 2001; Kuffner et al., 2006; Negri & Heyward, 2001).

2.1.2 Red Tide

One regional stressor that has been found to impact many different organisms in the southeastern United States is the episodic formation of red tide blooms. These harmful algae blooms (HAB) occur annually in the Gulf of Mexico from the proliferation of the toxin producing dinoflagellate, *Karenia brevis*. These algae are phototactic, tending to aggregate toward the surface of the water column in the presence of sunlight. Although ubiquitous at low concentrations throughout the year (<1000 cells L^{-1}), annual dense blooms tend to form near the coastline of the Gulf due to a multitude of factors (e.g. wind patterns, upwelling, nutrient loading, run-off); with some blooms lasting up to 18 months with densities of > 2.5×10^6 cells L^{-1} (Flewelling, 2008; Landsberg, 2002; Tester et al., 1993). Periodically, these dense blooms may migrate into the Atlantic Ocean and enter the Florida Gulf Stream, which can transport them up the Atlantic coastline as far north as North Carolina (Tester & Steidinger, 1997). There is growing evidence that suggests red tide events, along with other harmful algae blooms, have been increasing in duration and density over recent decades (Brand & Compton, 2007; reviewed in Hallegraeff, 1993; Magaña et al., 2003; U.S. Commission on Ocean Policy 2004). Most notably is a study by Brand and Compton (2007) who reported that bloom concentrations

have increased 13-18-fold in 1994-2002 compared to reports from 1954-1963, with the majority of blooms occurring 0-5 km from the shore line.

K. brevis produces a suite of harmful polyether neurotoxins called brevetoxins, given the acronym PbTx after the algae's former classification (*Ptychodiscus brevis*) (Baden, 1989; Brovedania et al., 2015; Poli et al., 1986). The neurotoxic effect of these lipophilic compounds comes from their affinity to site 5 of voltage gated sodium channels (VGSC) (Furey et al., 2007; Poli et al., 1986). Once bound, the result is a stable open conformation of the VGSC, which elicits an excitatory response of the cell (Atchison et al., 1986; Baden, 1989). These toxins are capable of bioaccumulation and being aerosolized, resulting in exposure through respiration and ingestion. Dense blooms have been known to cause human and environmental impacts such as neurotoxic shellfish poisoning, increased symptoms of airway related disease, mass fish kills, marine mammal die-offs, and impacts to benthic communities (Bossart et al., 1998; Davis, 1948; Geraci, 1989; Hoagland et al., 2009; Landsberg, 2002; Morris et al., 1991). Due to their economic importance, a considerable amount of effort has gone into assessing the effects of red tide blooms on bivalve species, of which the larval and gametic stages have shown to be particularly vulnerable to their effects. Negative impacts have included increased mortality, reduction in larval size, increased morphological abnormalities, reduction in gamete quality and a decrease in fertilization success (Leverone et al., 2006; Rolton et al., 2014; Rolton et al., 2015). Although effects on other invertebrates have been investigated, little is known of the potential implications these blooms may have on coral species; however, there is some evidence that red tide blooms may negatively impact corals as well. One study by Ross et al. (2010) demonstrated that short term exposure of *Porites astreoides* larvae to *K. brevis* or cell lysate resulted in

respiration inhibition and an increase in certain biomarkers of cellular oxidative stress. Unfortunately, this is the extent of our understanding of red tide's effects on coral species to date.

The detection of oxidative stress is a widely used approach in monitor sublethal stress responses in coral species (Downs et al., 2002; Rotchell & Ostrander, 2011; Rougée et al., 2006; Vijayavel et al., 2012). This is because reactive oxygen species (ROS), such as superoxide anion ($O_2{\cdot}^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$), can be induced from many different environmental perturbations and have been posited as an underlying mechanism in coral bleaching (Lesser, 1996; Lesser & Farrell, 2004). Typically, the cell produces antioxidants to quench these free radicals. However, a shift in balance to overproduction of ROSs can lead to damage of lipids, proteins, and DNA; as well as expulsion of zooxanthellae or initiation of apoptosis (Downs et al., 2002). There are many biomarkers of oxidative stress, such as the differential production of antioxidants or byproducts of oxidative and enzymatic reactions, which can be used to assess sublethal stress responses in coral following exposure to a stressor (Downs et al., 2012). Another common technique in identifying sublethal stress is estimating bleaching by assessing the reduction of photochemical efficiency of *in hospite Symbiodinium*. This can be done through pulse amplitude modulation (PAM) fluorometry, where the fluorescent yield of the photosystems is measured following dark adaptation (Genty et al, 1989). This can provide a snapshot of the photochemical capabilities of the algae and can be a better indication of coral bleaching then relying on qualitative observations of color change of the tissue (Fitt et al., 2001; Warner et al., 1999)

Because the majority of dense blooms form near coastal areas where corals are most commonly found, and the tendency for cells to aggregate near the water surface where they could potentially interact with the planula larvae, it is likely that corals are exposed to blooms at some point in their life cycle. With the alarming increase in HAB's it is important to determine if, and to what extent, *K. brevis* and their associated brevetoxins impact coral species. This study used a broad approach in order to determine if *K. brevis* or the brevetoxins they produce have a negative impact on different coral life history stages and coral species. More specifically, this study assessed the impacts of larval behavior, larval settlement and survival, photochemical efficiency of symbionts in larvae and adults, and the presence of biomarkers of oxidative stress; following acute exposure to high bloom densities of *K. brevis* or brevetoxins.

2.2 Methods and Materials

2.2.1 *Karenia brevis* Culture Maintenance and Brevetoxins

Cultures of *K. brevis*, strain CCFWC257, were acquired from Florida Fish and Wildlife Research Institute and reared to the experimental cell concentration and volume in a laboratory setting at University of North Florida. Cultures were maintained at room temperature under lighting with intensity between 100 - 123 µmol $m^{-2}s^{-1}$ on a 12:12 cycle in artificial seawater (≈ 35 ppt). Brevetoxin analogs (PbTx-2 and PbTx-3) that are commonly found in high abundance in *K. brevis* cells, were purchased from MARBIONC Development Group LLC (Wilmington, NC, USA). Brevetoxin solutions were made by first suspending PbTx-2 and PbTx-3 analogs in 100% methanol to a concentration of 0.1 µg $µL^{-1}$ and 0.01 µg $µL^{-1}$, respectively.

Subsequently, solutions were diluted in seawater for a final concentration of 0.018 µg mL^{-1} (PbTx-2) and 0.0018 µg mL^{-1} (PbTx-3). This falls into the range of concentrations previously described in water samples collected from *K. brevis* blooms off the coast of southwest Florida (Pierce & Henry, 2008).

2.2.2. Coral Collection

Porites astreoides

Forty colonies of *Porites astreoides* were collected off the Atlantic coastline of Summerland Key, Florida, transported to Mote Tropical Research Laboratory in coolers, and maintained in raceway flow tables with running seawater. Larvae were collected over the course of three nights, up until the new moon of May (May 23rd-May 25, 2017) (McGuire,1998). Brooded larvae were collected by placing each colony in a 3 L Rubbermaid Grip's Mix Bowl® with constant running seawater. Bowls were positioned to allow overflow into plastic tri-pour beakers fitted with 180 µm mesh to collect the positively buoyant larva. Following collection, larvae were pooled to control for possible maternal effects. Five adult colonies were chosen at random for exposure treatments and cut into 3 cm^2 fragments with a circular saw. One fragment from each colony was used per treatment and exposure time. The remaining colonies were later returned to the site of collection and re-adhered to the reef using Z-Spar Splash Zone Compound underwater epoxy.

Phyllangia americana and *Cladocora arbuscula*

Twenty colonies of *Cladocora arbuscula* and *Phyllangia americana* were collected from the Gulf of Mexico off the coast of Clearwater, FL (Rube Allyn Reef; collection permit obtained by K.

Lunz). Colonies were transported to the University of North Florida in coolers covered in wet bubble wrap. Viable colonies were cut into 2 cm^2 fragments with one colony used per replicate. Cut fragments were allowed to acclimate for 48 hours in aquaria prior to treatment.

Acropora cervicornis

Forty branches of *Acropora cervicornis* were procured from the Coral Restoration Foundation (Key Largo, FL). Branches were cut into fragments approximately 5 cm in length and allowed to acclimate in holding tanks for two weeks.

2.2.3 Larvae Behavioral Study

In order to test the ability of *P. astreoides* larvae to discriminate between red tide contaminated seawater and uncontaminated seawater, planula larvae were placed in a two channel Atema choice flume (Atema et al., 2002). The flume was designed to allow larvae an unhindered pairwise choice of treated seawater (*K. brevis* or brevetoxins) versus untreated seawater (Fig. 2.1). The chamber held 52 mL per channel, flowing at 4.2 mm s^{-1}, using the protocols outlined by Dixson et al. (2014). For each trial, treated water contained either *K. brevis* (5×10^5 cells L^{-1}, 2.5×10^6 cells L^{-1}, 3.8×10^6 cells L^{-1} and 7.6×10^6 cells L^{-1}) or brevetoxins (0.018 µg mL^{-1} (PbTx-2) and 0.0018 µg mL^{-1} (PbTx-3)). Water flow was measured by flow meters and maintained at 100 mL min^{-1} per channel for each trial. Dye tests were used to ensure laminar flow between the two channels, without turbulence or eddies. Preliminary tests were performed to ensure larvae were able to maintain position and make forward progress against the flow before any trials were conducted. For each trial a single larva was pipetted into the

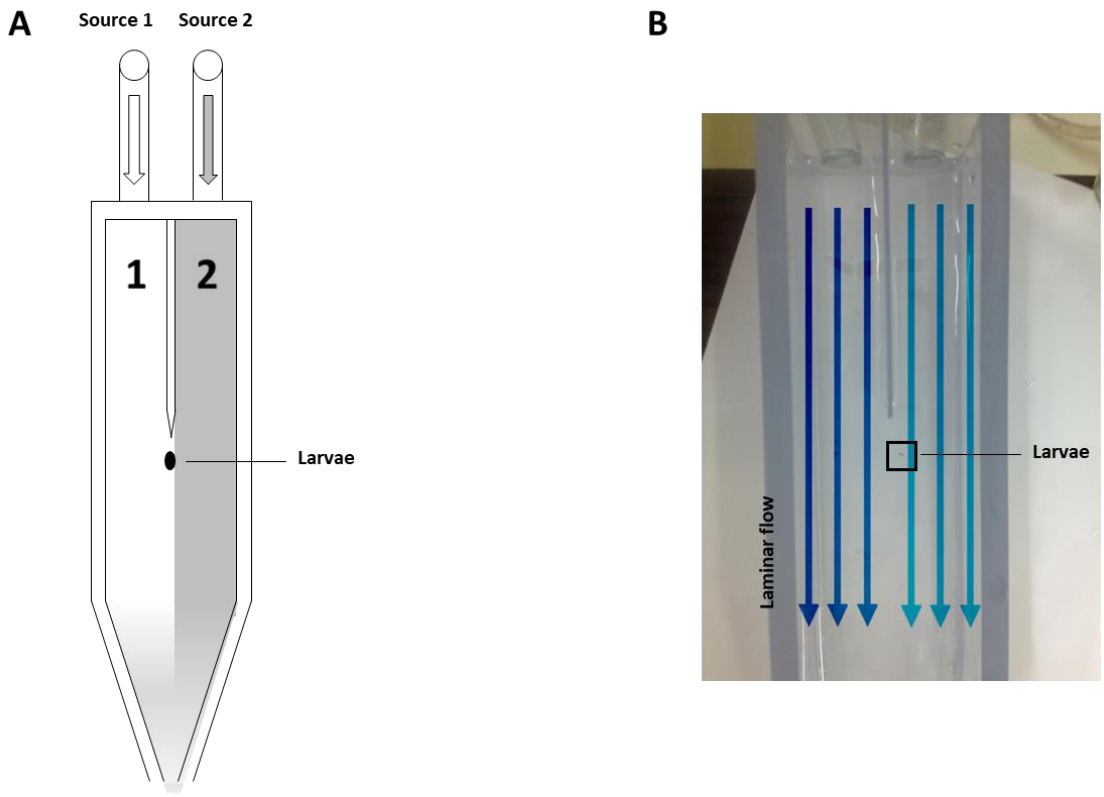

Figure 2.1 Diagram of the two-channel choice flume: (A) Schematic of the two-channel choice flume providing an illustration of larval placement, where larvae were allowed to make a pairwise choice between two water sources; (B) Photograph of larvae within the flume during experimentation where the arrows provide the visualization of laminar flow between the two sources of water

center of the flume where it was free to choose between sides or swim to preferred water source. Larvae were allowed to acclimate for 2 min, followed by a 2-mins testing period where the position (left or right) of the larvae was recorded at 5 s intervals. To ensure there was no side bias being observed, the larvae were given a 1-minute rest period. During this time water was flushed and the sources switched. The larvae were pipetted back into the flume for an additional 2-minute acclimation period followed by a 2-minute testing period.

2.2.4 Larvae Settlement Kinetics

To test the effects of *K. brevis* or brevetoxin exposure on larval settlement kinetics, 50 larvae were placed in a 400 mL tri-pour beaker containing a single terracotta tile (4.5 cm × 4.5 cm × 1 cm; Sunshine Pavers®), which served as a settlement substrate. Tiles were previously preconditioned at a depth of 6 m offshore for 4 weeks. Beakers were raised to a final volume of 300 mL. Larvae were left in the settlement beakers for either 24, 48 or 72 hours. Following exposure, larvae were scored as either swimmers or settlers. Percent swimmers, settlers and survivors was then calculated by dividing by 50.

2.2.5 Exposure of Coral Species

Porites astreoides

To test the impacts of red tide on photochemical efficiency, a two-factorial design was used consisting of time and treatment as factors. The treatments included: ambient seawater, methanol control (0.04% v/v), purified brevetoxins (0.018 µg mL^{-1} (PbTx-2) and 0.0018 µg mL^{-1} (PbTx-3)), and *Karenia brevis* (2.5 × 10^6 cells L^{-1}). Each replicate (n=5) was maintained in a 400

mL tri pour beaker at a final volume of 300 mL. Four Tri-pour beakers were randomly assigned to forty, 7 L plastic aquaria which were spread throughout two raceway flow tables and held in place using wood clothes pins. Temperature of the aquaria were continuously monitored using a YSI model 85 multiprobe meter (YSI, Yellow Springs, OH, USA).

To test photochemical efficiency, either 115 larvae or a single 3 cm^2 fragment were placed in 400 mL tri-pour beakers at a final volume of 300 mL seawater. Larvae or adult fragments were allowed to incubate for 24 or 48 hours. Post-exposure, fifteen larvae were used for analysis of photochemical efficiency, while the remaining 100 larvae were flash frozen in liquid N_2, transported to UNF and stored at -80C^o for future cellular stress analysis. Following exposure, adult fragments were analyzed for changes for photochemical efficiency, then flash frozen in liquid N_2 for future cellular stress analysis.

Acropora cervicornis

For *A. cervicornis,* four treatments were used as described before (n=5/ treatment). Each replicate consisted of a randomly selected 5cm fragment of *A. cervicornis* contained within a 300 mL glass exposure jar (275 mL of seawater). Fragments were exposed for either 24 or 48 hours. Jars were placed randomly on shelves in a lab setting under aquaria lights on a 12:12 light cycle at an intensity ≈ 30 µmol $m^{-2}s^{-1}$. Following exposure, fragments were measured for changes in photochemical efficiency then placed in -80 C^o freezer for future tissue analysis.

Phyllangia americana and *Cladocora arbuscula*

For *P. americana* and *C. arbuscula,* four treatments were used as described above (n=6/ treatment). Each replicate received a randomly selected 2 cm^2 fragment placed in a glass exposure jar containing 275 mL of seawater. Fragments were exposed for a single time point of

48 hours.[1] Jars were placed randomly on shelves in a lab setting under aquaria light on a 12:12 light cycle at an intensity ≈ 30 µmol $m^{-2}s^{-1}$. Following exposure, fragments were placed directly in -80 C^o for future cellular stress analysis.

2.2.5 Photochemical Efficiency

Following exposure, coral fragments and larvae were placed in 300 mL and 1 mL seawater, respectively. Photochemical efficiency of associated *Symbiodinium* within coral fragments or larvae was evaluated using pulse amplitude modulated (PAM) fluorometry (Diving PAM; Walz, GmbH, Germany).[2] Corals were dark adapted for 1 hour prior to analysis and changes in maximal quantum yield [$F_1/F_m = (F_m-F_o)/F_m$] of PSII were measured to indicate disruption of photochemical performance. For larvae, readings were taken by pipetting 15 larvae in 25 µL of seawater onto the tip of the fiber optic cable. The PAM fluorometer's gain and intensity were both set to 7. Following dark adaptation, adult fragments were submerged in seawater in a 3 L Rubbermaid Grip's Mix bowl ® with the PAM fluorometer's gain and intensity set to 3.

2.2.6 Analysis of Cellular Stress Biomarkers

For all coral fragments, tissue was removed from frozen samples with a Paansche airbrush (Chicago, IL, USA) using extraction buffer (50 mM phosphate buffer, pH 7.8 with 0.05

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[1] Both *P. americana* and *C. arbuscula* showed signs of necrosis prior to treatment. Fragments were cut so only viable tissue remained, limiting the number of samples for this study. Because of this only a 48-hour time frame was used to maximize possible effects of *K. brevis* and brevetoxins

[2] *C. arbuscula* and *P. americana* were not included in photochemical efficiency analysis. *C. arbuscula* display rough or irregular growth/branching patterns making it difficult to obtain accurate readings using the PAM probe, while *P. americana* is azooxanthellate.

mM dithiothreitol) over ice. Larvae and fragment cell lysis was performed by power homogenization on ice (Bio-Gen PRO200, CT, USA). Sample protein concentration was collected using a Quick Start TM Bradford Protein Assay Kit (Bio-Rad, Hercules, California, USA). Samples were then evaluated using commercially available biochemical assays as per the manufacturer's instructions. The OxiSelect^{TM} Hydrogen Peroxide/ Peroxidase Assay Kit (Colorimetric) (STA - 844) and OxiSelect^{TM} TBARS Assay Kit (MDA Quantitation) (STA – 330) were used to quantify hydrogen peroxide, peroxidase, and thiobarbituric acid reactive substances (TBARS) (Cell Biolabs, San Diego, CA, USA).

2.2.7 Statistical Analysis

Atema Choice Flume

For each trial a minimum of 10 random larvae were selected. Only larvae able to maintain their position in the flume were recorded. Kolmogorov-Smirnov tests were used to compare the proportion of time that individuals spent in the stream of water containing the biological cue (*K. brevis* or brevetoxins) compared to the proportion of time that individuals spent in one side of the chamber when no cue was present (ambient seawater vs ambient seawater).

Larval Kinetics Study

Data for larval swimmers, settlers and survivors were arcsine square root transformed. For settlers and swimmers, a two-way ANOVA was used with time and treatment as factors. Data for settlers failed the assumption of homogeneity of variance (Levene's test: $p = 0.021$). However, since equal sample sizes were used among groups, it was assumed that the ANOVA

was robust enough to handle the violation (Glass et al., 1972). For survivors, the data failed the assumption of normality and a Sheirer-Ray-Hare nonparametric two-way ANOVA was used. Because of these violations, settlement and survival data were analyzed using a Bonferroni post-hoc test.

Pulse Amplitude Modulated Fluorometry

Maximum quantum yield of *in hospite Symbiodinium* was analyzed using a two-factorial design. Photochemical efficiency of *P. astreoides* larvae and *A. cervicornis* adults were analyzed using a two-way ANOVA followed by Tukey's post-hoc test for differences among mean effects. However, photochemical efficiency of *P. astreoides* adult samples violated the assumption of normality and were analyzed using a Sheirer-Ray-Hare nonparametric two-way ANOVA. To further discern what factors were contributing to differences among groups, a Kruskal-Wallis one-way ANOVA was performed followed by Mann-Whitney U tests among groups and compared to statistical cut offs corrected using the Holm-Bonferroni method to reduce the effects of family-wise error rates (Holm, 1979).

Hydrogen Peroxide

Hydrogen peroxide concentrations within larvae, adult *P. astreoides* and *A. cervicornis* were analyzed using a two-way ANOVA or a nonparametric alternative where appropriate, with time and treatment as factors. For *C. arbuscula* and *P. americana* hydrogen peroxide concentrations were analyzed using a one-way ANOVA or nonparametric alternative where appropriate, with treatment as the factor. Adult *P. astreoides* failed the assumption of normality and a Sheirer-Ray-Hare nonparametric two-way ANOVA was used. To meet the assumption of ANOVA, data for *A. cervicornis* and *P. americana* were square root transformed

and $\log_{10}$ transformed, respectively. For *C. arbuscula,* the data failed the test of normality and a Kruskal-Wallis one-way ANOVA was used.

Peroxidase

Peroxidase activity for larvae, adult *P. astreoides* and *A. cervicornis* was analyzed using a two-way ANOVA or a nonparametric alternative when required, with time and treatment as factors. Based on the positive skewness of the data sets for larvae and *A. cervicornis,* a square root transformation was used in order to fit the data to a more normal distribution. Based on the positive skewness, adult *P. astreoides* was $\log_{10}$ transformed in order to fit a more normal distribution. For *C. arbuscula* and *P. americana*, peroxidase activity failed the test of normality and a Kruskal-Wallis one-way ANOVA was used.

TBARS

A two-way ANOVA or a nonparametric alternative was used to analyze the production of thiobarbituric acid reactive substances (TBARS) for larvae, adult *P. astreoides* and *A. cervicornis*, with time and treatment as the factors. For *P. astreoides* larvae, further analysis of a one-way ANOVA followed by Tukey's post-hoc test with a Holm-Bonferroni corrected alpha value was used to further evaluate the effect among treatments. For *C. arbuscula* and *P. americana,* a one-way ANOVA was used with treatment as a factor. Based on the positive skewness of the distribution of TBARS for *P. astreoides* larvae, a $\log_{10}$ transformation was utilized to transform the data to a more normal distribution. Since TBARS for adult *P. astreoides* and *A. cervicornis* was not normally distributed, a Sheirer-Ray-Hare nonparametric two-way ANOVA was used.

2.3 Results

2.3.1 Larvae Discrimination Between Ambient Seawater and Seawater Contaminated with *Karenia brevis* or Brevetoxins.

The proportion of time that individual larvae spent in a channel containing either *K. brevis* or brevetoxins was compared to the proportion of time that individuals spent in one side of the chamber when no cue was present (seawater vs. seawater). The mean proportion of time spent in one side of the chamber was near 50% for the control trial of seawater vs. seawater (mean = 49.31%, SE = ± 1.33). Larvae showed stronger preference for untreated seawater in each trial, with a larger percent time spent in the ambient seawater with increasing concentration of *K. brevis* (Fig. 2.2). At the highest concentration of *K. brevis* (7.6×10^6 cells L^{-1}), larvae displayed the most discernable avoidance with a mean time in the contaminated cue of 4.80% (SE = ± 1.03, $p < 0.0001$). Larvae exposed to 3.8×10^6 cells L^{-1}, 2.5×10^6 cells L^{-1} and 5×10^5 cells L^{-1} spent 6.25% (SE = ± 1.16), 15.42% (SE = ± 1.75) and 29.58% (SE = ± 1.67) of the time in those cues, respectively ($p < 0.0001$ per trial). Larvae exposed to brevetoxins (0.018 µg mL^{-1} PbTx-2 and 0.0018 µg PbTx-3 mL^{-1}) spent 3.75% of the time in that cue compared to the control (SE = ± 0.75, $p < 0.0001$).

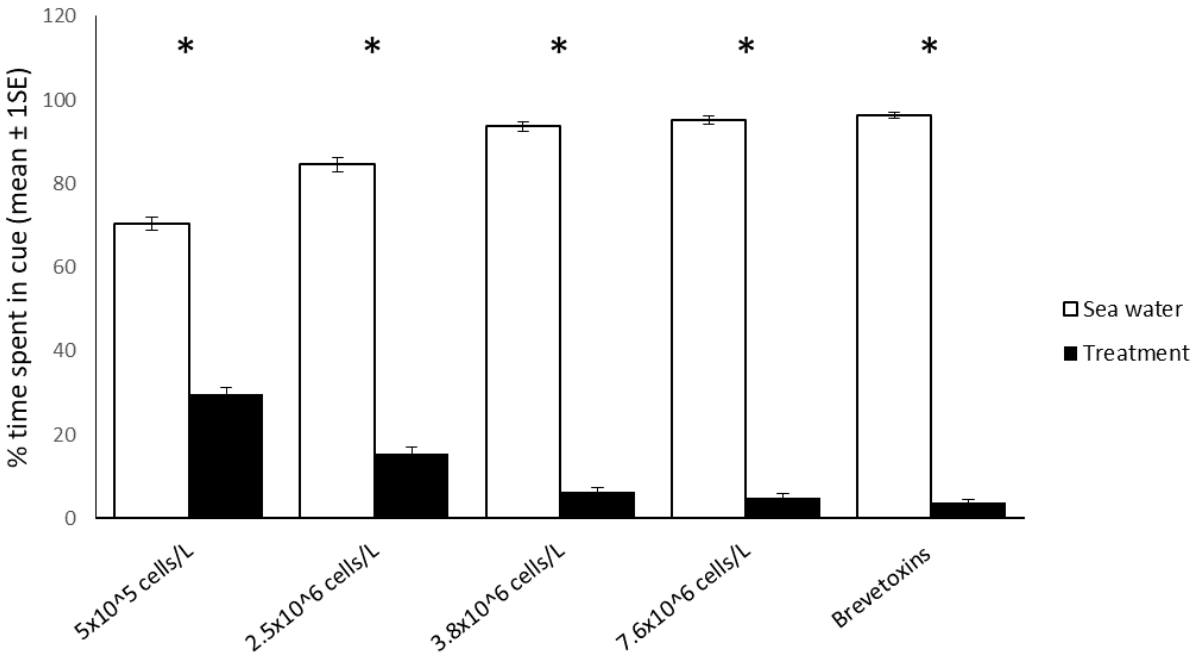

Figure 2.2 Percent time *P. astreoides* larvae spent in a channel when introduced to a pairwise choice between two water sources of ambient seawater and a biological cue. Trials consisted of seawater containing either *K. brevis* cells that represented medium (5×10^5 cells L^{-1}) and high (2.5×10^6 cells L^{-1}, 3.8×10^6 cells L^{-1} and 7.6×10^6 cells L^{-1}) bloom densities; or brevetoxins at 0.018 µg ml^{-1} (PbTx-2) and 0.0018µg ml^{-1} (PbTx-3). "*" indicates $p < 0.001$ via Kolmogorov Smirnov test when compared to control trials of ambient seawater vs ambient seawater. Bars represent ± 1 SE

2.3.2 Larvae Percent Settlement and Survival Following Exposure to *Karenia brevis* or Brevetoxins

The number of larval swimmers decreased over time ($F_{(2,48)} = 8.447$, $p = 0.001$); however, there was no difference among the treatments ($F_{(3,48)} = 2.366$, $p = 0.083$), nor was there an interaction (treatment*time) ($F_{(6,48)} = 1.699$, $p= 0.142$) (Fig. 2.3 A). Proportion of settlement and survivorship was impacted as a function of treatment ($F_{(3,48)} = 3.264$, $p = 0.029$; and $H = 9.079$, $p = 0.028$, respectively), while there was no interaction ($F_{(6,48)} = 1.172$, $p = 0.337$; and $H = 12.42$, $p = 0.053$, respectively). A Bonferroni post-hoc test revealed that both settlement and survival were reduced following exposure to *K. brevis* compared to ambient seawater. The number of settlers was reduced over time ($F_{(2,48)} = 3.782$, $p = 0.030$). However, this was not the case for survivors ($H = 5.729$, $p = 0.057$) (Fig. 2.3 B & C). There was no difference between percent survival of control and MeOH control treatments, yet there was a notable difference in percent settlement for the MeOH control at the 48-hour time frame. Since only three timeframes were used in this study, temporal resolution is limited. This may explain the discrepancy between the control and the MeOH control observed in larvae settlement. It is possible the majority of larvae in the MeOH control group settled shortly after the 48-hour time mark. This is likely since there was no difference between the control and the MeOH control at the 72-hour time frame (Fig. 2.3 B). It was believed that if temporal resolution of the data was increased by adding more time frames then the percent settlement in MeOH would be more accurately represented. Because of this, further analysis was conducted on control vs *K. brevis* treatments with the MeOH control and the PbTx/MeOH treatments excluded. Two-way ANOVAs were conducted of larvae kinetics between control and the *K. brevis* treatments. There

was a significant reduction of swimmers over time ($F_{(2,24)} = 10.107$, $p = 0.001$), while there was no significant difference between treatments ($F_{(1,24)} = 0.722$, $p = 0.404$), nor was there an interaction (treatment*time) ($F_{(2,24)} = 2.113$, $p = 0.143$) (Fig. 2.4 A). There was a reduction in settlement and survival in the *K. brevis* treatment compared to ambient seawater ($F_{(1,24)} = 9.466$, $p = 0.005$; and $F_{(1,24)} = 11.972$, $p = 0.002$, respectively). The proportion of settlement and survival did not differ as a function of time ($F_{(2,24)} = 3.295$, $p = 0.054$; and $F_{(2,24)} = 0.229$, $p = 0.797$, respectively), nor was there an interaction (treatment*time) ($F_{(2,24)} = 0.070$, $p = 0.932$; and $F_{(2,24)} = 0.741$, $p = 0.487$, respectively) (Fig. 2.4 B &C).

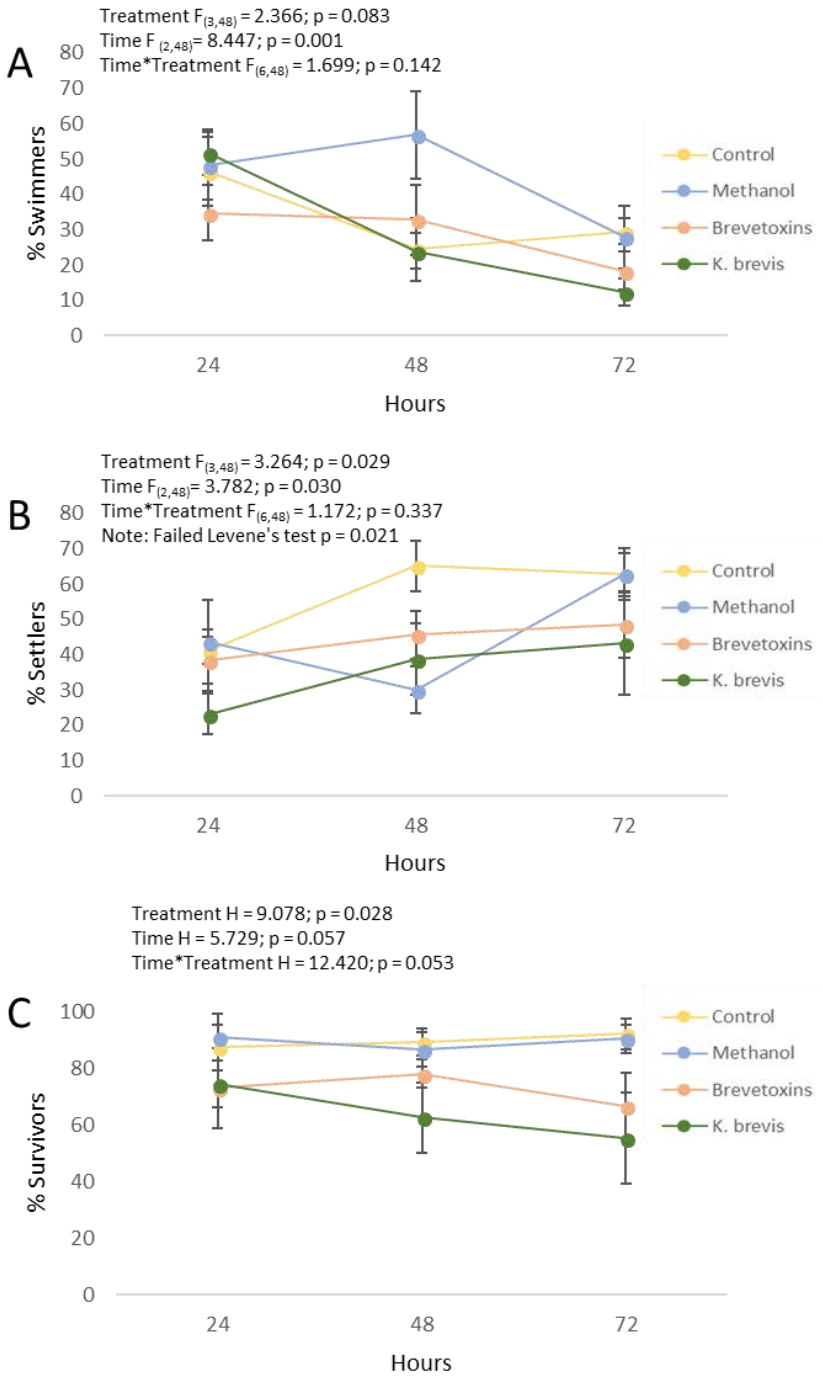

Figure 2.3 Kinetics of larval settlement and survival following exposure to either ambient seawater, MeOH, brevetoxins or *K. brevis*; for either 24, 48 and 72-hours: (A) percent swimmers, (B) percent settlers, and (C) percent survivors. Bars represent ± 1 SE

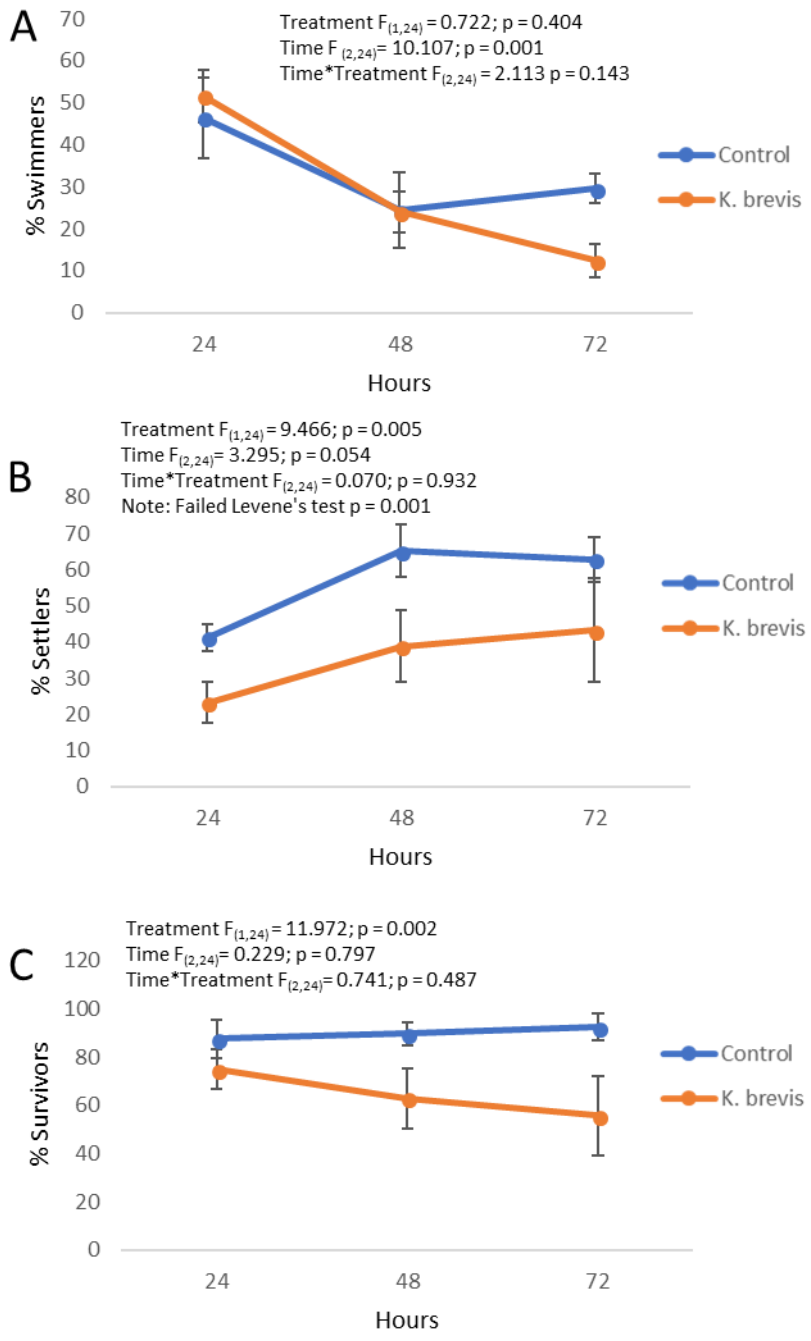

Figure 2.4 Kinetics of larval settlement and survival following exposure to either ambient seawater or *Karenia brevis*; for either 24, 48 and 72-hours: (A) percent swimmers, (B) percent settlers, and (C) percent mortality. Bars represent ± 1 SE

2.3.3 Photochemical Efficiency of *Symbiodinium* Following Exposure to *Karenia brevis* or Brevetoxins

Maximum quantum yield (F_v/F_m) was used to determine if *K. brevis* or associated brevetoxins had negative impacts on photochemical efficiency of *in hospite Symbiodinium*. In larvae there were significant differences in fluorescent yield as a function of treatment ($F_{(3,32)}$ = 27.531, $p < 0.001$) yet not time ($F_{(1,32)}$ = 4.110, p = 0.51) and interaction (treatment*time) ($F_{(3,32)}$ = 1.108, p = 0.360) (Fig. 2.5 A). Larvae exposed to *K. brevis* or brevetoxins had a reduced maximum quantum yield compared to controls (Tukey's post-hoc test). The photochemical efficiency of *Porites astreoides* adults was significantly impacted by treatment (H = 7.887, p = 0.048) and time (H = 20.916, $p < 0.001$) but there was no interaction(treatment*time) (H = 0.676, p = 0.879) (Fig. 2.5 B). Further nonparametric analysis among groups following the Holm-Bonferroni correction demonstrated a reduction in photochemical efficiency between *K. brevis* and control group at 48 hours. Finally, for *Acropora cervicornis,* there was no significant effect of time ($F_{(1,32)}$ = 0.467, p = 0.499) or treatment ($F_{(3,32)}$ = 1.615, p = 0.205) and there was no significant interaction (treatment*time) ($F_{(3,32)}$ = 0.596, p = 0.622) (Fig. 2.5 C).

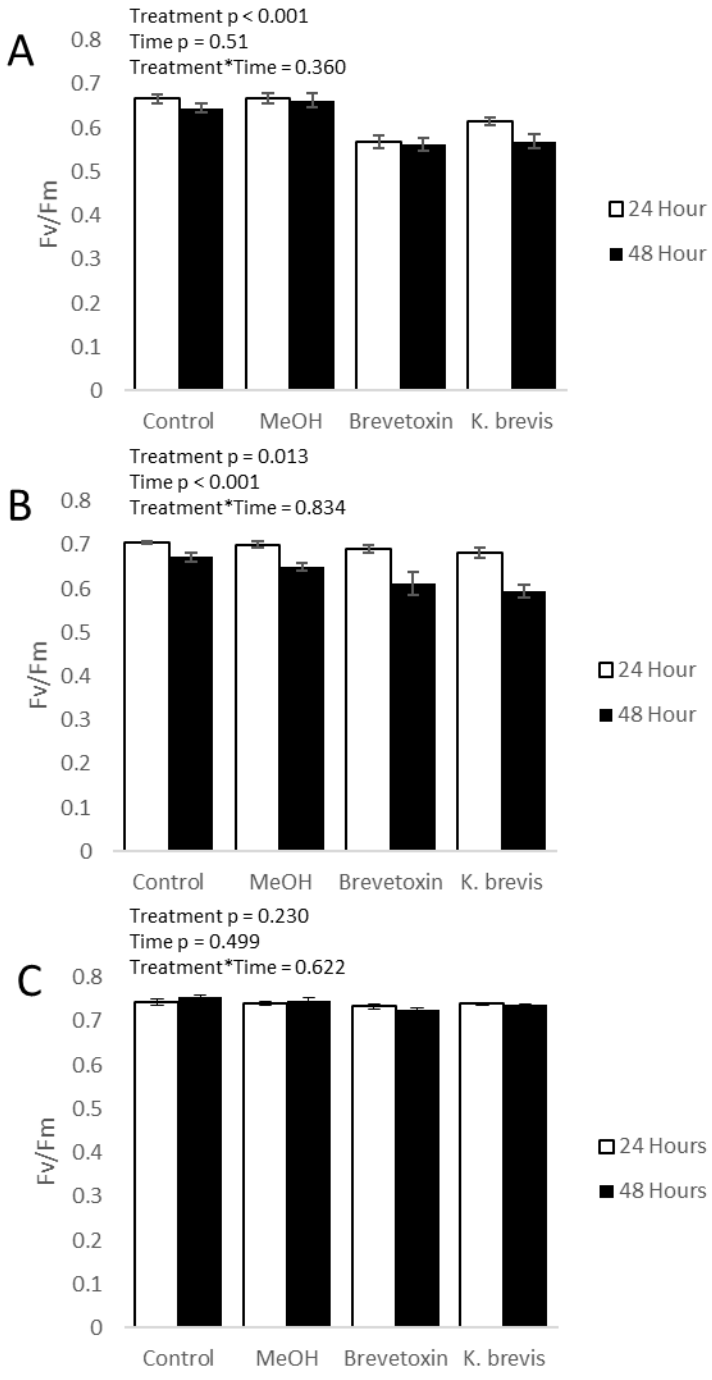

Figure 2.5 Maximum quantum yield of *in hospite Symbiodinium* within coral tissue following exposure to *K. brevis* or brevetoxins: (A) *P. astreoides* larvae, (B) *P. astreoides* adults, and (C) *A. cervicornis* (C). Bars represent ± 1 SE

2.3.4 Presence of Biomarkers of Cellular Oxidative Stress Following Exposure to *Karenia brevis* or Brevetoxins

Production of hydrogen peroxide following exposure to *K. brevis* or associated brevetoxins did not differ among treatments for either *P. astreoides* larvae, *P. astreoides* adults, or *A. cervicornis* ($F_{(3,32)} = 1.814$, $p = 0.164$; $H = 6.57$, $p = 0.087$; and $F_{(3,32)} = 0.906$; $p = 0.449$, respectively) as well as for adult *C. arbuscula* and *P. americana* ($H = 4.620$, $p = 0.202$; and $F_{(3,20)} = 0.357$, $p = 0.784$, respectively). Nor was there a significant interaction among treatment and time for *P. astreoides* larvae, *P. astreoides* adults, or *A. cervicornis* ($F_{(3,32)} = 0.747$, $p = 0.532$; $H = 1.975$, $p = 0.578$; and $F_{(3,32)} = 0.793$; $p = 0.507$) (Fig. 2.6). Correspondingly, following exposure to *K. brevis* or associated brevetoxins, peroxidase activity did not differ among treatments for *P. astreoides* larvae, *P. astreoides* adults, or *A. cervicornis* ($F_{(3,32)} = 0.515$, $p = 0.675$; $F_{(3,32)} = 1.180$, $p = 0.333$; and $F_{(3,32)} = 1.699$, $p = 0.187$, respectively), as well as for adult *C. arbuscula* and *P. americana* ($H = 3.240$, $p = 0.356$; and $H = 1.947$; $p = 0.584$, respectively). In addition, there was no significant interaction among treatment and time for *P. astreoides* larvae, *P. astreoides* adults, or *A. cervicornis* ($F_{(3,32)} = 0.747$, $p = 0.532$; $F_{(3,32)} = 0.473$, $p = 0.703$; and $F_{(3,32)} = 0.454$, $p = 0.716$, respectively) (Fig. 2.7). While hydrogen peroxide and peroxidase did not differ among treatments for *P. astreoides* larvae, there was a significant difference in lipid peroxidation levels ($F_{(3,32)} = 8.411$, $p < 0.001$, two-way ANOVA) with no interaction ($F_{(3,32)} = 1.012$, $p = 0.400$). This was also observed in adult *C. arbuscula* ($F_{(3,20)} = 3.613$, $p = 0.031$, one-way ANOVA). Further investigation of *P. astreoides* larvae following a one-way ANOVA and Tukey's post-hoc test with Holm-Bonferroni correction demonstrated that lipid peroxidation activity was higher in *K. brevis* treatment compared to controls following a 24-hour exposure. For adult *C. arbuscula*, a Tukey's

post-hoc test did not detect a significant difference among treatments. This was believed to be a result of small sample size and low power as a study limitation, so a Least Significant Difference (LSD) post-hoc test was used among treatments, which revealed higher lipid peroxidation activity in *K. brevis* compared to controls. There was no difference between lipid peroxidation among treatment for adult *P. astreoides* and *A. cervicornis* ($H = 4.93$, $p = 0.176$; and $H = 4.156$, $p = 0.245$, respectively), as well as for adult *P. americana* ($F_{(3,20)} = 2.689$, $p = 0.074$, one-way ANOVA). There was no interaction among treatment and time for adult *P. astreoides* ($H = 7.424$, $p = 0.060$). However, there was a significant interaction for *A. cervicornis* ($H = 13.736$, $p = 0.003$) (Fig. 2.8).

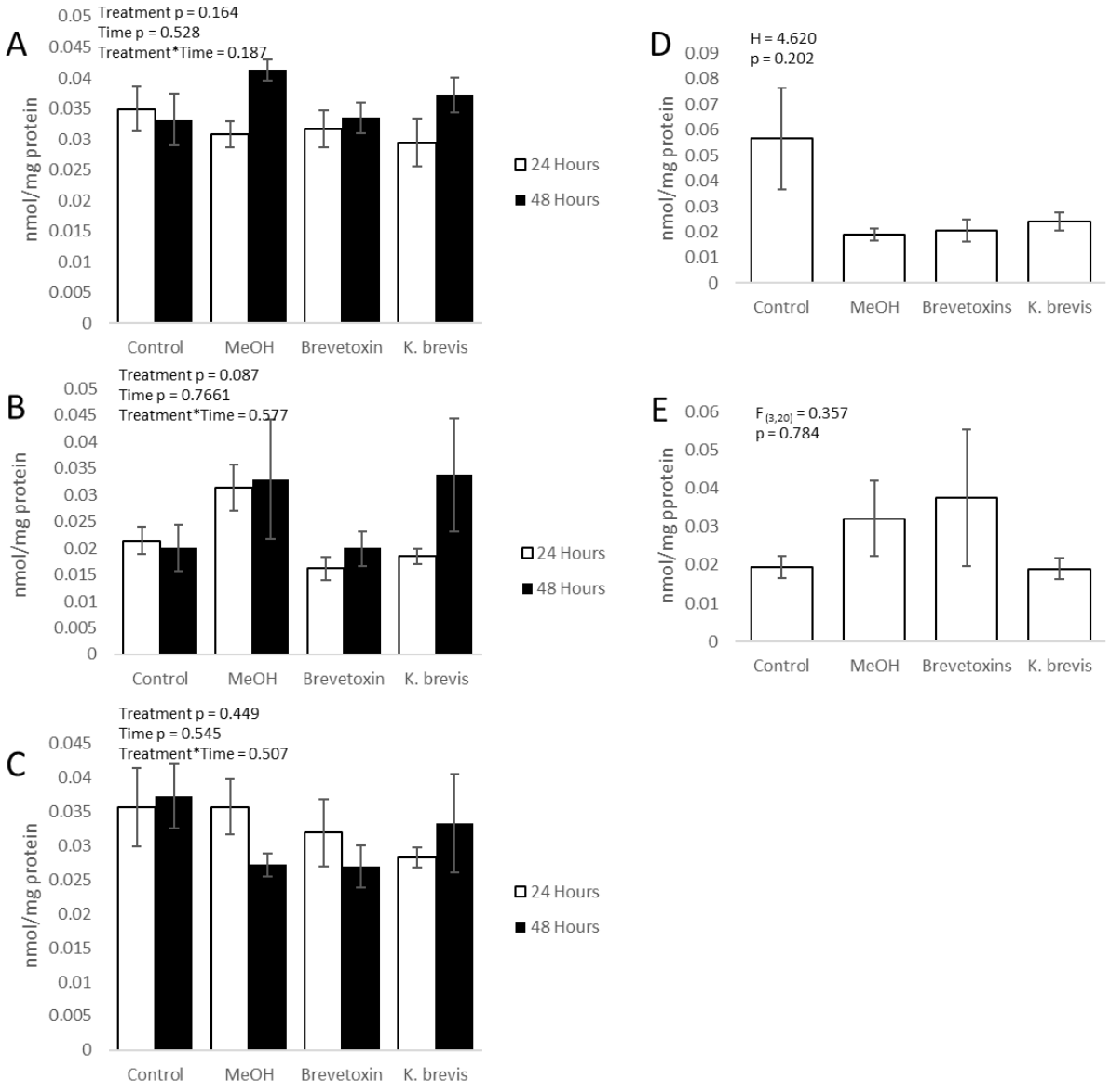

Figure 2.6 Hydrogen peroxide content in coral tissue following exposure to red tide: (A) *P. astreoides* larvae, (B) *P. astreoides* adult, and (C) *A. cervicornis* were exposed to either control, MeOH, brevetoxins or *K. brevis* for either 24 or 48 hours; (D) *C. arbuscula* and (E) *P. americana* were exposed to either control, MeOH, brevetoxins, or *K. brevis* for 48 hours. Bars indicate ± 1 SE

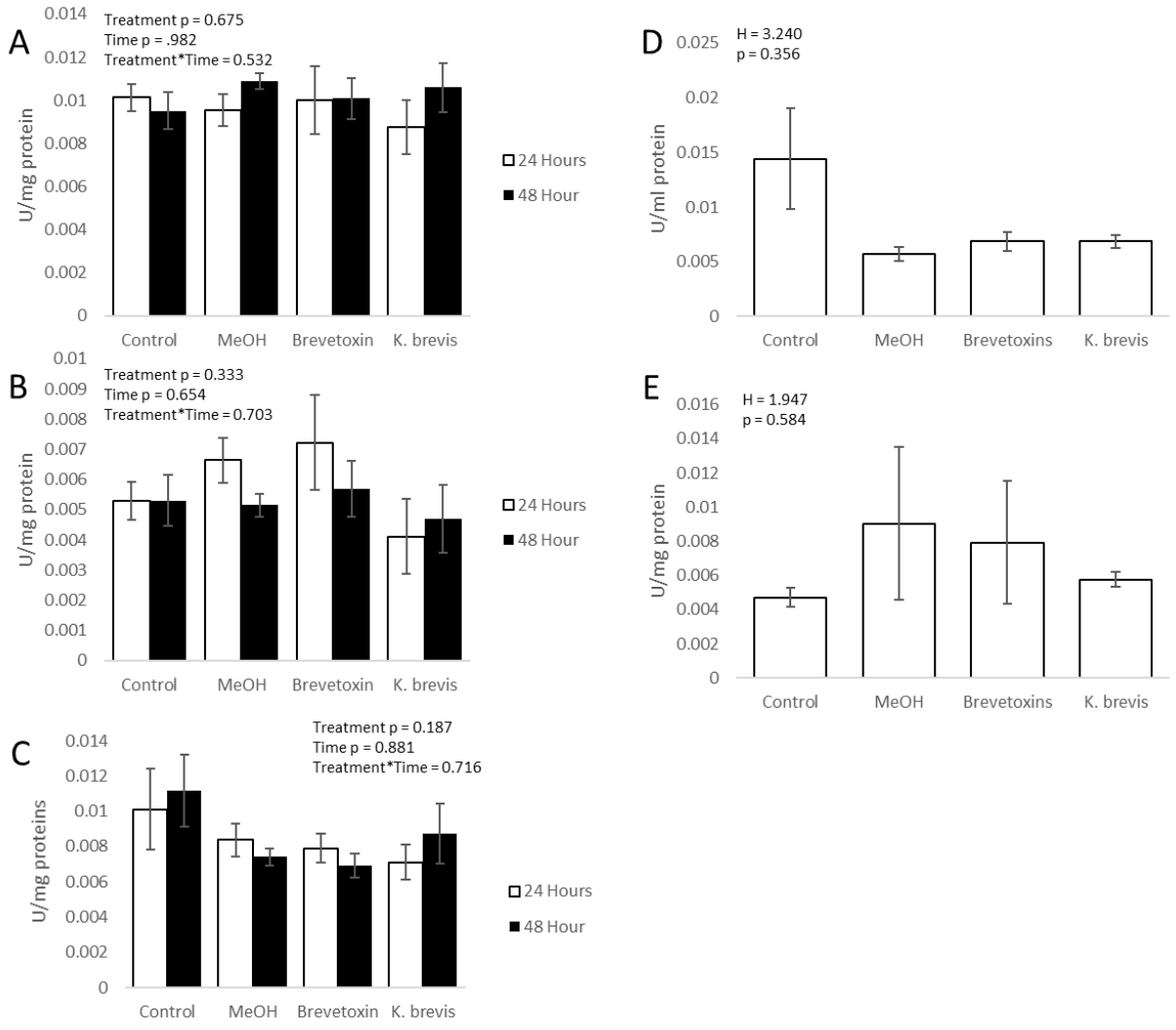

Figure 2.7 Peroxidase activity in coral tissue following exposure to red tide: (A) *P. astreoides* larvae, (B) *P. astreoides* adult, and (C) *A. cervicornis* were exposed to either control, MeOH, brevetoxins or *K. brevis* for either 24 or 48 hours; (D) *C. arbuscula* and (E) *P. americana* were exposed to either control, MeOH, brevetoxins, or *K. brevis* for 48 hours. Bars indicate ± 1 SE

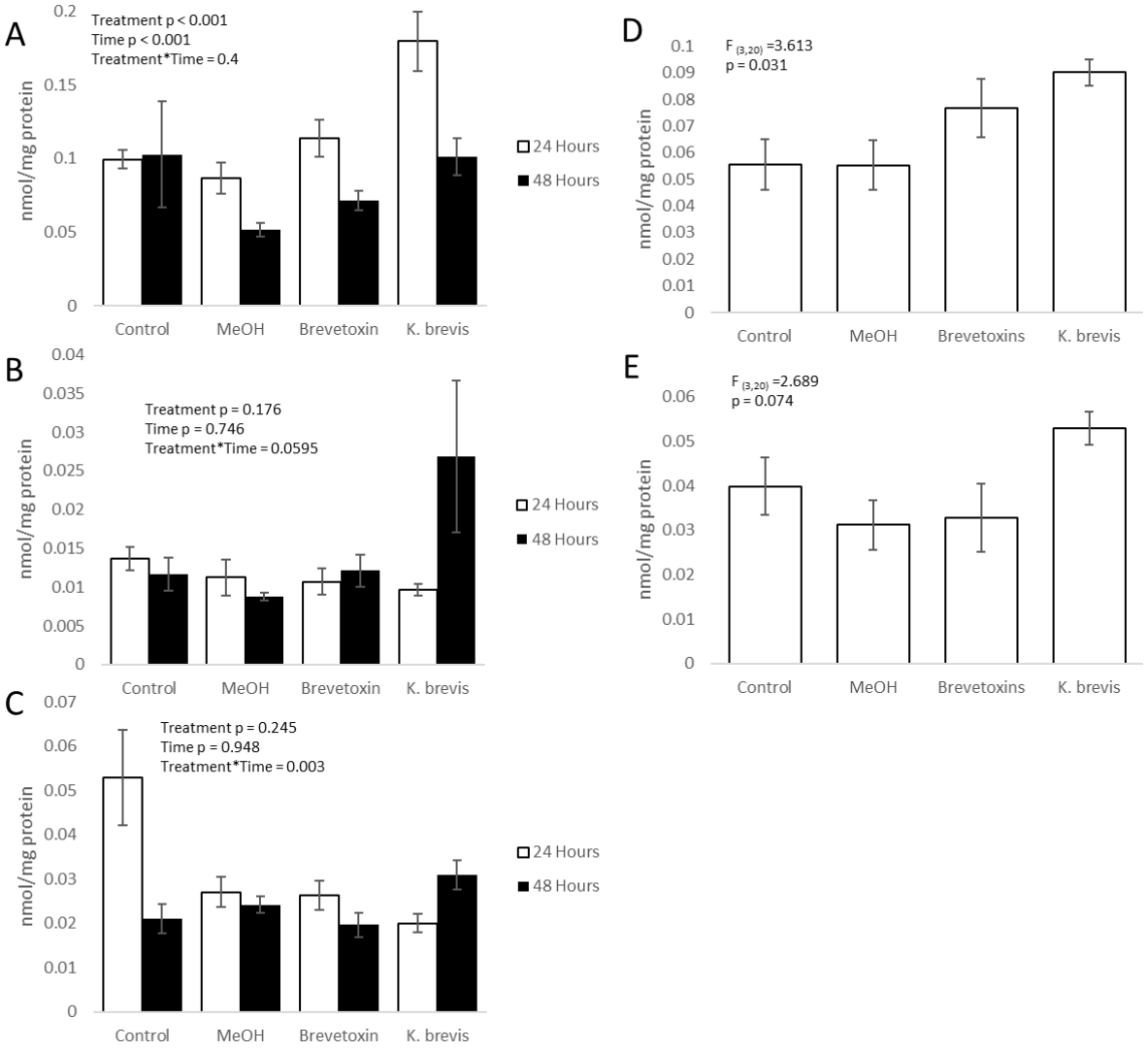

Figure 2.8 Lipid peroxidation in coral tissue following exposure to red tide: (A) *P. astreoides* larvae, (B) *P. astreoides* adult, and (C) *A. cervicornis* were exposed to either control, MeOH, brevetoxins or *K. brevis* for either 24 or 48 hours; (D) *C. arbuscula* and (E) *P. americana* were exposed to either control, MeOH, brevetoxins, or *K. brevis* for 48 hours. Bars indicate ± 1 SE

2.4 Discussion

2.4.1 *Karenia brevis* and Associated Brevetoxins Impact Larvae Behavior

In this study, *Porites astreoides* larvae actively avoided water contaminated with either *K. brevis* cells or brevetoxins. The effect was observed even at the lowest concentration assessed (5×10^5 cells L^{-1}), which is characterized as "medium bloom density" by the Florida Fish and Wildlife Conservation Commission (FWC) (http://myfwc.com/research/redtide/statewide/). The concentrations used in this study fall into the range of blooms that have been previously reported, most of which occurred within 15 km of the shoreline, in regions where corals are likely to inhabit (Brand & Compton, 2007). Larvae also significantly avoided seawater contaminated with brevetoxins (0.018 µg mL^{-1} (PbTx-2) and 0.0018 µg mL^{-1} (PbTx-3)), which is less than concentrations of brevetoxins that have been detected in medium bloom conditions (Pierce & Henry, 2008). Behavioral changes and avoidance of red tide blooms from planktonic organisms have been shown in other studies as well. Warlen et al. (1998), suggested larval avoidance of red tide explained recruitment patterns observed in estuarine fish in North Carolina, where there was a reduction in recruited larvae during blooms of *K. brevis*, followed by a surge of recruits later in the season. Cohen et al. (2007), demonstrated that exposure to low and moderate bloom densities of *K. brevis* negatively impacted swimming and phototactic behavior in copepods; while Lester et al. (2008) observed copepods aggregating in areas of lower *K. brevis* concentrations during dense blooms. Fiedler (1982), had demonstrated that zooplankton will display vertical migration, either above or below a bloom, in order to avoid the dinoflagellate *Gymnodinium splendens*, another red tide producing dinoflagellate. Even juvenile dolphins have been observed avoiding red tide blooms; however, it was unclear if it was a

direct response to the presence of the bloom or an indirect response due to a reduction in food availability (McHugh et al., 2011). *Symbiodinium* of *P. astreoides*, like most brooding species, are vertically transferred from parent to offspring and are capable of photosynthesizing during the larval stage (Chornesky & Peters, 1987; Richmond & Hunter, 1990; Baird et al., 2009). Because of this, larvae are capable of postponing settlement in unfavorable conditions; however, the duration of the larval stage is still limited. Exerting energy to avoid unfavorable water can cause energy reserves to deplete more rapidly. This can have many negative consequences such as hastened settlement to unfavorable substrate or increased larval mortality. In either case, energy exerted in avoiding the presence of red tide can result in a reduction of coral recruitment.

2.4.2 *Karenia brevis* and Associated Brevetoxins Impact Larval Settlement and Survival

This study demonstrated that red tide blooms can have a direct impact on settlement and survival of coral larvae. Along with dispersal, successful recruitment will depend upon the larvae's ability to locate a suitable substrate and complete metamorphosis. Here it was shown that exposure to high bloom concentrations of *K. brevis* cells impacted coral larvae percent settlement and survival, while not impacting the proportional number of swimmers. If larvae exhibited postponement of settlement to this stressor, it would be expected that there would be higher number of swimmers in the brevetoxin and *K. brevis* treatment. Alternatively, if larvae used settlement and metamorphosis as a way to escape being suspended in contaminated water, it would be expected that there would be fewer swimmers in the presence of the stressor. Since percent swimmers was relatively constant through each

treatment, it suggests that the presence of *K. brevis* did not force settlement postponement or larval escape, and induction of settlement was driven by other factors that were not considered in this study. Although there were more swimmers in the ambient seawater than in the *K. brevis* treatment at 72 hours, based on the number of settlers and survivors this difference is most likely due to larval mortality in the presence of the stressor. For both brevetoxins and *K. brevis*, there was a reduction in percent settlers and survivors for 48 and 72 hours, compared to ambient seawater. Settlement remained constant between the 48 and 72-hour exposures for ambient seawater and *K. brevis* treatments, suggesting settlement occurred early within the study. However, there was a clear decline in survivorship over time for brevetoxin and *K. brevis* while ambient seawater and methanol remained relatively constant. These trends became even more apparent when comparing ambient seawater to *K. brevis* alone. Since there was a similar decline in swimmers for each treatment, it can be inferred that the rate of attempted settlement was similar regardless of presence of a stressor. However, the fact that there was a reduction in both settlement and survivorship in the presence of a stressor suggests that *K. brevis* causes larval mortality shortly before or after settlement.

Settlement and metamorphosis can be energetically costly events and during this transition may leave larvae vulnerable to impacts of surrounding stressors (Wendt, 2000). Before settlement, the larvae may be able to sustain metabolic activities that allow them to tolerate cellular stress. When metabolic activities switch to promote settlement, the reallocation of energy and nutrients may result in a particularly susceptible period for the larvae. Regardless of the underlying cause, this study demonstrated that exposure to *K. brevis* can have negative impacts on larval survival. This is in contrast to Ross et al. (2010) who found

that a 20-hour exposure to low (6×10^5 cells L^{-1}) and high (4×10^6 cells L^{-1}) concentrations of *K. brevis* or cell lysate prior to being introduced to a settlement chamber did not impact *P. astreoides* larvae settlement or survival. There are a couple possibilities for this discrepancy. First, it might simply be that *K. brevis* or brevetoxins impact coral larvae following longer durations of exposure. In this study, the 24-hour exposure had very little difference in percent survivors and percent settlement for all four treatments, while at 48 hours there was a more discernible difference. At acute exposures, larvae may be resistant to the negative effects of *K. brevis*, while prolonged exposure results in a negative impact on recruitment success. This possibility warrants further investigation since red tide blooms can last up to 18 months (Tester & Steidinger, 1997). Also, in this study larvae settled in the presence of the stressor, which might impact the ability for the larvae to detect chemical cues that mediate larval settlement. In addition, another possible explanation to the differences between these studies is that *K. brevis* cell density does not necessarily correlate with production of brevetoxins (Pierce & Henry, 2008). Instead, prevalence of brevetoxins is dictated by other factors such as salinity or nutrient availability (Errera et al., 2010; Ransom Hardison et al., 2012. Finally, different strains of *K. brevis* were used between the two studies, which can result in variation of brevetoxin analog production. Regardless, this study demonstrates that ecologically relevant concentrations and acute durations of red tide exposure can have negative impacts on recruitment success. This coincides with results found in other studies. In 1987, a red tide outbreak in North Carolina was followed by a virtual failure of recruitment of the scallop species *Argopecten irradians concentricus* (Summerson & Peterson, 1990). Also, it has been found that recruitment of the mussel species *Perna viridis* is reduced during years of increased

K. brevis blooms (McFarland et al., 2016). The influences on behavior, settlement and survivorship of coral larvae in this study, along with impacts on recruitment of other species, warrants concern about coral recruitment when assessing environmental impacts following *K. brevis* blooms. With long term coral decline throughout the Caribbean, reproductive events that overlap with red tide blooms can hinder the recovery of these reefs (Gardener et al., 2003).

2.4.3 *Karenia brevis* and Associated Brevetoxins Cause Reduction of Photochemical Efficiency of *Symbiodinium* and Result in Variable Effects of Oxidative Stress in Coral

In this study, photochemical efficiency was reduced following acute exposure to red tide conditions. This is similar to Ross et al. (2010), who found a general trend of decreasing zooxanthellae density in larvae exposed to increasing concentrations *K. brevis* cells or cell lysate, although no statistical significance was detected. In this study, since there was a reduction in photochemical efficiency in *P. astreoides* adult fragments but not *A. cervicornis* suggests that some species of coral may be more sensitive to the effects of acute exposure of *K. brevis* than others. Such variation may be the result of adaptation within the host or the symbionts they possess (Abrego et al., 2008). It has been proposed that many stressors that impact coral bleaching do so via induction of oxidative stress, which can signal cellular pathways that can lead to the loss of the symbiont or cell death, such as exocytosis or apoptosis (Gates et al., 1992; Lesser, 1996; Lesser, 1997). Zooxanthellae are thought to be an important contributor to the release of reactive oxygen species (ROS) that result in dissociation of the host-symbiont relationship, with ROSs triggering an immune response in host tissue causing expulsion or degradation of the algae (Weis, 2008; Yakovleva et al., 2009). Impacts on

zooxanthellae in planula larvae can result in adverse effects on coral recruitment by causing a reduction in the photosynthesis:respiration ratio, leading to lower energy production, and causing a reduction in larvae survival or restricted planktonic dispersal (Coles & Brown, 2003; Coles & Jokiel, 1977).

The presence of excess ROSs, such as superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$), can damage lipids, proteins, DNA and may lead to cell death (Downs et al., 2002). Typically, cells generate defense mechanisms, such as antioxidants, to combat the production of ROSs. However, overproduction of ROSs can overwhelm these defense mechanisms and cellular damage can ensue (Lesser & Farrell, 2004; Richier et al., 2005). The presence of oxidative stress can be measured by changes in biomarkers, such as byproducts of oxidative damage and increased antioxidant enzymatic activity (reviewed in Tisthammer, 2017). In this study, commercially available assays of oxidative stress biomarkers showed variable indication of oxidative stress in coral tissue following exposure to brevetoxins and *K. brevis*. There was no detection of increased concentrations of hydrogen peroxide or peroxidase activity. However, there was an increase in thiobarbituric acid reactive substances (TBARS) at 24 hours for larvae and at 48 hours for *C. arbuscula* adults, following exposure to *K. brevis*. Thiobarbituric acid reacts with malondialdehyde, an end product to lipid peroxidation, indicating some oxidative damage was occurring within the cells. It is important, however, to note that while a one-way ANOVA detected a significance among treatments, Tukey's post-hoc test did not detect these differences among groups of *C. arbuscula* adults, which was believed to be an artifact of low sample size and statistical power as limitations to this study. Because of this a Least Significant Difference (LSD) post-hoc test was used. Further research using a larger

sample size is needed to validate these findings. Regardless, these results are similar to those reported by Ross et al. (2010), who also detected a mixed indication of oxidative stress in coral larvae following exposure to cells or cell lysate of *K. brevis*. They found an increase in catalase activity and lipid hydroperoxide content yet no change in superoxide dismutase content or levels of protein carbonyls. A few possibilities can be drawn from these varying results in this study: 1) only certain pathways of oxidative stress are induced by *K. brevis* and brevetoxins since evidence of lipid peroxidation was detected, while H_2O_2 and peroxidase was not; 2) the larvae stage may be more susceptible to their effects since lipid peroxidation was detected in *P. astreoides* larvae and not in adults; 3) that coral can recover from the oxidative damage produced by *K. brevis* since there was no difference between treatment and controls of larvae at 48 hours; and 4) that the effects may be species specific since only the species *C. arbuscula* showed signs of lipid peroxidation for adult fragments. Based on the design of this study it is not possible to discern which organism, either coral or symbiont, is contributing to the production of lipid peroxidation. Lipid peroxidation can occur from the production of ROSs in the chloroplasts of both plants and algae. In a study analyzing the thylakoid membrane of thermal tolerant and intolerant species of *Symbiodinium*, Tchernov et al. (2004) shown that the thermal threshold is determined by the amount of saturated lipids within different species membranes and that thylakoid integrity is a determinant factor to coral bleaching. This demonstrates that stressors that induce lipid peroxidation can result in symbiont expulsion and that resistance to this oxidative stress may be clade specific to the symbiont. Shimazaki et al. (1980) found that lipid peroxidation in spinach leaves induced by SO_2 resulted from the presence of singlet oxygen ($^1O_2$), a reactive oxygen species not measured in this study. $^1O_2$ is a

byproduct of photosynthesis, converted from triplet oxygen ($^3O_2$) by chlorophyll in the presence of light (Laloi & Havaux, 2015). Brevetoxin toxicity has traditionally been attributed to its affinity to VGSCs; however, recent discoveries have illuminated the notion that their toxicity may have broader effects (Radwan & Ramsdell, 2008; Perrault et al., 2014; Walsh et al., 2010). It may be that brevetoxins are inducing lipid peroxidation through unknown pathways, by either directly or indirectly causing increased production of ROSs such as $^1O_2$ or inhibiting antioxidants and quenching substances. Regardless, this study contributes to the growing body of knowledge that exposure to *K. brevis* and brevetoxins can prompt oxidative cellular stress (Perrault et al., 2014; Ross et al., 2010; Walsh et al., 2010; Walsh, 2014).

2.4.4 Conclusion

The data herein adds to results published by Ross et al. (2010), that red tide blooms of the dinoflagellate algae *Karenia brevis* and their associated brevetoxins can act as a regional stressor to coral species. Based on the results from oxidative stress and photochemical efficiency, it appears that the larval life stage may be more susceptible to the effects of *K. brevis* then the sessile adult stage. Also, red tide susceptibility may be species specific since *Cladocora arbuscula* was the only adult species in this study that showed signs of oxidative stress. Larval settlement and survival were found to be impacted by red tide, while larvae actively avoided red tide contaminated seawater. This can have negative implications to coral recruitment and have impacts on coral reef resilience and recovery. It is important to keep in mind that this study demonstrates conditions under acute exposure to red tide, whereas blooms have been recorded to last year-round. Long term exposure may demonstrate more notable effects which

merits future investigation of prolonged exposure to coral. Based on responses in larval behavior, settlement and survival along with impacts on oxidative stress and photochemical efficiency, red tide can act as a potential local stressor to coral species throughout the Gulf and Atlantic coastlines. These implications should be taken into consideration when analyzing environmental impacts following *K. brevis* blooms.

3.

Changes in the proteome of the non-model coral species, *Porites astreoides*, following acute exposure to the red tide producing dinoflagellate, *Karenia brevis,* and its associated brevetoxins

Abstract

A regional stressor that impacts many marine species in the Gulf of Mexico is the seasonal red tide events produced by dense blooms of the dinoflagellate *Karenia brevis*. The negative impacts of these algae result from the suite of lipid soluble neurotoxic polyethers they produce, known as brevetoxins. Unfortunately, effects of *K. brevis* and their associated brevetoxins on scleractinian coral species are poorly understood. Proteomic analysis provides a powerful technique in determining cellular responses. However, difficulties arise when applying these methodologies to coral species since most are non-model organisms with unmapped genomes. To circumvent this, part of the goal of this study was to test the feasibility of constructing a protein reference database from previously published transcriptomes of *Porites* coral species for protein identification in broad-scale proteomic analysis. To examine the cellular response of coral to red tide, adult fragments of the coral species *Porites astreoides* were exposed to ecologically relevant concentration of *K. brevis* (2.5×10^6 cells L^{-1}) or brevetoxin analogs (0.018 µg mL^{-1} (PbTx-2) and 0.0018 µg mL^{-1} (PbTx-3) and analyzed using iTRAQ labelling procedures. The custom constructed database allowed over 1,300 proteins to be identified. The majority of BLAST hits of proteins identified were associated with anemone and coral species, ensuring database integrity. Out of the proteome identified, 1,290 proteins

were quantified, revealing that 52 proteins in the *K. brevis* treatment and 15 proteins in the brevetoxin treatment were differentially expressed, containing statistically significant differential abundances compared to controls. Interestingly, only six proteins were shared between the two treatments. The results of differential expression, particularly of proteins related to oxidative stress, energy metabolism, and DNA binding are discussed.

3.1 Introduction

Over the past few decades, coral mortality events have been increasing on a world-wide basis. This has been attributed to impacts of a number of both global and regional stressors (e.g. global climate change, ocean acidification, diseases, overfishing, eutrophication, hurricanes, overpopulation, etc.) (Couch et al., 2017; Glynn, 1993; Hughes et al., 2017; Rodgers et al., 2015). In addition, it has been reported that cross-scale stressors (e.g. elevated sea surface temperature with increased salinity, irradiance, overfishing) have the capacity to work in combination, and may even yield synergistic effects when impacting coral (Ateweberhan et al., 2013; Coles & Jokiel, 1978; Darling et al., 2010; Porter et al., 1999). Although the effects of global stressors on coral species have been well documented, there has been a shift in emphasis to better understand regional stressors as these may be more directly mitigated through management practices and subsequently lessen the effects of global stressors (Hoegh-Guldberg et al., 2007). One of the regional stressors that affects many organisms in the Gulf of Mexico is the seasonal red tide forming dinoflagellate, *Karenia brevis*. The deleterious effects of these algae predominantly are a result from the neurotoxic polyethers they produce, known as brevetoxins. These lipid soluble toxins have a strong affinity to neurotoxin binding site five of

voltage gated sodium channels (VGSC) and stabilize the channel in the open formation, resulting in an excitatory response of the cell (Stevens et al., 2011). Unfortunately, very little is known of the effects of *K. brevis* and their associated brevetoxins on coral species. Ross et al., (2010) had shown that exposure of larvae of the coral species *Porites astreoides* to *K. brevis* cells or cell lysate resulted in an increase in certain biomarkers of oxidative stress, accompanied with inhibition of larval respiration. To date, this is the extent of our understanding of the effects red tide can elicit on coral species.

With the technological advancements in high-throughput sequencing and analysis of large scale biological data, there has been an increase in the use of -omics technologies to assess the cellular effects of environmental stressors on coral. The annotation and quantitation of transcriptomes through next-generation sequencing (NGS) technology has been a common strategy for studying gene expression in selected coral species (Bayer et al., 2012; Meyer et al., 2009; Palumbi et al., 2014; Pinzón et al., 2015; Rosic et al., 2014; Shinzato et al., 2014). This is because NGS enables a *de novo* assembly of transcriptome on non-model organisms without the need for a reference genome, and also can be used to examine the diversity of alternative spliced transcripts (Birol et al., 2009; Martin & Wang, 2011; Toonen et al., 2013; Traylor-Knowles et al, 2011; Vidal-Dupiol et al., 2013). This technique has been used to make many advancements in the understanding of numerous cellular processes in corals, such as molecular components of coral-symbiont interactions, gene activation in coral bleaching and biomarkers of certain stressors (Pinzón et al., 2015; Rosic et al., 2014; Shinzato et al., 2014). While transcriptomics has proven to be a useful tool in the study of coral gene expression, there are limitations. Relative abundance of transcripts can only be regarded as an estimate of cellular

response since post-transcriptional regulation and post-translational modifications can influence the final proteins expressed (Gygi et al., 1999). Because of this, proteomics allows for a more realistic “picture” of cellular responses following a stress event.

Although many studies have used transcriptomic techniques in corals and anemones, few studies have utilized the proteome to identify cellular responses to stress. Certainly, one aspect for the lack of proteomic analysis in coral research is the difficulty that arises from most species being non-model organisms. Proteomics requires a reference database of theoretic protein spectra in order to establish protein identification, while the robustness of proteins recovered is reliant on the inclusiveness of genes represented in the reference database. Transcriptome incorporation in proteomic analysis has shown to be an efficient approach to protein identification. One approach in incorporating transcriptome data in protein identification is to utilize RNA-sequencing techniques to generate a sample-specific protein database, which is known as Proteomics Informed by Transcriptomics (PIT) (Evans et al., 2012). This approach can be more efficient then using genome data alone in three main ways in that it can: 1) incorporate and identify nonsynonymous single nucleotide polymorphisms, which would otherwise alter peptide properties of mass and or charge; 2) incorporate mRNA isoforms that are absent from the reference genome; and 3) estimate transcript abundance to identify which transcripts are expressed, allowing the search databases to be trimmed, reducing the likelihood of type 1 error from multiple peptide comparisons (Sheynkman et al, 2014; Wang et al., 2011; Wen et al., 2014; Wen et al., 2016). The use of an RNA-sequence derived database in proteomics has also shown to be effective in protein identification in the absence of a genome reference for annotation. In a study on the non-model organism the black fruit bat, *Pteropus*

alecto; the PIT approach has shown to be effective in identifying possible cellular function in understanding the bats resistance to Hendra virus (HeV) (Wynne et al., 2014). In a proteomic study of frog eggs of the species *Xenopus laevis*, a genome-free construction of a protein reference database using both RNA-sequence data and publicly available transcript data allowed for a deeper recovery of the proteome (~11,000 proteins) than compared to proteins identified using a strictly genome constructed database (~9,000 proteins) (Wühr et al., 2014). With the popular use of transcriptomic analysis in coral research throughout the literature, there is a large amount of assembled transcriptome data publicly available. Constructing a protein search database through published RNA-seq data can provide a unique opportunity for protein identification in coral proteomics analysis.

Part of the aim of this study was to determine the feasibility of using accessible transcriptome resources to construct a database for proteomic quantification in *P. astreoides* following red tide exposure. To maximize the number of genes represented, three transcriptomes from two *Porites* species were used to create a custom protein search database, which included transcripts of host and symbiont (*P. australiensis*), three developmental stages (*P. astreoides*), and a thermal stress response (*P. astreoides*) (Kenkel et al., 2013; Mansour et al., 2016; Shinzato et al., 2014). Although gene expression that is unique to this stressor may not be represented in the database, it can be expected that many metabolic pathways have transferable responses to different stressors and that these proteins would therefore be detected (Petrak et al., 2008; Wang et al., 2009). One benefit to proteomic techniques in coral research is that the phenotypic responses of the coral and the symbiotic microorganisms they host, together known as the holobiont, can be analyzed as a whole. This is important because

coral phenotypic responses to environmental conditions can either be derived from the host, symbionts, or both (Abrego et al., 2008; Bay et al., 2009; Császár et al., 2009; Leggat et al., 2011; Rosic et al., 2014). Such observations have led to proposals such as the coral probiotic hypothesis and hologenomic speciation theory, which suggests that coral can change their microbiota in order to adapt to environmental conditions and adaptations provided by either the microbiota or the coral itself can be acted on by selective forces (Reshef et al., 2006; Rosenberg et al., 2007).

Since very little is known about the impacts of *K. brevis* and brevetoxins on corals at the cellular level, it is important to be able to identify what biochemical and physiological responses ensue following exposure to red tide blooms. Therefore, the specific goal of this study was to assess changes in the proteome of the coral species *P. astreoides* following acute exposure of ecologically relevant concentrations of *K. brevis* or their associated brevetoxins in order to determine which cellular functions are affected. The results herein provide direction in monitoring coral impacts following bloom exposure, as well as possible biomarkers that can be targeted in order to assess cellular damage.

3.2 Methods and Materials

3.2.1 *Karenia brevis* Culture Maintenance and Brevetoxins

Cultures of *K. brevis*, str. CCFWC257, were acquired from Florida Fish and Wildlife Research Institute and grown to experimental concentrations in a laboratory setting at Mote Tropical Research Laboratory. Cultures were maintained at room temperature under artificial lighting (intensity between 100 - 125 µmol $m^{-2}s^{-1}$) on a 12:12 cycle in artificial seawater at a

salinity ≈ 35 ppt. Brevetoxin analogs (PbTx-2 and PbTx-3) were purchased from MARBIONC Development Group LLC. Cultures of *K. brevis* were aliquoted to a final concentration of 2.5 × 10^6 cells L^{-1} for experimental treatments. Brevetoxin solutions were made by suspending brevetoxin analogs in methanol and aliquoted to a final concentration of 0.018 µg mL^{-1} (PbTx-2) and 0.0018 µg mL^{-1} (PbTx-3).

3.2.2 Coral Collection and Exposure to Red Tide

Colonies of *Porites astreoides* were collected of the Atlantic coast of Summerland Key, FL. Samples were transported to Mote Tropical Research Laboratory in coolers, where they were maintained in flow tables with running seawater. Colonies of *P. astreoides* were cut into 3 cm^3 fragments with a circular saw. Single fragments were placed in 400 mL tri-pour beakers at a final volume of 300 mL for each treatment, ensuring the same colony was not represented more than once in a treatment. Fragments were exposed to either ambient seawater (control), ambient seawater containing 0.04% methanol (MeOH), purified brevetoxins suspended in methanol (0.018 µg mL^{-1} PbTx-2 and 0.0018 µg mL^{-1} PbTx-3), or *K. brevis* (2.5 × 106 cells L^{-1}) for 48 hours in tetraplicate. Tri-pour beakers were placed into 7 L plastic aquaria in seawater to buffer possible changes in temperature, which was monitored using a YSI model 85 multiprobe meter (YSI, Inc, Yellow Spring, OH, USA) for the duration of the experiment. Following exposure, fragments were flash frozen in liquid N_2 and transported to UNF and stored at -80C°. Tissue from coral fragments were removed from the calcium carbonate skeleton over ice with a Paansche airbrush (Paansche, Inc., Chicago-, IL, USA) with extraction buffer (50 mM phosphate buffer, pH 7.8 with 0.05 mM dithiothreitol).

3.2.3 Protein Extraction

Total proteins were isolated from ≈2 g of coral tissue and were purified as described in Koh et al. (2012) with the following modifications. Tissue was ground in liquid N_2 with a precooled mortar and pestle in 6 mL of extraction buffer (1.2% β-mercaptoethanol, 0.1M Tris-HCl pH 8.8, 10 mM EDTA, 0.9 M sucrose) and 6 mL Tris saturated phenol pH 8.8, followed by overnight incubation at room temperature with shaking. Samples were centrifuged at room temperature for 40 min at 5,000 g. Protein pellets were dissolved in 4M Urea and 0.1% SDS in 10 mM Tris-HCl, pH 8.0. Protein concentration was measured using EZQ Protein Quantification Kit (Thermo Fisher Scientific, Inc., San Jose, CA, USA) with the SoftMax Pro Software v5.3 (Molecular Devices, Inc., San Jose, CA, USA).

3.2.4 iTRAQ Labelling

For each sample, 100 µg of proteins were reduced, alkylated to block cysteine, trypsin digested and labelled using the iTRAQ® Reagents -8plex kit as per the manufacturer's instructions (Sciex, Inc., Foster City, CA, USA)). Two independent samples of ambient seawater controls were labeled with iTRAQ tags 113 and 117, two independent samples of methanol control were labeled with iTRAQ tags 114 and 118, two independent brevetoxin treatments were labeled with iTRAQ tags 115 and 119, and two independent *K. brevis* treatments were labelled with iTRAQ tags 116 and 121. The combined peptide mixtures were lyophilized and a Solid Phase Extraction was performed in order to remove impurities by use of a SOLA SPE cartridge (Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. The resulting

purified peptide mixtures were dissolved in strong cation exchange (SCX) solvent A (25% (v/v) acetonitrile, 10 mM ammonium formate, and 0.1% (v/v) formic acid, pH 2.8). The peptides were eluted with a linear gradient of 0-20% solvent B (25% (v/v) acetonitrile and 500 mM ammonium formate, at pH 6.8) over 50 min followed by a ramp up to 100% solvent B over the course of 5 min and held for an additional 10 min using an Agilent HPLC system 1260 outfitted with a polysulfoethyl A column (2.1 mm × 100 mm, 5 µm, 300 Å, PolyLC, Columbia, MD, USA). The absorbance at 280 nm and 214 nm were monitored, and a total of 14 fractions were collected.

The fractions were lyophilized and resuspended in LC solvent A (0.1% formic acid). Samples were run on a hybrid quadrupole-orbitrap™ mass spectrometry (MS) system (Q Exactive Plus, Thermo Fisher Scientific, Bremen, Germany) using high energy collision dissociation (HCD) in each MS and MS/MS cycle as previously described (Koh et al., 2015).

3.2.5 Identification Database Construction, Protein Identification and Differential Expression Analysis

For protein identification, a nonredundant database was constructed from 3 annotated transcriptomes of *Porites* species previously published (Kenkel et al., 2013; Mansour et al., 2016; Shinzato et al., 2014). Peptide MS/MS data was thoroughly searched against the database with consideration to biological modification and amino acid substitution under the Paragon ™ algorithm using ProteinPilot version 4.5 software (AB Sciex, Inc.) (Shilov et al., 2007). Search parameters included iTRAQ 8plex quantification, cystine modification with methyl methanethiosulfonate, and trypsin digestion. Confidence levels of protein identification were set to 95% to establish a list of proteins with a 5% false discovery rate (FDR) (Tang et al., 2008).

Relative quantification of proteins detected by unique peptides was conducted using ratios from tandem mass spectra. For a protein to be considered significantly differentially expressed, it must have been quantified with at least three peptides in at least biological triplicate, with a Fisher's combined probability of < 0.05 (Fisher, 1948). Sequences of identified proteins were annotated using Blast2GO suite (Conesa et al., 2005) (http://www.blast2go.com/b2ghome).

3.2.6 Protein Network Analysis

Differentially expressed protein sequences were uploaded to the Search Tool for the Retrieval of Interacting Genes (STRING) to analyze protein network interaction (Szklarczyk et al., 2014)(https://www.string-db.org). Protein sequences were compared to the *Nematostella vectensis* network. Ten interactor proteins were selected for the second shell to detect interactions of the query proteins with proteins that may be absent from proteome recovery.

3.3 Results

3.3.1 Protein Database Construction and Protein Profiling

Three available transcriptomes of *Porites* species were translated into six-frame and constructed into one protein search database (*Porites* species concatenated nonredundant; Table 3.1). Search results based on the four concatenated databases showed that the constructed database provided the highest number of hits, which identified a total of 1,371 proteins with a 5% FDR. Out of the proteins identified, 1,290 proteins were quantified and further statistically analyzed. The Blast2GO search of the proteins identified resulted in 1,335 out of 1,371 proteins having BLAST hits, with 957 proteins annotated and 213 proteins mapped

(Fig. 3.1). The majority of sequences showed homology to sequences of anemone and coral species, as well as *Symbiodinium*, providing evidence of the database's integrity (Fig. 3.2).

Table 3.1. Database information for publicly available transcriptomes of *Porites* species and constructed database

Database	# of contig	Min. length	Median length	Max. length	# of hits	Reference
Porites australiensis host and symbiont	463,214	32	53	14,601	832	Shinzato et al. (2014)
Porites astreoides heat stress	80,604	32	51	2,398	847	Kenkel et al. (2013)
Porites astreoides life history stages	932,437	32	51	15,904	622	Mansour et al. (2016)
Porites concatenated nonredundant	837,531	32	54	1,692	1,371	This study

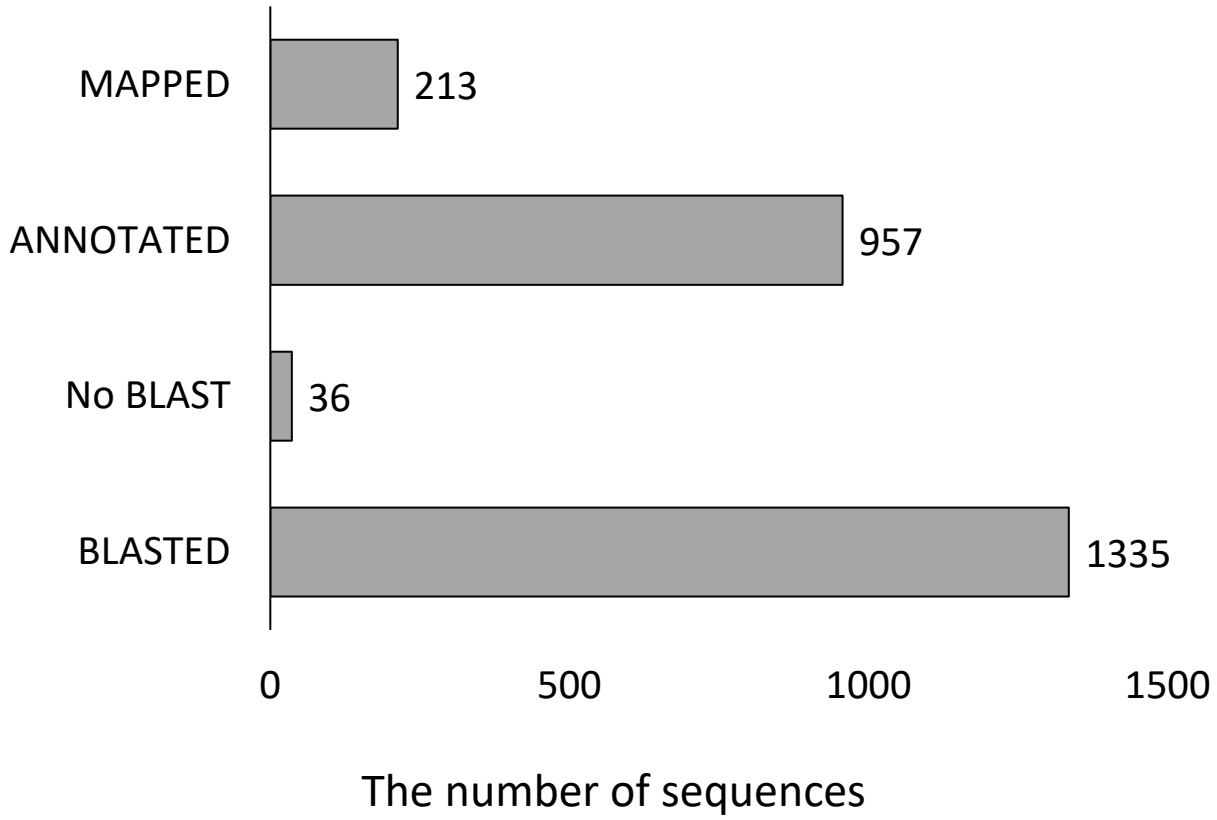

Figure 3.1 Distribution of Blast2Go search results of 1,371 proteins identified

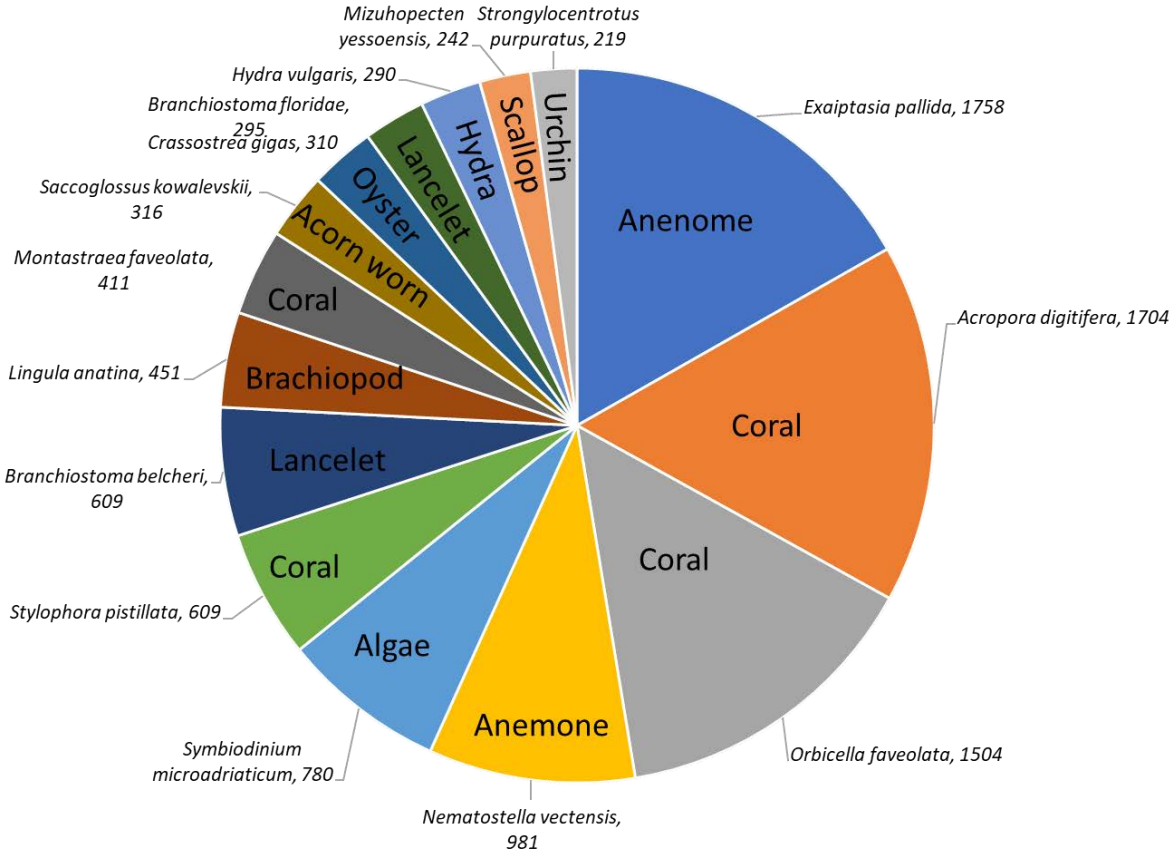

Figure 3.2 Distribution of species with number BLAST hits from proteins identified using the constructed database. The majority of hits showed homology to species of anemone, coral and *Symbiodinium*

3.3.2 Comparative Proteomics of Exposure to *Karenia brevis* and Brevetoxin Analogs

The iTRAQ labelling and the 2D LC-MS/MS methods allowed for the simultaneous protein quantitation of treatments (MeOH, brevetoxin, and *K. brevis*) compared to the control in order to determine the differential expression of proteins following exposure. Biological variation among replicates was determined by dividing the total number of variable proteins by 1,290 quantified proteins. Treatments of control, MeOH, brevetoxins and *K. brevis* had biological variation among replicates of 99 [7.7%], 24 [1.9%], 63 [4.9%], and 38 [2.9%], respectively. Interestingly, MeOH displayed 18 proteins that were differentially expressed, while brevetoxin and *K. brevis* treatments showed 15 and 52 proteins that were differentially expressed, respectively (Fig. 3.3). The *K. brevis* treatment had 15 downregulated and 37 upregulated proteins, while the brevetoxin treatments had 1 downregulated and 14 upregulated proteins, compared to the control. There were 6 differentially expressed proteins shared between the two treatments (Fig. 3.4). Out of the 61 differentially expressed proteins in both brevetoxins and *K. brevis,* 32 and 14 proteins were uniquely upregulated and downregulated in *K. brevis*, respectively (Fig. 3.4). In comparison, brevetoxins had 8 and 1 proteins that were uniquely, upregulated and downregulated, respectively (Fig. 3.4). Within the 6 differentially expressed proteins shared between the two treatments, 5 proteins were upregulated within both treatments and 1 protein was upregulated in brevetoxin and downregulated in *K. brevis* (Fig. 3.4).

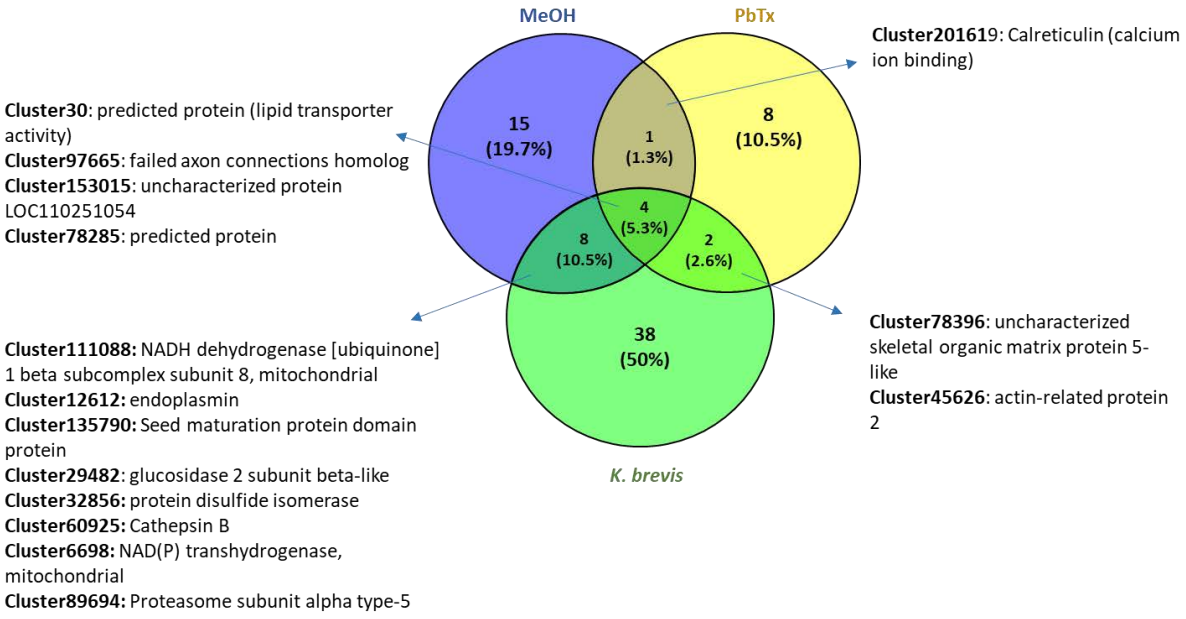

Figure 3.3 Venn diagram of differential expression between treatments. A total of 52, 15 and 28 proteins were recovered from K. brevis, brevetoxins or MeOH groups, respectively

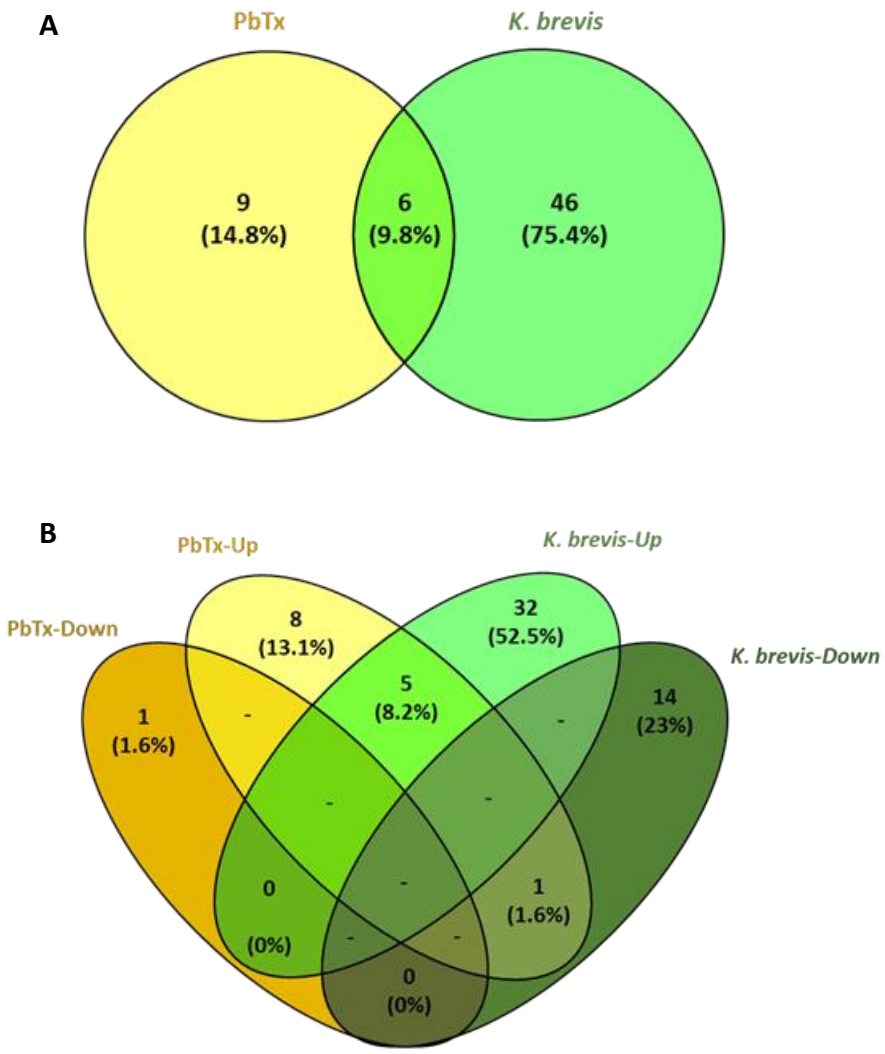

Figure 3.4 Venn diagram of number of proteins differentially expressed in coral tissue following exposure red tide: (A) total number of proteins differentially expressed in brevetoxin or *K. brevis* treatment; and (B) proteins anatomized into up/ down expression in brevetoxin or *K. brevis.* Six differentially expressed proteins were shared between brevetoxins and *K. brevis* treatments. Five of the six proteins were upregulated in both treatment while one protein was upregulated brevetoxin and downregulated in *K. brevis*

3.3.3 Functional Analysis of Differentially Expressed Proteins

Differentially expressed proteins categorized into functional groups demonstrated that cellular response varies depending on whether corals were exposed to brevetoxins or *K. brevis*. Coral tissue exposed to brevetoxins demonstrated upregulation in proteins associated with chromosomes, chromosome organization, DNA binding, non-membrane-bounded organelles, protein-containing complex assembly, pathogenesis, organelle organization, cellular component organization, intracellular non-membrane-bounded organelle, and protein-containing complex subunit organization (Table 3.2). In contrast, brevetoxin exposed corals demonstrated downregulation of proteins associated with catalytic activity, primary metabolic processes, and organic substance metabolic process (Table 3.2). Within *K. brevis* treatment, upregulated proteins were associated with cellular processes such as ubiquitin-like protein binding, DNA integration, vesicle-mediated transport, cytoplasmic vesicles, regulation of biological quality, membrane-bounded organelles, extracellular region, cofactor binding, flavin adenine dinucleotide binding, cell junction organization, vesicles, intracellular vesicles, homeostatic processes, and oxidoreductase activity, with downregulation mostly associated with biosynthetic processes (Table 3.2).

Table 3.2 List and annotation of 76 differentially expressed proteins of the coral species *P. astreoides* following exposure to MeOH, brevetoxin or *K. brevis* cells. Highlighted proteins are indicated as either upregulated (red) or downregulated (green) compared to the control

Accession	Protein ID	Species	PbTx/ Control Fold Change	PbTx/ Control P-value	*K. brevis*/ Control Fold Change	*K. brevis*/ Control P-value	MeOH/ Control Fold Change	MeOH/ Control P-value
gi|156221418	predicted protein	*Nematostella vectensis*	1.568439	0.030784	11.32181	0.004066	1.660407	0.029511
gi|1176107158	failed axon connections homolog	*Orbicella faveolata*	4.639326	0.011156	3.017394	0.037777	3.147614	0.013752
gi|1191069530	uncharacterized protein LOC110251054	*Exaiptasia pallida*	5.230125	1.66E-12	3.306682	1.33E-07	1.928633	0.001676
gi|1176094913	predicted protein	*Orbicella faveolata*	1.627073	0.00739	2.5335	8.24E-06	1.964641	0.000488
gi|1176105383	uncharacterized skeletal organic matrix protein 5-like	*Orbicella faveolata*	11.001	0.049381	9.719665	0.04666	2.954852	0.120531
gi|1270036269	glucosidase 2 subunit beta-like	*Stylophora pistillata*	0.914413	0.312526	1.908234	0.044758	1.548779	0.034758
gi|1263126539	Cathepsin B	*Stylophora pistillata*	1.319259	0.348046	2.284956	0.001255	1.963988	0.012666
gi|1005447951	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	*Acropora digitifera*	1.606491	0.194444	1.712802	0.009527	1.871895	0.010165
gi|1005469133	Seed maturation protein domain protein	*Acropora digitifera*	0.872206	0.088244	5.46086	0.019354	2.885382	0.006059
gi|1176110238	protein disulfide isomerase	*Orbicella faveolata*	1.21591	0.248198	1.562627	0.005998	2.204203	0.003874
gi|999985476	NAD(P) transhydrogenase, mitochondrial	*Exaiptasia pallida*	1.134247	0.713839	2.691382	0.001858	2.100305	0.003669
gi|156224519	Endoplasmin	*Nematostella vectensis*	1.253086	0.264599	2.732606	1.32E-07	1.737854	0.000155
gi|1270045038	Proteasome subunit alpha type-5	*Stylophora pistillata*	1.112558	0.861071	2.17942	0.012547	2.233484	6.07E-05
gi|1005485438	myosin-2 essential light chain-like	*Acropora digitifera*	1.194621	0.102289	2.42776	0.00075	1.221451	0.509782
gi|1176121623	cathepsin L1-like	*Orbicella faveolata*	0.998317	0.796067	2.178282	0.034611	1.120476	0.379714
gi|931444489	metal-dependent hydrolase	*Desulfobacterales bacterium*	1.406351	0.007914	2.556935	0.005811	2.038855	0.345334
gi|1263121217	Protein disulfide-isomerase A6	*Stylophora pistillata*	1.07236	0.599945	1.823862	0.003077	1.390737	0.338883
gi|944358134	Tubulin alpha-1C chain	*Alligator sinensis*	0.831663	0.893383	1.911361	0.029061	1.353872	0.333538
gi|1176120735	predicted protein	*Orbicella faveolata*	1.076222	0.999967	1.565964	0.013297	1.297601	0.295423
gi|1176099008	Intersectin-1	*Orbicella faveolata*	1.371581	0.12593	2.331115	0.011148	1.304206	0.217592
gi|1176090182	ras-related protein Rab-11A	*Orbicella faveolata*	1.008143	0.674206	1.89742	0.00982	1.281208	0.174059
gi|1263138886	protein disulfide-isomerase tigA precursor	*Stylophora pistillata*	0.971176	0.820692	1.698406	0.006504	1.72662	0.170405
gi|1005486638	phosphatidylserine decarboxylase	*Acropora digitifera*	0.997079	0.778768	1.677438	0.001829	1.184864	0.164853
gi|1176117806	predicted protein	*Orbicella faveolata*	0.825585	0.673549	1.725989	0.002305	1.243586	0.158336
gi|156376666	heterogeneous nuclear ribonucleoprotein A/B isoform X1	*Nematostella vectensis*	0.984816	0.478916	2.455394	0.040585	1.889163	0.133129
gi|999976565	succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial-like	*Exaiptasia pallida*	0.916131	0.539258	2.993336	0.023621	1.716752	0.124512
gi|1176081387	cleavage stimulation factor subunit 2-like	*Orbicella faveolata*	1.06318	0.377148	2.646103	0.035464	2.048871	0.123858
gi|828223890	THO complex subunit 2	*Hydra vulgaris*	0.872112	0.376587	2.464135	0.032166	1.264572	0.11359
---NA---	---NA---	---NA---	1.04729	0.336962	2.547305	0.015499	1.357509	0.10801
gi|1129196481	2-hydroxyacid dehydrogenase	*Symbiodinium microadriaticum*	0.707799	0.297647	2.48504	0.047882	0.976043	0.105731

gi|1176073868	predicted protein	*Orbicella faveolata*	1.212872	0.156919	2.426542	3.34E-05	1.30641	0.104707
gi|1176092520	neuronal pentraxin-2-like	*Orbicella faveolata*	1.259306	0.13019	1.720474	0.028857	1.508923	0.085836
gi|1176123940	cytochrome b-c1 complex subunit Rieske, mitochondrial-like	*Orbicella faveolata*	1.174942	0.834615	1.942884	5.88E-05	1.187696	0.010357
gi|156219690	coatomer subunit gamma-2	*Nematostella vectensis*	0.662037	0.066255	1.900929	0.000483	1.444461	0.007015
gi|749717734	Transposon Tf2-6 polyprotein	*Thelohanellus kitauei*	0.936283	0.071297	1.518932	0.000208	0.676355	0.000454
gi|1101333444	fructose-bisphosphate aldolase	*Bemisia tabaci*	0.872159	0.022244	1.521515	5.99E-07	1.295667	0.000253
gi|1176107568	dyp-type peroxidase family protein	*Orbicella faveolata*	1.025461	0.007599	1.640659	0.000984	0.99299	5.12E-05
gi|1176095254	actin-related protein 2	*Orbicella faveolata*	1.562503	0.02192	0.402453	0.013104	1.114702	0.092864
gi|1005436313	Calreticulin	*Acropora digitifera*	1.612738	0.02148	0.795859	0.063344	2.03298	0.008846
gi|156384837	histone H4-like	*Nematostella vectensis*	2.017123	3.53E-07	1.301568	0.426179	0.877138	0.695744
gi|1005492381	histone H2A-like	*Acropora digitifera*	1.714185	0.02424	1.564844	0.516272	0.893461	0.643855
gi|156308410	histone H2B	*Nematostella vectensis*	3.02019	0.012813	1.124397	0.178836	0.250718	0.204904
gi|1176106978	predicted protein	*Orbicella faveolata*	1.716772	0.028202	1.079154	0.987765	0.939825	0.128367
---NA---	---NA---	---NA---	1.850374	0.027425	0.587007	0.018967	2.511944	0.078861
gi|1129189947	photosystem I subunit II (chloroplast)	*Symbiodinium microadriaticum*	2.116115	0.016061	1.338062	0.202179	0.993901	0.025271
gi|134104063	chloroplast soluble peridinin-chlorophyll a-binding protein precursor	*Amphidinium carterae*	1.736329	1.21E-06	1.290355	0.001649	1.190654	3.67E-07
gi|1005435074	proteasomal ubiquitin receptor ADRM1-like	*Acropora digitifera*	0.720442	0.182179	0.395495	0.039624	1.241064	0.242936
gi|1176081911	spermatogenesis-associated protein 20	*Orbicella faveolata*	0.96128	0.375979	0.421346	0.043314	1.027921	0.195371
gi|1005455133	centromere-associated protein E-like	*Acropora digitifera*	1.062252	0.285289	0.478724	0.020745	1.476479	0.149443
gi|1176099874	protein kinase C and casein kinase substrate in neurons protein 1 isoform X3	*Orbicella faveolata*	1.216795	0.201559	0.443273	0.043739	0.840823	0.138901
gi|1176083171	centrosomal protein of 290 kDa-like	*Orbicella faveolata*	0.789164	0.02134	0.364405	0.008177	0.623285	0.137682
gi|452944860	photosystem I P700 apoprotein A2 (chloroplast)	*Symbiodinium sp*	1.218589	0.164473	0.325391	0.035873	1.359081	0.13494
gi|1176074793	3-phosphoinositide-dependent protein kinase 1	*Orbicella faveolata*	1.292585	0.268702	0.433083	0.046016	0.955474	0.130875
gi|1176102334	6-phosphogluconate dehydrogenase, decarboxylating-like	*Orbicella faveolata*	1.307389	0.130285	0.449164	0.049215	1.382212	0.129363
gi|1176083360	thiosulfate sulfurtransferase-like	*Orbicella faveolata*	1.295656	0.200228	0.465497	0.048299	1.070284	0.118301
gi|514686106	predicted protein	*Salpingoeca rosetta*	1.37703	0.207814	0.414586	0.040421	1.302305	0.108307
gi|1005478902	prohibitin-2 isoform X1	*Acropora digitifera*	1.372759	0.158176	0.384557	0.040269	1.617026	0.107319
gi|1176061994	Krueppel-like factor 16	*Orbicella faveolata*	1.138972	0.291996	0.321686	0.02145	1.19034	0.092665
gi|1263131715	Trifunctional enzyme subunit beta, mitochondrial	*Stylophora pistillata*	0.92571	0.124728	0.396252	0.002916	0.96744	0.066997
gi|1176065290	vacuolar protein sorting-associated protein 35	*Orbicella faveolata*	1.050479	0.022577	0.491381	4.79E-05	1.146204	0.003421
---NA---	---NA---	---NA---	0.182109	0.032211	1.635622	0.299677	1.52856	0.121255
gi|1005420261	Ribonuclease UK114	*Acropora digitifera*	0.877804	0.699209	1.390523	0.512789	1.593202	0.032474
gi|1176115663	putative gastrointestinal growth factor xP4 isoform X2	*Orbicella faveolata*	1.178351	0.053283	2.472908	0.267691	2.666926	0.030905
gi|1191040164	Tubulin-specific chaperone A	*Exaiptasia pallida*	1.102646	0.557875	2.06311	0.336127	2.498094	0.024159
gi|531865212	Cofilin	*Ophiocordyceps sinensis*	0.978832	0.892842	2.322191	0.100665	2.582119	0.019784
gi|1176067577	glutaredoxin-C4-like isoform X2	*Orbicella faveolata*	0.859274	0.966648	4.049493	0.172549	3.109092	0.017145
gi|1176081555	xaa-Pro aminopeptidase 1 isoform X1	*Orbicella faveolata*	0.646409	0.507543	8.942528	0.061219	4.640025	0.013715
gi|1176097593	neurogenic locus notch homolog protein 1-like	*Orbicella faveolata*	0.91009	0.182366	1.826818	0.104973	1.749404	0.011237
gi|1176118675	Eukaryotic translation initiation factor 5A-1	*Orbicella faveolata*	1.058862	0.65058	1.758509	0.332157	2.264461	0.006434
gi|156405900	10 kDa heat shock protein, mitochondrial-like	*Nematostella vectensis*	0.717782	0.72292	2.746301	0.822786	2.132616	0.005936
gi|1129200883	Phosphoenolpyruvate carboxylase, housekeeping isozyme	*Symbiodinium microadriaticum*	1.0622	0.145302	0.542766	0.000365	1.555658	0.005837
gi|1176061521	protein disulfide isomerase-like 2-2	*Orbicella faveolata*	0.927188	0.907186	1.803001	0.133813	1.820057	0.004572
gi|1005433753	epididymal secretory protein E1-like	*Acropora digitifera*	1.228128	0.700849	1.318009	0.921694	1.732306	0.001495
gi|1129179937	wd40 repeat-containing protein	*Symbiodinium microadriaticum*	1.280139	5.54E-05	0.692962	3.34E-05	2.005825	0.001042

gi|1176057982	ATP synthase subunit d, mitochondrial	*Orbicella faveolata*	1.191306	0.148965	1.070699	0.939175	1.841955	0.000659
gi|1176080457	glycosyl hydrolase family 31	*Orbicella faveolata*	0.715628	0.153408	1.281212	0.806443	0.396101	2.11E-05

3.3.4 Protein-Protein Interaction Network

Differentially expressed proteins of treatments were used to construct interaction networks to explore functional relationships, as well as to provide supporting evidence that differentially expressed proteins were a result of treatment and not type 1 error. Network analysis showed that proteins differentially expressed in brevetoxin treatments were not well connected (PPI enrichment p-value = 0.313), while a large proportion of proteins differentially expressed in *K. brevis* treatments contained interaction (PPI enrichment p-value =1.53×10^{-6})(Fig 3.5).

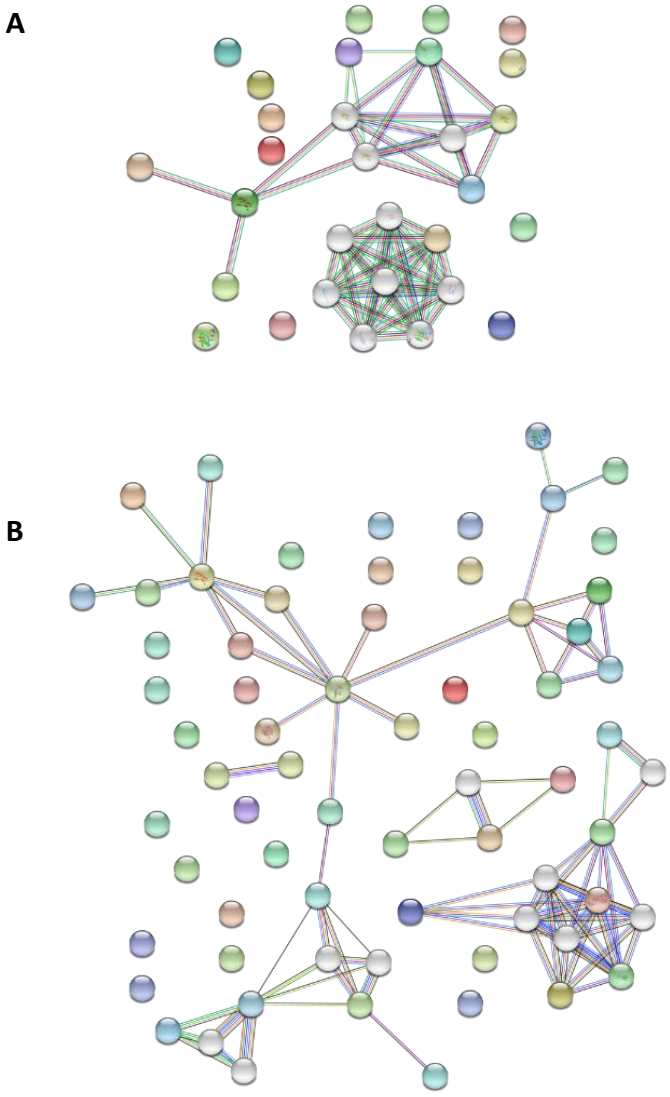

Figure 3.5 Protein-protein interaction network of differentially expressed proteins in *P. astreoides* following exposure red tide. Protein sequences following exposure to either (A) brevetoxins or (B) *K. brevis* were uploaded to the Search Tool for the Retrieval of Interacting Genes (STRING). Proteins were compared to the *Nematostella vectensis* network with ten interactors in second shell were selected to identify protein intermediates. Protein-protein interaction enrichment value was not statistically different for brevetoxin treatment ($p = 0.313$); however, was statistically significant for *K. brevis* ($p = 1.53 \times 10^{-6}$)

3.4 Discussion

3.4.1 Protein Profiling

Part of the goal of this study was to test the feasibility of using previously published transcriptomes of *Porites* species to construct a reference search database for protein identification in broad-scale proteomic analysis of the coral species *Porites astreoides*. Profiling the proteome of a biological sample requires a well-established database, which makes a proteomic approach less accessible for non-model organisms. The use of transcriptome data to construct a reference database would be an invaluable tool in proteomic analysis of non-model coral species and has shown to provide strong coverage of protein identification in other marine organisms (Tomanek, 2011). Due to a lack of mapped genomes in coral species, most broad-scale analysis of cellular responses occurs at the transcriptome level. However, many phenotypic responses arise from post-transcriptional and post-translational processes, resulting in the variation of transcript expression explaining less than half of the variation in protein abundance in some cases (Feder & Walser, 2005). Therefore, it is important to establish methodologies that can assess cellular responses at the protein level in non-model organisms.

This study demonstrated that a reference database constructed from three transcriptomes from two *Porites* species (*Porites astreoides* and *Porites australiensis*) allowed for the identification of a sufficient amount of proteins for quantitative proteomic analysis of the coral species *P. astreoides*. Using an iTRAQ technique, a total of 1,371 proteins were identified with 1,290 proteins quantified. This allowed differential expression to be determined in order to analyze the cellular response of coral following exposure so *K. brevis* or brevetoxins.

3.4.2 Differential Expression of Proteins Following Exposure to *Karenia brevis* or Associated Brevetoxins

Exposure of the coral species *P. astreoides* to red tide resulted in the differential expression of proteins associated with cellular function that varied depending on whether the coral was exposed to whole *K. brevis* cells or purified brevetoxin analogs. The brevetoxin treatment caused a notable upregulation in proteins associated with DNA binding and organization. Conversely, the *K. brevis* treatment chiefly caused the differential expression of proteins related to biosynthesis, vesicle-mediated transport, and homeostatic processes. Interestingly, only six of the identified proteins overlapped between brevetoxin and *K. brevis* treatments, while nine and 46 proteins were uniquely differentially expressed in brevetoxin treatments and *K. brevis* treatments, respectively. The lack of shared proteins may reflect certain limitations, suggesting methodologies used may not be sensitive enough to detect high resolution of the *P. astreoides* proteome. In addition, the constructed database can pose a limitation since it is based on transcriptomes that may not represent all genes present. Notably, as transcriptomes used for database construction were derived from other environmental stressors (i.e. thermal stress) or particular life stages, proteins with a unique functional response to red tide exposure would not be detected. Also, differences in homologs of *P. australiensis* in the constructed database may limit protein identification capacity. These restrictions would likely result in lower proteome coverage. In addition, differences may arise since brevetoxin concentrations are typically not correlate with *K. brevis* bloom density. Instead other factors dictate brevetoxin production, such as nutrient availability and salinity (Errera et al., 2010; Ransom Hardison et al., 2012). Finally, exposure to live *K. brevis* cells can add a layer

of complexity that may contribute to these differences in protein expression. For example, suspended brevetoxins most likely enter coral tissue through diffusion or respiration, whereas most coral are heterotrophic and live *K. brevis* cells might be ingested through filter feeding (reviewed in Houlbrèque & Ferrier-Pagès, 2009). In the case of the latter, metabolic processes can liberate toxic metabolites of brevetoxins, which could then cause cellular stress (reviewed in Plakas & Dickey, 2010). Network analysis of differentially expressed proteins compared to *Nematostella vectensis* pathways demonstrated that differentially expressed proteins from the *K. brevis* treatments had more interactions among themselves then would be expected from a randomly drawn sample of proteins of similar size from the genome; however, this was not the case for brevetoxin (Fig. 3.5). Because of the low PPI enrichment value (p-value = 0.313), differentially expressed proteins in the brevetoxin treatment should be interpreted with caution. The low connection may be a result of the small number of proteins being uploaded into the STRING search, the protein interactions are unknown, or the protein selection being due to random chance. Because of this, only proteins with known connections were interpreted herein. Regardless, because of the high connectivity of proteins in *K. brevis*, it can be interpreted with confidence that differential expression is related to cellular function and is in response to the treatment.

3.4.3 Coral Cellular Response to Ecologically Relevant Concentrations of *Karenia brevis*

There are many highly conserved proteins in the proteome of the cellular stress response that have evolved early in evolutionary history. Proteome comparison among humans, yeast, eubacteria (*Escherichia coli*), and archaebacteria (*Halobacterium* spp.) revealed

300 highly conserved proteins among the three domains, with 44 proteins having known function during cellular stress (Kültz, 2003; Kültz, 2005). Specifically, proteins of this conserved cellular stress proteome fall into functional categories of redox regulation and redox-sensitive proteins, sensing and repairing macromolecule damage, molecular chaperones, DNA repair enzymes, and energy metabolism (Gasch et al., 2000; Kültz, 2005). The broad protection that arises from the activation of these responses likely accounts for observed cellular cross-resistance to various stressors (Hohmann & Mager, 2007). However, proteins in cellular stress responses are not limited to this conserved proteome, with many other proteins involved that are not ubiquitously found throughout the four kingdoms.

The differential expression of proteins in *P. astreoides* following *K. brevis* exposure indicates that several processes associated with cellular stress were activated. In particular, processes associated with redox homeostasis, protein folding, energy metabolism, and production of reactive oxygen species (ROS) were altered (Table 2). Many cellular processes rely upon the maintenance of redox potential within the cell. Severe changes in redox homeostasis can result in cellular dysfunction and eventually lead to apoptosis. Particularly sensitive to redox fluctuations are pathways of protein synthesis in the endoplasmic reticulum since its oxidizing environment supports disulfide bond formation. In addition to impacts on redox state, a variety of insults such as changes in intralumenal calcium, altered glycosylation, nutrient deprivation and pathogen infection can lead to an unsuitable ER environment result in the misfolding of proteins. These stressors can result in the activation of the unfolded protein response (UPR), which ensures misfolded proteins are directed toward degradation pathways and not secreted into the cell (Malhotra & Kaufman, 2007). There were many proteins

upregulated following exposure to *K. brevis* that may suggests impacts on redox homeostasis and ER dysfunction (2-hydroxyacid dehydrogenase, disulfide isomerases, and endoplasmin) (Table 2). 2-hydroxyacid dehydrogenase is highly conserved throughout evolutionary history and is an important enzyme in the maintenance of redox homeostasis (Kültz, 2005). Disulfide isomerases are involved in formation and breakage of disulfide bonds during protein synthesis. This family of proteins has been shown to be induced and modified during oxidative stress, which is likely a response to ensure proper protein folding during oxidation in the ER (Huang et al., 2009; Lind et al., 2002). In addition, expression of endoplasmin has been shown to suppress oxidative damage and stabilize calcium homeostasis in the ER following stress (Bando, et al., 2004; Coe & Michalak, 2009).

Along with disruption of redox homeostasis, coral tissue exposed to *K. brevis* demonstrated differential expression of proteins related to increased energy metabolism. The induction of energy metabolism during the cellular stress response may be necessary for producing reducing agents needed for antioxidant systems (e.g. NADH, NADPH), as well as producing the energy needed for protein degradation, protein chaperoning, and DNA repair (Kültz, 2005). In addition, ER stress and activation of the UPR have been linked to expression of genes in glucose metabolism (Hotamisligil, 2010; Wang et al., 2009). Here, many proteins involved in energy metabolism were upregulated following exposure to *K. brevis*, including those that possess key roles in glycolysis, citric acid cycle, and electron transport chain within mitochondria (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, NAD(P) transhydrogenase, succinate-CoA ligase [ADP/GDP-forming] subunit alpha, cytochrome b-c1 complex subunit Rieske, and fructose-bisphosphate aldolase) (Table 2). Many of these proteins

are susceptible to oxidative damage, are targeted by ROSs, or have known roles in ROS defenses (Abbasi & Komatsu, 2004; Cadenas & Davies, 2000; Gameiro et al., 2013; Ghafourifar & Cadenas, 2005; Guzy & Schumacker, 2006*:* Heim et al., 2002; Jiang et al., 2016; Wen & Garg, 2004). For example*,* NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 is associated with the NADH dehydrogenase complex 1, which is the first enzyme in the mitochondrial electron transport chain. This enzyme is particularly susceptible to oxidative damage, being targeted by ROSs, which results in enzyme inactivation and protein damage (Cadenas & Davies, 2000). Other enzymes can further exacerbate ROS release in mitochondria following oxidative damage, such as cytochrome b-c1 complex subunit Rieske, which is a component of the mitochondrial ubiquinol-cytochrome c reductase complex dimer (complex III dimer) and is involved in the generation of the electrochemical potential in mitochondria for ATP synthesis. Not only is this protein found to be a target of oxidative damage but it's dysfunction is also implicated as a source of further release of ROSs in mitochondria (Guzy & Schumacker, 2006; Wen & Garg, 2004). Others proteins present have roles in a direct response to oxidative stress, acting as antioxidants or alternative pathways of energy production, such as NAD(P) transhydrogenase and fructose-bisphosphate aldolase (Abbasi & Komatsu, 2004; Gameiro et al., 2013; Heim et al., 2002; Jiang et al., 2016). Other ROS related proteins, in addition to those connected to energy production and oxidative stress within mitochondria, were glutathione S-transferase predicted protein and dyp-type peroxidae family protein, which are important in cellular resistance to oxidative stress (Veal et al., 2002; Yoshida & Sugano, 2015)

Proteins involved in protease activity that were upregulated following exposure to *K. brevis* (cathepsin B and cathepsin L1) suggests the induction of protein degradation, which can provide further evidence of ER and mitochondrial dysfunction, as well as oxidative stress. In addition to functioning in the UPR response in the ER, proteases actively recognize and degrade oxidative proteins, and replacements are then synthesized *de novo* (Davies, 1995; Grune et a., 1997; Grune et al., 1998; Sitte et al., 2000; Ullrich et al., 1999[a]; Ullrich et al., 1999[b]). Thus, the increased proteases observed may be the result of increased oxidative damage following ER and mitochondria dysfunction.

A possible unifying explanation of the observed cellular responses following exposure to *K. brevis* is that there was a disruption in intracellular Ca^{2+} maintenance processes. ER stress and oxidative damage can result in the release of Ca^{2+} from the ER into the cytosol, which in turn, due to their close proximity, can cause production of ROSs in mitochondria (Barrige, et al., 2003; Görlach et al., 2006; Jacobson & Duchen, 2002; Lizák et al., 2006; Malhotra & Kaufman, 2007). This can result in feedback, causing further Ca^{2+} release from the ER (Viner et al., 2000). There is growing evidence that brevetoxin analogs may directly or indirectly influence Ca^{2+} channels in addition to voltage gated sodium channels. For example, it has been shown that brevetoxins inhibit Ca^{2+} uptake and stimulated Ca^{2+} -Mg^{2+} ATPase activity in the sarcoplasmic reticulum of rabbit skeletal muscles (Kim et al., 1978). The process by which brevetoxins affect Ca^{2+} is not well understood, which may result from direct brevetoxin-Ca^{2+} channel interaction or indirectly from changes in membrane potential from influx of Na^{+} from open VGSCs. Studies have shown that Na^{+} influx from brevetoxin exposure can subsequently result in activation of *N*-methyl-D-aspartate receptors and IP_3 messengers, leading to an increase in intracellular

Ca^{2+}(George et al., 2009; Liberona et al., 2008). Furthermore, brevetoxins have been found to competitively inhibit the binding sites of other structurally similar biotoxins that are known to directly impact Ca^{2+} channels (de la Rosa et al., 2001; Gusovsky & Daly, 1990; Konoki et al., 1998). In this study there is evidence for increased cytosolic Ca^{2+} following exposure to *K. brevis* with downregulation of 3-phosphoinositide-dependent protein kinase 1, which functions in the regulation of the release of sequestered calcium ions into the cytosol, and upregulation of myosin-2 essential light chain, which functions in calcium binding. This falls in line with finding by Tian et al. (2011), who reported that proteomic analysis of gill and brain tissue of fish following exposure of PbTx-1 brevetoxin analog resulted in an increase of expression in calcium binding proteins (Myosin-like proteins). Based on the overall cellular function observed in this study, *K. brevis* may be impacting cytosolic Ca^{2+}, resulting in subsequent ER/mitochondrial dysfunction, increased oxidative stress, and protein damage. Such changes in Ca^{2+} can directly impact ER oxidative homeostasis, resulting in misfolded proteins, induction of UPR, as well as increased production of ROSs within mitochondria.

3.4.4 Coral Cellular Response to Ecologically Relevant Concentrations of Brevetoxin Analogs

Interestingly, much of the differential expression in the proteome was not shared between *K. brevis* and brevetoxin treatments. The majority of proteins expressed from exposure to brevetoxin analogs PbTx-2 and PbTx-3 were related to DNA organization, chromatin formation and transcription expression (e.g. histone H4, histone H2A, histone H2B) (Table 2). The complex folding pattern of eukaryote DNA packaging into chromosomes begins with the DNA-protein complex made from the interaction of negatively changed DNA and

positively charged histones proteins (Van Holde, 1988). The histones involved in the formation of nucleosomes come from four histone families (H2A, H2B, H3, and H4) and they are highly conserved throughout evolutionary history across eukaryotic taxa (Thatcher & Gorovsky, 1994). Histone variants also play an important role in differential gene expression. Histone modification can alter nucleosome conformation and change accessibility of certain transcriptional regulatory proteins (Berger, 2002; Lee et al., 1993; Norton et al., 1989; Struhl, 1998; Vettese-Dadey et al., 1996). Deregulation and overexpression of certain histones have been associated with cancerous cells and have been known to cause abnormal expression of oncogenes, which can be suppressed by degradation of the histone through upregulation of tumor suppressors (Baptista et al., 2013; Dryhurst et al., 2012; Hua et al., 2008; Svotelis et al., 2010; Valdés-Mora et al., 2012).

In this study, the histones upregulated are likely the result of cell proliferation, since overexpression and excess accumulation of histones outside of DNA synthesis can be toxic to the cell, restricting histone expression to S phase of the cell cycle (Mei et al., 2017). Interestingly, the concentration of brevetoxin PbTx-2 (2.01×10^{-8} M) used in this study was similar to that used by Murrell & Gibson (2009), who found that exposure to low concentrations of PbTx-2 (10^{-8} M) resulted in proliferation of Jurkat E6-1 cells and only at higher concentrations was cell division negatively impacted. The phenomena of enhanced cell proliferation at low doses of toxins has been well documented throughout the literature, known as a hormetic dose response (Reviewed in Calabrese & Blain, 2005). Hormesis is thought to be an adaptive compensatory response of the cell; however, the mechanisms that regulate this are still poorly understood (Mattson, 2008). Since a single concentration and time frame

was used in this study, it cannot be determined if the coral tissue was exhibiting a hormetic response following brevetoxin exposure. Future research would be needed that takes into consideration varying concentrations and exposure duration.

3.4.5 Study Limitations and Future Direction

There are several limitations to this study that may prevent detection of cellular impacts of *K. brevis* and associated brevetoxins on coral tissue. As aforementioned restrictions in the construction of the reference database could result in lower protein identification, the use of a more encompassing database may result in higher resolution of the proteome. For example, the utilization of RNA-sequencing (following the PIT technique) to create a sample-specific reference database can help increase the amount of proteins identified by accounting for nonsynonymous SNPs and protein isoforms, as well as providing the ability to trim database searches by taking into consideration transcript expression. Also, the utilization of parameters that take into consideration of post-translational modifications can further elucidate cellular responses to the stressor. For example, histones undergo many different modifications including acetylation, phosphorylation, methylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerization; reflecting their versatile roles in chromosome packaging, transcriptional regulation, and DNA damage and repair (reviewed in Kouzarides, 2007). The role of histone modification in DNA damage is mostly related to signaling and accessibility of repair enzymes (Masumoto et al., 2005; Paull et al., 2000; Rogakou et al., 1998; Rogakou et al., 1999; Sanders et al., 2004; Thatcher & Gorovsky, 1994; Van Attikum et al., 2004; Wang et al., 2006). Along with neurotoxic effects, there is growing evidence that

brevetoxins may result in DNA damage. Metabolization of brevetoxins can results in many different metabolites. However, of particular interest are epoxides, since they are highly reactive with nucleophilic sites and form adducts with macromolecules such as DNA and histones (Groopman et al., 1980; Koskinen & Pilna, 2000; SooHoo et al., 1994). Brevetoxins have been found to result in, single and double stranded DNA breaks, cause chromosomal aberration and alter cell proliferation; which can be prevented with the introduction by the antagonistic metabolite brevenol (Murrell & Gibson, 2009; Murrell & Gibson, 2011; Sayer et al., 2005; Sayer et al., 2006). Also, intratracheal exposure of rats to brevetoxins have been found to result in clastogenic activity in the liver (Leighfield et al., 2009). By incorporating the possibility of post-translational and post-transcriptional modifications into the scope of the analysis, protein identification can more accurately indicate whether or not cellular impacts such as DNA damage are occurring.

In addition to changes in methodology, future research should take into consideration prolonged exposure to *K. brevis* or associated brevetoxins. The goal of this study was to assess sublethal stress in coral tissue following acute exposure. However, blooms have been reported to last up to 18 months (Tester & Steidinger, 1997). Prolonged exposure may result in more discernible effects. Regardless, the results of this study demonstrate that a reference database constructed from previously published transcriptomes is efficient enough to allow protein recovery for large-scale proteomic analysis, and that exposure of the coral species *Porites astreoides* to red tide conditions can result in differential expression of proteins related to a cellular stress response.

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Vita

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Education

Master of Science, Biology, 2018
College of Arts and Sciences; University of North Florida; Jacksonville, FL
Area of Concentration: Ecology/Environmental Biology
Graduating GPA: 4.00;

Bachelor of Science, Biology, 2014
College of Arts and Sciences; University of North Florida; Jacksonville, FL
Area of Concentration: Coastal Environmental Science
Graduating GPA: 3.85
Graduated with: *Summa Cum Laude* Institutional Honors

Associate of Arts, 2011
Palm Beach State Community College; Lake Worth, FL
Graduating GPA: 3.96
Graduated with: High Academic Distinction, Phi Theta Kappa Honors Society, 28 additional credits in environmental-based classes

Presentations

"The effects of the red tide producing algae, *Karenia brevis*, on *Porites astreoides*: a potential regional stressor to coral reefs"
Oral Presentation at Benthic Ecology Meeting; Corpus Christi, TX March 2018

"Behavioral and physiological responses of the coral *Porites astreoides* to high bloom densities of *Karenia brevis* and associated brevetoxins."
Poster Presentation at Benthic Ecology Meeting; Myrtle Beach, SC April 2017

"Effects of the red tide producing dinoflagellate *Karenia brevis* and associated brevetoxins on viability and sublethal stress response in coral."
Poster Presentation at the SE Phycological Colloquy Meeting; Valdosta, GA November 2016

"Effects of the red tide producing dinoflagellate *Karenia brevis* and associated brevetoxins on viability and sublethal stress response in coral."

Poster Presentation at the UNF Natural Science Poster Session; Jacksonville, FL — November 2016

Grants, Scholarships and Awards

Coastal Biology Travel Award Grant — April 2017
Biological Research Enhancement Scholarship — January 2017
Florida Pell Grant — 2013, 2014, 2015
Graduated with Summa Cum Laude Institutional Honors — December 2014
Transformational Learning Opportunity Presidential Scholarship — 2013 – 2014
Awarded by UNF College of Arts and Sciences for Coral Reef *Development Research*

Research Experience

Proteomics and Mass Spectrometry Training Experience — 2017
Interdisciplinary Center for Biotechnology Research; University of FL; Gainesville, FL
Over 70 hours of personal training by Dr. Jin Koh, the Scientific Director for Proteomics and Mass Spectrometry at the University of Florida, and other members of the facility on an array of broadly applicable proteomic techniques and concepts.

Graduate Research — 2015 – 2018
Biology Department; University of North Florida; Jacksonville, FL
Master's Thesis Project: *"Effects of the red tide producing dinoflagellate* Karenia brevis *and associated brevetoxins on viability and sublethal stress responses in scleractinian coral."*

Undergraduate Research — 2014
Biology Department; University of North Florida; Jacksonville, FL
Research Assistant in Dr. Cliff Ross's Lab
Area of Research: Factors affecting Caribbean coral larvae recruitment
Research Assistant in Dr. Matthew Gilg's Lab]
Area of Research: Heritability of Heat Tolerance in Caribbean corals.
Skills obtained: DNA isolation, PCR amplification, DNA band isolation and extraction, DNA purification.

Teaching Experience

Graduate Teaching Assistant
Biology Department; University of North Florida; Jacksonville, FL
Graduate teaching assistants lead laboratory sections of general biology courses for undergraduate students majoring in biology. Responsibilities include: presenting of information in a lecture setting,

providing instruction and assistance during laboratory experiments and activities, administering quizzes and exams, evaluating student performance, and assigning course grades.

General Biology I Lab (BSC 1010 C) 2015-2016
General Biology II Lab (BSC 1011 C) 2017-2018

Skills and Qualifications
SSI Open Water SCUBA Certification Since 2015