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# THE EFFECT OF NAACL ON AKINETE DIFFERENTIATION IN THE CYANOBACTERIUM NOSTOC PUNCTIFORME

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THE EFFECT OF NaCl ON AKINETE DIFFERENTIATION IN THE  
*CYANOBACTERIUM NOSTOC PUNCTIFORME*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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## Abstract

### THE EFFECT OF NAACL ON AKINETE DIFFERENTIATION IN THE CYANOBACTERIUM *NOSTOC PUNCTIFORME*

A thesis is submitted by Jonathan Heekin to fulfill the requirements for the degree of Master of Science at Virginia Commonwealth University.

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*Nostoc punctiforme* is a nitrogen-fixing, symbiotic/free-living cyanobacterium. There has been a great deal of research conducted on the genomic nature of *N. punctiforme* as it pertains to its ecologically important role in the nitrogen cycle in varied environments around the world. My study concentrated on the dormant cell type known as the akinete. Increasing concentrations of NaCl were used to follow the growth phases from germination to akinete formation (lag phase-logarithmic growth phase-stationary phase). I found that increased salt concentrations caused *N. punctiforme* to form akinetes faster when compared to the control. Germination rates were not greatly increased or shortened by salt concentrations at or below 40 mM NaCl. Damage to cells due to NaCl was observed between 105 mM and 500 mM. Physiological studies, such as this one, enable better quantifiable field research since the organism's limitations under laboratory conditions are known. This research allows researcher to more accurately plan and pick study sites, develop field studies and gives a solid basis for comparison to the natural environment.

## **Introduction**

Cyanobacteria, once known as blue-green algae, inhabit every known environmental niche around the world (Lumpkin and Plucknett, 1982; Chiu et al., 2005, Faithfull et al., 2006; Rai et al., 2006). These organisms have been a cornerstone of research for over fifty years in the areas of cellular differentiation, DNA sequencing, and the relationship between DNA, RNA/chemical signaling. Their environmental importance centers on their ability to carry out nitrogen fixation (Granhall, 1978; Newton and Johnson, 1980, Argueta et al., 2004; Argueta and Summers, 2005; Argueta et al., 2006).

This study used *Nostoc punctiforme* as the experimental organism. *N. punctiforme* forms symbiotic relationships with different bryophytes, gymnosperms and angiosperms (Bergman and Osborne, 2002; Chiu et al., 2005) and can be grown in pure axenic culture. Although there has been a great deal of work done to understand their cellular differentiation and niche capabilities, there has not been much research to determine their ecological potentials in global ecosystems. Salt tolerance is a very important area of study for many microecologists, microbiologists and researchers interested in sustainable agriculture (Zhang et al., 2000). It is natural that the question arises regarding the range and capabilities of a versatile organism such as *N. punctiforme*. Researchers are interested in its ability to fix nitrogen, thus increasing this limiting nutrient in an ecosystem or crop field setting. Researchers want to take this important N<sub>2</sub> fixing process into more limiting environments so that they can better understand nitrogen, carbon, phosphorous and sulfur cycling in environments that are either in danger of being destroyed by human activity or

are being affected by global ecological changes (Foster et al., 2003). These environments include salt marshes, wetlands and unusable farmland that was once productive. It is known that cyanobacteria are primary controllers of nutrient cycling in such areas by controlling nitrogen availability (Mikkat et al., 1996).

A major roadblock in current research involves the understanding of how micro and macro environments work together and separately to alter ecosystems. It is crucial that research identifies natural/limiting habitat nutrient cycling, interactions and capabilities of both the inhabitants and the environment. The global and niche dependence on fixed nitrogen emphasizes the need to better understand cyanobacteria and their varied roles in different environments. Laboratory research can relate to the natural world only so far, since it is a controlled environment. Yet, this provides a benefit to researchers by clarifying the natural and artificial limits under which organisms can survive and thrive. *N. punctiforme* is a key nitrogen fixing organism in varied ecosystems around the world. Understanding its life cycle and dormant capabilities under stress is imperative if researchers wish to extrapolate data to find where and why nitrogen becomes a limiting nutrient factor. Once researchers have a better picture of the controls on cyanobacteria development they will better understand limitations to niche diversity, microbe soil cycling and the cellular signals utilized in cell differentiation of one of the world's oldest inhabitants. Such information is critical to understand the world created and continually effected by microbes. This will aid ongoing work being done to redevelop land, to protect flora and fauna diversity, increase land productivity and maintain suitable habitats for endangered species.

Most recent research has focused on the ability of different cyanobacterial strains/species to continue fixing nitrogen under salt stress---in salt marshes and farmland desiccated by excess salts (Katoh et al., 2004; Higo et al., 2006; Rai et al., 2006).

Countries that do not yet have easy access to artificial fertilizers through lack of funds or through their own legislation (to prevent nitrate contamination) study cyanobacterial salt stress in an effort to open more land to agriculture for exploding human populations.

India and China use cyanobacteria as a research model for this purpose because their land is under intense industrial development and they have to feed a larger population on less farmland (Zhang et al., 2000).

In Europe and the US, researchers are trying to unravel the intricate food webs, nutrient flows and the organisms involved in ecosystem dependant interactions (Cavagnaro et al., 2007). The data sets collected in these studies could be used in future studies in the area of cyanobacteria development and salt tolerance. The study of salt marshes has been an intense area of study in recent years (Ferjani et al., 2003; Marin et al., 2006; Mishra et al., 2006). Ecologists are very interested in making sure that these special ecosystems, like salt marshes and wetlands, are preserved and protected. These researchers need to better understand how the nutrient system operates and which organisms are contributing what and when. Salt marshes, estuaries and wetlands have become economic and politically sensitive subjects resulting in efforts to restore and protect them. In order to do this properly, the ecosystem, on both the macro and micro level must be completely evaluated and understood. It has been found that in a salt marsh

ecosystem cyanobacteria are responsible for the fixed nitrogen needed by other organisms (Miosander et al., 2005).

It is important for researchers to discover the mechanisms organisms use to survive in these unique environments. This present project may further our understanding of how salt stress is met by survival mechanisms. Cyanobacteria research may, one day, help restore these vital, sensitive ecosystems if we have a better understanding of dormant cycles and stressors leading to dormant cell types.

There was a great deal of research around the world in the 1970's and 80's with efforts concentrating on a way to create a symbiont fertilizer for crops (Peters and Mayne, 1974; Granhall, 1978; Lumpkin and Plucknett, 1982). The one problem agriculturalists and academics still have today is a way of renitrifying soils that have been ravaged by salt or other barriers to crop production. This problem has been prevalent in recent years with soils being destroyed by destructive farming techniques and the overuse of artificial fertilizers. This research brings the scientific community closer to understanding if a nitrogen fixing microbe can survive or thrive in such salt stressed ecosystems. Also, it is equally important to better understand how these early precursors to other life on earth have the facility to endure varied climates around the world and return from dormancy.

This currently reported project continues a chapter on cyanobacterial research opened by Dr. Argueta and Dr. Summers (Argueta et al., 2004; Argueta and Summers, 2005; Argueta et al., 2006). It used different physiological quantifications to evaluate akinete differentiation and correlated it to the developmental profile under a stressor not

yet critically evaluated. There have not yet been experiments published that use these tools to study akinete development. Most experiments that have been done, (Argueta et al., 2004; Argueta and Summers, 2005; Argueta et al., 2006) have concentrated on heterocyst formation, making these cells better understood. They have been important to researchers because of their ability to fix atmospheric nitrogen. This present study also complements work that was recently completed having analyzed the salt stress capability of the symbiotic cyanobacterium *Anabaena azollae* and other *Anabaena* species (Faithfull et al., 2006; Rai et al., 2006). My experimental model is also of symbiotic origin and adds to the scientific knowledge to identify if it is as salt sensitive as *A. azollae*.

Both akinetes and heterocyst are believed to share genes regulating differentiation; gaining knowledge about akinete development, thus may aid heterocyst research as well. This research allows us to ask better questions and narrow our research scope when investigating dormant cell formation/function in cyanobacteria and other organisms. This study focused on the effect of NaCl on the growth and akinete development of *N. punctiforme*.

## **Materials and Methods**

### Culture Source:

*Nostoc punctiforme*, a nitrogen-fixing cyanobacterium of symbiotic origin was used in all control and experimental studies conducted during this thesis project. The original culture was obtained from Dr. Wan Ling Chiu of the Virginia Commonwealth University Department of Biology in January, 2007 and has been subcultured ever since.

### Culture Maintenance Methods:

Stock cultures were subcultured biweekly using sterile transfer techniques. During these transfers, 2 ml of culture were added to 75 ml of sterile AA/8 medium (Allen and Arnon, 1955) contained in 125 ml Erlenmeyer flasks.

Samples used to start vegetative studies were transferred from seven day old stock cultures to ensure that the cells used were in the logarithmic growth phase. Five milliliters of culture were transferred into 75 ml of AA/8 medium in 125 ml Erlenmeyer flasks containing various concentrations of NaCl (0 mM, 20 mM, 30 mM, 40 mM) and monitored over 21 days. Growth was assessed as an increase in chlorophyll *a*. Akinete percentages were monitored using a research microscope. This documented the growth and development changes during the 21 day study period. Sample pictures were taken of the control and salt-treated cultures the same days akinete frequencies were determined.

Control cultures that had already formed akinetes (aged cultures), usually older than 90 days, were used as stock cultures for akinete germination studies. Five milliliters of culture were transferred to 75 ml of AA/8 medium contained in 125 ml Erlenmeyer

flasks containing various concentrations of NaCl (0 -500 mM) and followed over a 28 hour period. Germination was monitored as a decrease in akinetes present. Akinete percentages were monitored by counting 300 cells (3 counts of 100 each) per flask every 4 hours. Sample pictures were taken of the control and salt-treated cultures the same hours akinete frequencies were determined.

Aged cultures were also used as stock cultures in the growth profile studies. Five milliliters of culture were transferred to 75 ml of AA/8 in 125 ml Erlenmeyer flasks containing various concentrations of NaCl (0 mM, 20 mM, 30 mM, 40 mM) and followed over a 21 day period. Growth was monitored as a change in chlorophyll a concentration. Akinete percentages were monitored by counting 300 cells (3 counts of 100 each) per flask. As before, sample pictures were taken of the control and salt-treated cultures the same days akinete frequencies were taken.

In all studies, transfers were done in a sterile laminar flow hood using sterile transfer pipettes (Fisher Scientific). All cultures were maintained on an orbital shaker (New Brunswick Scientific) set at 40 rpm.

#### Microscopic Observations:

Microscopic observations were made using either a Miles Corporation Microscope with an attached Super Image Digital Camera or an Olympus BX41 microscope with an attached Olympus America Digital Camera using the Magnifier 2.1 image capture program. Slides were prepared by taking 1 ml of culture using sterile technique and placing the sample in a 2 ml polypropylene centrifuge tube (Corning). A 2

ml transfer pipette (Fisher Scientific) was used to place a drop of sample on a slide. A cover slip (18X18mm, Corning) was then put on the slide. Samples were observed at 100, 400 and 1000 (oil) times magnification.

No fewer than 3 pictures were taken for each slide prepared. More pictures were taken of each sample as akinete differentiation proceeded. All pictures were taken at 1000X. A new slide was made for each 100 cell count (3 slides made per sample per day monitored).

Cells of cultured *N. punctiforme*, as they age, will clump making getting definitive pictures more challenging. Due to this (cells being in different planes and on top of each other) it was necessary, in some microphotographs, to gratingly change the fine focus in order to annotate different cell layers. This was specifically useful in identifying akinetes in clumps of cells, since the microscope could pinpoint reflective granules regardless of cell layers. This fine focus technique was very useful when taking pictures of damaged cells in the higher NaCl concentration treated cultures.

#### Chlorophyll a Extraction:

To extract chlorophyll a from samples, 1 ml of sample was transferred (using sterile technique) to a 15 ml polypropylene centrifuge tube (Corning). The sample was centrifuged for 5 minutes in a Lourdes Clinical Centrifuge (1200 rpm) and the supernatant was discarded. The pellet was then re-suspended in 3 ml of 100% methanol (Fisher Scientific). The tube was then vortexed to resuspend the pellet in the methanol.

Samples were then placed in the dark for 15 minutes to allow for chlorophyll a extraction. After 15 minutes, the samples were centrifuged again at 1200 rpm for 5 minutes.

Sample supernatants were transferred to a cuvette (path length of 1 mm) for reading in the spectrophotometer (Varian DMS-80 UV Visible). Methanol (100%) was used as the blank for each reading. Both were placed in the dual beam spectrophotometer and the absorbance was read at 665 nm. The chlorophyll a concentration was calculated using the following equation using an extinction coefficient of 13.43 and a dilution factor of 3:  $\text{ug Chl } \underline{a} / \text{ml of original culture} = (13.43) \times (\text{absorbance at 665 nm}) \times (3)$ .

#### Statistical Analysis and Design:

All experiments were run in triplicate and repeated at least three times. Means and standard deviations were calculated, when appropriate, and Null Hypotheses were tested using ANOVA and associated post-hoc tests (using InStat Software, Graphpad Inc.). Prism (Graphpad Inc.) was used to graphically display the results.

## Results

### Vegetative Study:

Cultures were treated with various concentrations of NaCl (0, 20 mM, 30 mM, and 40 mM) to assess the effects of salt on the growth and development of *N. punctiforme*. Growth, in each case, was monitored as an increase in chlorophyll a content and the developmental parameter that was followed was akinete differentiation. The inocula for these studies were log phase vegetative cultures that lacked akinetes (Plate 1-A, D)

Growth in the various salt concentrations was followed over a 19 day growth period. During this time interval, 20 mM was not significantly different from the control (0 mM NaCl) treatment on any day assessed. The control was, however, significantly different from the 30 mM from day 7 on and from the 40 mM treatment from day 2 to the end of the assessed period. In all cases, the difference was a reduction of growth caused by the salt treatment (Fig. 1). In addition, the 40 mM treatment resulted in a significantly greater growth reduction than either the 20 or 30 mM treatment from day 2 on and the 30 mM treatment grew significantly slower than the 20 mM treatment from day 7 until the end of the experiment (Fig. 1).

Akinete differentiation was monitored, as percent akinetes, over a 21 day observation period. Over this 21 day period, no akinetes were seen in control cultures. On the other hand, all salt treatments induced akinete differentiation and had significantly higher induction rates than the control from day 15 to the end of the observation period. There were no differences between the 20 and 30 or 20 and 40 mM treatments although

the 30 and 40 mM treatments differed on days 13 and 16 of the study. No salt treatment seemed to be more inductive than the other but all were greater than the control (Fig. 2).

In all studies, the possible differences between means were tested at the 0.05 level using Analysis of Variance (ANOVA) and a Tukey-Kramer Multiple Comparisons Test.

#### Akinete Germination Studies:

Cultures were treated with various concentrations of NaCl (0, 80 mM, 90 mM, 100 mM, 105 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 200 mM, 250 mM, 300 mM, and 500mM) to assess the effects of salt on the germination of *N. punctiforme* akinetes (Plate 1-C). Germination was monitored visually using the research microscope. The inocula for these studies were aged cultures that had already formed akinetes. Even though some germination and cell division was observed at concentrations as high as 500 mM NaCl, it was impossible to determine if the increasing damage observed were cells being lysed/shriveled as they attempted germination/cell division or if they were being damaged directly. Therefore, no reliable data could be obtained because of this cellular damage (Plate 1-E).

Akinete germination, therefore was followed in detail at lower NaCl concentrations (0 mM, 20 mM, 30 mM and 40 mM). Germination was followed over a 28 hour period. The salt treated-cultures germinated at significantly slower rates than the control (Fig. 3). In addition, all salt-treated cultures germinated less than the control by hour 28 (Fig. 3).

### Akinete Differentiation Studies and Overall Growth Profile:

Cultures were treated with various concentrations of NaCl (0, 20 mM, 30 mM, and 40 mM) to follow the effects of salt on the akinete differentiation of *N. punctiforme*. Growth, in each case, was monitored as an increase in chlorophyll a content and the developmental parameter that was followed was akinete differentiation as percent akinetes. The inocula for these studies were aged cultures that had already formed akinetes (Plate 1-B, F).

Growth in the various salt concentrations was followed over a 19 day growth period. During this time interval, the control growth (0 mM NaCl) was significantly higher than the 30 mM and 40 mM treatments from day 10 and the 20 mM treatment from day 12 (Fig. 4). Akinete germination was slower in the salt-treated cultures with the 40 mM treatment not reaching 100% germination by day 9. The 20 mM treatment did not differ from the control throughout the experimental time period (Fig. 5). After day 12, the 30 mM and 40 mM treatments developed akinetes significantly faster than the control (Fig. 5a and 5b).

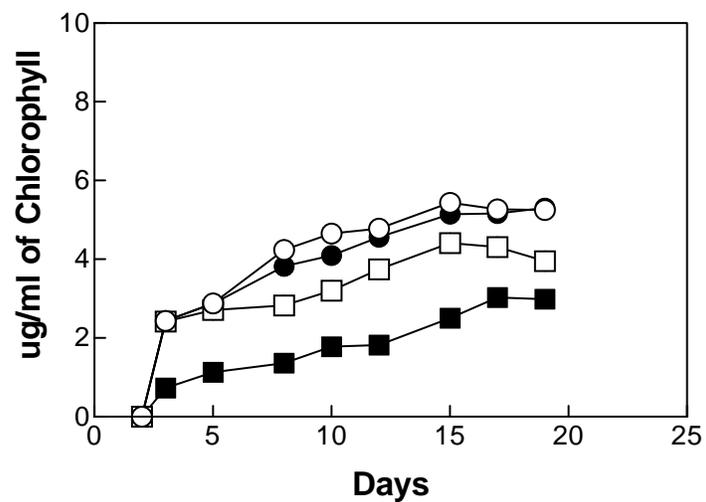


Fig. 1. The effects of salt (open circles=control, black circles=20 mM, open squares=30 mM, black squares=40 mM) on the growth (ug Chl a/ml original culture) of *N. punctiforme* over a 19 day growth period. P value was significant at  $p < .05$ .

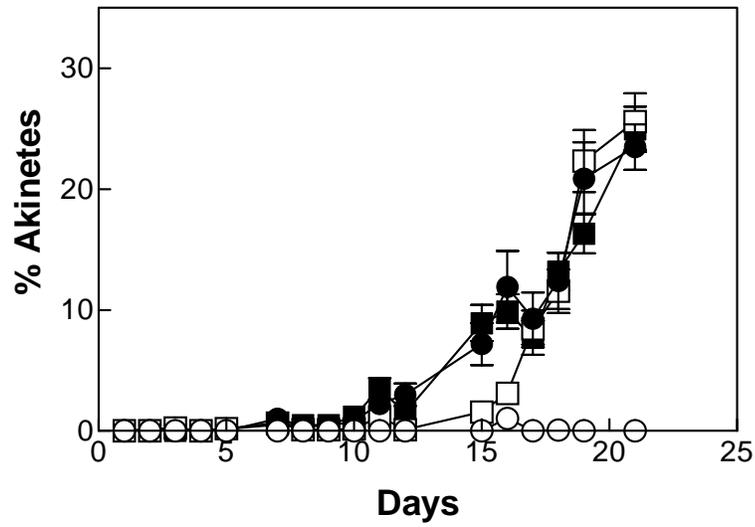


Fig. 2. The effect of salt (open circles=control, black circles=20 mM, open squares=30 mM, black squares=40 mM) on akinete differentiation in *N. punctiforme* over a 21 day growth period. P value was significant at  $p < .05$ .

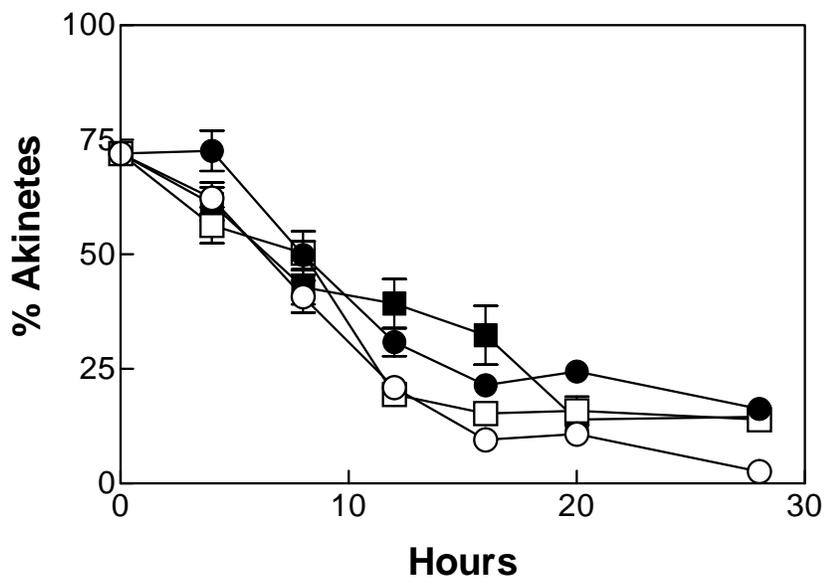


Fig. 3. The effect of salt (open circles=control, black circles=20 mM, open squares=30 mM, black squares=40 mM) on akinete germination in *N. punctiforme* followed over a 28 hour observation period. P value was significant at  $p < .05$ .

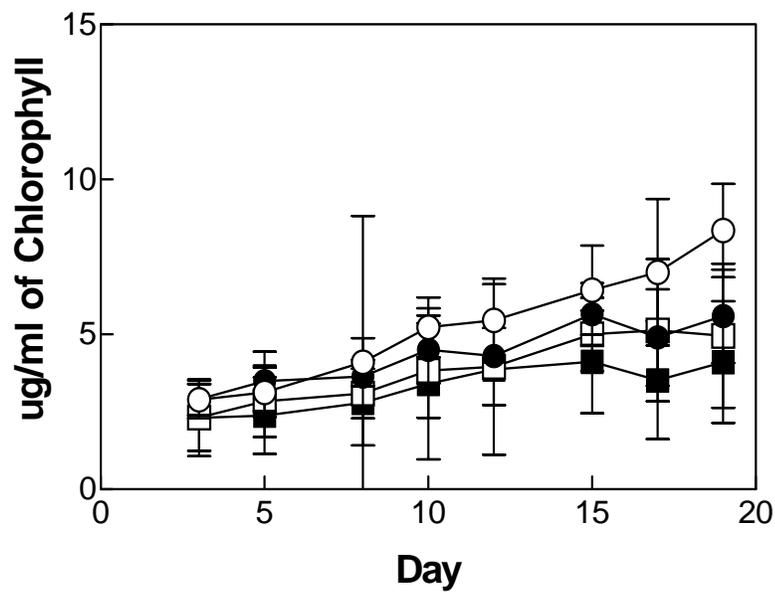


Fig. 4. The effect of NaCl on the growth of cultures of *N. punctiforme* (open circles=control, black circles=20 mM, open squares=30 mM, black squares=40 mM) started from akinetes. P value was significant at  $p < .05$ .

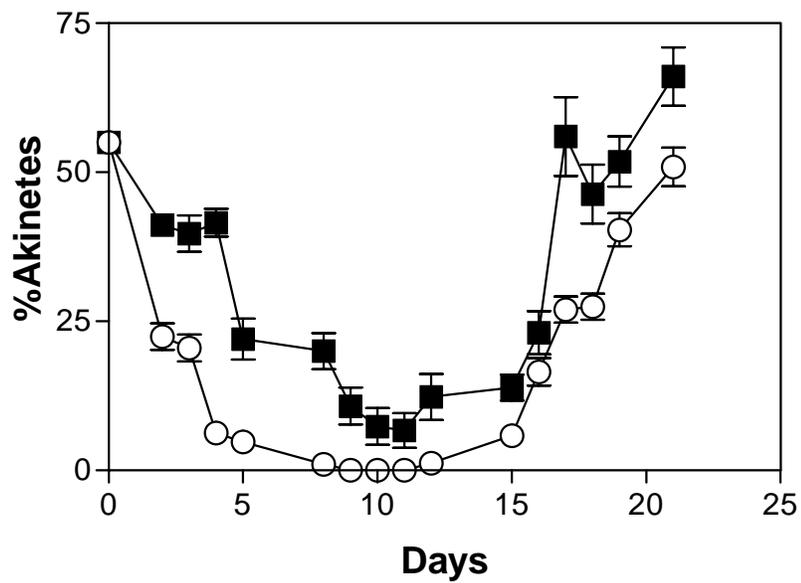


Fig. 5a. The effect of NaCl on akinete germination and/or differentiation (open circles=control, black squares=40 mM) in cultures of *N. punctiforme*. P value was significant at  $p < .05$ .

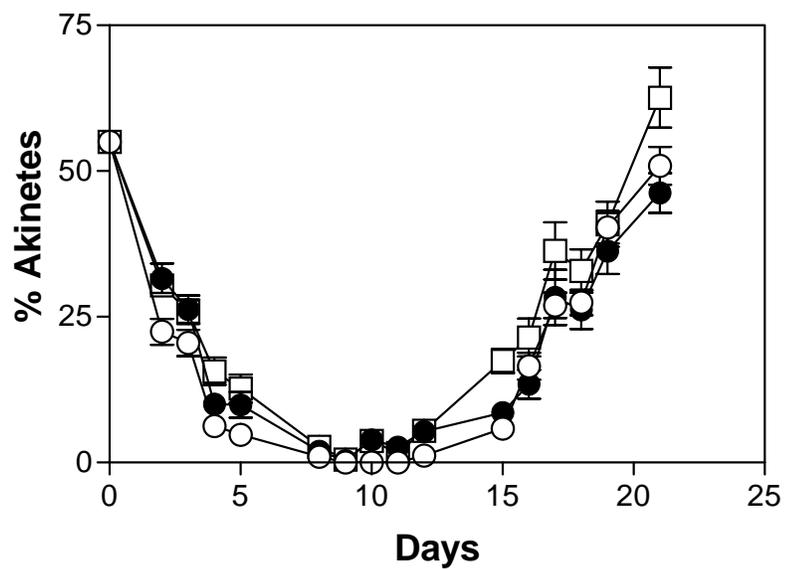


Fig. 5b. The effect of NaCl on akinete germination and/or differentiation (open circles=control, black circles=20 mM, open squares=30 mM,) in cultures of *N. punctiforme*. P value was significant at  $p < .05$ .

## Plate 1

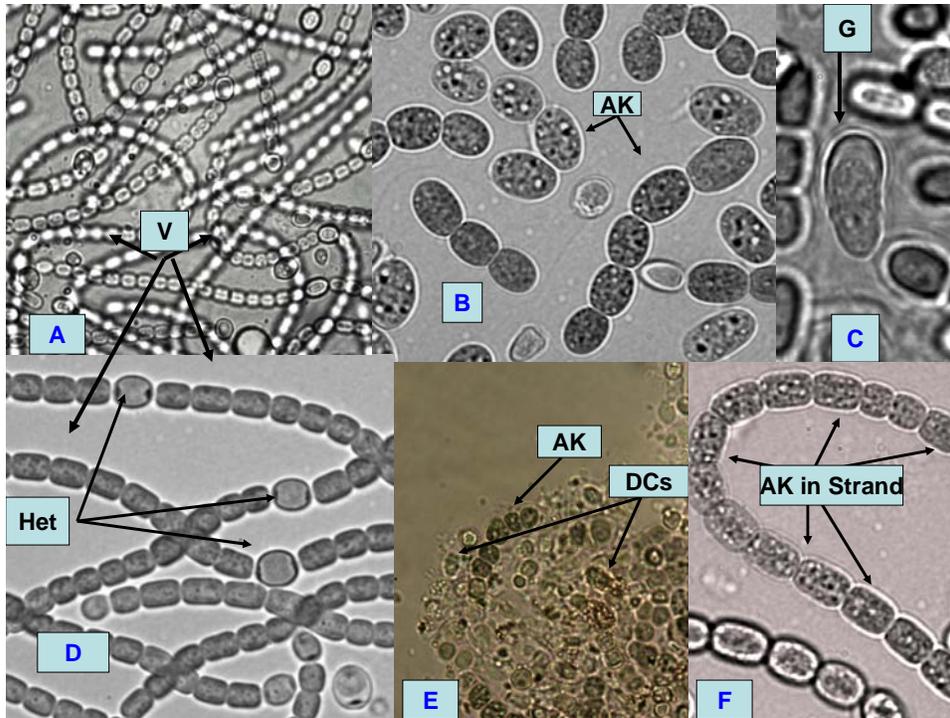


Plate 1: **A.** Vegetative logarithmic growth, arrows point to photosynthetic vegetative cells (400X); **B.** Akinete cells maturing, can easily see reflective granules (1000X); **C.** Germinating (G) akinete, the new vegetative cell discards the old akinete cell wall (1000X); **D.** Heterocyst with vegetative cells, can easily see polar nodules in heterocysts (1000X); **E.** Damaged cells (DCs) at higher NaCl concentrations >105mM, akinete strand (AK) surrounded by dead or damaged cells (400X); **F.** Akinetes in a strand before they mature and break away as individual akinetes or smaller strands (1000X).

## Discussion

This project focused on dormancy. The physiological aspects of dormancy under stress are not as well documented as the genetic aspects of dormancy. Cyanobacteria are inherently important to many ecosystems because they are not only primary producers supporting food webs, but many are capable of fixing atmospheric nitrogen, thus allowing ecosystems to grow in complexity. Most researchers (Hutchinson, 1959; Ricklefs, 1987; Goldberg and Barton, 1992; McCann, 2000; Gotelli and Colwell, 2001; Mittelbach et al., 2001; Kneitel and Chase, 2004; Wiens et al., 2006) view communitarian ecological questions with little regard to dormancy and its crucial role in ecosystem diversity and stability. This project sought to establish a baseline, from which, to ask such questions and used an organism whose ecological role and significance is well documented. This research and other research like it will enable field researchers to better understand nutrient cycling, food web matrixes and how important dormancy is to every biological discipline (microbiology, ecology, evolution biology and numerous disciplines centered on nutrient cycling—bottom up control factors). However, field work will be important for establishing *N. punctiforme*'s nutrient cycling control and dormancy capability in different stressful environments employed to support their diversity.

Microbiologists and microecologists have been very interested in why and how cells keep the ionic balance necessary for cellular function, including theories such as intracellular accumulation of inorganic ions, salt tolerance using salt-dependant enzymes, accumulation of “compatible” ions, and either by synthesis or transport—the accumulation of a negative or neutral ion to offset the intracellular [K<sup>+</sup>] (Martin et al., 1999).

Most of these mechanisms have been examined before in cyanobacteria (Warr et al., 1984). Their research looked at the osmotic affects of NaCl and KCl on the cells of cyanobacteria. There are other findings in the field of cyanobacteria salt tolerance that are useful in evaluating my findings. Marin et al. reported in 2006 that glucosylglycerol accumulation occurred in *Synechocystis* sp. strain PCC 6803 (a fresh water cyanobacterium) when it was shocked with high levels of NaCl. This strain did become acclimated to the salt concentrations used, but the mechanism was not understood. It is known that certain glycerols, sugars and zwitterionic solutes are used in salt tolerance in the Kingdom Archaea (Martin et al., 1999). In 1996 this same team found that it was the active transport of glucosylglycerol that was involved in PCC 6803's salt acclimation (Mikkat et al., 1996). However, other avenues have been studied including putrescine transport, the GgpS enzyme and the corresponding ggpS gene (Ferjani et al., 2003). It is not clear if salt acclimation can be attained by *N. punctiforme*. However, it is a useful concept when thinking about dormancy as a whole. Dormancy is a way for the organism to retain its internal environmental conditions while the outside conditions change. Thus dormancy can be viewed as a way these organisms have acclimated to stress. This makes the study of akinetes and their development that much more important.

Since the revolution of microbiology by PCR, gene products and the functions of genes has been a continued focus of research on cyanobacteria and *N. punctiforme* in particular. Researchers have described genes involved in salt acclimation (Apte and Haselkorn, 1990; Fernandes et al., 1993; Apte and Alahari, 1994). These works were conducted on *A. torulosa* and indicated a possible 100kb gene responsible for salt stress

survivability. Later work continued on the same path, researching the K<sup>+</sup>/Na<sup>+</sup>, K<sup>+</sup>/NaCl relationships. In 1998 the Wolk laboratory at Michigan State University (Schwart et al., 1998) used *Anabaena 7120* (also studied extensively in our laboratory) to show that the same transcribed products from gene *lti2* were seen in light limitation, cold incubation and salt (NaCl) stress. Their work has helped to bring the genetic aspect of the equation in line with the observed phenotypes documented for many stress reactions in cyanobacteria. It started to paint the picture that environmental stress reactions were linked with the same or common genes coding for many different stress responses.

New observations were made in 2003 (Chauhan et al., 2003). Using a K<sup>+</sup> uptake mutant this team found that salt tolerant strains of *Anabaena variabilis* had a greater uptake capability than the strains without the modified/increased K<sup>+</sup> uptake pathway. This has made the strongest correlation so far between K<sup>+</sup> channels/uptake (influx) and the offset of NaCl toxicity to the cell by its influx. In 2004 there was another study that closely followed what Dr. Wolk's laboratory had done in 1998, but used new arrays to evaluate the data (Kato et al., 2004). The results from this study confirmed that those genes upregulated under salt stress in *Anabaena* were also upregulated when other forms of stress were applied. This may be part of the mechanism used by *N. punctiforme* as it moves rapidly to the dormant akinete stage as salt concentrations reach mM concentrations it is not able to handle. As seen in my study, akinete formation occurs quicker as the NaCl concentration increases (Fig. 5a). This upregulation of gene products parallels the physiological growth pattern observed in my study.

Recently, a trehalose synthesis coding gene has been found to be upregulated in salt stressed *A. 7120*. Other stressors (heat shock, desiccation) also cause a big increase in this gene product. There has been great debate whether *A. 7120* is a *Nostoc* or *Anabaena* species. Based on the work done to date on both *A. 7120* and *N. punctiforme*, it is clear that they are similar enough to allow some cross extrapolations on the overall question of stress response and specifically regulating the NaCl stress response. Therefore, it is possible that trehalose was upregulated in my studies.

My data indicate that the threshold concentration with the first significant effect for normal phase growth is below 20 mM NaCl for *N. punctiforme*. Rai, however (Rai et al, 2006) did not find this for *Anabaena azollae*. They studied salt tolerance on the *Azolla/Anabaena* relationship and each organism separately. These researchers found that the more sensitive partner was the *A. azollae* cyanobacterium, with a maximum salt tolerance of no more than 40 mM of NaCl before the symbiotic relationship ended. *A. azollae* was not able to expel excess Na<sup>+</sup> ions and lost its positive K<sup>+</sup> gradient at increased NaCl concentrations. Since *N. punctiforme* is found in harsher terrain areas; it might be conceivable that it would be better able to handle stresses, such as salt, but apparently not.

*A. azollae* is an aquatic symbiont and may actually be more able to handle ion changes across the cell membrane better than a terrestrial cyanobacterium. It could be that the NaCl tolerant mechanism found in *A. azollae* (discussed under salt tolerance mechanisms) is absent, or not as proficient in *N. punctiforme*. Since many cyanobacteria have genes they are not seen to be used such, as *A.7120* not utilizing hormogonia genes

(Argueta et al., 2006), then it is possible that the genes for NaCl tolerance are similar in *N. punctiforme* and *A. azollae*, but *N. punctiforme* is not able to utilize the gene product (if it is made) as well as *A. azollae*. Figures 2 and 5a, in my study, distinctly show that *N. punctiforme*'s dormant phase is induced under salt stress.

This emphasizes the importance of environmental conditioning of genetic products; how, where in the cell, when, efficiency, capability of use. It could be that, since the laboratory is not a natural environment, that the laboratory studies will have limited applications. It is also possible that NaCl tolerant mechanisms are more efficient in a microenvironment affected by natural environmental conditions. Yet, it is more restrictive and controlled in the laboratory, where the researcher needs to investigate one variable at a time and needs a protocol with which to do this without any outside variables.

All the research discussed above has greatly added to our understanding of the underlying cellular capabilities/tools evolution has developed to aid cyanobacterial invasions to almost all known ecosystems of the planet. My research is just as important and informative as the DNA/RNA research. My physiological study has enabled us to see and quantify akinete developmental changes under salt stress. It is highly likely that the above stated mechanisms and involved genes are also important in akinete germination and subsequent vegetative growth.

My studies also (germination in the Vegetative Study and Growth Development Study) followed Dr. Faithfull's (Faithfull and Burns, 2006) results closely with almost full germination occurring after 7-9 days. The germination studies were probably the

most important studies conducted when considering the ecological significance of dormancy and viability in nature. Natural sea salinity is approximately 460 mM. Salt marsh salinity can range anywhere from 50 mM to 300 mM. As seen in my results (Fig. 3), germination is not stopped or even significantly slowed by 40 mM NaCl. Salinities, as high as 300 mM, did allow growth and germination even though cell damage was observed. However, the damage was comparable to lower concentrations, unlike the wide cell damage observed at 500 mM.

In 1991, it was reported that cyanobacteria could take a shock of NaCl over 600 mM by producing certain proteins in great abundance (Hagemann et al., 1991). It was believed that some of these proteins had salt enzymatic activity and others could have been made by the cells to do active transport of ions into or out of the cell. The same proteins were observed during heat shock in the same set of experiments. These findings parallel what I found for *N. punctiforme*. We know that it can handle saline shocks up to 500 mM NaCl, not as high as other cyanobacteria, but still higher than sea salt—460 mM NaCl. As can be seen by these experiments, the mechanisms used by cyanobacteria are similar to other prokaryotes. However, *N. punctiforme* cannot be shocked with as high a NaCl concentration. My results indicate the 105 mM NaCl may be the growth/germination limit in laboratory based controlled studies.

Microbial mats, found in brackish water, are known to have many different microbes at different levels doing different biological cycling events (Parodi, 2002) (sulfur reduction, nitrogen reduction and nitrogen fixation). This is the place that akinetes

could be found, even at levels of the mat not yet involved in nitrogen fixation (upper section), ready for a turnover event, disturbance of the mat matrix, or change in salinity.

Medium salt soils of estuaries range from 50 mM up to 1 molar NaCl depending on soil depth (Marcum et al., 2007), partially the range shown in my experiments, in which, *N. punctiforme* akinetes can germinate and grow. This could be evidence that *N. punctiforme* akinetes could form in high salt environments before the vegetative cells die, to await an influx of fresh water. This is a possible cycle/survival mechanism for microbes in salt gradient affected estuaries and salt marshes. My experiments show quantifiable laboratory results that salt affected environments (barrier islands, salt marshes and estuaries) could use cyanobacteria to provide fixed nitrogen.

The extrapolation of these results may not be pertinent to all nitrogen-fixing cyanobacteria, but from my results, it can be concluded that there is a high probability that akinetes (and dormancy in general) could be major operators in halophytic systems. Akinetes enable these organisms to re-establish ecosystem biodiversity and dynamics and confer a certain level of resilience after natural disturbance. The body of knowledge of natural anthropogenic disturbances is a major area of current research. Many theorists in several disciplines (Community Ecology and Landscape Ecology) overlook dormancy as a coping mechanism on the ecosystem level. My research adds to this body of knowledge enabling future researchers to incorporate this ecosystem coping mechanism with regards to nutrient cycling (nitrogen cycling specifically) and the resulting trophic response capabilities. My research also gives other researchers a template to use when evaluating cyanobacteria in extreme environments and their ability/inability to adapt.

Nature has enabled redundancy in key chemical cycling events through niche diversity. It is possible that the dormant akinete can survive in 250-300 mM NaCl (based on my results) salt near sea spray areas on barrier islands and germinate with limited vegetative cell density providing some of the needed fixed nitrogen plants cannot make themselves. To test these areas we need to conduct these types of physiological/growth laboratory studies to make sure that, when we look for such organisms, we can narrow or widen our search criteria and area as needed.

One tool, Denaturing Gradient Gel Electrophoresis (DGGE; R. Franklin, VCU Biology, personal communication), can be used to find cyanobacteria among thousands of other microbes that might be present. This research tool can find the presence of akinetes in any sample from any environment. All that is needed are specific primers to indicate the presence of akinetes and the knowledge of whether such cells should/could be present.

The major research on akinete gene expression and differentiation in the past few years has come from Dr. Summers and Dr. Argueta at California State University, Northridge. They have published 3 papers since 2004, all building off each other (Argueta et al., 2004; Argueta and Summers, 2005; Argueta et al., 2006). The focus of this research has been akinete formation by *N. punctiforme* under dark heterotrophic conditions. Their research has recently produced new insight into the regulation of akinete formation via the genes involved. In his most recent paper, Dr. Summers identifies 3 new genes that affect akinete differentiation (Argueta and Summers, 2006). Primers used in these experiments can be used in DGGE research to identify if akinetes,

and thus, cyanobacteria are present so that nutrient cycling of the researched system can be more fully understood and explored.

The first of the genes he has described is named NpF0062 (Argueta and Summers, 2006) and is important in making the akinete lipopolysaccharide cell layer thicker. His Real-Time PCR (RT) results showed an 11 fold increase in the gene product over three days as akinetes formed. The second confirmed gene was NpR4070. This gene showed a big increase (112 fold) using RT PCR in both wild type strains tested under phosphate limiting and light limiting stress—*zwf* induced akinetes. This gene was also expressed in hormogonia, a motile cellular unit in *N. punctiforme* and some other cyanobacteria.

The third gene identified was interlinked with gene NpF5999 (an aminopeptidase in vegetative cells also expressed in akinetes) and was designated NpF6000. It is thought to be a nitroreductase coder that is expressed in akinetes. Primers for these three genes were included in their publications. Any of these primers could be used in the DGGE method. Such studies may be the future of ecological dynamic mapping. Such mapping is possible only with both the physiological and DNA knowledge.

My results (Fig. 2 and 5a/b) indicate that akinete percentages increased as the time progressed; it was thought that the akinete percentages would be correlated to the concentrations of NaCl. However as the NaCl concentrations increased, growth stalled while the controls continued their exponential growth until their granulated cells were comparable to the experimental concentrations. It was thought, at the onset of the experiment, that the controls would not indicate much cellular differentiation into akinetes. The controls were able to develop akinetes, however, because they were started

with highly akineted, cellular dense stock cultures, and thus there was a magnitude increase in cells added in the Akinete Development Studies than there were added in the Vegetative Studies. This forced *N. punctiforme* to go through the natural life cycle from full dormancy (akinetete) to dormancy (akinetete) again. This fact could explain why the akinete percentages were not more significantly different in my studies. The chlorophyll a concentrations do show that the salt concentrations did have a significant growth cycle difference from the controls and from each other as the NaCl concentration increased. This raises little doubt that NaCl did have an impact on *N. punctiforme* (Fig 4).

In conclusion, my studies indicate that *N. punctiforme* had limited logarithmic growth under NaCl stress. Akinetes were developed faster as the salt concentration was increased and they can handle substantial NaCl shock. My studies show *N. punctiforme* (and other nitrogen-fixing cyanobacteria) can conduct important ecological activities in saline stressed ecosystems, which should be explored further using field studies and other cyanobacteria species.

It is very possible that akinetes exist in harsh environments such as salt marshes, based on my data and the wealth of research providing genetic stress regulation stated above. Cyanobacteria may not proliferate to the visible population densities researchers can easily measure (via spectrophotometry or acetylene reduction assays using GC), but akinete germination and growth of some vegetative cells may be possible in these environments, thus providing fixed nitrogen to the limited plant and microbial species present. Sea spray ecosystems (barrier islands) may provide an example of this growth capability and should be studied further in the field. Field samples can be studied in the

laboratory using DGGE analysis to verify the presence of important nutrient cycling organisms such as cyanobacteria.

Only some threshold ranges were determined by my studies. Future studies to test for threshold NaCl tolerance should start at 0.1mM and increase or decrease depending on results. This will allow further research to pinpoint the minimal amount of NaCl stress needed to cause a growth developmental change in *N. punctiforme* and create a baseline for other environmentally important nutrient cycling studies. Physiological profiles, such as the ones followed in my studies, should be important in designing future experiments.

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**Leadership Practice Experience:**

- 2002-Present Teaching assistant for Dr. Fisher at VCU. Helped organize, teach and mentor students, most of whom were seniors in the honors program
- 2001- Present Lab Manager for Dr. Fisher's Lab. Duties include maintaining all safety documentation, mentoring students and keeping lab up to date in accordance with OSHA regulations. Under my leadership the lab has never received an unsatisfactory rating by the university or during any State/Federal organizational inspection.
- 2005- 2007 Performed duties as Platoon Leader/ XO and Commander. During Annual Training 2006 I performed XO duties which included detailed planning of main training objectives for AT and mentoring and guiding new, junior officers. I also stepped in for the commander when he was unavailable.
- 2002-2005 Led a Platoon of 73 soldiers. Duties included planning missions (acted as Company Operations Officer), leading soldiers in the field during combat operations/movements. I had 4 Maintenance Teams in hot spots around the country and traveled several times a month to visit and take care of my soldiers-- -Najaf (during the Najaf offensive), Scania—area of operations included Baghdad.
- 1995-1999 Served four years active duty In the USN. Became a qualified electrician on the P3 Orion Sub-Hunter (Patrol Squadron 46 "Grey Knights"). Successfully completed 2 deployments to Misawa, Japan acting as a valued member of both the AE shop and Line Shop supporting 14 crews and keeping 12 aircraft fully mission capable.

**References:** Available upon request