The Influence of Gene Transfer Agents in *Symbiodinium* Associated Microbial Communities and the Coral Holobiont

By

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CERTIFICATE OF APPROVAL

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Abstract:

Coral reefs serve an imperative role in the formation of marine ecosystems. They act as the foundation for complex food webs by supporting fish, sponges, algae, and invertebrates. However, coral bleaching and climate change have become major concerns in the conservation of ocean species over the past few decades. As a result, new methods of restoration are necessary for the propagation of these endangered coral species. This study focuses on the critical role that the symbiotic algae *Symbiodinium*, as well as their associated bacteria, play in the health of coral. A specific type of bacteria, α-proteobacteria, use virus-like particles called gene transfer agents as a method of diversification. This study indicates that the application of these gene transfer agents may increase the fitness of the coral holobiont's associated bacteria. Coral degradation can perhaps be lessened if inhabiting microbiota can survive stressful conditions by diversifying their assemblage. This study attempts to understand the complex coral system by observing the effects of gene transfer agents on symbiotic algae as well as its associated bacteria.

INTRODUCTION:

I: Importance of Corals

Coral reefs are known for their beauty and high marine productivity as well as their ability to act as the foundation for hundreds of thousands of species (Hoegh-Guldberg, 1999). They offer the most diverse of shallow-water marine ecosystems by harboring complex food webs filled with an array of species essentially undescribed by science (Roberts et al., 2002). These reefs are underwater structures of calcium carbonate that edge one sixth of the world's coastlines (Hoegh-Guldberg, 1999). Due to the high habitat heterogeneity of a coral system, reefs assist in niche diversification and offer opportunities for new species evolution (Moberg & Folke, 1999). These stunning coral colonies are formed through growth of many identical coral polyps (Spalding, 2001). Each polyp uses the water's available calcium carbonate to construct its rigid skeleton (Spalding, 2001). Although they cover less than 0.1% of the ocean floors, corals provide habitats for 25% of all marine species (Spalding, 2001). As a result, the reefs serve a number of important roles that benefit both humans and the marine organisms that live on them.

The presence of coral reefs in the oceans serve an important economic and resourceful role for society. Half a billion people live within 100 kilometers of coral systems (Hoegh-Guldberg, 1999). People rely on these reefs to provide a means of economic development. Communities surrounding coral habitats depend on the income and business of visiting tourists. Coral reefs generate the fine coral sand that characterize many of the white sand tropical beaches (Moberg & Folke, 1999). The mere existence of coral generates 1.6 billion dollars each year solely in tourism of the Floridian reefs alone (Hoegh-Guldberg , 1999) This income contributes to the economic stimulation of nearby hotels, boat operators, diving instructors, and local

restaurants (Spalding, 2001). The fisheries linked with the reefs also generate significant wealth for countries with reef coastlines. In some cases, coral reefs are the only means of monetary wealth for impoverished countries, in addition to providing essential protein in the human diet (Hoegh-Guldberg, 1999). Humans rely on reef spawning sites for the propagation of these nourishing fish species. Because of the natural weather buffering system, reefs can also protect the shorelines by breaking the power of waves from storms and hurricanes (Spalding, 2011). They provide a means of erosion prevention that can be especially important in regions with surrounding residential areas and businesses (Spalding, 2001). This allows cities and towns to save billions of dollars each year by preventing the cleanup of floods and damaged shorelines. Lastly, coral reefs harbor pharmaceutically useful substances with anticancer, antimicrobial, anti-inflammatory, and anticoagulation properties among the mollusks, seaweeds, and other sea animals that reside among reefs (Moberg & Folke, 1999).

II: The Coral "Holobiont:" A Complex Host

Coral consists of a complex holobiont coupled with both internal and external microbiota (Bourne et al. 2009). *Symbiodinium*, a dinoflagellate algal symbiont critical to the survival of coral, exist intracellularly in exceptionally high densities and provide the coral animal with up to 90% of the dietetic requirements (Berkelmans & Van Oppen, 2006). The holobiont also includes many types of bacteria, fungi, archaea, and viruses that perform highly specialized roles in this mutualistic system (Bourne et al. 2009). Any changes or disturbances in the coral associates can deteriorate the health of the animal (Bourne et al. 2009). In order to fully understand the mechanisms of the coral holobiont, it is necessary to have an in-depth knowledge of each connected member.

A healthy coral reef normally contains more than ten billion algal symbionts per square meter (Baker, 2003). The close association of the primary producing algae and the coral consumer permit the tight nutrient recycling that generates the high productivity which is signature to the coral reef system (Hoegh-Guldberg, 1999). Understanding the fragile relationship between the algae and their hosts is essential to the conservation of both the coral and their symbiotic dinoflagellates (Baker, 2003). The persistent growth and division of animal and algal cells can be attributed to the success of this symbiosis. These algae live within the coral polyp and assist the coral in retrieving nutrients from the nutrient-poor water while also providing necessary oxygen (Glynn, 1996). The algae also increase the calcification of the corals, mediate nutrient flux, and provide photosynthetically fixed carbon to the coral animal (Glynn, 1996). *Symbiodinium* do this by catching solar energy and nutrients through photosynthesis to provide the majority of metabolic requirements. The algae selectively transport amino acids, sugars, and carbohydrates across the symbiont gradient (Hoegh-Guldberg, 1999). As a result, the *Symbiodinium* contribute to the coral's ability protect its skeletal components by maintaining high calcification rates (Hoegh-Guldberg et al., 2007). In return, the coral animal provides the zooxanthellae with ammonium and phosphate (Hoegh-Guldberg, 1999). These nutrients stem from the waste metabolism of the invertebrate host and are vital to the survival of the primary producer (Hoegh-Guldberg, 1999).

The symbiotic algae are maintained by the presence of various microbes. Microbial associates of corals and *Symbiodinium* provide a variety of benefits to the coral host. Associated bacteria can assist in the fixation and passage of nitrogen and carbon as well as provide antimicrobial compounds that can inhibit the growth of potentially harmful micro-organisms (Littman et al., 2010). Some compounds, such as dimethylsulfoniopropionate (DMSP), provide

nutrient sources for the bacterial populations as well as offer them structure and stability (Bourne et al. 2009). Comprehensive research of microbial multispecies mutualism in the coral holobiont can help identify the key species in the regulation of coral health (Bourne et al. 2009). These interactions are vital to the study of commensal relationships because holobiont species interactions, such as antimicrobial properties in coral mucus, often time determines spatial distributions of the bacteria populations (Bourne et al. 2009).

One of the most prevalent types of bacteria in coral systems are a group known as α-proteobacteria. This important group of bacteria use gene transfer agents as a survival technique in the diversification of their population. Gene transfer agents, or GTAs, are defective bacteriophages that can package bacterial host DNA and transfer the DNA to other α-proteobacteria (Paul 2008). Microbes rely on these beneficial mutations and processes of horizontal gene transfer in order to evolve new traits (McDaniel et al., 2012). Gene transfer agent-like sequences are found in almost all species of α-proteobacteria and are important in understanding mechanisms of symbiosis.

III: Risks Facing Coral Reefs

Coral bleaching has become a major concern in the conservation of ocean species over the past few decades. The cost of losing more than half of the world's coral reefs can be estimated at about 90 billion dollars lost in tourism alone (Hoegh-Guldberg , 1999). Sea temperatures in coral habitats have increased by almost 1°C over the past century and currently increasing between 1-2°C each century (Hoegh-Guldberg, 1999). More than half of the world's reefs are being threatened by increases in global temperatures due to escalation of human activities and global warming (Roberts et al., 2002). Corals have an obligate requirement for low levels of nutrients and high water clarity. As a result, widespread changes in ocean water can be

detrimental due to the corals natural low tolerance for changes in their environment. Since the coral reef is dependent on countless other organisms like bacteria and algae, fluctuations in the abundance of any of the species can be fatal to the coral animal and all of its inhabitants (Brown, 1997). The bleaching phenomenon is due to the loss of the symbiotic algae. *Symbiodinium* possess chlorophyll and carotenoids which create a brown coloration in the animal (Douglas, 2003). When corals are exposed to levels of stress, they expel their zooxanthellae symbiont and, consequently, lose their pigment (Douglas 2003). These bleached corals turn white, the color of their calcium carbonate skeleton (Douglas 2003). During the bleaching event, the *Symbiodinium* become metabolically compromised and are no longer capable of providing the coral animal with its necessary synthetic carbon (Douglas, 2003). When the algae are no longer useful to the coral system, they are labeled as foreign substance and dismissed from the system. Thermal tolerance of the holobiont is partially dependent on the thermal tolerance characteristics of the zooxanthellae associated with the coral. The zooxanthellae are essentially the "weakest-link" in the symbiotic partnership and the survivalship of the coral often depend on the health of the algae (Berkelmans & Van Oppen, 2006). These bleaching events typically result in the loss of 60-90% of the corals zooxanthellae. In addition, the zooxanthellae loses about 50-60% of their photosynthetic pigments. If the stress causing the expulsion of the zooxanthellae is not permanent, the corals can usually regain their algae over the course of a few weeks. However, if the anthropogenic stress is prolonged, the zooxanthellae are unlikely to return, and the coral host will die (Glynn, 1996).

There are environmental factors that can contribute to coral bleaching occurrence. Increase in human activity has led to a number of irreversible consequences that affect the health of the oceans, its inhabitants, and the food web itself. Corals can be sensitive to water

temperature, nutrient loading, increases or decreases in salinity, and pH changes. However, the leading identified cause of this widespread epidemic is the elevation of sea temperatures. About 25% of the world's coral reefs have already been damaged, which can be attributed to these increases in global temperatures (Roberts et al., 2002). The drastic changes in coral heath and composition today can be credited to coral adaptation to highly stable sea temperatures over the course of the past thousands of years. Evidence suggests sea temperatures have not varied more than 2°C in the previous 18,000 years. In addition, seasonal and daily fluctuations in tropical seas temperatures are typically very small and, in the past, have not triggered significant stress in the reefs (Hoegh-Guldberg, 1999). As a result, the rapid increases in sea temperature over the past few decades have made it difficult for coral and zooxanthellae to adapt accordingly. These bleaching events occur most during sudden temperature drops followed by strong heat waves. Corals bleach when sea temperatures exceed their normal seasonal threshold (Brown, 1997). It is believed that recent increases in temperature and intensifications in ultraviolent rays disrupt the algae's ability to participate in photosynthetic activities, which can lead to the dissociation of the symbiotic relationship. This happens when the ultraviolent radiation decreases dinoflagellate growth rates and cellular chlorophyll concentrations (Hoegh-Guldberg, 1999). *Symbiodinium* species commonly live in habitats 1-2 degrees below the temperature in which the dissociation of the symbiosis can be triggered (Douglas, 2003). Since these zooxanthellae live so close to their upper thresholds in tropical environments, the smallest change in temperature can disrupt their role in the coral system and induce bleaching (Glynn, 1996).

Another important disturbance in the delicate coral system is the increasing rate of ocean acidification. The reduction of ocean pH is the product of increased dissolution of carbon dioxide in the sea due to the rise of atmospheric carbon-dioxide concentration following the increase in

human fossil fuel combustion and deforestation (Doney et al., 2009). The concentration of carbon dioxide in the Earth's atmosphere today exceeds 380 parts per million. This concentration has increased more than 80 parts per million since industrialization and exists at an all-time high since the age of humans.

Carbon dioxide in the ocean exists as carbonic acid. This carbonic acid dissociates into bicarbonate ions and protons which reduce the availability of carbonate to biological systems (Hoegh-Guldberg et al., 2007). This alteration in the biogeochemical cycle often results in the lowering of the calcium carbonate saturation state. This lack of carbonate promotes erosion of shell-forming marine organisms like corals (Doney et al., 2009). This often times results in a decrease in density of the skeletal component of the coral polyps. Since these carbonate shells serve as backbone and protection of the coral animal, decreases in solidity can have detrimental effects on the sustainability of the creature. The dissociation of strong acids and bases used in fuel combustion and agriculture produce atmospheric byproducts of strong acids like sulfuric and nitric acid that enter ocean system and contribute to ocean acidification (Doney et al., 2009). In addition, this increase in ocean acidity has correlated with the rise in global ocean temperatures by about 0.74 degree Celsius (Hoegh-Guldberg et al., 2007).

It has also been suggested that rapid dilution of reef waters with polluted freshwater from storm runoff may be a cause of reef bleaching. Nutrient loading is an indirect problem facing these reefs. The changes in the ecosystems in the coral reef network can be heavily influenced by the transition between virtually undisturbed beaches and coasts to developing human-dominated landscapes. There have been declines in water quality, habitat, and biological diversity in these systems with an increase in agricultural land and urban development (Alan, 2004). The increase in land use has caused run-off pollutants, nutrients, and sediment particles that contribute to the

stress on the coral reefs (Moberg & Folke, 1999). Both rural farmland and developing cities for human sprawl are the principal contribution to nitrogen runoff into coastal and fresh waters. This oftentimes results in excessive eutrophication. Although an increase in nitrogen in a coral system does not directly affect the coral animal, unnatural increases in nutrient availability are believed to lower coral resilience and increase their susceptibility to disease (Hoegh-Guldberg et al., 2007). This loss of resilience often leads to an irreversible alteration in the state of the ecosystem. Coral reefs that are exposed to eutrophication often lead to an ecosystem "flip." These corals may suffer from the invasion of non-reef building organisms and algae. When algae begin to govern a coral system, the community shifts from highly diverse to macroalgae dominated. An abundance of invasive macroalgae in a coral system often choke out native species and diminish reef functionality as well as lead to anoxic conditions (Moberg & Folke, 1999).

IV: The Functionality and Benefits of Horizontal Gene Transfer

Microbial gene transfer processes are beneficial because they allow microbes to evolve to changing conditions despite their lack of a sophisticated sexual reproduction system. Horizontal gene transfer is the primary source of intraspecies diversification of the bacterial genome (Heur et al., 2007). Bacteria rely on processes of gene transfer to adapt to variable environments (McDaniel et al. 2012). Bacteria develop resistance through the exchange of genetic material with organisms that possess beneficial characteristics (Tenover, 2006). This mechanism of gene exchange comprises of three classic mechanisms: conjugation, transduction, and transformation. In conjugation, a bacterium uses an extended structure called a *pilus* in order to transfer plasmid-encompassing genes to a nearby bacterium (Tenover, 2006). This gene exchange can be facilitated by the prevalence of transposons or the incorporation of genes into host genome

(Tenover, 2006). Transformation is the processes in which bacteria obtain genetic factors from other bacteria that have released their DNA into the environment following the breakdown of the cell. This allows for the exchange of resistance genes and beneficial genetic material to strains of bacteria that were previously vulnerable (Tenover, 2006). The process is facilitated by proteins found in some naturally transformable bacteria, unlike conjugation, which requires independently replicating genetic elements (Frost et al. 2005). Resistance genes can also be transferred by independently replicating bacterial viruses via transduction. This process allows bacteria to transfer their genetic material via bacteriophages (Tenover, 2006). These bacteriophages package segments of host DNA in their capsid and can introduce this genetic material into a new host (Frost et al. 2005). This allows for the recombination with the chromosomal DNA which can then be inherited and passed on to the next generation (Frost et al. 2005).

A variety of bacteria that associate with the *symbiodinium* dinoflagellate and the coral animal practice a form of horizontal gene transfer including its high densities of α-proteobacteria. In order to create genetic variation among its species, α-proteobacteria use gene transfer agents as a method of diversification. Gene transfer agents are known to transfer genetic material between bacteria. This suggests these bacteria swap DNA and beneficial genes between other bacterial groups, and even cross-phylum transfers have been documented (McDaniel et al., 2010). In the past, it has been revealed that lab-grown gene transfer agents from *Symbiodinium* associated α-proteobacteria have demonstrated high rates of gene transfer in coral-associated environments (reef water, coral mucus) (McDaniel et al. 2010). Preliminary research suggests that gene transfer agents may have direct effects on *symbiodinium* and coral larval settlement (McDaniel, unpublished).

Coral degradation can perhaps be mitigated if their inhabiting populations can survive stressful conditions by diversifying their assemblage (Hoffman & Sgro, 2011). A GTA-introduced slice of DNA may carry new genes that can bestow the recipient bacterium with a new phenotype that may be advantageous for survival in their environment (Stanton, 2007). For example, the process of transduction between bacterium can be beneficial in transferring antibiotic resistant genes which ensure their survival against exposure to one or more antibiotics (Lang & Beatty, 2001). This evolutionary adaption of the bacteria can also help the species of the coral holobiont system counter stressful conditions arising from climate changes (Hoffman & Sgro, 2011). This adaption may be one way the bleaching corals can persist through the severe changes in habitats associated with human interference and climate change. The most important environmental factors that alter virus-host interactions are damages to host DNA and metabolic activity. DNA damage and declines in metabolic activity of bacteria often occur most through periods of stressing solar UV light (Miller, 2000). However, gene transfer agents can maintain their transfer ability even after exposure to harsh conditions in which their bacterial hosts would likely die (Stanton, 2007).

Previously, it was assumed that gene exchange in natural environments was unlikely due to the low probability of occurrence between the phage and the host. However, concentrations of bacteriophages, and most notably those infecting α-proteobacteria, are very high in marine environments and coral reef systems (Miller, 2007). As a result, understanding the interactions between gene transfer agents, the α-proteobacteria, the *symbiodinium,* and the coral animal can be beneficial to the newly developing process of coral conservation. The presence of gene transfer agents to possibly promote microbial and coral fitness may possibly be used as a form of preservation management. Research indicates that gene transfer treatment to coral larvae may

promote settlement and resilience of these animals suggesting the potential utility in coral culture and restoration efforts (McDaniel, unpublished). However, if the reef system is continually affected by the fragmentation and invasion of habitats, the transfer process in which genes move throughout bacterial populations can be impaired (Hoffman & Sgro, 2011).

The fate of the abundance of coral is dependent on the corals ability to undergo substantial adaption over the next few decades. Because bacteria reproduce rapidly, their ability to adapt and evolve can occur in a matter of days to years (Hoegh-Guldberg, 1999). Due to the natural symbiotic relationship within the system, stress acclimation of any of the coral residents, especially bacteria, would increase the entire holobiont system's chance of survival. Although the existence of gene transfer within the coral endosymbiont is likely to increase the coral resilience to high temperatures, populations of coral and their zooxanthellae are likely to take several hundred years to produce a system better capable of tolerating high ocean temperatures (Hoegh-Guldberg, 1999). The instability of the changing climate makes it difficult for scientists to make any further predictions about coral health. However, the contribution toward a more resilient reef system throughout stressful times is the goal of this generation in coral research.

V: The Mechanism of Gene Transfer Agents

This phenomenon of horizontal gene transfer is made possible by the viral capability to form their own symbiosis with a host bacterium, in this case, an α-proteobacteria. Viruses contribute large amounts of extracellular nucleic acids into marine environments (Lang & Beatty, 2010). Most of these viruses typically package viral genomes exclusively and can be either lytic or temperate viruses. Viral replication often occurs through lytic cycles in which viruses inject their nucleic acids into a host cell. Host cells misguidedly copy the viral acids instead of its own which result in the destruction of the host cell as well as its membrane

(Matigan 2005). A functional prophage (temperate phage) is activated to enter a lytic cycle in which the bacterial host is stressed, causing the bacterial cell to be destroyed and viral offspring are released (Stanton, 2007).

However some of these viral particles, including GTAs, do not behave like a normal functioning prophage. These defective phages are morphologically similar to prophages but they are incapable of the self-transmission to the host cell, meaning they cannot transmit their own nucleic acid to a bacterial host (Lang & Beatty, 2010). Instead, these phage-like particles package pieces of host DNA. These pieces of host DNA are then transported outside the cell and toward a new bacterium. The genetic information is generally thought to be incorporated in a new bacterium through the process of homologous recombination or by the establishment of new plasmid within the cell's cytoplasm (Miller, 2000). When this host DNA reaches another organism, genes can be exchanged and transduction is completed. Unlike transformation by naked DNA, gene transfer agents package their DNA in capsids. These capsids offer protection from nucleases and other chemicals that have the capability of deteriorating host DNA (Stanton, 2007). Certain strains of gene transfer agents appear to have a common ancestor present in all α-proteobacteria. Thus, it can be suggested that presence of gene transfer agents may have been the result of a cellular evolution from a prophage that evolved in concert with the bacteria, rather than a mere mutated defective phage (Lang & Beatty, 2010).

VI: Research Objectives

It has been documented that lab-created gene transfer agents are taken up and active in marine microbial assemblages. Repeated experiments have also documented that GTAs from some types of α-proteobacteria enhance larval settlement in the lab setting (McDaniel, L unpublished). It can be concluded that GTAs stimulate larval settlement as well as increase the fitness and survival of these corals in a laboratory setting. It has already been recognized that gene transfer agents are beneficial among water column microbes, coral mucus-associated microbes, and larvae associated microbes (McDaniel, L 2010 and unpublished). As well, gene transfer agents induced important transcriptional changes within the coral larvae as well as the microbial community associated with the larvae. However, the mechanism of how these changes persist is not yet understood.

In order to examine the position in the coral symbiotic relationship where this genetic alteration is occurring, isolated *Symbiodinium* culture was treated with gene transfer agents. With the purpose of pinpointing the level at which this beneficial symbiosis transpires, the presence of gene transfer agents was tested collectively with the algae as well as its *Symbiodinium*-associated microbial population. This study was performed with the hopes of gaining insight about GTA's influence on *Symbiodinium*, if any, as well as precisely understanding the extent of their impact. Due to gene transfer agent's history in coral fitness, the treatment of *Symbiodinium* cultures with active gene transfer agents is predicted to increase the metabolic activity and/or growth of the cells.

Methods

I: The Growth and Purification of the Gene Transfer Agents

One characteristic of GTAs is the fact that they are produced spontaneously by the bacterial donor strain once it enters stationary phase growth. Gene transfer agents were grown and harvested from marine α-proteobacteria donor strain *Reugeria mobilis* 45A6. This strain was chosen because it was initially isolated from a *Symbiodinium* strain, its ability to reproducibly produce GTA particles during stationary phase-growth (McDaniel, 2010), as well as observed effects on coral larval settlement. The α-proteobacterial GTA donor strain was inoculated from frozen stocks and incubated for three and half days before the viral purification was begun. At the day of maximal GTA production, the culture was centrifuged at 9,500 x G to separate the cell pellets from the viral supernatant. The lysates were further purified by passage through a Nalgene filter tower with a 0.2 µm pore size filter with gentle vacuum. RG-1 DNase and RNase One were added at 2.5µl/ml 0.1 µl/ml respectively to degrade any non-viral (non-encapsidated) nucleic acids from the sample. Sodium chloride (1 M concentration) and polyethylene glycol (10% w/v) were then added to precipitate the viral particles and left on ice overnight. The samples were again centrifuged at ultra-high speed to produce a viral pellet. A sterile Pasteur pipette was used to aspirate the supernatant to separate the PEG from the viral pellet. The samples were inverted and drained for several minutes before re-suspending the pellet in 400µL of SM buffer. The pellet was left to soak for an hour and then combined with an equal volume of chloroform in a phase-lock gel tube. The phase-lock tube was then centrifuged for five minutes at room temperature. The remaining PEG combines with the chloroform to leave the purified gene transfer agents in the top (aqueous) layer of the tube. The viral like GTA particles were then enumerated using SYBR-Gold staining with epifluoresence microscopy.

II: Heat-killed GTAs as a Control Group

Heat-killed gene transfer agents were used as a control group in this study and active gene transfer agents as a treatment group. The volume of purified GTAs were separated in half and the control group was agitated by microwaving for a few seconds until the sample tube was warm to the touch and condensation was observed forming inside the tube. For each experiment, an equal volume of gene transfer agents were added to a fixed amount of well mixed *Symbiodinium* culture. Replicate number and volume as well as GTA concentration varied for each experiment.

III: Measuring Cell Activity using an MTT Assay

In order to the test cellular viability and health of the *Symbiodinium* cells, a 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide assay (MTT) was performed. The assay, in addition to cell counts, was used to determine a cell specific activity value. This allowed for the direct comparison of metabolic activity between replicate samples within each experiment as well as between experiments. This assay is based on the conversion of a soluble terazolium salt into an insoluble formazan product within cells by the reducing activity found in actively metabolizing mitochondria (Promega Corporation 2012). This tests the samples for metabolic competence by the assessment of mitochondrial performance.

Heat-killed replicates were prepared using a hot water bath at 70°*C* for one hour. 45µL of MTT dye solution was added to each of the 300µL samples to give a blue colored formazan product when absorbed by metabolically active cells. After a three hour foil-covered static incubation period, the cells were then disrupted with 300µL of a solubilization solution and incubated for an hour in a 37°*C* shaking incubator. This allowed the assay to be read on a small volume spectrophotometer at 570 nm (Promega Corporation 2012). The spectrometer was

blanked with sterile artificial salt water. The cell specific activity values for experimental and control groups were compared to distinguish possible stimulatory effects resulting from the presence of active gene transfer agents.

IV: Light Incubations

Symbiodinium grows photoautotrophically. As a result, they synthesize everything they need using inorganic nutrients and energy gained from light exposure. Due to the *Symbiodinium's* photosynthetic abilities, the first few experiments within the study were done in a lighted, temperature controlled photosynthetic incubator. This allowed the measurement of cell growth as well as the cell specific activity of the autotrophs as they grow. Cell counts were measured at the start of the treatment on December 11th 2013 and after 48 hours on December 13th, 2013. Counts were evaluated using a hemocytometer. Lugol's solution was added to fix the samples to prevent movement and further growth. In the first light incubation, six replicates were used for each treatment. Two replicates for each of the twelve samples was measured for cell counts. The entire grid of the hemocytometer was counted with little or no dilutions needed. Cell specific activity was measured using the absorbance of the samples at 570nm as well as their corresponding cell counts. This experiment was then repeated using the six replicates of each treatment at three different time points with a new set of purified GTAs. Each sample during the 2nd light incubation contained a concentration of 2.8×10^{9} gene transfer agents (GTA's) ml^{-1}. A sample was separated for counts upon inoculation. The first measurement was taken on January 22nd, 2014 after being treated for 24 hours. The procedure was repeated the following days at 48 hours and 72 hours.

V: Dark Incubations

Dark incubations were then performed to measure the *Symbiodinium's* activity when it is not efficiently growing. The first dark incubation was performed on January 7th, 2014. Instead of a photosynthetic incubation after exposure to the treatment, the samples were incubated in a dark, temperature controlled bacterial incubator. This experiment again used six replicates for each treatment group and one measurement at 48 hours. The assay was performed in the same manner using identical quantities of reagent as well as incubation times. The 2nd dark incubation was performed including the prokaryotic protein synthesis inhibitor chloramphenicol as a second control. This control was meant as a bacterial inhibitor to measure the sole activity of the *Symbiodinium* algae. Like the treatment, these samples were treated with active GTAs. Six replicates for each of the three treatments were used and measured at 24, 48, and 72 hours. T = Zero counts were again taken upon inoculation. Chloramphenicol was used at 34µg/ml in methanol. Each of the six control samples were treated with 7µl of the inhibitor. The GTA dose for each of the samples was 2.03×10^{9} gene transfer agents (GTA's) ml^{-1}, slightly lower than the lighted experiment.

VI: *Symbiodinium* Filtrate

An MTT assay was then performed using the *Symbiodinium* bacterial filtrate in order to measure the bacteria's response to the GTA treatment without the presence of the zooxanthellae. This experiment was begun on March 4th, 2014. The samples were highly agitated using electromagnetic pulsing and as well as vortexing in order to separate the bacteria from the algal cells. The samples then were run through a 2.0 µm filter to split the *Symbiodinium* cells from their associated bacteria. The *Symbiodinium* filtrate was then inoculated with the GTAs and measured using the MTT assay as well as A+PY media petri plate counts to determine bacterial

counts for three sampling days. The full plates were counted using a 10^{-5} dilution. Twelve replicates for each treatment were plated at 24, 48, and 72 hours. Colony numbers were used in conjunction with absorbance measurements to calculate cell specific activity of the bacteria. Bacterial direct counts were performed using SYBR Gold staining. The bacterial cells were enumerated using staining fluorescence to determine what fraction of the present bacteria were culturable.

VII: Data Analysis

In order to interpret the data, the two different treatment groups were compared to another using a two-tailed t-test in order to determine whether the two sets of data were statistically different from one another. A significance value of 0.10 was used to ensure that differences within the data were not a product of chance. This test was used to compare the differences in cell counts between the treatment and control groups as well their differences in cell specific activity. Cell specific activity was measured by subtracting the heat-killed *Symbiodinium* absorbance value from active *Symbiodinium* value and dividing by the average cell count for each sample. Bacterial plate counts were measured by counting the colonies on the plate and multiplying by the dilution factor as well as the volume factor to determine colony forming units (CFUs).

Results and Discussion:

The application of active GTAs obtained from *Ruegeria mobilis* strain 45A6 to lab cultured *Symbiodinium* samples under light incubations, did not appear to have any direct effect on the metabolic activity or growth of the samples. There are little differences in growth of both of the sample types after the addition of GTAs during the first lighted incubation (Figure 1). There were also no significant differences in cell specific activity between the treatment and the control group samples (Figure 2). This evidence is supported in the repetition of the lighted incubation as shown by Figures 3 and 4. The light incubations produced similar results among the samples at three different time frames following the inoculation.

The GTAs were then applied to *Symbiodinium* culture and incubated in the dark. Due to the nature of the photosynthetic algae, the samples grew at a slower rate as expected (Figure 5). The two sample types remained similar in growth throughout the incubation time. However, the cell specific activity of the treatment samples was significantly higher than the control group replicates with a p-value of 0.099746 (Figure 6). This indicated that the GTAs were improving the fitness of the *Symbiodinium* samples. The experiment was then repeated only to produce conflicting results. During the second dark incubation, the cell specific activity of the control group was higher than the treatment at 24 hours with no differences at 48 and 72 hours (Figure 8). The growth rates of the samples were also inconsistent. The chloramphenicol-treated *Symbiodinium* counts were significantly higher than the heat-inactivated GTA control at 24 hours with a p value of 0.03089. The chloramphenicol-treated counts were also significantly higher than the active GTA treatment and control at 48 hours with p-values of 0.00431 and 0.00216 respectively.

They continued to show higher counts than the treatment group and the control group at 72 hours with p-values of 0.01338 and 0.00780 respectively. The treatment counts were significantly higher than the control at 48 hours with a p-value of 0.03109 (Figure 7). The low absorbance values of the chloramphenicol control samples shown by the MTT assay would typically suggest the chloramphenicol acted as an inhibitor for the algae as well as for its associated bacteria (Figure 8). However, the high *Symbiodinium* cell growth in the chloramphenicol controls indicate that the MTT assay was likely measuring the metabolism of the *Symbiodinium*-associated bacteria rather than the algae itself. It was hypothesized that the inconsistency in the results between the two experiments was due to the activity of the associated microbial populations. This is supported by the fact that the metabolic activity of the whole cultures continued to increase over the duration of the experiment (Figure 8) despite the fact that the *Symbiodinium* cell counts declined (Figure 7).

In order to test this hypothesis, the *Symbiodinium* filtrate was assessed in the same manner. The active gene transfer agents led to a significant increase in the metabolic activity of the bacterial fraction at 24 hours (Figure 10), although the effect did not persist through the 48 and 72 hour time periods. Interestingly, the bacterial plate counts showed lower colony counts in the GTA treated samples yet equivalent cell-specific metabolic activity (Figure 9). It is not clear whether the lower colony counts are a result of GTA treatment or separation from the *Symbiodinium* culture. The bacterial cells were enumerated using staining fluorescence to determine what fraction of the present bacteria were culturable. Direct bacterial counts via SYBR Gold staining indicated that an average of 48% of the present bacteria were capable of growing on the A+PY media petri plates. These viable but nonculturable bacteria may be influential in the overall cell-specific activity value. As a result, direct counting may be a more appropriate way to

determine cell-specific activity of the bacterial filtrate. A repetition of this experiment is needed in order to draw any further conclusions about the influence of GTAs on *Symbiodinium* associated microbial communities. It is apparent that GTAs are influencing the functioning of coral holobiont and the effect appears to be most likely on the microbial components of the system. However, the exact mechanism by which this occurs is yet to be understood.

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Tables and Figures:

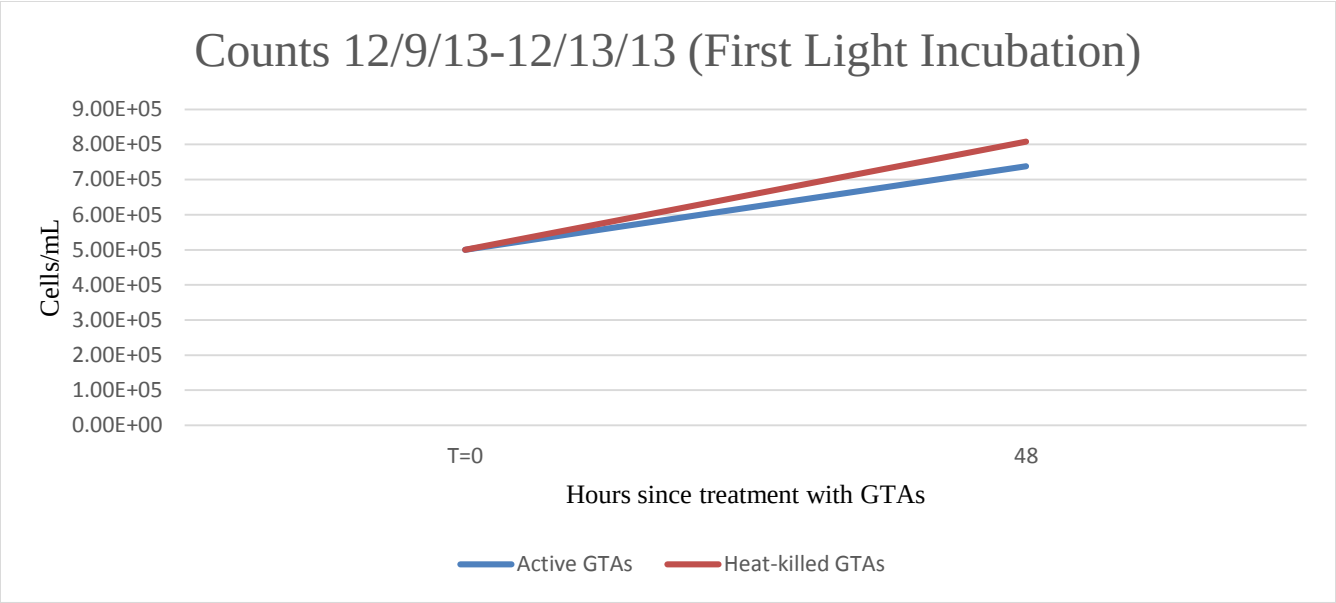

Figure 1: No significant differences in *Symbiodinium c*ell counts between the treatment and control at 48 hours during the first lighted incubation. Counts were recorded at T=0 and 48 hours after exposure to a lighted, temperature controlled photosynthetic incubator. The entire grid of the hemocytometer was counted with little or no dilutions needed.

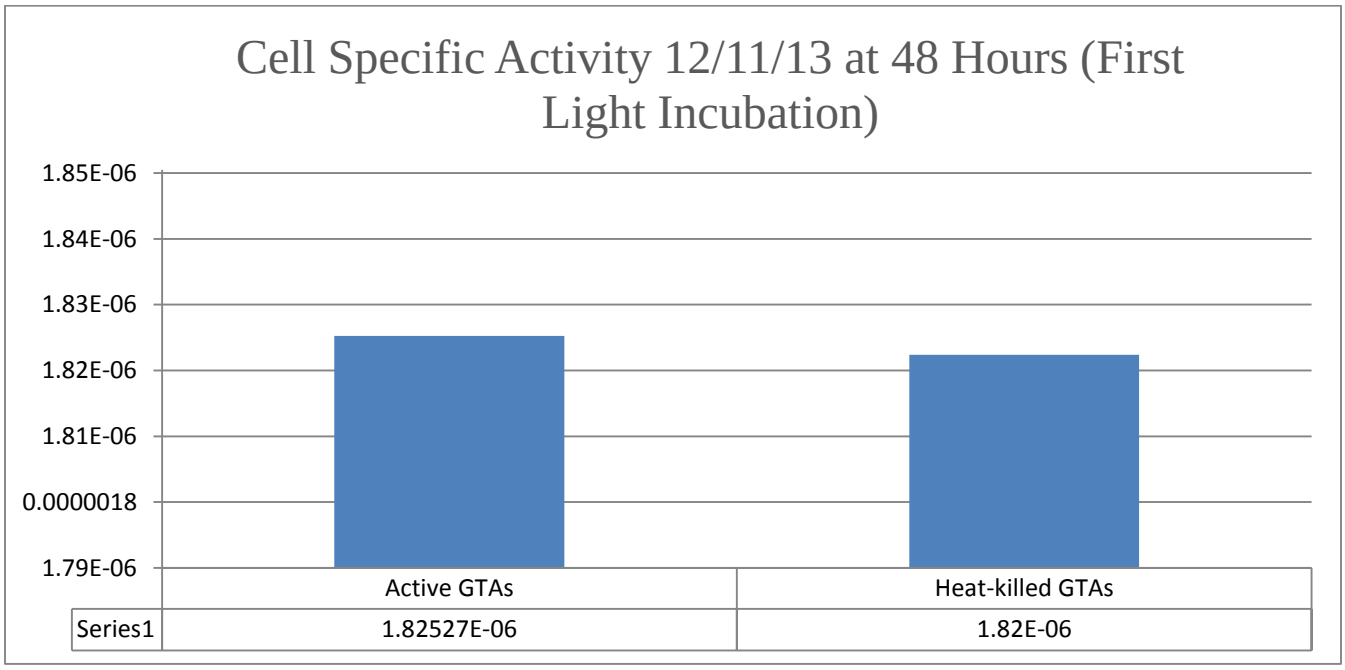

Figure 2: No significant differences in cell specific activity of *Symbiodinium* samples between active GTA treatment and heat-killed control at 48 hours during first lighted incubation. Six replicates were used and measurements were taken once at 48 hours. Cell specific activity was measured using the absorbance of the samples at 570nm as well as their corresponding cell counts.

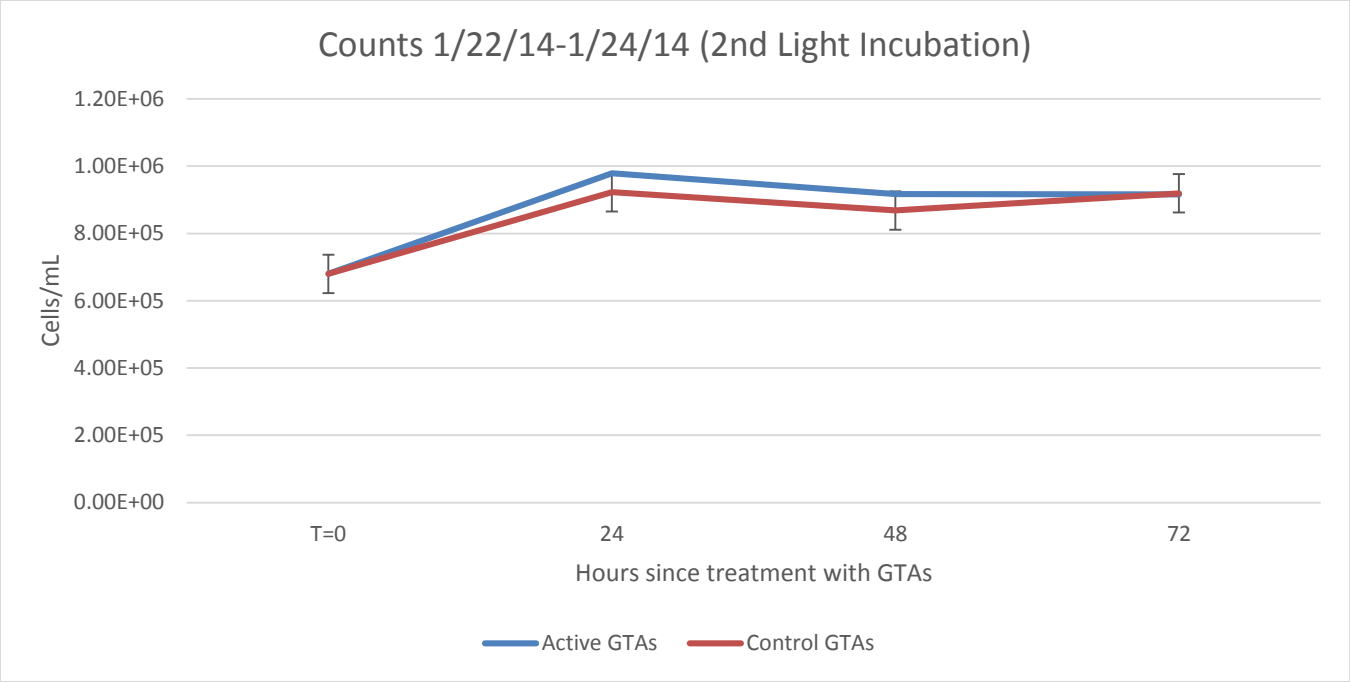

Figure 3: No significant difference in *Symbiodinium* cell counts between active GTA treatment and control at 24, 48, and 72 hours during the second lighted incubation. Counts were recorded at T=0 , 24, 48, and 72 hours after exposure to a lighted, temperature controlled photosynthetic incubator. The entire grid of the hemocytometer was counted with little or no dilutions needed.

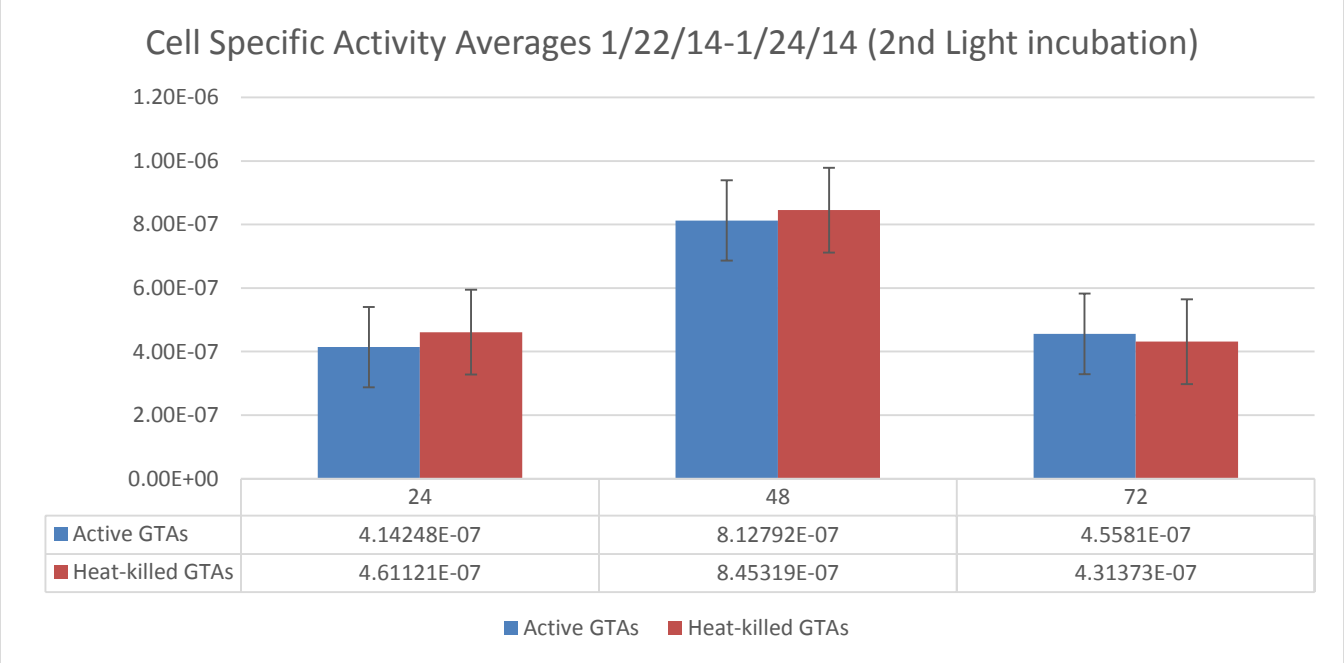

Figure 4: No significant differences in cell specific activity of *Symbiodinium* samples between heat-killed control and active GTA treatments at 24, 48, or 72 hours during the second lighted incubation. Cell specific activity was measured for each of the twelve replicates using the absorbance of the samples at 570nm as well as their corresponding cell counts.

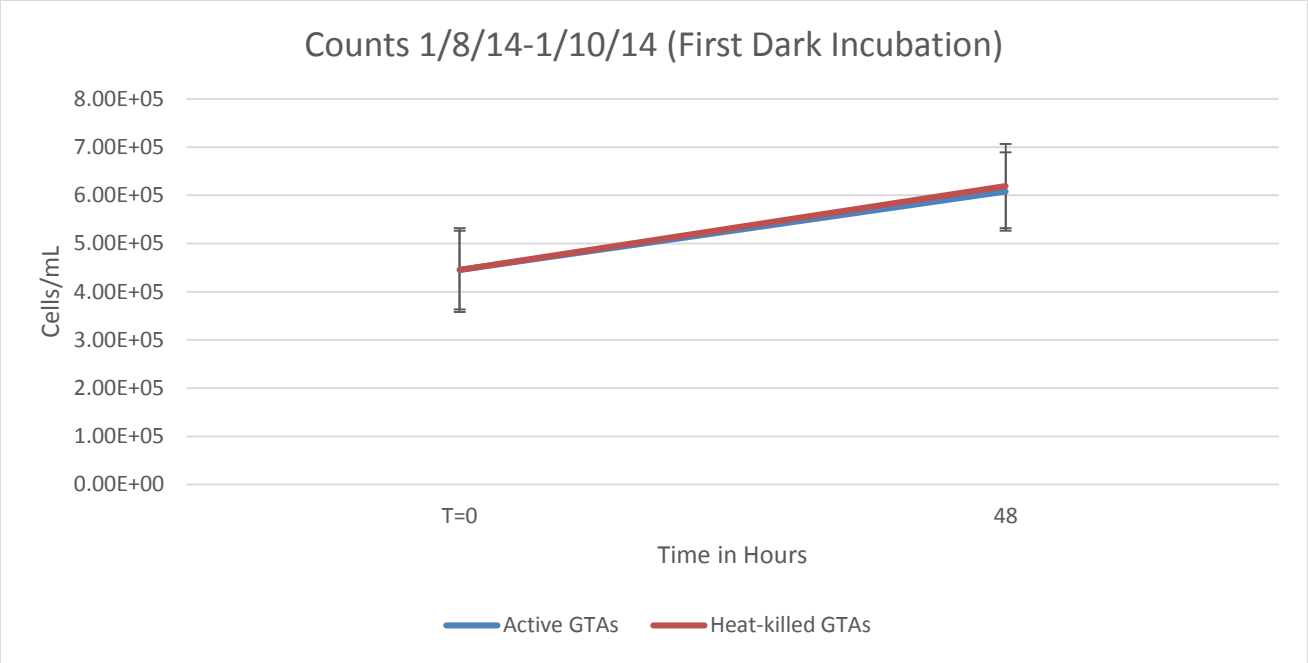

Figure 5: No significant differences in *Symbiodinium* cell counts between active GTA treatment and heat-killed control at 48 hours during the first dark incubation. Counts were taken at T=0 and 48 hours after the samples were exposed to a dark, temperature controlled bacterial incubator.

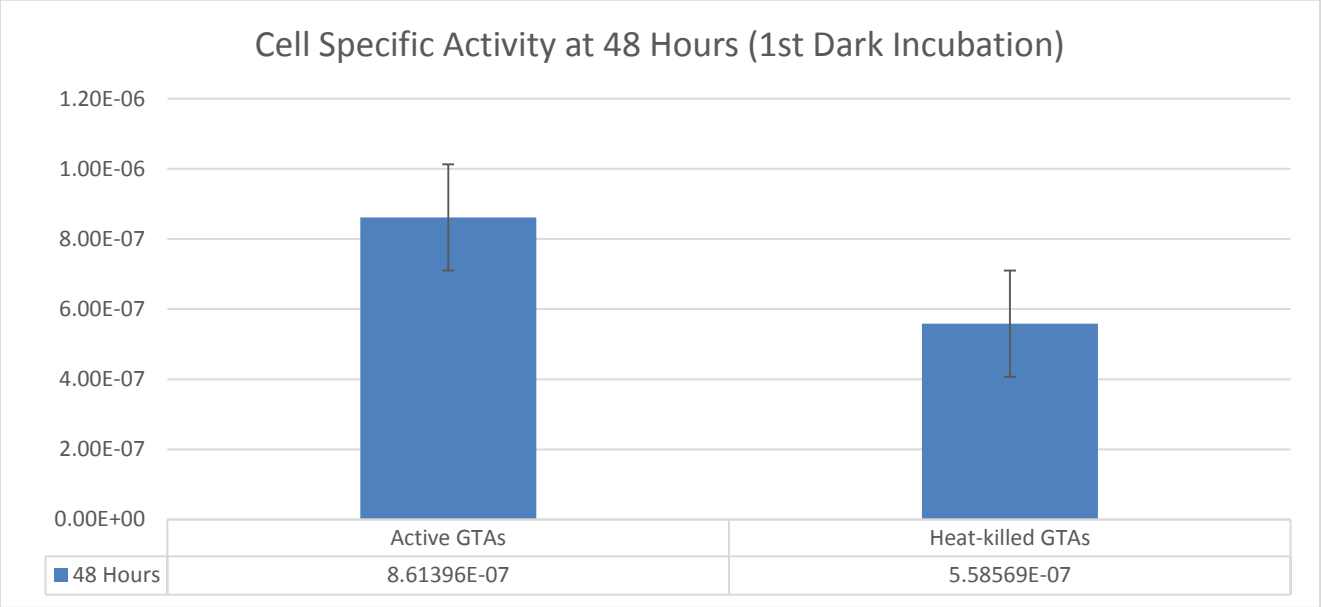

Figure 6: Active GTA treatment significantly higher than heat-killed control in *Symbiodinium* samples at 48 hours during the first dark incubation, with a p-value of 0.099746. Cell specific activity was measured for each of the twelve replicates using the absorbance of the samples at 570nm as well as their corresponding cell counts.

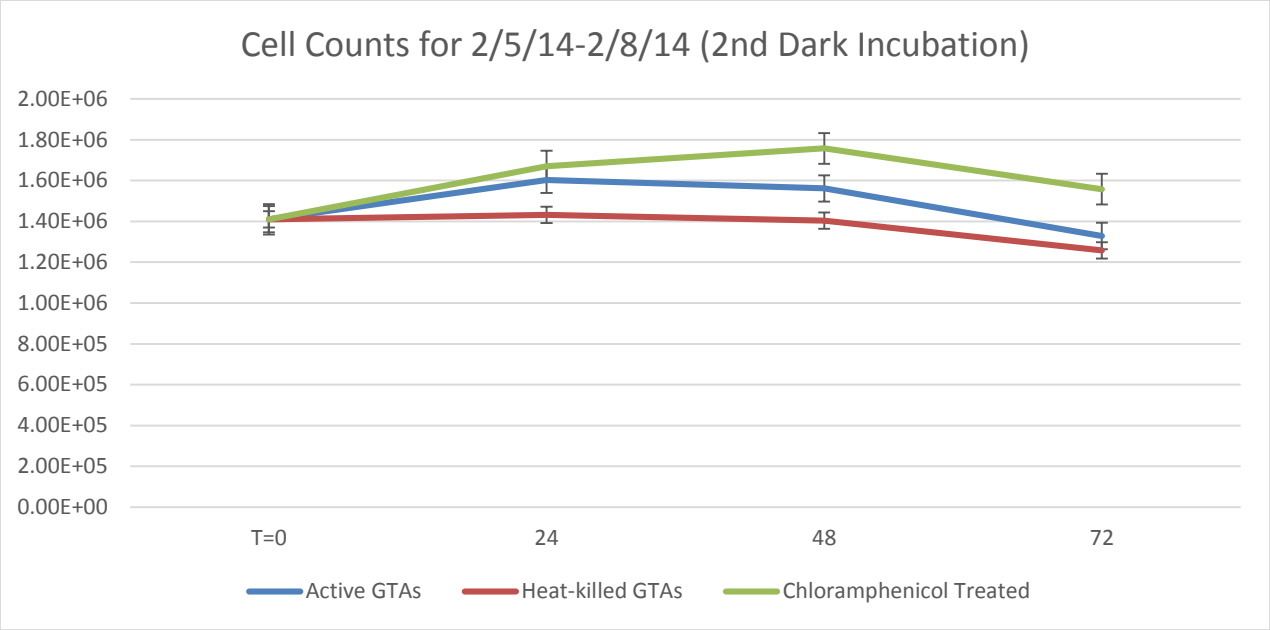

Figure 7: Chloramphenicol treated *Symbiodinium* counts are significantly higher than the control at 24 hours with a p value of 0.03089. Active GTA *Symbiodinium* counts are significantly higher than the heat-killed control at 48 hours with a p-value of 0.03109. Chloramphenicol treated *Symbiodinium* counts are significantly higher than the treatment and control at 48 hours with p-values of 0.00431 and 0.00216 respectively. Chloramphenicol treated *Symbiodinium* counts are significantly higher than the treatment and the control at 72 hours with p-values of 0.01338 and 0.00780 respectively. Six replicates for each of the three sample treatments were measured at 24, 48, and 72 hours. Chloramphenicol was used at 34µg/ml in methanol. The GTA dose for each of the samples was 2.03×10^{9} gene transfer agents (GTA's) ml^{-1}, slightly lower than the lighted experiment.

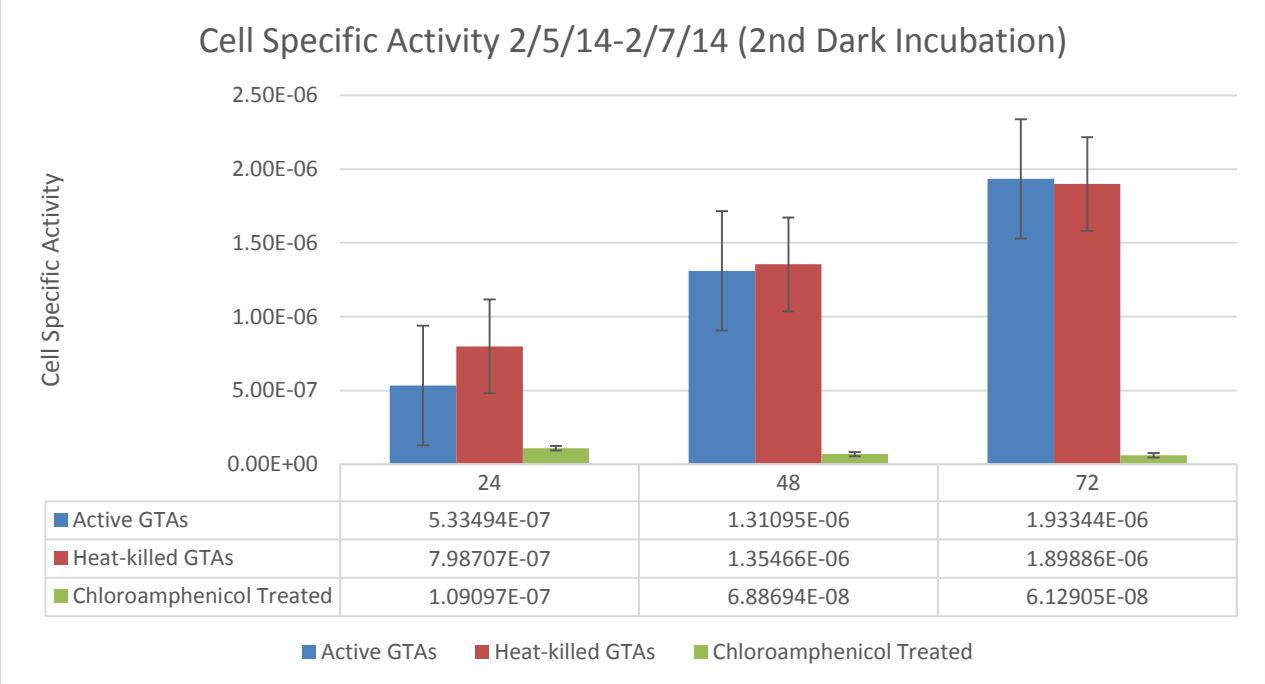

Figure 8: Heat-killed control is significantly higher than the active GTA treatment at 24 Hours in the Symbiodinium samples *with a p-value of 0.02917. Cell specific activity was measured for each of the eighteen replicates using the absorbance of the samples at 570nm as well as their* corresponding cell counts.

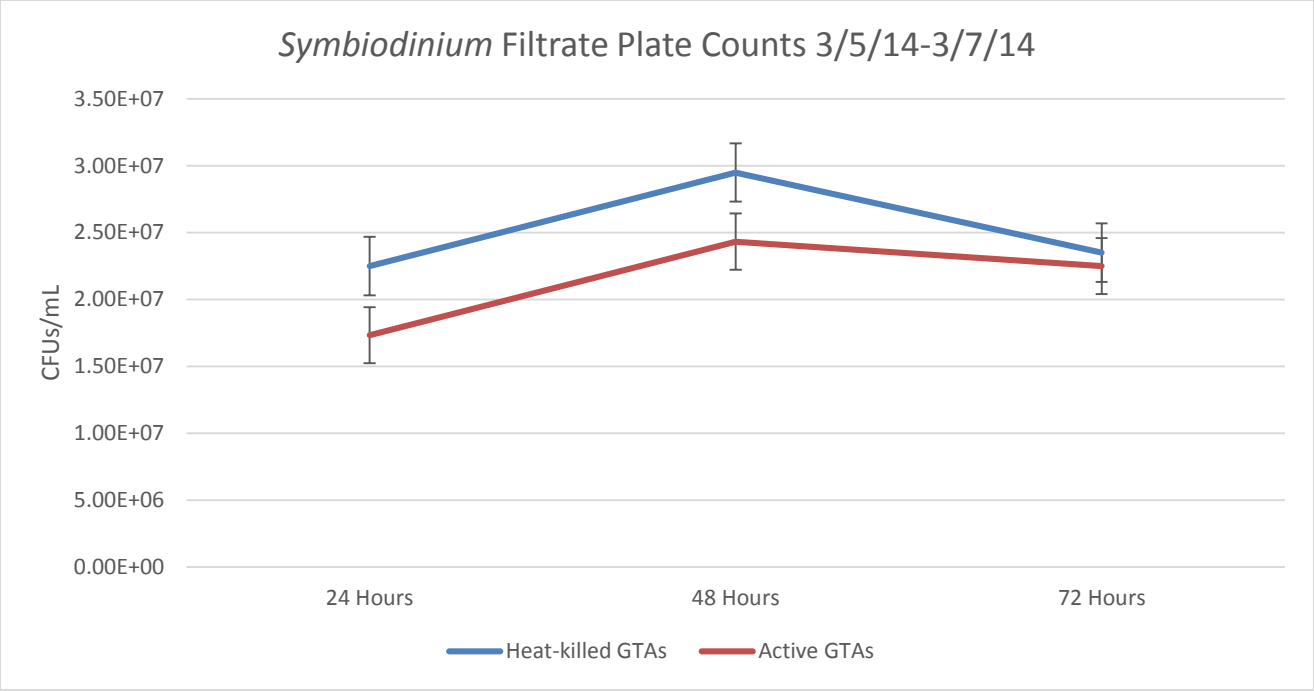

Figure 9: Heat-killed control bacterial plate counts are significantly higher than active GTA treatment in *Symbiodinium* filtrate samples at 24 Hours with a p-value of 0.07926. Heat-killed control bacterial plate counts are significantly higher than active GTA treatment in *Symbiodinium* filtrate at 48 Hours with a p-value of 0.04629. The full plates were counted using a 10^{-5} dilution.

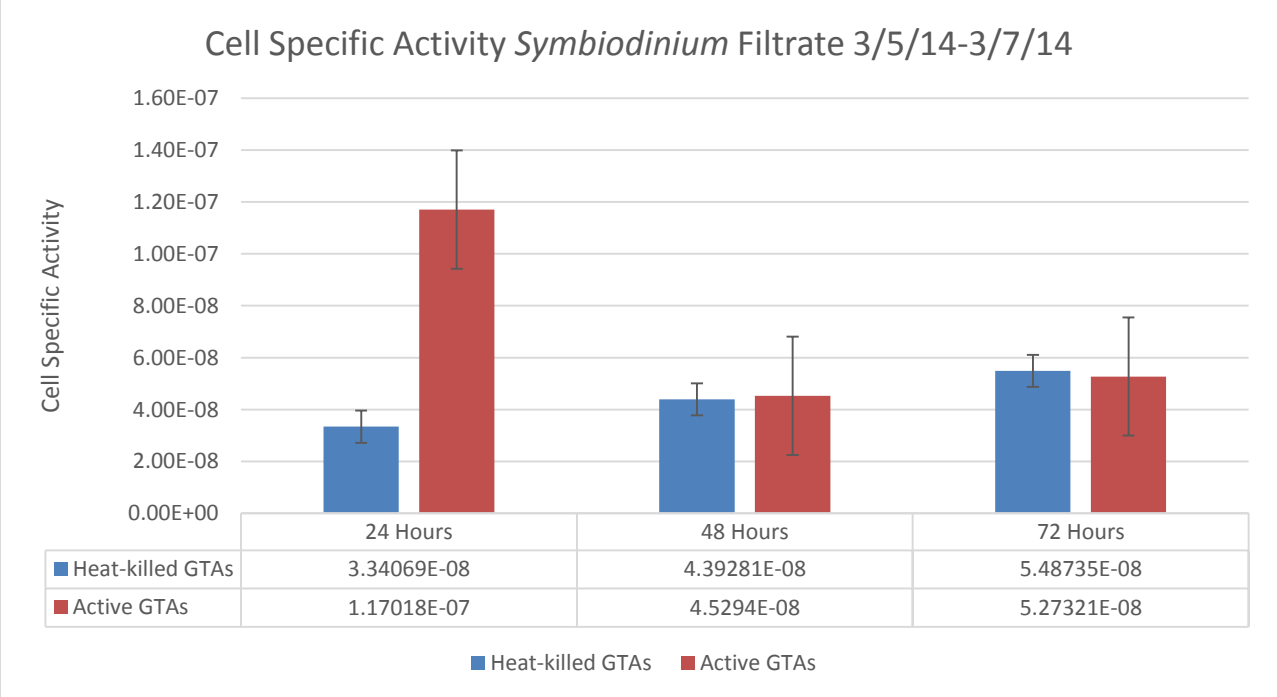

Figure 10: Active GTA treatment is significantly higher than heat-killed control in *Symbiodinium* filtrate at 24 hours with a p-value of 0.09848. Colony numbers were used in conjunction with absorbance measurements to calculate cell specific activity of the bacteria.

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