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Growth and Toxin Production by *Microcystis Aeruginosa* PCC 7806 (Kutzing) Lemmerman at Elevated Salt Concentrations

Ken Black, Mete Yilmaz, Edward J. Phlips

School of Forest Resources and Conservation, University of Florida, Gainesville, USA.
Email: Phlips@ufl.edu

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ABSTRACT

One of the most common and widespread bloom-forming cyanobacteria associated with toxin production is Microcystis aeruginosa (*Kutzing*) *Lemmerman. While normally associated with fresh water environments, this toxigenic species has been observed at bloom concentrations in a number of major estuaries worldwide. This study examined the effect of salinity on growth and toxin production by M. aeruginosa strain PCC* 7806 *under controlled laboratory conditions. Salt concentrations above* 12.6 *ppt resulted in total cessation of growth. Toxin production was similarly affected, with cultures grown in salt concentrations of* 4.6 *ppt and above yielding less toxin than the control after* 20 *days of culture. Toxin concentrations after* 20 *days of culture were* 40% *of the control at* 4.6 *ppt. The relative proportion of extracellular to intracellular toxin increased over time in cultures with salt concentrations greater than* 4.6 *ppt. Extracellular toxins persisted in the media long after the cessation of growth. The results suggest that the influence of M. aeruginosa and/or its toxins can extend well out into estuarine environments under the influence of significant freshwater inputs.*

***Keywords*:** *Microcystin*, *Cyanobacteria*, *Estuaries*

1. Introduction

Cyanobacterial blooms are common across the globe, affecting both freshwater and marine ecosystems [1-3]. Among these bloom-forming species, there are a number of toxigenic strains [4-6]. One of the more common and widespread bloom-forming cyanobacteria associated with toxin production is *Microcystis aeruginosa* (Kutzing) Lemmerman. The toxin most often associated with *M. aeruginosa* is microcystin, a hepatotoxin which can negatively impact aquatic animal and human health on the cellular and organ level [4,7,8]. Blooms of toxic *M. aeruginosa* have been implicated in mass mortalities of aquatic animals and the destabilization of food webs [3,9, 10]. Consumption of microcystin contaminated drinking water and tainted food items pose potential human health risks, especially in third world countries where effective treatment practices are not uniformly applied [4,11-14]. Because of the potential harmful effects of *M. aeruginosa* it has become a focus of efforts to control harmful algae blooms.

Although *M. aeruginosa* is most commonly associated with freshwater environments, blooms have been observed in mesohaline regions of estuaries, such as the Chesapeake Bay [15], the Neuse River in North Carolina [2], the Neva Estuary in the Gulf of Finland [16], the Guadina Estuary in Spain [17], the Swan River in Australia [18], and the St. Lucie Estuary [19] and St. Johns River [20] estuaries in Florida. The highest reported salt concentrations at which *M. aeruginosa* survives ranges from 2 to 17 ppt [2,21-24]. Isolates from blooms can vary in toxicity [25,26]. The appearance of toxic strains of *M. aeruginosa* in saline environments has become a serious issue for the management of affected coastal environments, particularly those where there is extensive utilization of marine resources for fishing, recreation or consumption of potable water after desalinization [27-29]. Previous work indicates that elevated salt concentrations result in the lysis of cells and the release of microcystins into the supporting water [23,24].

This study examined the response of a toxic strain of *M. aeruginosa* to a range of salt concentrations, in terms of survival, growth, toxin production and the fate of the toxins produced over the growth cycle. Most previous studies have focused on cells from discrete portions of

the growth cycle or from natural bloom samples. The objectives of this study were: 1) To determine changes in cell abundance and microcystin content of *M. aeruginosa* grown over a range of salinities, 2) To evaluate the fate of microcystin in terms of its relative distribution within cells and the surrounding media over the growth cycle, and 3) To examine the longevity of microcystin in saline media.

2. Methods

Microcystis aeruginosa PCC 7806 cells were grown in Hoagland's medium buffered to 8.0 with HEPES [30], yielding a baseline salt concentration of 0.6 ppt. Cultures were grown at 25˚C, and light was provided by cool-white fluorescent bulbs at approximately 60 μmol·photons·m^{-2}·s^{-1} PAR irradiance, on a 12:12 light: dark (L:D) photoperiod.

Treatment groups were based on culture media (salinity of 0.6 ppt), to which NaCl was added to reach additional final salinities of 2.6, 4.6, 6.6, 8.6, 10.6, 12.6, 14.6, 20.6, 25.6, 30.6, and 35.6 ppt. Treatment groups were set up in triplicate in 500 ml Erlenmeyer flasks. Flasks were inoculated with *M. aeruginosa* cells in the exponential growth phase. Inoculums were added at a ratio of 1:10, culture to media, yielding a starting concentrations of approximately 40 μg·L^{-1} chlorophyll *a*, 10^6 cells·ml^{-1}, and 35 μg·L^{-1} of microcystin.

Two methods were used to quantify changes in cell biomass, chlorophyll concentrations and cell counts. *In vivo* chlorophyll *a* was determined fluorometrically using a Turner fluorometer [31] at two day intervals from $t = 0$ to $t = 20$ days. Fluorescence values were converted to chlorophyll *a* concentrations using standard relationships obtained from replicate samples analyzed for chlorophyll *a* using spectrophotometric analysis [32] after methanol extraction [33] using a Hitachi dual beam spectrophotometer.

Samples for cell counts were collected at $t = 0$, 2, 8, 14 and 20 days. Samples were preserved with Lugol's solution [32]. Cell counts were carried out microscopically using the Utermöhl sedimentation method [32,34]. Subsamples were allowed to settle in an Utermöhl chamber for 24 hours. Cells were counted on a Nikon inverted light microscope at 400x magnification.

Both intracellular and extracellular microcystin concentrations were determined for samples collected from the 0.6, 4.6, 8.6, 12.6 and 20.6 ppt salt treatment groups at $t = 0$, 2, 8, 14 and 20 days of culture. Two separate five ml aliquots were collected during each sampling. To obtain the extracellular fraction, one of the samples was filtered through a 0.7 μm pore size glass fiber filter. Filtrates were frozen and stored until toxin analysis. The other whole water sample was separately frozen and analyzed for toxin concentration.

Samples were thawed and boiled in a 100˚C water bath for 60 seconds [35]. Cell debris was pelleted by centrifugation and discarded. Toxin concentrations were determined via Enzyme Linked Immuno-Sorbent Assay (ELISA). Envirologix© Competitive ELISA kits for the quantification of Microcystin-LR and congeners were utilized, following the methods outlined by the manufacturer. Toxin concentrations were measured using a Stat Fax 3200 microplate reader. If necessary, samples were diluted in order to accommodate the ELISA's assay range of 0.16 to 2.5 μg·L^{-1} Microcystin-LR. The concentration of the intracellular microcystin was calculated by subtracting the filtered sample values from the corresponding unfiltered sample.

ANOVA and Tukey's (HSD) post-hoc tests were utilized to determine the significance of the salinity effects. Statistical analyses were done with a SPSS Version 17.0 statistics package.

3. Results and Discussion

Rates of increase in chlorophyll *a* concentration and cell numbers of *M. aeruginosa* decreased with increased salt concentrations (**Figure 1**). After two days growth, cultures grown at salt concentrations of 8.6 ppt or higher varied significantly from the control in terms of chlorophyll *a* concentration (ANOVA, $F = 11.84$, $p < 0.05$, $n = 39$) and cell density (ANOVA, $F = 10.41$, $p < 0.05$, $n = 39$). After twenty days exposure, cultures grown at 4.6 ppt or higher exhibited significantly lower chlorophyll *a* concentrations compared to the 0.6 ppt and 2.6 ppt groups (ANOVA, $F = 64.64$, $p < 0.05$, $n = 39$). In terms of cell numbers at 20 days, cultures grown at 2.6 ppt or higher had significantly lower cell densities than cultures grown at 0.6 ppt (ANOVA, $F = 85.30$, $p < 0.05$, $n = 39$). Cultures grown at or above 16.6 ppt showed no significant increases in chlorophyll *a* or cell numbers over the incubation period, similar to the observations of Verspagen *et al.* [29]

At two days of culture, no significant differences were observed in total microcystin content of cultures grown at different salt concentrations (ANOVA, $F = 2.467$, $p = 0.113$, $n = 15$) (**Figure 2**). After twenty days, total toxin content of cultures grown at 4.6 ppt salt or greater were significantly lower than in the 0.6 ppt group (ANOVA, $F = 123.977$, $p < 0.05$, $n = 12$). A strong increase in total microcystin concentration was observed in the 0.6 ppt and 4.6 ppt treatment groups between 8 and 14 days of culture growth, reaching mean concentrations of up to 1500 μg·L^{-1} after 20 days. At salinities of 0.6, 4.6 and 8.6 ppt the mean percentage increases in cell numbers and

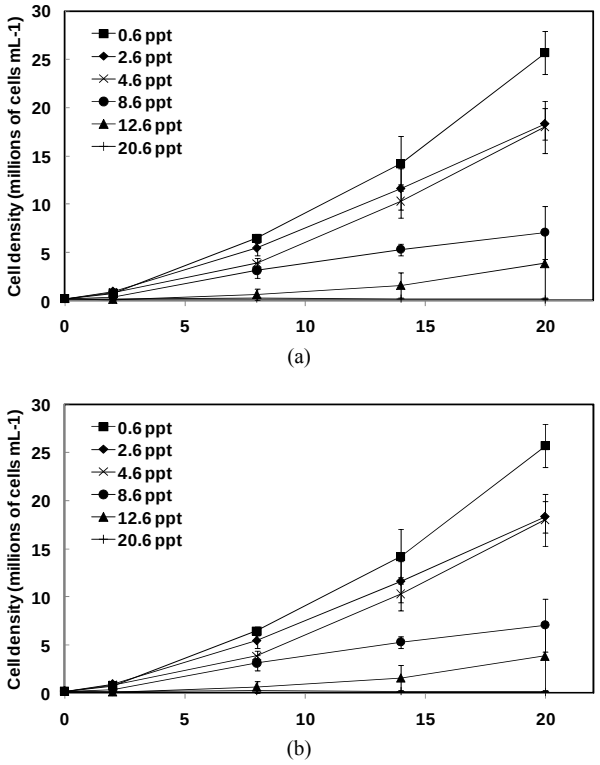

Figure 1. Changes in abundance of *M. aeruginosa* over time at a range of salt concentrations in terms of mean chlorophyll *a* concentrations (a) and cell numbers (b). Standard deviations are shown as vertical bars. All treatment groups at or above 16.6 ppt salt showed no increase in chlorophyll *a* or cell numbers.

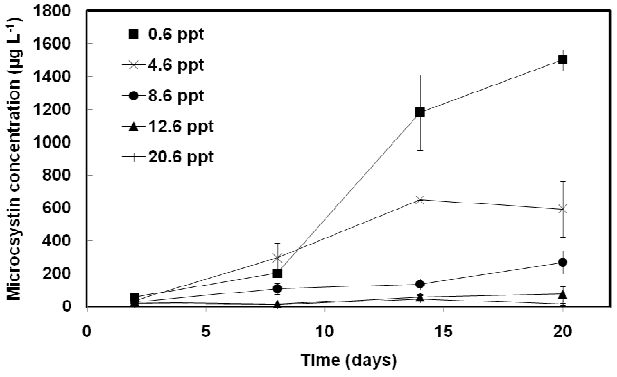

Figure 2. Changes in total microcystin concentration over time, under a range of salt concentrations. Standard deviations are shown as vertical bars.

total microcystin concentrations over twenty days of culture were similar, i.e. within 10% of each other.

Differences were observed over the culture period and between treatment groups in the distribution of toxins within (intracellular) and outside (extracellular) of the *M. aeruginosa* cells. The percentage of intracellular microcystin increased over time in cultures grown at salt concentrations up to 12.6 ppt, reflecting increases in cell density (**Figures 3**). At twenty days, cultures grown at 12.6 ppt or greater showed a greater proportion of extracellular than intracellular microcystin (ANOVA, F = 5.865, p <0.05, n = 15), as observed for *M. aeruginosa* blooms entering San Francisco Bay in California [15].

Extracellular microcystin concentrations in the ambient media persisted for the entire 20-day culture period. Over 80% of the initial extracellular toxin concentration remained after 20 days of culture in the 20.6 ppt treatment group, despite a lack of cell growth and rapid degradation of cells (i.e. initial concentration = 15.81 $\mu g \cdot L^{-1}$, SD = 1.00, N = 3; final concentration = 13.21 $\mu g \cdot L^{-1}$, SD = 3.21, N = 3).

The relatively high tolerance of toxic *M. aeruginosa* to elevated salt concentrations highlights the potential importance of this species in terms of the ecology of estuaries, including the health of aquatic animals [11]. Toxic cells consumed through the gastrointestinal tract can enter the blood stream and effect internal organs [36]. Microcystin has been shown to bioaccumulate in the tissues

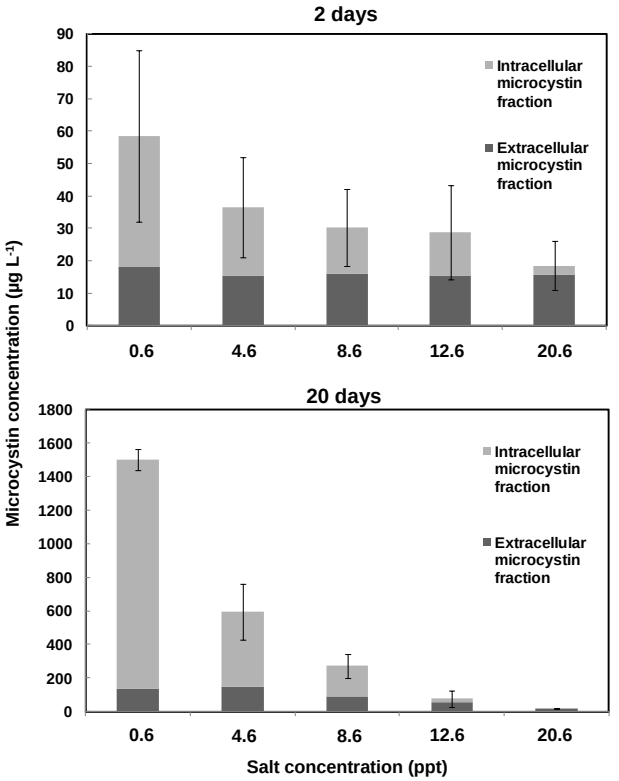

Figure 3. Total microcystin concentrations divided into intracellular and extracellular fractions, after 2 (top) and 20 (bottom) days of growth at salt concentrations of 0.6, 4.6, 8.6, 12.6, and 20.6 ppt. Standard deviations are shown as vertical bars.

of a wide range of organisms [4,8], including zooplankton [37] ,shellfish [37-40], and fish [36,41], thereby potentially exposing all trophic levels of the food web to microcystin [42]. Soluble microcystins in the water have been shown to affect the gills of fish, reducing the capacity for gas and ion exchange [43].

The presence of intracellular and extracellular microcystins may also impact human health. One of the major concerns is microcystin contamination of potable water [4,13]. In estuaries, concerns center on the increasing use of desalinated water for human consumption [44]. Management options depend on whether the toxins are principally intracellular or extracellular [24,27,29]. If the toxin is primarily intracellular, removal of cells can substantially reduce the toxin threat, but if the toxin is primarily extracellular chemical treatments may be necessary.

Another human health concern is the consumption of shellfish and fish containing microcystin, however, considerable uncertainty remains over the potential risks. Several researchers have observed significant levels of microcystin in the tissues of commercially important fish, such as tilapia [36], and shellfish, such as the blue mussel [45]. The highest concentrations tend to be localized in gastrointestinal organs [36].

In addition to consumptive issues, recreational use of estuarine waters might also be affected by the presence of toxic *M. aeruginosa* blooms [7]. Irritation due to contact of microcystin with epithelial tissues has been observed in humans, including blistering of affected tissues and hepatoenteritis [4,46]. Toxin exposure can occur through direct exposure to water via inhalation of aerosolized cells and contaminated water particles. Anecdotal evidence has linked several cases of pneumonia to recreational usage of waters during a *M. aeruginosa* bloom [47]. A microcystin LR concentration of 20 $\mu g \cdot L^{-1}$ has been suggested as a threshold level of concern for recreational exposure [4], but more definitive guidelines remain an issue of debate and continued research [48].

The longevity of the effects of toxic *M. aeruginosa* blooms in different ecosystems depend on factors that accelerate the degradation or dilution of the toxin, such as ultraviolet radiation, strong oxidizers, naturally occurring bacteria which deactivate or otherwise eliminate microcystins [37], and hydrologic considerations, such as tidal flushing and water residence time, which define the rates of dilution of both toxic cells and extracellular toxin. Greater rates of exchange with coastal marine water also decrease the spatial and temporal window of salinities favorable for survival. The results suggest that the influence of *M. aeruginosa* and/or its toxins can extend well out into estuaries, particularly those with restricted water exchange with coastal waters where mesohaline conditions can persist for extended periods of time.

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