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Effects of Elevated Salinity and Oxidative Stress on the Physiology of the Toxigenic Cyanobacterium Microcystis Aeruginosa

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Effects of elevated salinity and oxidative stress on the physiology of the toxigenic cyanobacterium *Microcystis aeruginosa*

by

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CERTIFICATE OF APPROVAL

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Abstract

Harmful algal blooms (HABs) are found worldwide, particularly in places where warm, well-lit, and stagnant waters are common. HABs can have negative effects on aquatic plants and wildlife due to the reduction in light availability associated with turbidity, decrease in O_2 availability, and the production of secondary metabolites that can harm or even prove lethal. Aquatic ecosystems are regularly being affected by elevated salinity because of recent water management strategies, episodes of drought, and salt water intrusion. This research focused on how salinity levels ranging from 0-10ppt affected physiological attributes such as cellular growth and abundance, cell mortality, toxin release, and oxidative stress in the toxigenic cyanobacterium, *Microcystis aeruginosa*. It was determined that salinity treatments of 7ppt and above caused a decrease in both cellular growth and abundance, as well as an increase in toxin release due to cell mortality. *M. aeruginosa* was able to survive in salinities up to 7ppt. A pattern of caspase activity in response to elevated salinity was shown, but whether cellular mortality was due solely to programmed cell death (PCD) was not definitive. A strong antioxidant response, measured through catalase activity, was noted when salinity was enhanced to 7ppt. Above this value, the damaging effects of salinity caused elevated levels of reactive oxygen species (ROS) production and cell death. It was determined that the maximum amount of hydrogen peroxide that *M. aeruginosa* could withstand without significant impact to growth and abundance was below 250µM. Salinities of 7ppt and above had a negative impact on the physiology of *M. aeruginosa*, leading to cell death and an increase in microcystin release into the environment. These two factors can lead to fish kills, poor drinking water, and other recreational and commercial problems for an aquatic ecosystem. By determining the precise salinity that HAB

cellular mortality is imminent, predictive models can be employed to predict the impacts of salt intrusion and groundwater management.

1. Introduction

1.1 Harmful algal blooms (HABs)

Harmful algal blooms (HABS) are a natural phenomena that have significant effects on aquatic ecosystems, human health and coastal economies (Landsberg, 2002). Although coastal environments have undergone an increase of HABs over the last couple of decades, their presence and sometimes toxic effects have been known throughout history. Native tribes in America knew to not gather and consume shellfish from certain areas, such as stagnant lakes or ponds. Records kept by the early sea captain James Cook noted poisonous shellfish and the effects of water discoloration (Anderson *et al*., 2002). A review by Codd *et al.* (1999) highlighted blooms recorded as early as 1188, in particular where Geraldus Cambrensis remarked on cyanobacteria blooms in Llangorse Lake, Wales. It is now known that stagnant waters may induce algal bloom formation which can lead to discoloration and cyanotoxin production. However, that characteristic discoloration of coastal waters can be caused simply by the presence of the chlorophyll and carotenoids in the phytoplankton in which they reside, rather than by algal blooms. Also, because many shellfish are filter feeders, it stands to reason that they may bioaccumulate toxins present in the water and pass them on to the higher trophic levels.

Cyanobacterial (blue-green algal) harmful algal blooms are becoming more numerous, widespread and persistent throughout coastal ecosystems. These blooms are typically a result of nutrient-loaded and stagnant waters, as those conditions are highly conducive towards bloom formation. Reports of cyanobacterial harmful algal blooms (cyanoHABs) on a worldwide scale have increased over the past 20-30 years (Carmichael, 1992; Hallegraeff, 1993; Hayes *et al*., 2001; Burns *et al*., 2002). While this may be biased due to increased public awareness and more intensive sampling and monitoring strategies, global climatic shifts in conjunction with ensuing

anthropogenic stress have no doubt played a role in this phenomenon (Hayes *et al.*, 2001; Roelfsema *et al.*, 2001; Paul, 2008). The ecological consequences of toxic and bloom-forming species are numerous with significant impacts to human health (Ressom *et al*., 1994; Chorus & Bartram, 1999; Carmichael, 2001), aquatic organisms, and environmental ecosystems (Boesch, 2002). The fate of the compounds produced by cyanoHABs in their natural environment and potential accumulation in aquatic food webs are still largely unknown (Turner & Tester, 1997).

It is the remarkable ecophysiological adaptational capabilities of cyanobacteria that are the focus of this study. They dominate a wide variety of ecosystems, from oligotrophic oceans to hypertrophic lakes and ponds, and their blooms can stretch from polar to tropical regions (Plickney & Paerl, 1998; Stomp *et al.*, 2007). The ability to fix atmospheric nitrogen, which gives them practically an unlimited amount for biological use, gives them an advantage over other competitors, particularly since they are the only known oxygen-using phototrophs to do so (Paerl & Ustach, 1982; Knoll, 2003). Introduction of nutrients, whether anthropogenic or otherwise, that are typically limiting for them allows for massive blooms to emerge and cause drastic changes in their local ecosystem (Carpenter *et al.*, 1998). These changes include increased turbidity, odor problems, decreased light availability for macrophytes and phytoplankton and nocturnal depletion of oxygen. These negative effects can limit aquatic macrophytic growth, cause fish kills, and even create human health problems through the production of cyanotoxins (Scheffer, 1998; Izaguirre & Taylor, 2004; Uwins *et al.*, 2007).

Microcystis aeruginosa is a cosmopolitan freshwater cyanobacterium that contains a suite of toxic heptapeptides that have detrimental impacts on environmental health. *M. aeruginosa* can be found globally in freshwater, such as lakes and rivers, and the slightly brackish waters found at the entry to estuaries. The blooms they form are considered

cyanobacterial harmful algal blooms because the surface scums produced possess many of the characteristics of other cyanoHABs, such as lower dissolved O_2, taste and odor problems in drinking water, and fish kills (Carmichael, 1995). The decaying scums formed can measure up to 1m thick, contain billions of cells/ml^{-1}, and produce chlorophyll-A up to 3,000 μg l^{-1}. In turn, they lower the recreational and commercial value of any body of water they are found in. Ecologically, the toxins produced by *M. aeruginosa* blooms can impact other photoautotrophs, heterotrophs such as zooplankton, and fish through bioaccumulation of those toxins in the food web (Kotak *et al.*, 1996; Sedmak & Elersek, 2005). They also impact food quality for zooplankton and fish and can shift an aquatic community from large to smaller species of zooplankton (Fulton & Paerl, 1987; Malbrouck & Kestemont, 2006). Their potential impact on an aquatic ecosystem should not be underestimated.

Unlike some other cyanobacteria, *M. aeruginosa* cells do not contain heterocysts that some photoautotrophs require to convert atmospheric nitrogen to the nitrates needed for biological use. In order for blooms to develop, external input from nutrients such as nitrogen and phosphorus are typically required in high concentrations (Pearl *et al*., 2001). Water temperature is also important for initial bloom formation as *M. aeruginosa* is preferential towards water temperatures exceeding 20°C. Furthermore, for actual sustained growth, water bodies with a long residence time are required as well (Reynolds, 1997; Jöhnk *et al.*, 2008). The blooms tend to thrive in more stagnant waters which facilitate *M. aeruginosa's* vertical movement throughout the water column (Walsby *et al.*, 1997). The ability for *M. aeruginosa* to modify its buoyancy allows it to outcompete many other phytoplankton for sunlight (Huisman *et al*., 2004). Once the bloom is established and growing, lower CO_2 and higher pH and turbidity can help boost growth, but none of those factors are necessarily required (Shapiro, 1990).

The monocyclic heptapeptides they produce (termed microcystins) represent one of the most abundant families of hepatotoxins found in aquatic systems. To date, over 80 analogs have been characterized (CEPA, 2009). Most microcystins have a common structure that contains three D-amino acids (alanine, *erythro*-β-methylaspartic acid and glutamic acid), two L-amino acids (which are variable) and two unusual amino acids, *N*-methyldehydroalanine and a hydrophobic, 20-carbon chain, unique to these toxins, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA)**.** The toxins are named referring to the one-letter abbreviations that represent the two variable amino acids present. The dominant analogs are Microcystin: -LA, -YR, -RR, and -LR. The hepatotoxic forms of microcystins are known to inhibit protein phosphatase types 1 and 2A, both of eukaroytic origin (MacKintosh *et al.*, 1990; Yoshizawa *et al.,* 1990). This inhibition has led to deaths in humans and both wild and domestic animals due to hepatic hemorrhaging (Beasley *et al*., 1989; Pouria *et al.*, 1998).

While hepatotoxic variants of microcystins are directly associated with the deaths of fish, domestic livestock, and even human mortalities (Skulberg *et al.*, 1984; Gunn *et al*., 1992; Codd *et al*, 1999), not all *M. aeruginosa* strains produce toxins. In some bloom-specific cases, one species or strain can be morphologically identical to the next yet vary in toxicogenicity (Baker *et al.*, 2001, Lyra *et al.*, 2001). In other cases, some species are known to upregulate or downregulate their toxicity under varying laboratory conditions (Utkilen & Gjølme, 1992; Kaebernick *et al.*, 2000). It is not known why such natural variation in toxicity exists. The use of molecular probes that target toxin-associated genes, in conjunction with immunoassays, has led to advances in the identification of toxic strains (Kaebernick & Neilan, 2001; Bittencourt-Oliveira, 2003). Microcystin production has been reported to be influenced by the rates of cell division, in addition to levels of nutrients and light (Orr & Jones, 1998). However, even if a

strain of *M. aeruginosa* is found to contain toxin associated genes or low levels of cell-bound toxins, it is not exactly clear what environmental conditions may induce toxin release.

1.2 Environmental effects

Several environmental factors influence harmful algal bloom growth, such as salinity, temperature, nutrient accumulation and solar irradiation. An influx of nutrients into a body of water, whether from anthropogenic or natural sources, can promote growth in the nutrient-limited algal cells already present. Of all of the macronutrients required by freshwater photoautotrophs, phosphorus (P) is typically regarded as the "most growth limiting" since it is found in the smallest quantity (Schindler, 1997). Thus, introduction of this nutrient can lead to dramatic bloom growth. In many polar, coastal and temperate marine and estuarine environments, however, nitrogen (N) is the growth limiting nutrient for primary production (Glibert, 1988). This means the external input of nitrogen can produce the same growth effect in the slightly brackish water found at the boundary between marine and freshwater ecosystems. A combination of the two nutrients can promote growth in lower estuaries as well (Fisher *et al*., 1992). Although nitrate and phosphate-limited environmental conditions have no effect on some toxin production, such as microcystins by *M. aeruginosa*, reduction of N and P input from point and non-point sources is needed to limit algal growth in both freshwater and estuarine environments (Utkilen & Gjølme, 1995).

Aquatic ecosystems are being impacted by episodes of increased salinity in response to periods of drought, agricultural practices, water management strategies and salt water intrusion associated with climate change (Nielsen *et al.,* 2003; Kundzewicz *et al*., 2008). Salinity levels have a profound influence on fish and wildlife, aquatic vegetation, water quality and the

composition of phytoplankton communities (Mallin *et al.*, 1991; Montague & Ley, 1993). While *M. aeruginosa* is predominantly a freshwater species, several reports have demonstrated that selected strains are capable of surviving under brackish water conditions (Orr *et al*., 2004, Tonk *et al.,* 2007). The precise threshold for salt tolerance in this species likely depends upon the genotype of the strain as well as the duration and intensity of salinity exposure. Increasing demand for fresh drinking water by resident populations, irrigation, and the impact of intruding seawater near estuary thresholds can lead to many freshwater ecosystems with elevated salinities, especially during warmer months. Some countries, such as the Netherlands, have considered or implemented conversion of freshwater lakes and ponds to more brackish waters in an effort to potentially reduce growth of algal blooms (Verspagen *et al*., 2006). Very little is known, however, concerning how elevated salinity affects the cell growth and toxin production of *M. aeruginosa* and other HAB-forming cyanobacteria.

Due to the large demand on groundwater by the population of Central Florida, a current interest in the supplemental withdrawal of surface water has emerged. Most recently a request has been granted that will permit Seminole County Florida to withdraw 5.5 million gallons from the St. Johns River system on a daily basis (SJRWMD, 2008). Predictions state that 262 million gallons day $^{-1}$ can be removed from the headwaters in the near future (SJRWMD, 2008). In conjunction with the Seminole county water withdrawal, plans are currently under development to deepen the navigation channel of the lower St. Johns River basin which will drastically enhance salt water intrusion (SJRWMD pers. comm.). While preliminary salinity modeling has been undertaken, the potential effects of salt water intrusion and sea level rise on coastal ecosystems of the St. Johns River are not known. Due to several physical features of the St. Johns River (SJR; Northeast Florida, United States), selected regions are regularly exposed to

bouts of elevated salinity. The large tidal amplitudes yield semidiurnal tidal exchanges of 4250 $m^3 s^{-1}$. Tide signatures can be detected up to 200 km upstream. Because of the large tidal exchanges, habitats in the lower estuary can experience salinity changes of 12ppt, or more, over several hours. It is not understood how shifts in salinity could impact the physiology, population densities and toxin release of harmful cyanobacterial species.

Exposure to elevated NaCl has been reported to inhibit CO_2 fixation in photoautotrophs, which subsequently causes the disruption of electron flow in electron transporting processes (e.g. photosynthetic transport chains) (Moller, 2001). When these biochemical pathways become uncoupled, electrons that have a high-energy state are transferred to molecular oxygen (O_2) to form reactive oxygen species (ROS) (Mittler, 2002). The buildup of ROS, such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) can damage a number of intracellular targets including proteins, lipids, and nucleic acids leading to the physiological failure of the organism (Halliwell, 2006; Lesser, 2006). Each of the three oxygen-reduction intermediates have varying toxicities, reactivities, and potential targets. The unpaired electron in superoxide anions and hydroxyl radicals make them particularly reactive with biomolecules. Through the Fenton reaction, H_2O_2 and $O_2^{\bullet -}$ can produce a hydroxyl radical that can create lesions in DNA, effectively making the molecules mutagens (Imlay, 2003).

Most photoautotrophs, including cyanobacteria, have the capacity to eliminate potentially destructive ROS via enzymatic antioxidant mechanisms. (Fig. 1.1). Non-enzymatic components include small molecules such as tocopherol, ascorbic acid, carotenoids and glutathione. The predominant antioxidant enzymes include catalase, superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase (Apel & Hirt, 2004, Latifi *et al*., 2009). When oxidant levels in a cell

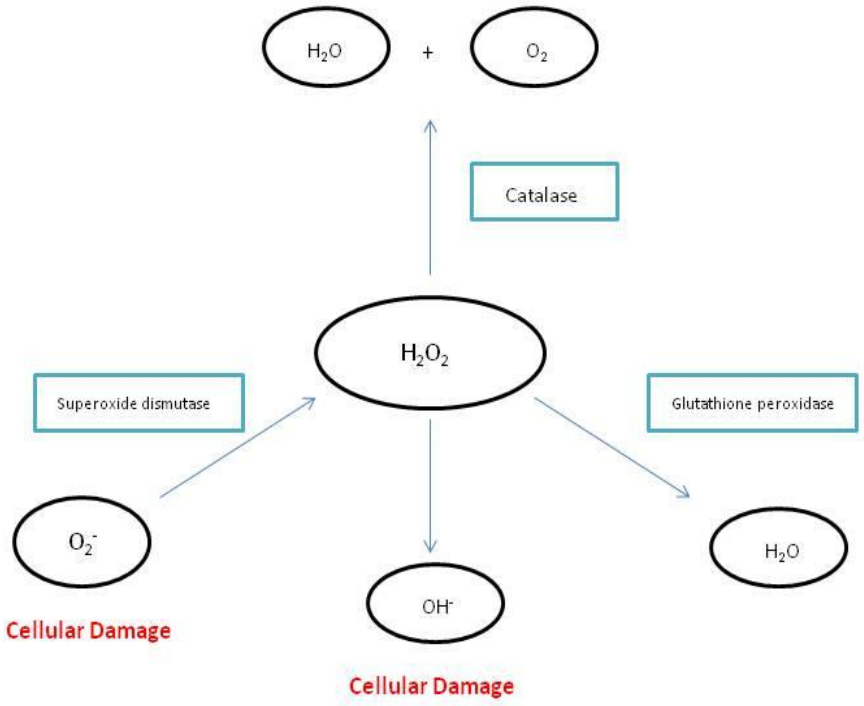

Figure 1.1 Degradation of reactive oxygen species (ROS) by antioxidant enzymes

overwhelm its antioxidant capabilities, however, the oxidative stress produced can cause serious damage or even cellular death. Antioxidant capabilities have been found in nearly all subcellular regions of plant tissue, highlighting the importance of ROS detoxification for cellular survival (Choo *et al.*, 2004; Mittler *et al*., 2004). In the case of cyanobacteria, the cells not only must compensate for oxidative stress produced through typical oxygen reduction, but also through the electron transport chain in photosynthesis. This must be done in part by preventing electron escape during electron transfer to oxygen.

The effects of ROS overproduction in relation to salt stress (as toxic byproducts and as intricate signal transduction molecules) have been well studied in agriculturally relevant and model plant species (Miller *et al*., 2010). Conversely, studies examining the antioxidant responses in toxic cyanobacteria,
particularly in response to salt stress, are much more limiting. As *M. aeruginosa* encounters water of elevated salinity, blooms may terminate abruptly or they may adapt and persist. Few studies have addressed the cellular mechanisms by which *M. aeruginosa* adapts to changing environmental challenges associated with hypersalinity exposure and how this relates to toxin release into the environment.

1.3 Programmed cell death (PCD)

As adverse environmental conditions start to overwhelm the capacity for the organism to adapt (i.e. hypersalinity exposure for extended duration), cell death will be eminent. While numerous studies have examined the environmental conditions and mechanisms that drive phytoplankton bloom formation, there is much less work characterizing the cellular mechanisms that drive bloom collapse. It is important to understand these regulatory processes in

phytoplankton in light of their pivotal roles with respect to primary production, biogeochemical cycling and negative effects on coastal ecosystems. Recent work has shown evidence for the activation of an autocatalytic cell death mechanism, in both eukaryotic and prokaryotic phytoplankton representatives, in response to selected environmental stressors such as nutrient deprivation, cell age, temperature, light intensity and oxidative stress (Berges & Falkowski, 1998; Berman-Frank *et al*., 2004; Ross *et al*., 2006; Bidle and Bender, 2008; Bouchard & Purdie, 2011). This cellular self-destruction mechanism is analogous to programmed cell death (PCD) or apoptosis found in higher eukaryotes. PCD is a genetically controlled form of cell suicide that is essential for the proper function and ultimate survival of organisms. It is critical for the regulation of cellular homeostasis by selectively eliminating certain individuals in a population following sublethal cellular damage. The selective advantage of PCD may be population benefit (i.e. eliminate damaged cells from a population and simultaneously supply surviving cells with organic nutrients). The causes and biochemistry of cell death, and the quantitative significance of cell death in the ecology of cyanobacterial populations are not well understood. A better understanding of cyanobacterial cell death will potentially provide insight into bloom development and population regulation.

The overall goal of this research was to understand how elevated salinity influences the physiology, bloom dynamics and toxin release of *M. aeruginosa*. Research objectives were: 1) to experimentally grow *M. aeruginosa* under varying salinities to determine the effects on cell growth, photochemical efficiency and toxin release into the environment; 2) to determine if elevated salinity induces oxidative stress which in turn may trigger PCD; and 3) to determine if *M. aeruginosa* has the capability of upregulating an antioxidant response to counteract the negative effects associated with exposure to enhanced salinity.

2.

Elevated salinity and oxidative stress promotes programmed cell death in the toxigenic cyanobacterium, *Microcystis aeruginosa*

Abstract

This study examined the effect of elevated salinity on the physiology, growth dynamics, toxin release, and antioxidant response of the cyanobacterium, *Microcystis aeruginosa*. It was found that elevated salinity had a negative impact on cellular abundance and growth, but had little effect on photochemical efficiency. Cellular mortality increased with elevated salinity, which caused an increase in microcystin release into the surrounding environment. Caspase activity increased as well as a function of salinity, although production stabilized for all salinities by 10 days. ROS production and SYTOX-positive cells were most pronounced in the 10ppt salinity treatment. The antioxidant response and oxidative damage, assessed via catalase activity and lipid peroxidation, respectively, were highest at 7ppt. It was likely that the truncated growth rate at the 7ppt treatment was in part due to that oxidative cellular damage. Cells subjected to 10ppt treatments were unable to withstand the osmotic stress and therefore cellular mortality occurred. Overall, increasing salinity to 7ppt and above negatively impacted the cyanobacteria's ability to grow and caused cell mortality, an increase in both lipid hydroperoxides and antioxidants, and an increase in toxins released into its immediate environment.

2.1 Introduction

Harmful algal blooms, in particular cyanobacterial harmful algal blooms (cyanoHABs), are ubiquitous and their negative impacts on humans, animals, and plants are well known

(Oberholster, 2004). These blooms are sensitive to water temperature and nutrient input, and as a result are becoming more numerous worldwide due to climate change and the increase in nutrients from both natural and anthropogenic sources (Elliot *et al*., 2006). CyanoHABs are considered harmful because they produce toxins that can harm or kill plants and wildlife. Some toxins produced by cyanoHABs are cyclic peptides such as microcystins and nodularins. Alternatively, other common toxins such as saxitoxins, cylindrospermopsins, and anatoxin-A fall into the alkaloid family. In addition to toxins, cyanoHABs cause hypoxia, odor, an increase in water column turbidity, and a decrease in the availability of light for both phytoplankton and macrophytes such as seagrasses. The toxins also tend to accumulate in filter feeders and can be passed on to higher trophic levels.

In these experiments, the cyanobacterium *M. aeruginosa* was used based upon its ubiquitous presence in fresh and slightly brackish waters worldwide. In addition, some strains produce microcystins, a family of monocyclic heptapeptides that can be lethal to plants and wildlife alike (Smith & Gilbert, 1995; Rohrlack *et al*., 2005). *M. aeruginosa* also produces surface scums that can cause fish kills due to O_2 depletion, poor drinking water taste, and lower recreational and commercial value of the water it resides in. This species thrives in warm, eutrophic, stagnant water with high exposure to light, which is found in abundance in the southern US (Coles & Jones, 2000; Wiedner *et al.*, 2003). Florida, in particular, is known for both industrial and residential run-off, warm and stagnant water, and changes in salinity in rivers that flow into the Atlantic Ocean or the Gulf of Mexico.

It is the effect of those salinity changes that these experiments focus on. Removal of groundwater feeding into a river as well as tidal changes downstream can cause dramatic changes in the salinity of a given section of water. Because cyanobacteria, and *M. aeruginosa* in particular,

produce toxins that can be released upon death, it was of interest to determine what level of salinity causes cell death and the associated release of toxins into the surrounding water. The overall goal of this research was to quantify the effects of elevated salinity on chlorophyll-A content, photochemical efficiency, oxidative stress, toxin release and cell death.

2.2 Materials and Methods

2.2.1. General culture maintenance

Microcystis aeruginosa (strain LB 2385) was obtained from the Culture Collection of Algae at the University of Texas at Austin. Upon arrival to UNF, the cyanobacteria was seeded into four 125-mL Erlenmeyer flasks containing 80mL of standard BG-11 medium at 23°C. These stock culture flasks were grown under a 12:12 light:dark (L:D) cycle at an irradiance of 53 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. Cultures were transferred to fresh media every 3-4 weeks. Cultures were periodically swirled to promote growth and to prevent the formation of surface aggregates.

2.2.2. Cellular growth, photochemical efficiency and toxin release

To evaluate the effects of elevated salinity on *M. aeruginosa*, samples of stock cultures that reached the mid-exponential phase were distributed into twenty 125mL autoclaved Erlenmeyer flasks which, in turn, were exposed to selected salinity treatments (n=5/treatment). The following salinity treatments were used: 1.4ppt (control [standard BG-11]), 3ppt, 7ppt and 10ppt. In all treatments, 10mL of cell culture was mixed with 40mL of BG-11 media that was previously supplemented with the appropriate concentration of salt (Instant Ocean Reef Salt; Blacksburg,

VA, USA). Salinity of the media was determined using a YSI meter. All replicates were randomized and grown under light and temperature conditions listed above for a period of 11 days for cellular abundance and photochemical efficiency. In a separate experiment, using the same experimental design described above, levels of extracellular microcystins were quantified over the course of 7 days. Cultures were manually stirred once per day. At each sampling time point, 50µL of each sample was preserved in Lugol's for fixing and preservation for cell counts. An additional 1 ml and 20µL was taken for chl-A readings and photochemical efficiency measurements, respectively.

Cellular abundance

Chlorophyll A content was measured with a laboratory fluorometer (Turner Designs Trilogy 7200; Sunnyvale, CA, USA) using standard techniques (Parsons *et al*., 1984; Lee *et al*., 1994). Cell counts were performed on Lugol's fixed samples using a hemocytometer and Leica DM500 microscope (Wetzlar, Germany). To calculate cell growth for each treatment, the following equation was used: $\mu = \mathrm{Ln}\,(N_T - N_0) / (\Delta t)$. N_0 represents the population cell density at the initial time interval, N_T represents the cell density at the end of the time interval, and Δ is equivalent to the length of the time interval in days ($t_t - t_0$).

Photochemical efficiency

Photochemical efficiency of *M. aeruginosa* was examined using pulse-amplitude modulation (PAM) fluorometry (Diving-PAM; Walz, GmbH, Germany). Changes in dark adapted maximal quantum yield [Fv/Fm = (Fm-Fo)/Fm'] of PSII (Genty *et al*., 1989) were assessed with this method. Twenty microliters of sample was gently pipetted directly on to the tip of the fiber-optic

cable. Both intensity and gain was set to ‘‘8.’’ All samples were dark adapted for 15 minutes prior to analysis.

Microcystin quantification

To quantify the levels of microcystins at selected time points, an Enzyme-Linked Immunosorbent Assay (ELISA) was employed. The Envirologix© Competitive ELISA kits (Portland, ME, USA) for the quantification of Microcystin-LR and congeners was used as per the manufacturer's instructions. Toxin concentrations were measured using a Thermo Scientific Biomate 3 spectrophotometer (Madison, WI, USA). Samples were diluted, as needed, in order to accommodate the ELISA’s assay range of 0.16 to 2.5 $\mu g \cdot L^{-1}$ Microcystin-LR.

2.2.3. Reactive oxygen species production and programmed cell death

The probe 2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA; Invitrogen, Carlsbad, CA, USA) was used to measure intracellular ROS levels in the cyanobacteria over a 10-day period. This probe is a nonfluorescing, nonpolar compound. When this compound reacts with cellular esterases, the diacetate group is cleaved off to yield the polar compound DCFH. Oxidation of DCFH by ROS yields the fluorescent product DCF. Following the selected salinity treatments, cells were washed in PBS (0.01M, pH 7.4), and DCFH-DA was added to the cells at 5 μM (final concentration) from a 5 mM stock solution in DMSO. Cells were allowed to incubate in the dark at room temperature for 30 minutes, then were centrifuged at 7,000xg for 30 seconds. After the supernatant was poured off, the cells were resuspended in 100μL of DI water. Cells were then viewed and photographed using an Olympus epifluorescence microscope (Center Valley, PA, USA) equipped with fluorescein isothiocyanate (FITC) and Texas Red filter sets (Ex: 465–495

nm; Em: 515–555 nm and Ex: 540-580 nm; Em: 600-660 nm) and a digital camera (DP 21; Olympus). Three replicates per sample and five fields of view per replicate were used for analysis. All cells were counted using NIH ImageJ software (Bethesda, MD, USA). ROS-positive cells (green) were compared to the total cell count (red associated with chlorophyll) to determine the percentage of ROS-positive cells.

Cellular mortality

To assess percent cell death as a function of salinity exposure, a 10 day time course experiment was run using the methods of Bouchard & Purdie (2011). Briefly, cell death was quantified using SYTOX-green (Invitrogen), a nucleic-acid-specific stain that only passes across the plasma membrane of dead or compromised cells, staining the DNA green under blue-light illumination. SYTOX-green working stocks were prepared in Milli Q water (to a final concentration of 50 μM). Two μL of the SYTOX-green working stock was added to 1.0mL of culture (final concentration of 0.1μM) and incubated in the dark at room temperature for 30 min. Cells were centrifuged at 7,000xg for 30 seconds then resuspended in 100μL of DI water. Three replicates per sample and five fields of view per replicate were used for analysis. SYTOX-positive cells were observed and photographed using an Olympus epifluorescence microscope (Center Valley, PA, USA) equipped with fluorescein isothiocyanate (FITC) and Texas Red filter sets (Ex: 465–495 nm; Em: 515–555 nm and Ex: 540-580 nm; Em: 600-660 nm) and a digital camera (DP 21; Olympus). SYTOX-positive cells (green) were compared to the total cell count (red associated with chlorophyll) to determine percent cell mortality.

Caspase activity

To evaluate the effects of salinity and time on the initiation of programmed cell death, *M. aeruginosa* was incubated in an independent 10 day experiment and assayed for caspase proteolytic activity. Following incubation, *M. aeruginosa* cells were centrifuged at 6,600 × g for 5 min at 4 °C on a Beckman TJ-6 centrifuge, resuspended in 50uL cell lysis buffer, then homogenized with a Fast-Prep 24 (MP Biomedicals; Santa Ana, CA, USA). The supernatant was collected and protein concentration was quantified with a BCA Protein Assay Kit (Pierce Biotechnology; Rockford, IL, USA) according to the manufacturer's instructions. The Enzchek® Caspase-3 Assay Kit #2 (Invitrogen) was utilized to quantify programmed cell death activity in *M. aeruginosa*. This assay exploits the specific proteolytic cleavage of the amino acid sequence Asp–Glu–Val–Asp (DEVD). Aliquots (1 ml) of the supernatant was combined with 990 µl of 1× reaction buffer (Caspase-3 Assay Kit #2, Invitrogen) and 10 µl Z-DEVD-R110 substrate (final substrate concentration, 25 µM). Samples were incubated at room temperature for 25 min and subsequently assayed for the appearance of the caspase-catalyzed fluorescent cleavage product Rhodamine-110 on a FLx 800 fluorescent microplate reader (Biotek)(Ex/Em: 496/520 nm; N = 5). The reversible aldehyde caspase inhibitor Ac-DEVD-CHO (Invitrogen) was used as a negative control.

2.2.4 Oxidative stress and antioxidant response assays

As a function of salinity level and time, the activity of the antioxidant enzyme catalase was evaluated using the Amplex Red Catalase Assay Kit (Life Technologies; Carlsbad, CA, USA) as per the manufacturer's instructions. In addition, to assess ROS-induced cell damage, lipid

peroxidation levels were quantified using the Lipid Hydroperoxide Assay Kit from Cayman Chemical (Ann Arbor, Michigan, USA) as per the manufacturer's recommendations. Finally, to quantify levels of H_2O_2 production in response to elevated salinity, the Peroxoquant Quantitative Peroxide Assay Kit (Pierce Biotechnology) was used as per the manufacturer's instructions. All assay values were normalized by protein content.

To assess the damaging effects of ROS on *M. aeruginosa* growth, samples were incubated with selected concentrations of H_2O_2 over the course of 4 days. Chlorophyll-A content was measured with a laboratory fluorometer (Turner Designs Trilogy 7200) using standard techniques (Parsons *et al*., 1984; Lee *et al*., 1994). A working solution of 5mM H_2O_2 was used to create treatments of 0, 250, 500, and 750µM of H_2O_2. Cell counts were performed on Lugol's fixed samples using a hemocytometer and Leica DM500 microscope (Wetzlar, Germany). Photochemical efficiency for the hydrogen peroxide treatments was determined using the methods listed above.

2.2.5 Statistical analysis

Data for chlorophyll-A content, photochemical efficiency, cellular abundance, microcystin, ROS, and SYTOX content were all analyzed using two-way repeated measures ANOVAs. Mauchly's test of sphericity was used to assess sphericity in repeated-measures ANOVAs, and Greenhouse-Geiser estimates of sphericity were used to correct degrees of freedom for data that violates Mauchly's test. The purpose of Mauchly's test of sphericity was to ensure the variances of the differences between all possible pairs of groups were equal. Degrees of freedom were corrected since violation of sphericity can lead to over- or underestimation of significance. In cases where significant interactions were found in the repeated measures ANOVAs, one-way ANOVAs were

performed on salinity treatments at each respective time point. Following the one-way ANOVAs, a Tukey's post hoc test was conducted to determine significant groupings. One-way ANOVAs were performed on data for caspase, catalase, lipid hydroperoxides, and hydrogen peroxide treatments. The data was normally distributed as determined by the Shapiro-Wilk test. All statistical analyses were conducted using IBM SPSS Statistics 20 (IBM Corp.; Armonk, NY, USA).

2.3 Results

2.3.1 Elevated salinity decreases cellular growth and increases toxin release

Elevated salinity had a significant impact on the chlorophyll-A content found in *M. aeruginosa* over time, and an interaction was found between salinity and time (Fig. 2.1, two-way repeated measures ANOVA, $F = 84.428$, $p = 0.000$). Mauchly's test indicated that the assumption for sphericity had been violated, X^2 (27) = 188.013, $p = 0.000$, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.231$). Cellular chlorophyll-A, and in turn cellular growth, increased in the control and 3ppt treatments over the 11 day period. In the 7ppt treatment, chlorophyll-A content did not increase as quickly as the control and 3ppt treatments. Cellular death was observed in the 10ppt treatment, starting from day 3 and decreasing throughout. By the end of the experiment, there was no significant difference between the control and 3ppt treatments ($p = 0.866$). However, those groups contained significantly higher chl-A content compared to the 7 and 10ppt treatments (Fig. 2.1, one-way ANOVA, $F = 90.863$, $p = 0.000$).

Increasing water salinity appeared to have an impact on the photochemical efficiency of *M. aeruginosa* from day 3 to day 6, but there was no significant difference among the salinity treatments by the end of the experiment (Fig. 2.2). Mauchly's test indicated that the assumption for sphericity had been violated, X^2 (27) = 53.327, p = 0.000, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = 0.482). An interaction between salinity and time was found (two-way repeated measures ANOVA, F = 20.738, p = 0.000). The control and 3ppt treatments had very similar growth and decline curves for Fv/Fm, suggesting water with a salinity of 3ppt did not compromise the cells' photochemical efficiency over the time span monitored in this study. Conversely, the 10ppt treatment was negatively affected between day 3 and day 4. Afterwards, it began to slowly recover until the end of the experiment. After an initial dip in Fv/Fm in the first 24 hours, the 7ppt treatment showed a relatively consistent Fv/Fm value throughout the experiment. There was a statistically significant difference between the 10ppt treatment and the other three treatments between day 4 (one-way ANOVA, F = 97.658, p = 0.000) and day 9 (one-way ANOVA, F = 16.494, p = 0.000). By the end of the experiment, however, there was no significant difference among the four salinity treatments for photochemical efficiency (one-way ANOVA, F = 2.122, p = 0.138).

Elevated salinity had a significant effect on the cellular growth and total cell count of *M. aeruginosa*, but an interaction was found between salinity and time (Fig. 2.3, two-way repeated measures ANOVA, F = 29.063, p = 0.000). Mauchly's test indicated that the assumption for sphericity had been violated, X^2 (27) = 112.086, p = 0.000, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = 0.322). Starting cell concentrations had mean values of ~9.35×10^6 cells/mL. As shown in the experiment with chl-A and photochemical efficiency, the control (BG-11 only) and 3ppt treatments had similar mean

cellular growth rates (1.63 and 1.62, respectively) with the control treatment finishing with a slightly higher total number of cells. The 7ppt treatment had a lower cell growth rate of 1.52 and finished the 11-day experiment at a third of the control and 3ppt cell counts. There was an initial dip in total cell count between day 3 and day 4, but the cells recovered until day 7 where the growth reached a plateau. The 10ppt treatment had the only negative cellular growth rate in this experiment, ending with -1.47 as the mean. This led to the initial 10ppt mean total cell count of $9.35x10^6$ cells/mL decreasing to $9.7x10^5$ cells/mL. By the end of the experiment, there was no significant difference between the control and 3ppt treatments (one-way ANOVA, $F = 130.996$, $p = 0.931$). Both 7ppt and 10ppt treatments were significantly different from the control ($p = 0.001$ and 0.000, respectively) and each other ($p = 0.000$).

The amount of microcystins released was affected by an increase in salinity (Fig. 2.4). An interaction between salinity and time was found (two-way repeated measures ANOVA, $F = 4.520$, $p = 0.003$). Mauchly's test indicated that the assumption for sphericity had been violated, $X^2 (20) = 49.028$, $p = 0.001$, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.414$). Prior to 48 hours into the experiment, the control and 3ppt treatments were not significantly different from each other (one-way ANOVA, $F = 5.359$, $p = 1.000$) and the 7ppt treatment ($p = 0.508$ and 0.487), but they did differ from the 10ppt treatment ($p = 0.36$ and 0.34). There was no significant difference between the 7ppt and 10ppt treatments ($p = 0.268$). The highest levels of microcystins were released by the 10ppt treatment, then 7ppt, followed by 3ppt and control until the end of the experiment. Differences were greatest at day 6, where the 10ppt treatment had a mean of 6.74 ppb, 7ppt showed a mean of 5.63 ppb, and the 3ppt and control groups had means of 5.33 and 5.1 ppb, respectively. By the end of

this 7-day experiment, however, there was no significant difference among all four salinity treatments ($p = 0.290$).

2.3.2 Elevated salinity increases reactive oxygen species (ROS) production

An increase in salinity had a relatively dramatic affect on ROS production in *M. aeruginosa* (Fig. 2.5). An interaction was found between salinity and time (two-way repeated measures ANOVA, $F = 9.451$, $p = 0.000$). Mauchly's test indicated that the assumption for sphericity had been violated, X^2 (5) = 24.430, $p = 0.000$, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.804$). All salinity treatments started out with 0% ROS detected on day 0. After three days, the 10ppt treatment had a mean of 7.65% of total cells containing detectable ROS, making it significantly different from the other three salinities (one-way ANOVA, $F = 5.357$, $p = 0.003$). There was no difference among the 3ppt, 7ppt, and control treatments at that time point. This difference between 10ppt and the other salinities increased starting at day 6 ($F = 49.002$, $p = 0.000$), but it was found that the 7ppt treatment also differed from the control and 3ppt treatments. The 10ppt treatment still had the highest ROS mean of all salinities by the end of the experiment, although the difference was smaller ($F = 23.440$, $p = 0.000$). The greatest abundance of ROS positive cells were detected at day 6 associated with the 10ppt treatment. H_2O_2 levels associated with that treatment were calculated to be 1.839×10^{-5} umol/ug^{-1} protein.

In the SYTOX experiment, dramatic cell death was not evident until day 10 (Fig. 2.6). An interaction existed between time and salinity (two-way repeated measures ANOVA, $F = 12.720$, $p = 0.000$). Mauchly's test indicated that the assumption for sphericity had been violated, X^2 (5) = 29.970, $p = 0.000$, therefore degrees of freedom were corrected using Greenhouse-Geisser

estimates of sphericity (ε = 0.781). There was no significant difference in the SYTOX-positive means among the four salinities on the first day ($p = 0.086$).

By the third day, the 10ppt treatment had significantly lower numbers of SYTOX-positive cells compared to the other salinities ($p = 0.000$). This trend reversed for the higher salinity treatments by day 6 ($p = 0.000$) and remained that way until the end of the experiment ($p = 0.000$). There was a very large difference in the mean percentage of SYTOX-positive cells in the 10ppt treatment (11.43%) when compared to the lower salinities. These data did not show whether the cell death was caused naturally due to osmosis or through PCD, only that there were dead cells in the culture.

Elevated salinity caused a significant increase in caspase activity as a function of time (Fig. 2.7). By day 3, the 10ppt treatment possessed a significantly higher amount of caspase activity when compared to the lower salinities (one-way ANOVA, $F = 20.532$, $p= 0.000$). This trend continued until day 10, where there was no significant difference in enzyme activities among the 3, 7, and 10ppt treatments ($F = 5.003$, $p = 0.012$). Caspase activity in the control decreased over the ten day period, as did the 3ppt treatment until the last day. The 7ppt and 10ppt treatments both had relatively high caspase activity means throughout the entire experiment, suggesting that the elevated salinity induced enhanced caspase activity.

2.3.3 Elevated salinity induces an antioxidant response to salt stress

Increasing salinity appeared to have mixed results on catalase activity in *M. aeruginosa* over the ten day period (Fig. 2.8). Catalase activity remained relatively low until day six. At that point,

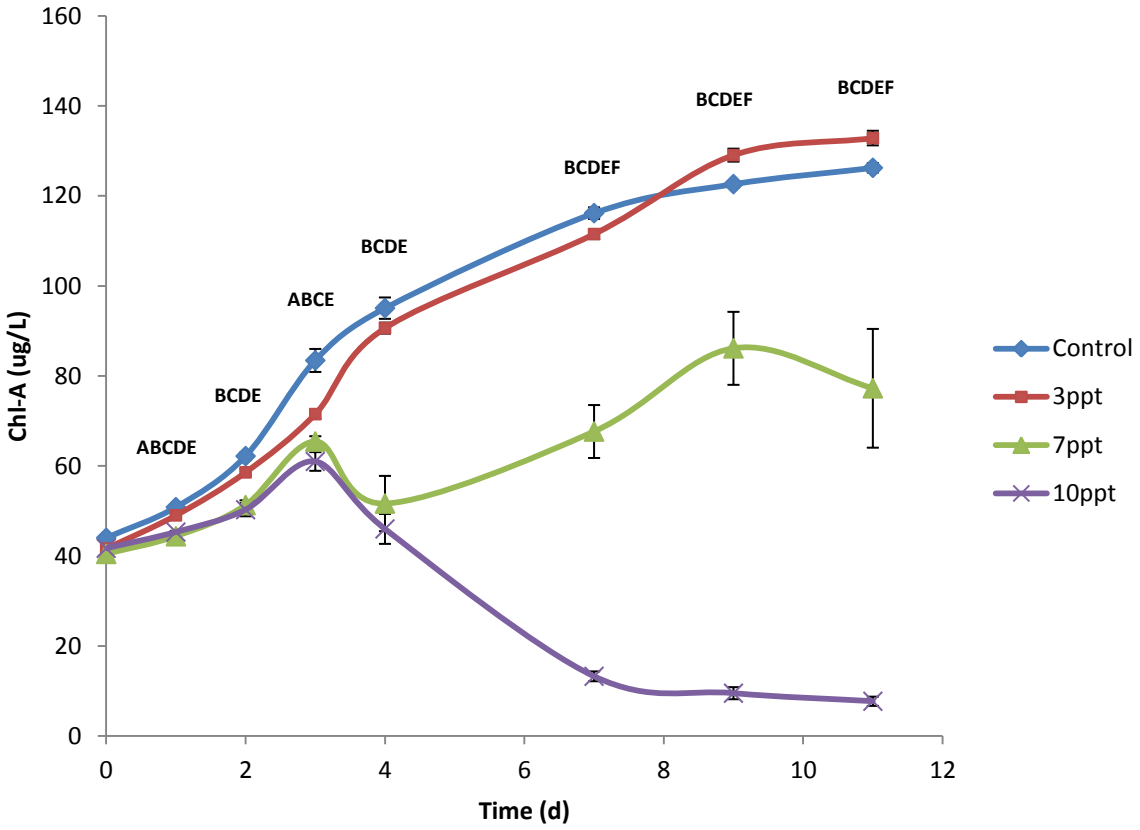

Figure 2.1 Effect of increasing salinity on chlorophyll-A in *M. aeruginosa*. Cell cultures were subjected to four salinity treatments and chl-A concentrations were used to determine cellular growth over a 11-day experiment. Data points are the mean of 5 replicates (±1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

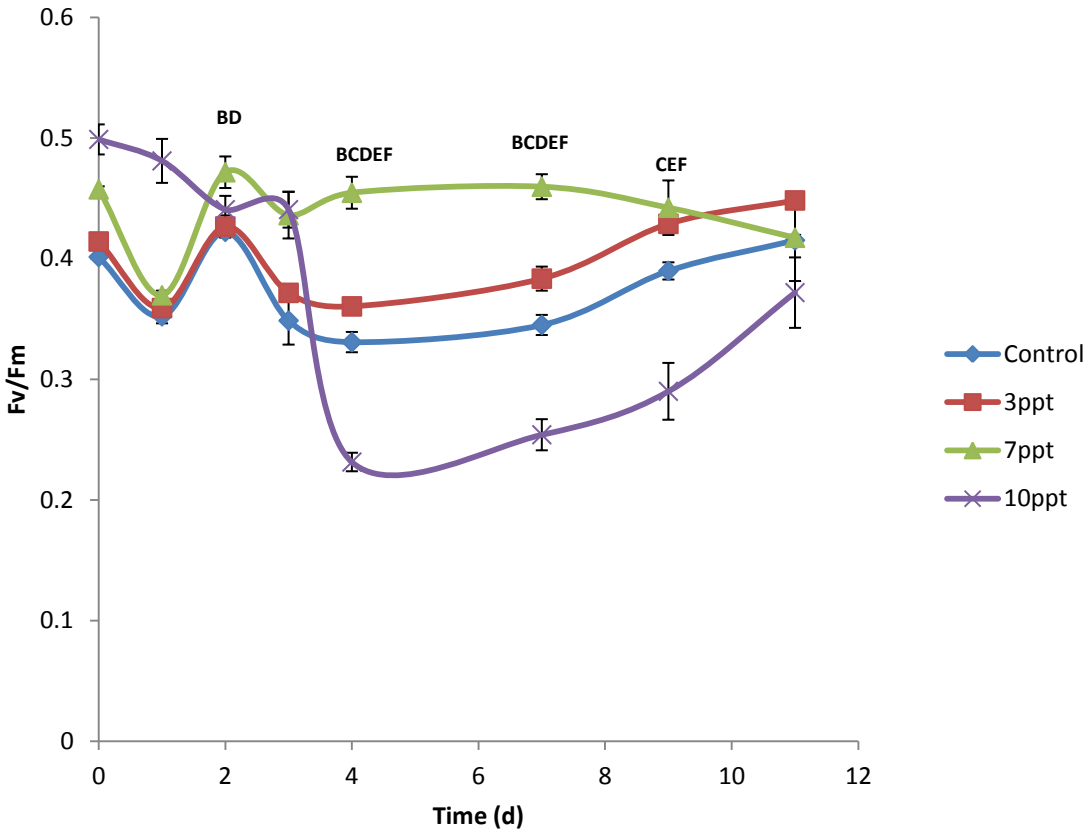

Figure 2.2 Effect of elevated salinity on the photochemical efficiency of *M. aeruginosa*. Cell cultures were subjected to four salinity treatments to determine how the photochemical efficiency of *M. aeruginosa* was impacted over a 11-day experiment. Data points are the mean of 5 replicates (± 1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

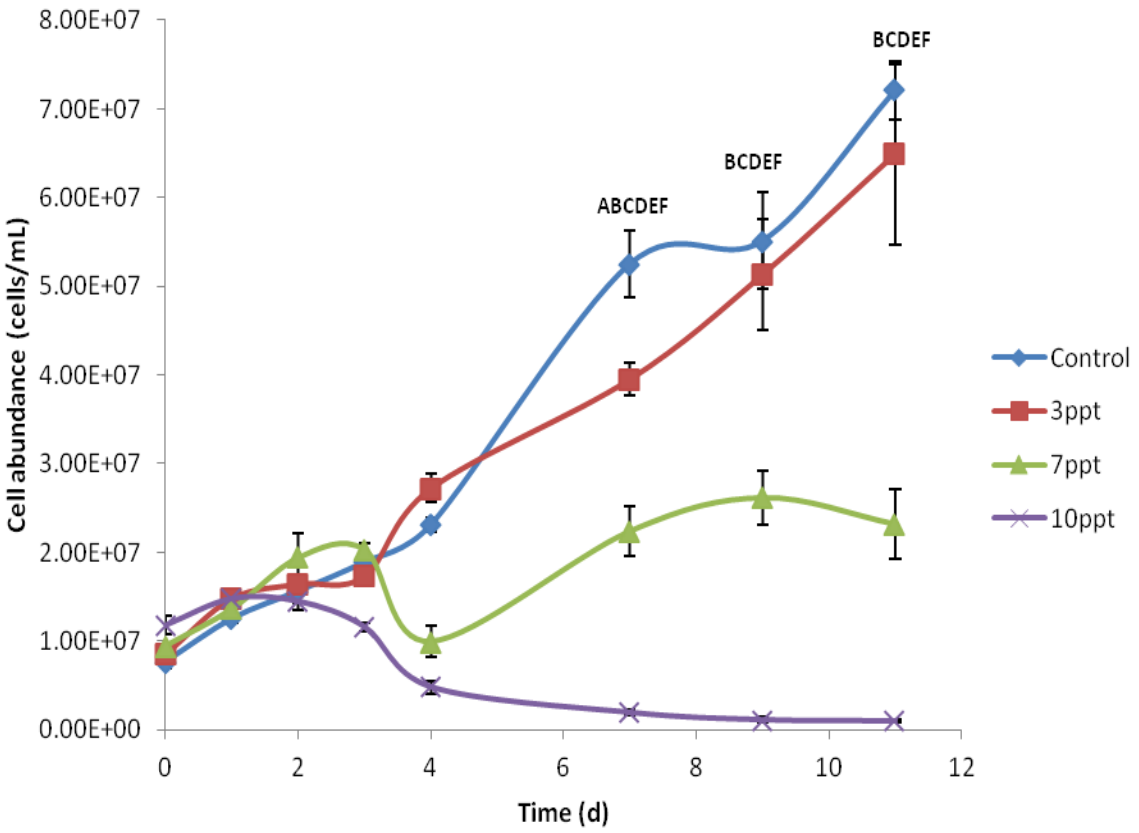

Figure 2.3 Effect of increasing salinity on cell abundance of *M. aeruginosa* over an 11-day period. Cell cultures were subjected to varying salinities to determine if cellular abundance is impacted by higher salinities. Data points are the mean of 5 replicates (±1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

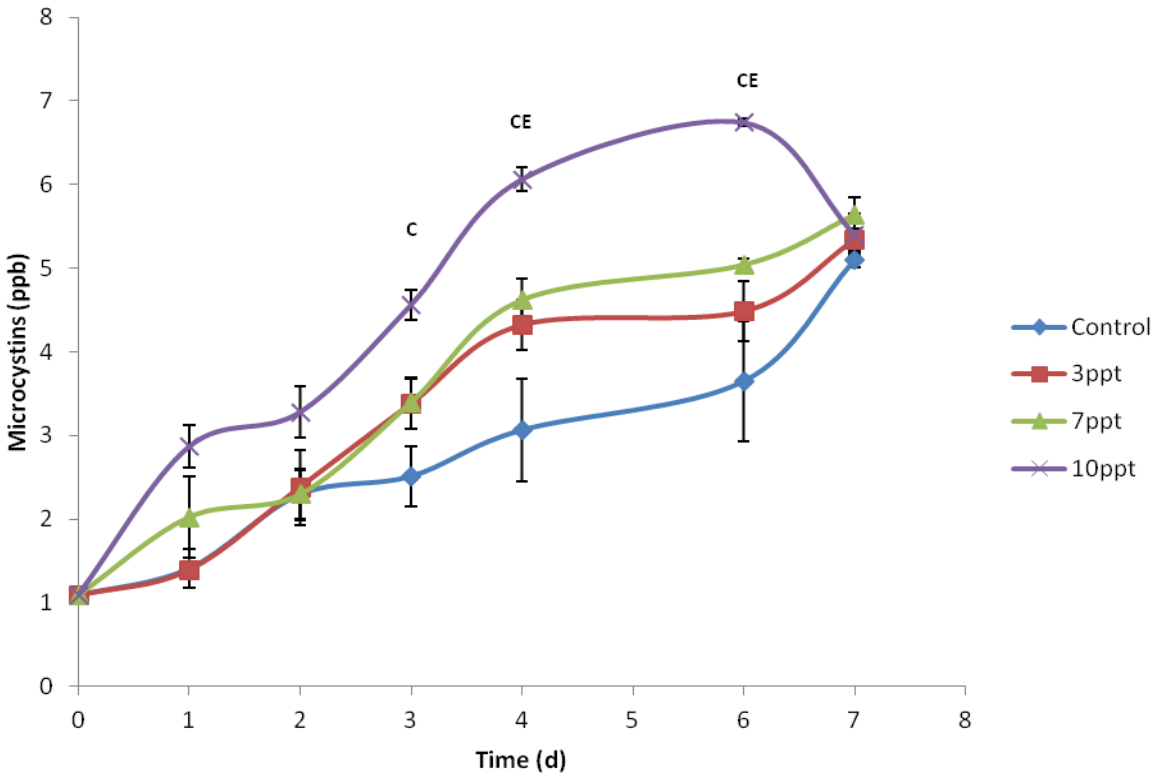

Figure 2.4 Effect of elevated salinity on microcystins released from *M. aeruginosa* over a 7 day period. Cell cultures were exposed to varying salinities to determine if elevated salinity led to increasing extracellular microcystin concentrations. Data points are the mean of 5 replicates (± 1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

the catalase activity in the 7ppt treatment was significantly higher than the other salinities (one-way ANOVA, $F = 9.960$, $p = 0.001$). The gap between the amount of catalase in the 7ppt treatment and the amount found in the other treatments increased by day 10 ($p = 0.000$). Catalase activity in the 7ppt treatment was much greater than the 3 and 10ppt salinities.

Similar results were found in the levels of lipid peroxidation in *M. aeruginosa* (Fig. 2.9). The third day of the experiment yielded a significantly large disparity in lipid hydroperoxide content among the 7ppt treatment and the three other salinities (one-way ANOVA, $F = 34.070$, $p = 0.000$). This trend continued throughout the 10 day experiment. By the end, the 7ppt treatment had a significantly higher amount of lipid hydroperoxide content in the cells compared to other treatments ($p = 0.003$). After the first day, the control and 3ppt treatments had lower amounts of lipid hydroperoxides in their cells, signifying that they were able to handle the salinity-induced oxidative stress. This also happened in the 10ppt treatment where the opposite was expected. The amount of lipid hydroperoxides in the 7ppt treatment started high and stayed relatively stable at that concentration until the end, meaning that perhaps the amount produced overwhelmed the cells' ability to break it down.

Exposure to exogenous hydrogen peroxide had a significant impact on chlorophyll-A content in *M. aeruginosa* over a 4-day period (one-way ANOVA, $F = 108.583$, $p = 0.000$). Deviation from the control was noted beginning on the first day, and that downward trend continued until the end of the experiment (Fig. 2.10). By day 3, there was no significant difference among the 250, 500, and 750µM treatments, but all three significantly differed from the control ($p = 0.000$). Hydrogen peroxide began to have a significant impact on chlorophyll-A content starting with

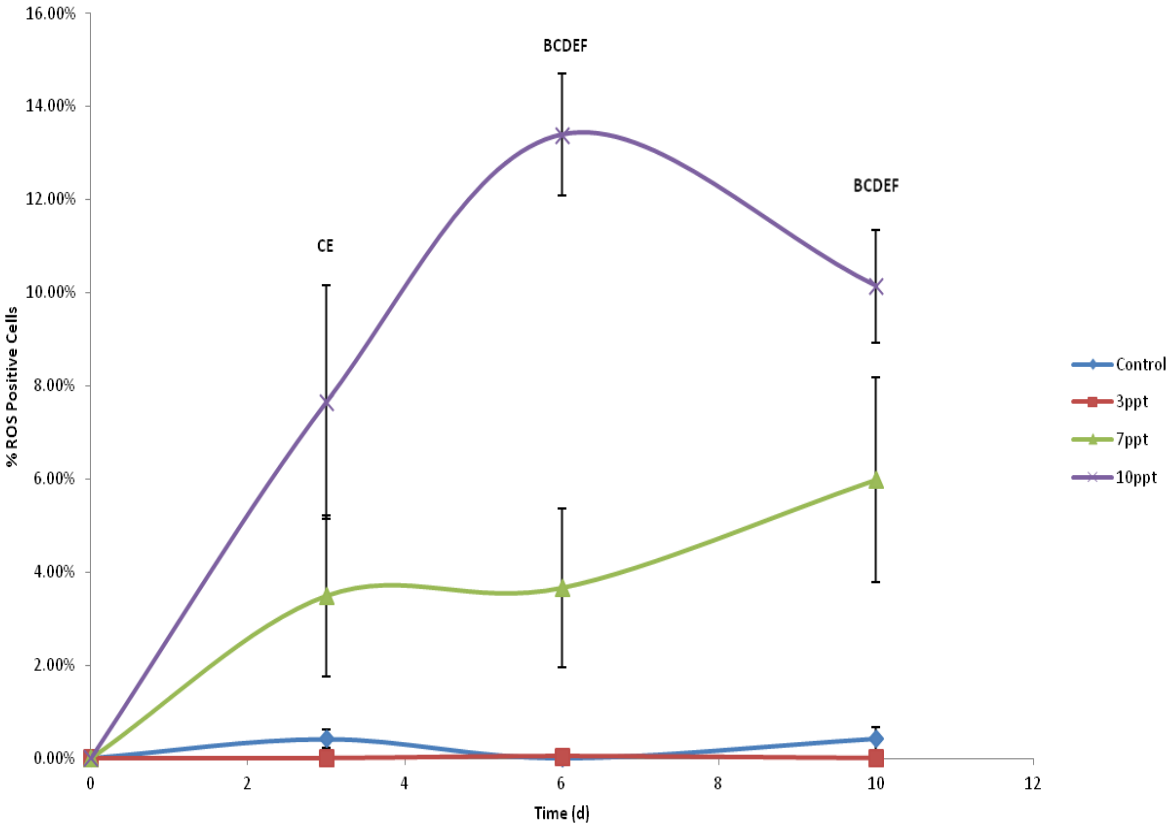

Figure 2.5 Effect of increasing salinity on ROS production in *M. aeruginosa* over an 10-day period. Cell cultures were exposed to varying salinities to determine if elevated salinity induces intracellular ROS production. Data points are the mean of 3 replicates using 5 fields-of-view per replicate (±1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

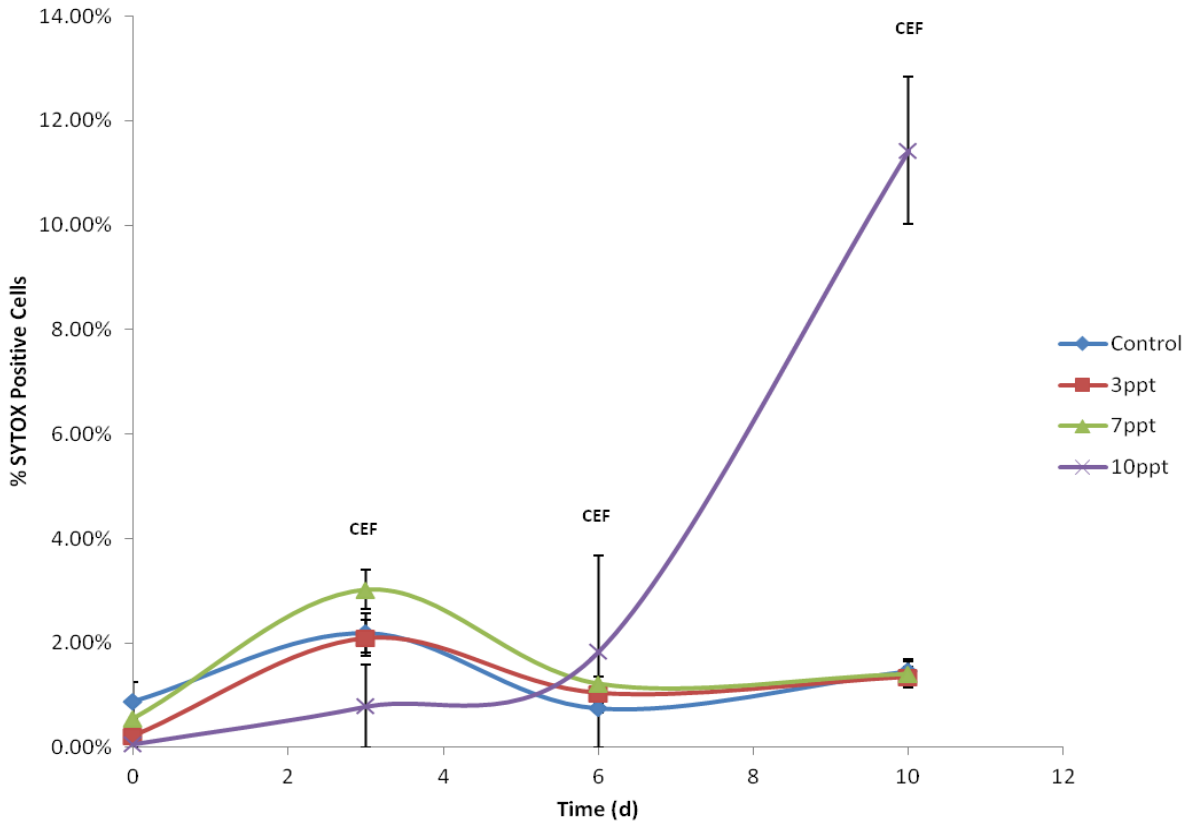

Figure 2.6 Induction of cell death by elevated salinity in *M. aeruginosa*. Cell cultures were exposed to varying salinities to determine if higher salinities induce cellular mortality. Data points are the mean of 3 replicates using 5 fields-of-view per replicate (± 1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

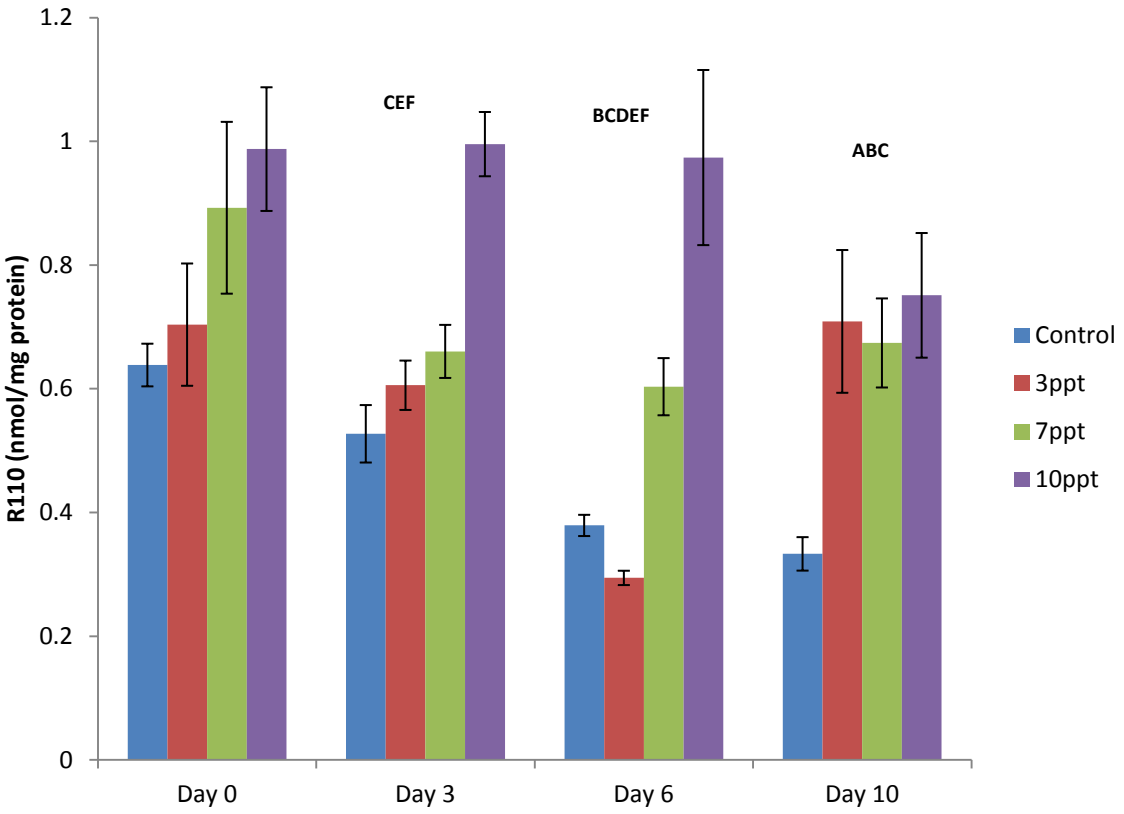

Figure 2.7 Effect of increasing salinity on caspase activity in *M. aeruginosa* over a 10-day period. Cell cultures were exposed to varying salinities to determine if elevated salinity led to increasing caspase activity, which is an indicator of PCD. Data points are the mean of 5 replicates (±1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

250µM after 1 day of incubation. A previous experiment using 0, 1, 10, 100, and 1000µM of hydrogen peroxide showed that there was no effect on chlorophyll-A (or photochemical efficiency) below 100µM prior to day 4. Therefore, it was determined that 100µM-250µM was the threshold range for impact.

Increasing hydrogen peroxide also had a significant effect on photochemical efficiency in *M. aeruginosa* over the same 4-day period as the chl-A experiment (one-way ANOVA, $F = 15.482$, $p = 0.000$). The Fv/Fm values for the control treatment remained relatively stable throughout the experiment, while the 250µM treatment steadily decreased from day 0 until day 4, where the Fv/Fm value was calculated as zero (Fig. 2.11). Fv/Fm values for the 500µM treatment decreased after day 0 and stayed low through the experiment but never reached the zero mark. Fv/Fm values for the 750µM treatment stayed low and stable until day 3 where they reached zero.

As with the chl-A experiment, hydrogen peroxide had an impact on photochemical efficiency starting with 250µM after 1 day of incubation. Cell counts were performed on the chl-A and Fv/Fm experiment. The number of cells for day 0 was calculated to be 3.6×10^6 cells/mL. The control finished the 4-day experiment at 1.1×10^7 cells/mL. Final cell counts for 250, 500, and 750µM H_2O_2 were 9.2, 4.8, and 4.8×10^{-5} cells/mL, respectively.

2.4 Discussion

2.4.1. Elevated salinity decreases cellular growth and increases toxin release

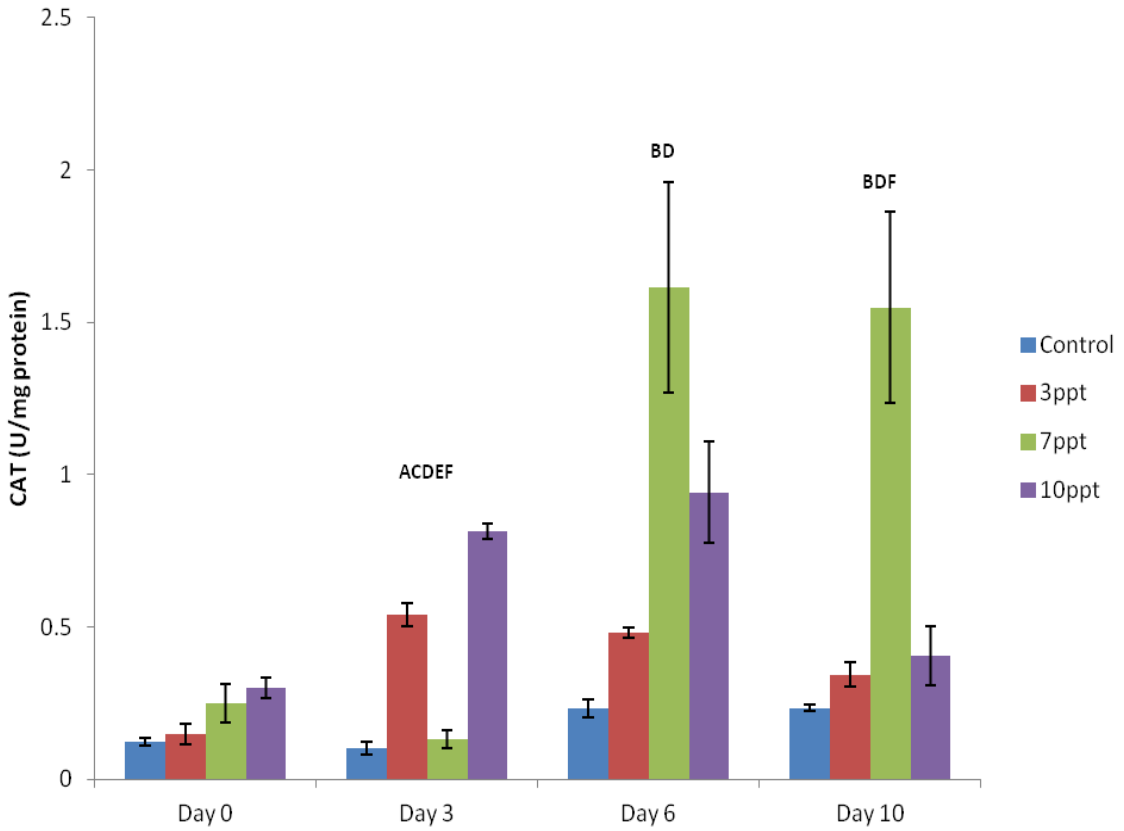

Figure 2.8 Effect of increasing salinity on catalase activity in *M. aeruginosa* over a 10-day period. Cell cultures were exposed to varying salinities to determine if higher salinities induced a greater antioxidant response in the form of catalase. Data points are the mean of 5 replicates (±1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

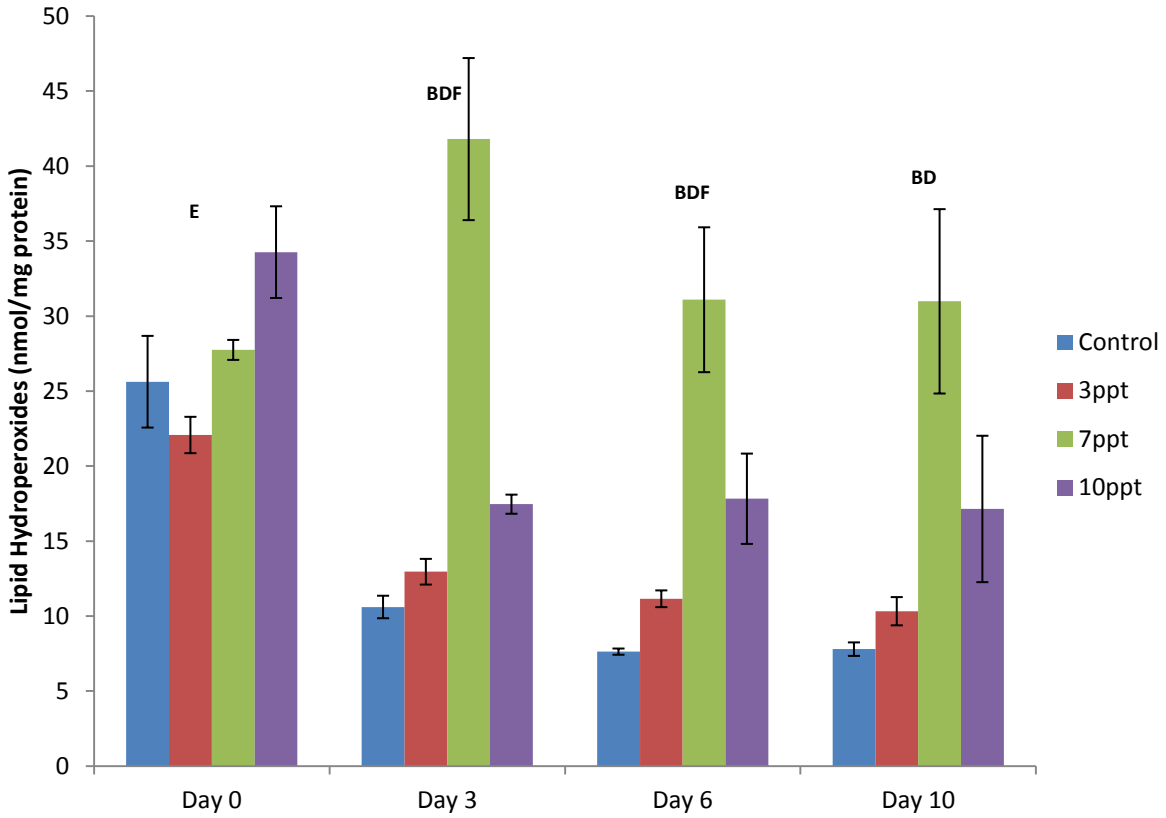

Figure 2.9 Effect of increasing salinity on lipid hydroperoxide production over a 10-day period. Cell cultures were exposed to varying salinities to assess oxidative damage to lipids in the form of lipid hydroperoxides. Data points are the mean of 5 replicates (±1 SEM). Significant differences are noted by "A" (control vs. 3ppt), "B" (control vs. 7ppt), "C" (control vs. 10ppt), "D" (3ppt vs. 7ppt), "E" (3ppt vs. 10ppt), and "F" (7ppt vs. 10ppt).

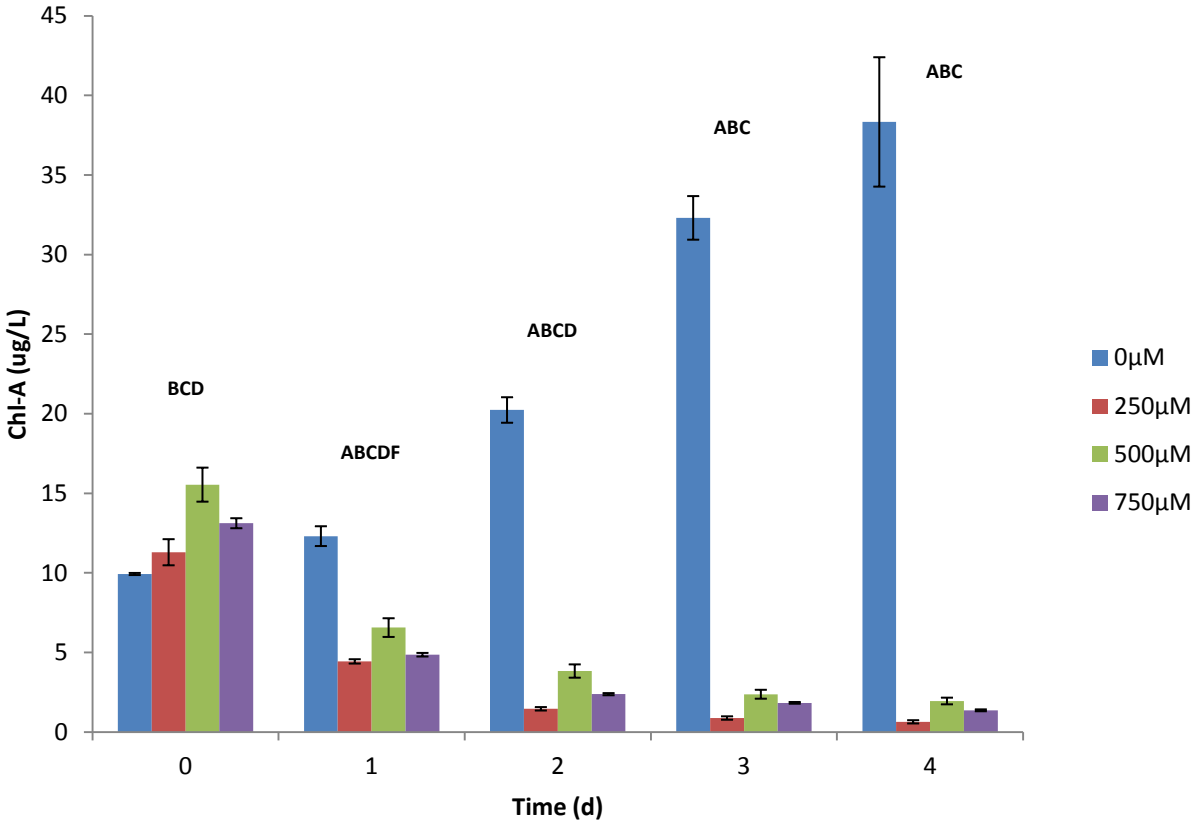

Figure 2.10 Effect of increasing hydrogen peroxide on chlorophyll-A content in *M. aeruginosa* over a 4-day period. Cell cultures were exposed to hydrogen peroxide concentrations to determine the concentration needed to affect cell growth and abundance. Data points are the mean of 5 replicates (±1 SEM). Significant differences are noted by "A" (0µM vs. 250µM), "B" (0µM vs. 500µM), "C" (0µM vs. 750µM), "D" (250µM vs. 500µM), "E" (250µM vs. 750µM), and "F" (500µM vs. 750µM).

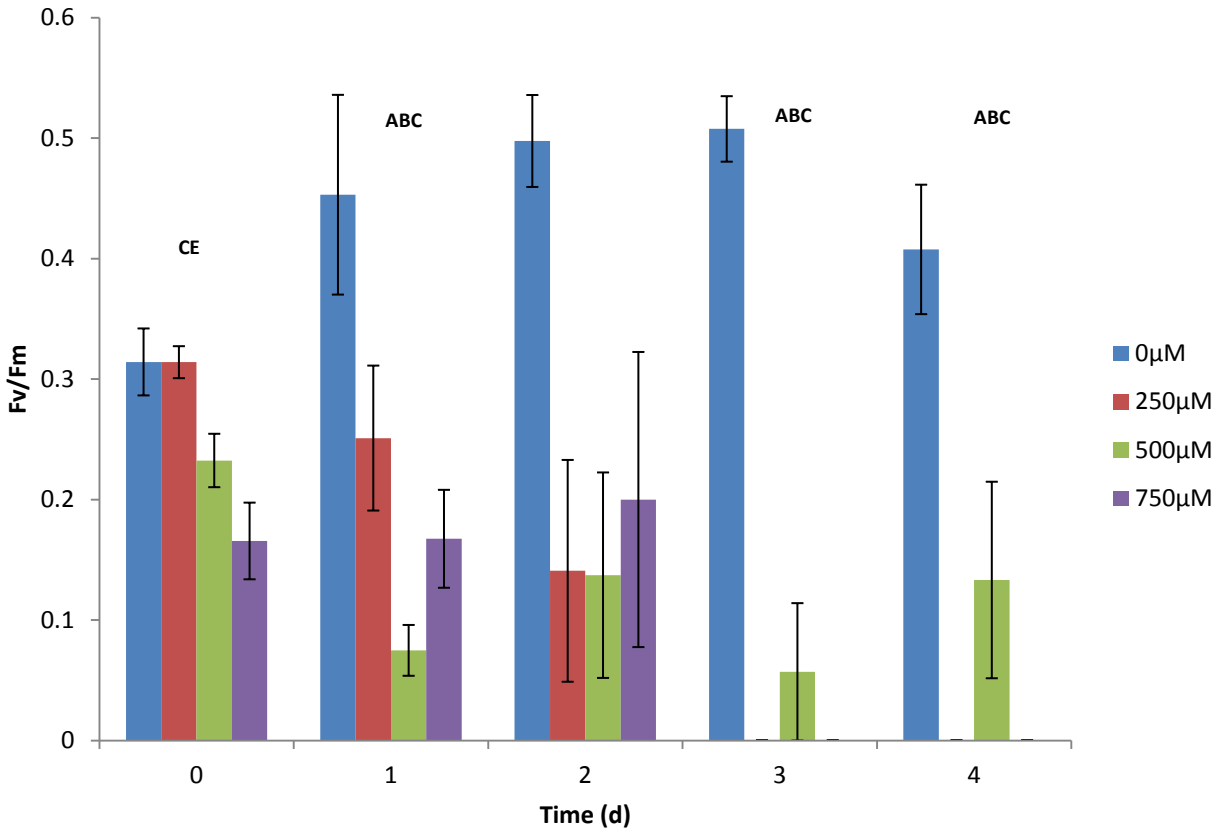

Figure 2.11 Effect of increasing hydrogen peroxide on photochemical efficiency in *M. aeruginosa* over a 4-day period. Cell cultures were exposed to hydrogen peroxide concentrations to determine the concentration needed to impact photochemical efficiency in *M. aeruginosa*. Data points are the mean of 5 replicates (±1 SEM). Significant differences are noted by "A" (0µM vs. 250µM), "B" (0µM vs. 500µM), "C" (0µM vs. 750µM), "D" (250µM vs. 500µM), "E" (250µM vs. 750µM), and "F" (500µM vs. 750µM).

The cellular growth and abundance of *M. aeruginosa* was directly affected by elevated salinity. In this study there was no significant difference in chl-A concentration over the 11-day time frame between the control treatment, with a salinity of 1.4ppt, and the 3ppt treatment (Fig. 2.1). Once the salinity increased to 7ppt, however, the amount of salt stress on the cells had a dampening effect on cellular chl-A content.

Production of chl-A decreased after day 3 then stayed fairly stable until the end of the experiment. This showed that the elevated salinity may have been killing cells, which would lower chl-A fluorescence. The greatest impact on chl-A fluorescence was in the 10ppt treatment. A normal growth pattern was shown until day 3, then the amount of chl-A decreased dramatically until day 11. Such a high amount of salt in the 10ppt treatment caused the vast majority of cells to die, which in turn affected the fluorescence output of the cells' chl-A.

These results differed from the results of Tonk *et al.* (2007), where their selected strain of *M. aeruginosa* was reported to grow under higher salinities (10, 15, and 17.5ppt) over a similar time frame. In their study the 10ppt treatment stayed relatively stable in terms of growth rate with rates decreasing as salinity increased. The 17.5ppt treatment was the only one that showed higher cellular mortality, comparable to results found in the 10ppt treatment in this experiment. It is possible that the strain used in that research was more halotolerant than the strain used here, thereby allowing the cells to respond with the salinity more efficiently before succumbing to cell death. Orr *et al*. (2004) provided evidence that another strain of *M. aeruginosa* was salt-tolerant until reaching 9.8ppt. Salinity treatments higher than 9.8ppt caused decreasing cellular concentrations, much like what was found in this current study. Despite the difference in salinity thresholds and results of Tonk *et al.* (2007), increasing salinity to 10ppt had a negative impact on the cellular growth of *M. aeruginosa* in this research.

Another variable analyzed was the maximum photochemical efficiency of *M. aeruginosa*'s photosystem II fluorescence, termed Fv/Fm. Use of the variable Fv/Fm is common in studies analyzing environmental effects such as temperature, light, and salinity on cyanobacteria and phytoplankton (Lu & Vonshak, 2002; Berman-Frank *et al.*, 2004; Affenzeller *et al.*, 2008; Bouchard & Purdie, 2011). Typical values for Fv/Fm for *M. aeruginosa* have been reported to range from 0.25 to 0.35 (Bouchard & Purdie, 2011; Ding *et al.*, 2012). In this study, mean Fv/Fm values ranged from 0.23 to 0.5. Similar to data shown in the chl-A experiment, there was little difference between the control and 3ppt treatment in regards to Fv/Fm, but as salinity increased, values for Fv/Fm decreased accordingly (Fig. 2.2). Elevated salinity in the 10ppt treatment caused a decrease in *M. aeruginosa* photochemical efficiency until the 11th day, but the cyanobacteria was able to recover from the stress and match the efficiency of the other salinities. In contrast, salt stress actually increased the photochemical efficiency of the 7ppt treatment throughout the experiment until the last day. This type of resiliency has been shown in *M. aeruginosa* in previous research. In research by Tonk *et al.* (2007), the growth rate of *M. aeruginosa* subjected to 10ppt salinity initially decreased in the first three days of the salt-shock experiment but had recovered to near-control levels by day 4.

It was unclear why mean Fv/Fm for *M. aeruginosa* at 7ppt would be higher than the control treatment, particularly after seeing the effects of salt stress at 10ppt. It was likely that, in the 10ppt treatment, the salinity stress caused damage to photosystem II thereby lowering the fluorescence given off by the cells. Going by the highest salinity treatment alone, it could be surmised that salt stress negatively impacted photochemical efficiency of *M. aeruginosa* for a short time, which lowered growth and possibly caused cell death. To quantify that decrease in growth, cellular abundance (cells/mL) was measured across all four treatments. This was

performed directly through counting *M. aeruginosa* cells in a given treatment. Using the slope, the actual growth rate for each salinity was determined. It was found that the cellular abundance and growth rate of *M. aeruginosa* was impacted at a threshold of 7ppt. Once the salinity reached 10ppt, the vast majority of cells were dead by day 4. Mean growth rate for the control treatment was 1.63 which declined in each subsequent treatment. Tonk *et al.* (2007) found the specific growth rate of *M. aeruginosa* to be 0.4 in the control samples, which had a salinity of 0.6ppt. Furthermore, growth rates for their 10, 15, and 17.5ppt treatments were negatively impacted as salinity increased over 9 days. The 10ppt treatment recovered by the end of that experiment, but the remaining two treatments remained in a state of decline.

These data for cellular abundance and cell growth show that elevated salinity had a direct impact on the total number of *M. aeruginosa* cells and their ability to multiply over time. As it was shown in the previous experiments, the line between growth and death appeared to fall between 7 and 10ppt. This observation coincided with the data provided by the microcystin experiment. Much like the data found for Fv/Fm, there was not a significant difference in microcystin output among the salinities by the end of the experiment (Fig. 2.4). However, prior to that, the 10ppt treatment consistently released enhanced levels of microcystins into the environment when compared to the other three salinities. The apex of microcystin release occurred at day 6 where the extracellular microcystin concentration yielded a mean of 6.74ppb. It was likely that much of the microcystins exuded were from cells that died early on in the experiment, which was why the 10ppt treatment had higher microcystin concentrations than the other treatments.

Microcystins are exuded naturally from living *M. aeruginosa*. However, higher levels of toxins may be released upon cell death (Ross *et al*., 2006). As was shown with the cellular abundance data, the 10ppt treatment had far more dead cells by day 7 compared to the other salinities used

in this study. It was assumed that the higher amounts of microcystins detected in the media were a reflection of salinity-based cell death. Similar findings were reported by Tonk *et al.* (2007) who found that extracellular microcystins increased once the salinity reached 10ppt and higher. Microcystin production and cellular growth decreased after this salinity threshold . Similarly, Ross *et al.* (2006) showed an increase in extracellular microcystin concentrations when *M. aeruginosa* was exposed to a salinity of 32 ppt. In this research, it was surmised that elevating salinity past 7ppt for *M. aeruginosa* caused a decrease in cellular abundance and growth and caused an increase in microcystins released into the media.

2.4.2. Elevated salinity increases reactive oxygen species (ROS) production

Hydrogen peroxide (H_2O_2) is a strong oxidizing agent that is naturally formed in aquatic ecosystems, particularly in surface waters that are able to obtain sufficient sunlight or by photosynthetic organisms that produce it due to environmental stressors (Ross *et al.*, 2006). Hydrogen peroxide is known to be an effective algaecide due to its toxicity against cyanobacteria and other algae that form blooms. Application of H_2O_2 to *M. aeruginosa* can lead to decreased cell growth and abundance as well as increased cellular mortality (Dziallas & Grossart, 2011; Mikula *et al.*, 2012). Furthermore, photosynthetic organisms that are exposed to elevated salinity tend to produce hydrogen peroxide and other reactive oxygen species due to the uncoupling of biochemical pathways and subsequent electron transfer to molecular oxygen (Mittler, 2002). Over time, the ROS produced, especially hydroxyl radicals and superoxide anions, can cause damage to proteins, lipids, and nucleic acids, as well as lesions in DNA. The buildup of ROS and damage to the cells can lead to cellular mortality (Halliwell, 2006; Lesser, 2006).

Because it was known that H_2O_2 is a very effective algaecide (Qian *et al*., 2010), it was of

interest to determine if salt stress causes the production of intracellular ROS, which in turn causes cell death. In this experiment, the focus was on whether salt stress from elevated salinity caused an increase in reactive oxygen species production in *M. aeruginosa* and in turn whether ROS caused cellular death through natural and/or programmed means. Quantification of ROS in *M. aeruginosa* and other cyanobacteria has been performed by others, and the procedures used are similar (Tang *et al.*, 2007; Affenzeller *et al.*, 2008; Bouchard & Purdie, 2011). It was assumed that the greater the percentage of cells containing ROS, the more damage that was done to the overall cell community which can ultimately lead to cell mortality. Both the 7ppt and 10ppt treatments had higher numbers of cells marked as ROS-positive throughout the experiment, with percentages reaching as high as 14% of all cells in the samples containing ROS (Fig. 2.5). Such a high percentage of cells containing intracellular ROS helped to explain why there was such a high cellular mortality shown in the first few experiments.

Examples of ROS production increasing due to elevated salinity have been found in other cyanobacteria and algae. Using the cyanobacterium *Scytonema javanicum*, Tang *et al.* (2007) found that ROS levels increased in the first 3 days then slowly decreased following exposure to 50mM NaCl . Affenzeller *et al.* (2008), analyzing salt stress on the green alga, *Micrasterias denticulata*, showed that ROS levels increased when cells were exposed to both 200mM NaCl and 200mM KCl. ROS concentrations began decreasing within 30 minutes of treatment, rather than taking days to be reduced (Affenzeller *et al.*, 2008). It is likely that both *S. javanicum* and *M. denticulata* are more halotolerant than *M. aeruginosa*, and therefore are able to counter ROS production more efficiently. Finally, in an experiment by Bouchard and Purdee (2011), where *M. aeruginosa* was exposed directly to 0.5mM H_2O_2, ROS contents per cell increased over the span of the four day experiment. The addition of hydrogen peroxide tipped the balance between

oxidizing agents and antioxidants in favor of the oxidizers, which led to intracellular damage. That damage affected chl-A content, photochemical efficiency, and cellular growth, much like the results shown in this research.

One method of quantifying cellular mortality due to elevated salinity was by using the SYTOX-green assay. Higher percentages of 'green' cells in a sample indicated higher cellular mortality. Similar to the data gathered in the ROS experiment, higher salinities yielded higher percentages of cells marked as 'dead'. The 10ppt treatment, in particular, had relatively high values for 'green' cells in this experiment. This directly showed that there was a high cellular mortality in the 10ppt treatments for the previous experiments, which explained the decrease in cell abundance and growth, and the increase in microcystin output. These results coincide with results found in other studies, where increasing salt stress led to an increase cellular mortality in both *M. aeruginosa* as well as other cyanobacteria and green alga (Tang *et al.*, 2007; Tonk *et al.*, 2007; Affenzeller *et al.*, 2008).

Another focus was on whether salt stress from elevated salinity induced PCD in *M. aeruginosa*. Several other studies have determined that caspase-like proteases are responsible for cellular death in other cyanobacteria and chlorophytes, as well as *M. aeruginosa* (Ning *et al.*, 2002; Berman-Frank *et al.*, 2004; Segovia *et al.*, 2003; Ross *et al.*, 2006; Bouchard & Purdie, 2011). Caspases are vital to the role of PCD, necrosis, and inflammation of cells. The caspase-catalyzed cleavage of the amino acid compound Asp–Glu–Val–Asp (DEVD) into the fluorescent compound Rhodamine 110 (R110) was analyzed. Higher values for R110 indicated higher caspase proteolytic activity in a given sample. Experimental results were mixed in regards to PCD, as it was expected that the 10ppt treatment would have much higher caspase activity if cellular mortality was directly linked to PCD and not due to environmental causes. It is possible

that as time progressed, cells died and lysed which would prevent accurate detection of caspases. This would have been a problem particularly at higher salinities where cell mortality was more pronounced. There was a pattern of increasing caspase activity in higher salinity treatments, but the differences between them were not significant enough to provide a definitive answer on whether *M. aeruginosa* induced PCD in response to elevated salinity.

Although results involving salt stress induced PCD in *M. aeruginosa* are scarce, research of PCD caused by salt stress in other cyanobacteria, as well as by other forms of stress have been conducted. In an experiment involving the marine cyanobacterium, *Trichodesmium* spp., it was found that an increase in oxidative stress led to an increase in caspase activity that led to cellular death. The oxidative stress was induced by exposing the cyanobacteria to 10μM H_2O_2 for 3 hours (Berman-Frank *et al.*, 2004). Research has found that increasing salt stress in the cyanobacteria, *Anabaena* spp., also induces PCD through an increase in caspase activity. This was done by exposing the cells to 0.1 mol l^{-1} of KCl and $CaCl_2$ over an eight-day period. By the 8th day, 70% of cells exposed to KCl for 4 days and $CaCl_2$ for 4 days were found to be TUNEL-positive, indicating PCD (Ning *et al.*, 2002).

However, in the green alga *Micrasterias denticulata*, salt stress actually decreased caspase-like activity to between 60 and 80% of control levels in cells exposed to 200mM KCl and 200mM NaCl over 30 minutes. After 3 hours of exposure to the same treatments, only the NaCl treatment showed caspase activity approaching control levels, which may indicate that the green alga does not implement PCD in response to salt stress (Affenzeller *et al.*, 2008). Finally, in a study by Ross *et al.* (2006), stress caused by addition of 100μM H_2O_2 to *M. aeruginosa* cells over a 24 hour period led to an increase in caspase-3 activity and, subsequently PCD, when compared to control treatments. In research by Bouchard and Purdee (2011), stress caused by *M. aeruginosa*

cells exposed to 5μM H_2O_2 over a 96 hour period also led to elevated caspase-3 activity when compared to the control treatment. These results are similar to what was found in this research, as a pattern emerged where caspase activity was significantly higher in the 10ppt treatment compared to the control until day 10. However, due to lack of significant difference among the four treatments at the end of the experiment, PCD may not be considered the definitive answer to the cause of cellular mortality. It was likely that osmotic stress was the major culprit of cellular mortality.

2.4.3 Elevated salinity induces an antioxidant response to salt stress

Antioxidant enzymes are readily available in aquatic photosynthetic organisms to handle ROS that are naturally generated (Choo *et al.*, 2004; Mittler *et al*., 2004). Enzymes such as superoxide dismutase, glutathione peroxidase, and catalase are all used to break down hydrogen peroxide into compounds that are not inherently lethal to the organism. Here, it was determined if *M. aeruginosa* induced an antioxidant response to the ROS generated in the cells by salt stress. One way this was done was to look at catalase activity in the treatments. The role of catalase is to break down hydrogen peroxide (H_2O_2) into the components of H_2O and O_2, which prevents damage to the cell. Although higher catalase activity likely signifies higher concentrations of intracellular ROS, lower catalase activity can either mean lower concentrations or total breakdown of the enzymes needed to reduce ROS.

The results of the experiment can be explained by recognizing the purpose of catalase, which is to break down H_2O_2 into compounds that are not harmful to the cells. The higher activity in the 7ppt treatment indicated that catalase activity and subsequent decomposition of H_2O_2 was high, meaning that the strong antioxidant response was enough to handle the salt stress. It is likely that

the lower catalase activity values for 10ppt indicated that the amount of H_2O_2 produced far exceeded any antioxidant response the cells could muster, thus overwhelming the machinery and ultimately caused cellular death due to ROS damage. ROS production in the control and 3ppt treatments was handled efficiently by the catalase response, therefore cellular mortality was much lower in those treatments. It should be noted that other antioxidants, such as superoxide dismutase and glutathione peroxidase, were likely at work in conjunction with catalase, but they were not assayed in this experiment.

Although data on the antioxidant response of *M. aeruginosa* to ROS caused by salt stress is scarce, studies showing antioxidant responses in *M. aeruginosa* and other cyanobacteria to other forms of stress are available. In a study involving the application of the allelochemical gramine to *M. aeruginosa*, catalase activity increased as the exposure to the chemical increased, regardless of the gramine concentration (Hong *et al.*, 2009). Another study by Hong *et al.* (2008) involved the application of the allelochemical ethyl 2-methyl acetoacetate (EMA) to *M. aeruginosa*. It was also found that the length of the exposure to the chemical directly affected catalase activity and not the concentration of the chemical itself. Finally, a study analyzing the antioxidant response of the cyanobacteria, *Anabaena doliolum*, to copper found significant increases in both lipid peroxidation and catalase activity in response to increasing copper concentration (Mallick & Rai, 1999).

Because oxidative stress from intracellular ROS tends to cause damage to lipids in the cells, it was of interest to determine if cells subjected to higher salinities produced greater concentrations of lipid hydroperoxides. As was seen with the catalase activity results, the 7ppt treatment had higher values for lipid hydroperoxides than the other salinities. The same high catalase activity shown in Fig 2-5a was used to counter the high lipid hydroperoxide concentrations caused by

salt stress to prevent cellular damage and/or mortality. It was likely that the 10ppt treatment was overwhelmed by intracellular damage done by the lipid hydroperoxides after the first day of the experiment. This would lead to cell death, so overall lipid hydroperoxide values would be lower due to decomposition when released into the water. Oxidative stress due to other environmental variables have been shown to cause damage to lipids in cyanobacteria. In a study performed by Zeeshan and Prasad (2009), increasing doses of UV-B radiation were applied to three different cyanobacteria: *Nostoc muscorum*, *Plectonema boryanum*, and *Aphanothece* spp. Oxidative stress was measured by analyzing concentrations of malondialdehyde (MDA), a compound generated by ROS and typically used as a bio-marker for oxidative stress. Increasing levels of UV-B radiation over 15 minutes led to 3-10% increases in MDA concentration, indicating that the oxidative stress caused by the radiation led to increasing lipid peroxidation in all three cyanobacteria. As exposure time increased to 60 minutes, MDA concentrations, and therefore lipid peroxidation, also increased.

Determining the amount of hydrogen peroxide needed to affect physiological attributes of *M. aeruginosa* was useful, so the cells were treated with 250, 500, and 750µM H_2O_2 concentrations over a 4-day period. It was found that not only did increasing H_2O_2 concentration negatively affect cell growth and photochemical efficiency, but it only required 24 hours to show effects. Impact on chl-A content and Fv/Fm was visible by the first day of the experiment. Chl-A continued to decrease in all treatments (except the control) until the termination of the experiment. The Fv/Fm values decreased in the 250µM and 750µM treatments to the point of reaching zero, whereas the 500µM treatment simply decreased and stayed low. It was determined that the threshold H_2O_2 concentration was 100µM in a previous experiment, meaning that was the maximum amount of hydrogen peroxide that could be added to the cells before

having a negative impact on cellular growth and abundance. This was reinforced by the data analyzed in this experiment, as negative impact on cell growth and abundance was evident in the 250μM treatment within 24 hours of exposure.

Research analyzing how hydrogen peroxide affects the physiological attributes of *M. aeruginosa* yielded similar results to this experiment. Addition of hydrogen peroxide concentrations as low as 50nM caused significant decreases in chlorophyll A in toxic, non-toxic, wild-type and mutant *M. aeruginosa* strains (Dziallas & Grossart, 2011). Similar research performed by Mikula *et al.* (2012) showed that application of increasing hydrogen peroxide concentrations led to lowered photochemical efficiency and cellular abundance, as well as increased cellular mortality in both light and dark environments. Results provided by Bouchard and Purdee (2011) also showed decreases in chlorophyll-A, Fv/Fm, and cellular abundance when hydrogen peroxide was applied to *M. aeruginosa*. It was also found that cellular mortality, intracellular ROS concentrations, and caspase 3-like activity increased due to H_2O_2. The results from these experiments all match what was found in this research. Increasing salinity led to oxidative stress due to production of H_2O_2 which caused cellular mortality in *M. aeruginosa*. In addition, as shown in Bouchard and Purdee (2011), direct application of H_2O_2 to *M. aeruginosa* caused an increase in caspase activity indicating induction of PCD. A pattern of increasing caspase activity as salinity increased was also shown in this research, so it is possible that cellular mortality was a combination of osmotic stress and induction of PCD in some *M. aeruginosa* cells.

3. General Conclusion

Elevated salinity directly impacted the cellular abundance, growth, and microcystin release of this strain of the cyanobacterium *M. aeruginosa*. Increasing salinity led to a lower number of cells due to cell mortality, lower cellular growth, and an increase in the concentration of microcystins released into the external environment. In this research, it was found that elevating salinity past 7ppt for *M. aeruginosa* caused a decrease in cellular abundance and growth and caused an increase in microcystins released into the media. The cyanotoxin output and dead cells from salt stress can lead to harmful effects on the environment, such as fish kills, higher turbidity, and anoxic zones after depletion of oxygen by consumption of dead cyanobacteria cells by heterotrophic bacteria. Cellular death appeared to be caused by a combination of osmotic stress and induction of PCD due to oxidative stress. *M. aeruginosa* had the ability to produce an antioxidant response to ROS generated by salt stress, but the threshold was found to be between 7 and 10ppt before the cell machinery was overwhelmed and cell death occurred. Any dramatic increases in salinity that turn a freshwater environment into one with a salinity above 7ppt can cause environmental and recreational harm due to bloom demise. A hydrogen peroxide concentration of 250µM or above directly decreased cellular growth and abundance. This data could be useful when applying H_2O_2 as an algaecide.

Future research may include looking at how elevated salinity affects *M. aeruginosa* and other cyanobacteria over a longer period of time to determine if the cyanobacteria are able to eventually counter cellular mortality through antioxidant means. As these experiments were considered salt-shock experiments, it would be useful to expose *M. aeruginosa* to increasing concentrations of salt over time rather than subjecting the cells to the new salinity in a matter of minutes. A period of acclimation may be crucial in allowing the cells to adjust to the new salinity

and perhaps survive the stress. This would give a clearer picture of how removing groundwater or tidal influxes affect a cyanobacteria population in a given area, as the change is typically gradual when compared to the experiments in this research.

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Vita

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Education

2011	B.S. Biology, University of North Florida, Jacksonville, FL Upper-level GPA – 3.53/4.0; Cumulative GPA- 3.26/4.0
2014 (anticipated)	M.S. Biology, University of North Florida, Jacksonville, FL Thesis title: Effects of elevated salinity and oxidative stress on the physiology of the toxigenic cyanobacterium, *Microcystis aeruginosa*

Research Experience

2011 - present	Graduate Research: Characterization of the impacts of elevated salinity on toxigenic *Microcystis aeruginosa* (Advisor: Dr. C. Ross)
2010 - 2011	Undergraduate Research: Effects of environmental factors on the seed germination of the hooded pitcher plant, *Sarracenia minor* (Advisor: Dr. Tony Rossi)

Grants and Awards

2009	Undergraduate SMART Grant, University of North Florida **($2000)**

Teaching Experience

Graduate Teaching Assistant – University of North Florida, Department of Biology (Fall 2011 - Spring 2013) - General Biology I Lab

Presentations

Warhurst, C. Lentz, M. and Ross, C. Exploring the impacts of environmental stressors on toxic cyanobacterial blooms. Scholars Transforming Academic Research Symposium (S.T.A.R.S.). April 11, 2012. University of North Florida, Jacksonville, Florida

Warhurst, C. Huff, C. and Ross, C. Effects of elevated salinity on the physiology of the toxigenic cyanobacterium, *Microcystis aeruginosa*. Southeastern Phycological Colloquy (SEPC). October 20, 2012. University of North Florida, Jacksonville, Florida

Warhurst, C. Huff, C. and Ross, C. Effects of elevated salinity on the physiology of the toxigenic cyanobacterium, *Microcystis aeruginosa*. Biology, Chemistry, and Physics (BCP) poster session. October 19, 2012. University of North Florida, Jacksonville, Florida. *Awarded best Graduate poster.

Warhurst, C. Huff, C. and Ross, C. Effects of elevated salinity on algal blooms and oxidative stress on the toxigenic cyanobacterium, *Microcystis aeruginosa*. Showcase of Osprey Advancements in Research and Scholarship (SOARS). April 19, 2013. University of North Florida, Jacksonville, Florida.

Professional Memberships

- Association for the Sciences of Limnology and Oceanography (ASLO)

Community Outreach

- NOAA Phytoplankton Monitoring Network (2011 - 2013)