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Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium *Microcystis aeruginosa*

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Abstract

An unprecedented bloom of the cyanobacterium *Microcystis aeruginosa* Kütz. occurred in the St. Lucie Estuary, FL in the summer of 2005. Samples were analyzed for toxicity by ELISA and by use of the polymerase chain reaction (PCR) with specific oligonucleotide primers for the *mcy*B gene that has previously been correlated with the biosynthesis of toxic microcystins. Despite the fact that secreted toxin levels were relatively low in dense natural assemblages (3.5 $\mu g\,l^{-1}$), detectable toxin levels increased by 90% when *M. aeruginosa* was stressed by an increase in salinity, physical injury, application of the chemical herbicide paraquat, or UV irradiation. The application of the same stressors caused a three-fold increase in the production of H_2O_2 when compared to non-stressed cells. The application of micromolar concentrations of H_2O_2 induced programmed cell death (PCD) as measured by a caspase protease assay. Catalase was capable of inhibiting PCD, implicating H_2O_2 as the inducing oxidative species. Our results indicate that physical stressors induce oxidative stress, which results in PCD and a concomitant release of toxin into the surrounding media. Remediation strategies that induce cellular stress should be approached with caution since these protocols are capable of releasing elevated levels of microcystins into the environment.

Keywords: Caspase; Cellular stress; Hydrogen peroxide; *Microcystis aeruginosa*; Microcystins; Programmed cell death

1. Introduction

The prevalence of toxic cyanobacterial blooms in the state of Florida has received considerable attention in the past 20 years since first being recorded in Lake Okeechobee and Lake Istokpoga (Carmichael, 1992; Burns et al., 2002). Long-term studies in three major marine ecosystems (Florida Bay, Indian River Lagoon, and the Suwannee Estuary) and five freshwater ecosystems (Lake Okeechobee, the St. Johns River, Lake Griffin, the Rainbow River, and the Suwannee River) have provided informative data on trophic states, water exchange rates, light availability, and measurements of growth-limiting nutrients of planktonic assemblages (Phlips et al., 1993, 2002; Phlips, 2002). Aside from increasing anthropogenic input, cyanobacterial blooms can form in eutrophic water masses simply from abiotic natural sources such as surface or ground water input from naturally nutrient-rich sediments (Phlips et al., 2002). As urbanization and agricultural expansion have led to increases in nutrient effluxes into Florida water systems, cyanobacteria have been quite opportunistic in exploiting these available nutrients (Canfield et al., 1989).

Lake Okeechobee is one of best known sources of cyanobacterial blooms in the United States (Phlips et al., 2002). The Florida hurricane season of 2004 resulted in a major sediment disturbance to the lake resulting in the release of high levels of inorganic phosphorus. Periods of high rainfall followed by the release of water from district canals most likely resulted in a washout of freshwater cyanobacteria into the St. Lucie River (SLR) Estuary. The SLR Estuary is one of the largest brackish water systems on the east coast of Florida. Encompassing 780 square miles, the estuary represents an indispensable asset both biologically and economically.

Sporadic colonies of *Microcystis aeruginosa* were first identified in the SLR Estuary in June 2005. By July 2005, *M. aeruginosa* abruptly emerged as a dense bloom covering the St. Lucie waterway within both St. Lucie and Martin counties, Florida (Fig. 1A and B).

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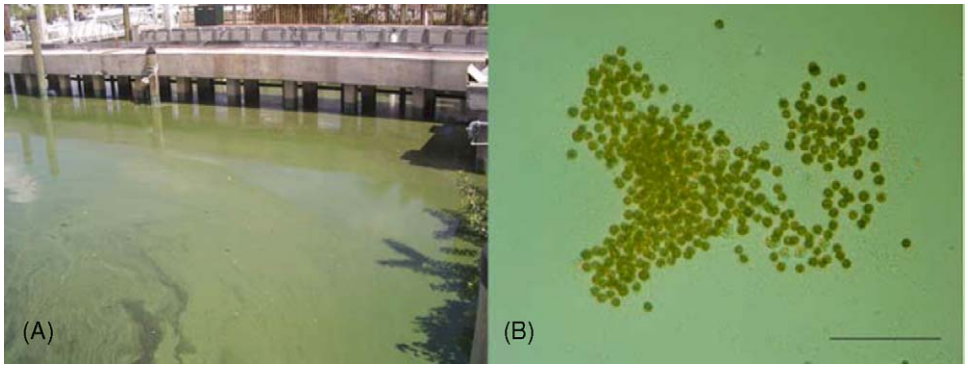

Fig. 1. (A) Bloom of *M. aeruginosa* in the St. Lucie River, August 2005. (B) Colony of *M. aeruginosa*. Scale bar, 45 μm.

M. aeruginosa contains a suite of toxic heptapeptides that have detrimental impacts on environmental health. Hepatotoxin variants of microcystin are directly associated with the deaths of fish, domestic livestock, and even human mortalities (Skulberg et al., 1984; Gunn et al., 1992; Rodger et al., 1994; Jochimsen et al., 1998; Codd et al., 1999). Interestingly enough, not all *Microcystis* strains produce toxins. In some bloom-specific cases, one species can be morphologically identical to the next yet may vary in toxicogenicity (Baker et al., 2001). In other cases, some species are known to upregulate or downregulate their toxicity under varying laboratory conditions (Kaebernick and Neilan, 2001). It is not known why such natural variations in toxicity exist. The use of molecular probes that target toxin-associated genes, in conjunction with imunoassays, have led to advancements in the identification of toxic strains (Bittencourt-Oliveira, 2003; Kaebernick and Neilan, 2001; Vaitomaa et al., 2003). 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 clear exactly what environmental conditions may induce toxin release.

There are no previous reports directly relating the cellular stress of *M. aeruginosa* with microcystin release into the environment. In cyanobacteria, H_2O_2 is commonly produced via photochemical reactions where concentrations vary in proportion to the amount of sunlight (Palenik et al., 1987; Xue et al., 2005). However, the biological production of H_2O_2 may reflect an imbalanced state of redox within the chloroplast and thus may serve as a proxy for cellular stress (Twiner and Trick, 2000; He et al., 2002; Choo et al., 2004). Among the approaches used to quantify H_2O_2, the fluorogenic method is quite successful and has subsequently been used to monitor oxidative stress in a variety of photosynthetic organisms (He and Hader, 2002; Ross et al., 2005).

The purpose of this study was to verify toxicity in the SLR Estuary assemblage of *M. aeruginosa* and determine if certain environmental conditions or potential remediation strategies could induce stress and lead to a significant release of soluble toxins into the surrounding water column. In addition, it was of interest to evaluate whether toxin release was associated with oxidative stress and subsequent programmed cell death (PCD).

2. Materials and methods

2.1. Microcystis collections

Specimens of *M. aeruginosa* were collected from surface waters (salinity 0.2‰, temperature 32.7 °C) adjacent to the Riverwatch Marina, Stuart, Florida (27°27.962′N, 80°19.747″W) on 12 August 2005. For programmed cell death experiments, specimens were collected from a nearby unnamed freshwater pond (27°54.288′N, 80°37.234″W). Cyanobacteria were transported to the Smithsonian Marine Station (SMS) at Fort Pierce for immediate analysis after collection.

2.2. DNA extraction and amplification

Total genomic DNA was prepared using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Typical DNA yields ranged from 1 to 10 μg ml^{-1}. Absorbances (A_{260}/A_{280}) to determine quantity and quality were measured using a SmartSpec Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA). PCR amplification was conducted by following the protocol previously reported by Bittencourt-Oliveira (2003) using the *mcy*B primers (forward and reverse) designed by Neilan et al. (1999). The amplification process was carried out in 25 μl volumes on a MJ PTC-200 cycler (Bio-Rad) using Taq Master Mix (Qiagen). The following cycling parameters were used: 94 °C for 2 min, followed by 35 cycles at 94 °C for 10 s; 40 °C for 20 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Aliquots of the PCR reaction product were run on a 1.2% agarose gel containing 10 μg ml^{-1} ethidium bromide, and documented with a Typhoon 9410 high performance gel and blot imager (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

2.3. Sequencing analysis and accession numbers

*Mcy*B gene amplicons were sequenced in both directions using the PCR primers *mcy*B and Big Dye Terminator v3.1 technology (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were viewed and edited using ChromasLite2000