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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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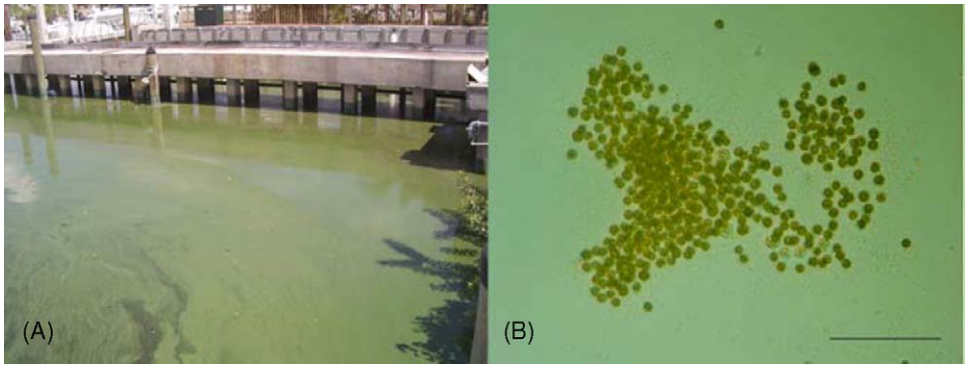

Fig. 1. (A) Bloom of *M. aeruginosa* in the St. Lucie River, August 2005. (B) Colony of *M. aeruginosa*. Scale bar, 45 μm.

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

(www.technelysium.com.au/chromas.html) and aligned using Biology Workbench (http://workbench.sdsc.edu). Sequences were also compared to those in databases using the Basic Local Alignment Search Tool (BLAST) algorithm to identify known sequences with a high degree of similarity. Homology to other published *mcy*B gene sequences was conducted with MegAlign (Lasergene, DNASTAR, Madison, WI, USA). The *mcy*B gene sequence was submitted to GenBank and assigned the accession number DQ218313.

2.4. Detection of toxins

Detection and quantification of microcystin toxins released into the water column were measured using an Envirologix Microcystin Tube Kit (Envirologix Inc., Portland, ME, USA) according to the manufacturer's instructions. This ELISA based assay is capable of reacting with four microcystin toxin variants (Microcystin LR, LA, RR, and YR) as well as Nodularin. Absorbance measurements (450 nm) were recorded on a Shimadzu UV-265 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). A standard curve was constructed using commercially available Microcystin LR (Sigma, St. Louis, MO, USA). The measurement of toxins released from 1.05×10^8 cells in 50 ml of in situ estuary water (salinity 0.2‰, temperature 32.7 °C, maintained at a constant irradiance of 10 $\mu E\, s^{-1}\, m^{-2}$) was used as a control for all experiments ($n = 3$). Experimental stress conditions are described below.

2.5. Measurements of cellular stress

To establish a link between cellular stress and toxin release, cells of *M. aeruginosa* were assayed for H_2O_2 production as a direct correlate of stress response. This type of assay has been commonly employed as a marker for stress response in higher plants (Orozco-Cardenas and Ryan, 1999) and marine algae (Collen and Pedersen, 1994; Küpper et al., 2002; Ross et al., 2005). The response is based on the reaction of hydrogen peroxide with the fluorogenic probe dichlorofluorescein diacetate (DCFH-DA, Invitrogen Corp., Carlsbad, CA, USA). DCFH-DA is a non-fluorescing, non-polar compound. When this compound reacts with cellular esterases the diacetate group is cleaved off to yield the polar compound DCFH (2′7′-dichlorodihydrofluorescein). Oxidation of DCFH by hydrogen peroxide yields the fluorescent product DCF (Collen and Davison, 1999). DCFH-DA was dissolved in dimethylsulfoxide in 10 mM aliquot stocks (stored at −80 °C).

The measurement of H_2O_2 released from 1.05×10^8 cells in 50 ml of in situ estuary water was used as a control value. Irradiance was measured on a LI-188B Integrating photometer (LI-COR, Lincoln, Nebraska, USA). At selected time points (upon application of a stressor), 1 ml of media surrounding the cyanobacteria from each replicate was collected and added to a cuvette containing 0.40 U/ml esterase and 25 μM of DCFH-DA. The total volume was increased to 2000 μl in filtered seawater. H_2O_2 was quantified fluorometrically on a Biorad VersaFluor fluorometer (Bio-Rad; excitiation: 488 nm, emission: 525 nm) using the methods of Ross et al. (2005). Biologically relevant concentrations of commercially obtained H_2O_2 (Sigma) were used as standards.

To examine the effect of increased salinity on the release of microcystins, *M. aeruginosa* cells were counted via hemocytometer and subsequently diluted to 1.05×10^8 cells per replicate ($n = 3$ per treatment). Cells were transferred to beakers containing 50 ml of full strength seawater (32‰) and incubated at room temperature for 5 h. Post-incubation, 80 μl of the surrounding water was assayed for secreted toxins.

To examine the oxidative stress initiated by solar radiation *M. aeruginosa* samples (1.05×10^8 cells immersed in 50 ml estuary water) were exposed to full sunlight for predetermined time intervals ($n = 3$ per treatment). Experiments were conducted outside under a constant solar irradiance of 1600 $\mu E\, s^{-1}\, m^{-2}$. All samples were placed in 50 ml conical tubes which were in turn placed in a plastic container filled with chilled water to prevent any increase in temperature during the course of the experiment. Acrylic filters were used to obtain the desired transmittance of solar radiation reaching the samples: UF 96 (absorbs UV-A, -B, and -C) and UVT (transmits UV-A and -B but not UV-C) (Spartech Polyclast, Stamford, CT, USA). At selected time points, 80 μl of the surrounding water was assayed for secreted toxins.

Paraquat (Sigma) is a non-selective contact herbicide that destroys plant tissue by interrupting the steady flow of electrons from photosystem I to NADPH. Instead, electrons are donated directly to molecular oxygen yielding highly powerful oxidants. To examine the effect of toxin release upon herbicide treatment, 1 mM paraquat was added to a 50 ml falcon tube containing 1.05×10^8 cells in estuary water. Following an incubation period of 5 h, 80 μl of the surrounding water was assayed for secreted toxins.

To examine the effect of sonic injury, cells (1.05×10^8 cells/50 ml estuary water) were subjected to three 10 s pulses of 20 kHz sonic sound on a Branson 1510 ultrasonic cleaner (Branson, Danbury, CT, USA) as previously described by Mydlarz and Jacobs (2004). Post-injury cells were gently mixed for 5 h and subsequently analyzed for toxin release as described above.

2.6. Induction of cellular programmed cell death by oxidative stress

To evaluate the relationship between oxidative stress and programmed cell death, 1.05×10^8 cells of *M. aeruginosa* were incubated in 50 ml of in situ estuary water with selected concentrations of H_2O_2 and assayed for caspase proteolytic activity as a function of time. Cells were incubated with H_2O_2 and 50 units of catalase (Sigma) to verify that H_2O_2 was indeed the specific molecule promoting PCD. Following incubation with H_2O_2, *M. aeruginosa* cells were collected via filtration, flash frozen with liquid N_2 and soluble proteins were extracted in 20 ml of 100 mM phosphate buffer (pH 7.8). The extract was centrifuged at $6600 \times g$ for 5 min at 4 °C on a Beckman TJ-6 centrifuge. The supernatant was collected and protein concentration was quantified with a Quick Start™ Bradford Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions.

The Enzchek® Caspase-3 Assay Kit #2 (Invitrogen) was utilized to quantify programmed cell death activity in stressed cells of *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 were 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 Biorad VersaFluor fluorometer (Ex/Em: 496/520 nm; $N = 3$). The reversible aldehyde caspase inhibitor Ac-DEVD-CHO (Invitrogen) was used as a negative control. Cyanobacterial samples were preincubated with 100 μM Ac-DEVD-CHO for 20 min prior to the addition of H_2O_2. Background fluorescence was subtracted for no-enzyme controls.

2.7. Statistics

To determine if the treatments had an effect on toxin release a one way ANOVA was conducted followed by an LSD post-hoc test. The data were rank transformed since they did not meet the assumptions of normality nor equal variances. The analysis for H_2O_2 production was conducted in the same manner. All figures show untransformed data. A Pearson product moment correlation was used to correlate toxin secretion and H_2O_2 release. All analyses were conducted using Statistix 7 (Analytical Software, Tallahassee, FL, USA).

3. Results

3.1. Detection of mcyB

Samples of *M. aeruginosa* were collected in situ (salinity 0.2‰, temperature 32.7 °C) from a highly dense area as shown in Fig. 1A. The presence of the *mcy*B gene was established by the PCR-amplification of *M. aeruginosa* DNA with *mcy*B gene-specific primers. A PCR amplicon of the expected size (759 bp) was obtained (Fig. 2). The PCR product was sequenced and its identity was confirmed by BLAST analysis. This sequence was analyzed for its homology to other *mcy*B gene sequences. Alignments with other published *mcy*B sequences resulted in almost identical identity matches ranging from 97.6% to 99.7% identity (compared to published *mcy*B gene sequences with accession numbers AB092806, AJ224717, AJ224726, AJ492554, AY568035). This data confirms that the presence of the *mcy*B gene, responsible for the production of microcystins, was present in the St. Lucie River *M. aeruginosa* assemblage.

3.2. Detection of extracellular toxins

Direct measurement of toxins from 1.05×10^8 cells in 50 ml of in situ water, resulted in associated values around 3.5 μg l^{-1}. When cells were subjected to an osmotic change in media (32‰ seawater) an 80% increase in toxin level was detected within 5 h when compared to the 3.5 μg l^{-1} toxin value observed in non-stressed cells (Fig. 3). The absorbing ability of ultraviolet filtering panels (UF 96UV) was capable of preventing any significant increase in soluble toxin over the same time course. Conversely, when cells were incubated under ultraviolet transmitting plates (UVT) a 55% increase in soluble toxin was detected when compared to the 3.5 μg l^{-1} toxin value detected in non-stressed cells (Fig. 3). The use of paraquat resulted in a 90% increase in detectible toxins. Physical injury yielded the highest release of soluble toxins with spectrophotometric values 95% greater than the control samples. All stressors (aside from the use of UF

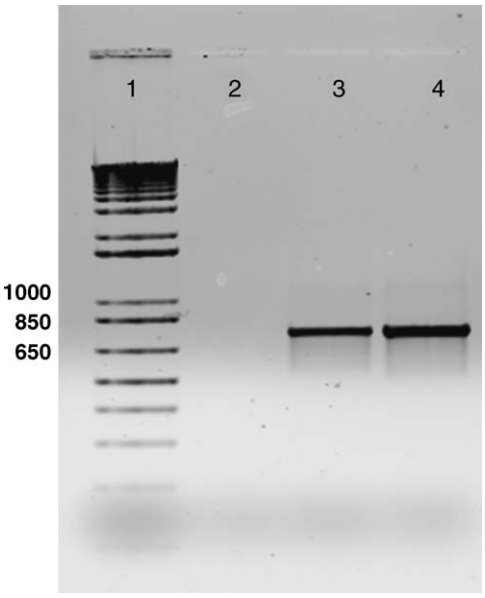

Fig. 2. Ethidium bromide-stained 1.0% agarose electrophoresis gel showing microcystin synthetase gene amplicons (~750 bp) from toxic *Microcystis* using oligonucleotide primers FAA and RAA (Neilan et al., 1999). Lane 1, molecular marker 1 kb DNA plus ladder (Invitrogen, CA); lane 2, negative (no template) control; lane 3, St. Lucie River species; lane 4, 1:10 dilution of St. Lucie River species DNA stock.

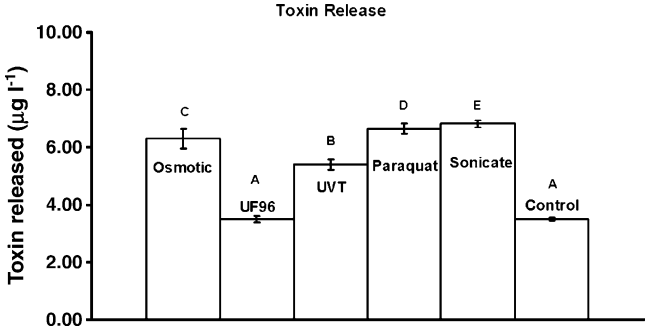

Fig. 3. Measurement of toxins (as microcystin LR equivalents) secreted from stressed cells compared to undisturbed cells maintained in natural estuary water. Cells of *M. aeruginosa* were osmotically stressed by being transferred to 32‰ seawater for 5 h (Osmotic). Cells were stressed by full exposure to solar radiation (UVT) and compared to Ultraviolet filtered solar irradiation (UF 96). Cells were incubated with a lethal dose of the herbicide (Paraquat), and physically disrupted by ultrasonication (Sonicate) ($n = 3$ for each experiment). Data bars are represented as the mean with error bars of ±1. Significant groupings are indicated by the letters above bars, analyses were conducted by a one-way ANOVA followed by an LSD post-hoc test.

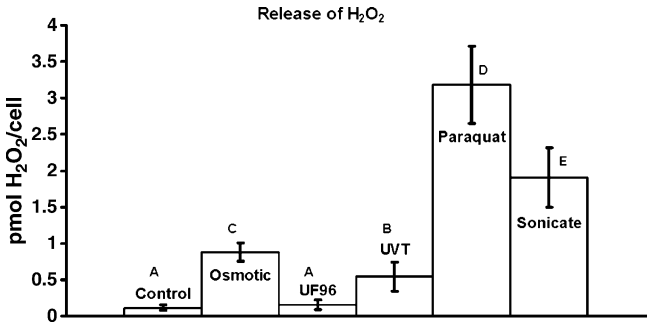

Fig. 4. Measurement of H_2O_2 released as a function of stress treatment. Data bars are represented as the mean with error bars of ±1. Significant groupings are indicated by the letters above bars, analyses were conducted by a one-way ANOVA followed by an LSD post-hoc test.

96UV) resulted in a significant increase of toxin concentration ($p < 0.001$).

3.3. Hydrogen peroxide production in response to cellular stress

H_2O_2 was released by *M. aeruginosa* following an increase of salinity, physical injury, application of paraquat, or UV irradiation (Fig. 4). Control cells were shown to release ~0.25 pmol H_2O_2 cell^{-1} after sitting undisturbed for 5 h. This would be expected as basal levels of reactive oxygen species, such as H_2O_2, are exuded as simple byproducts of photosynthesis. When cells were placed in 32‰ seawater, H_2O_2 levels elevated to 0.75 pmole H_2O_2 cell^{-1}. The use of the UV absorbing material UF 96UV prevented any change in H_2O_2 release when compared to the control. The ability for UV radiation (UVT) to reach the cells resulted in H_2O_2 levels reaching 0.60 pmol H_2O_2 cell^{-1}. The use of paraquat to compromise cellular homoestasis resulted in a considerable release of reactive oxygen species (3.25 pmol H_2O_2 cell^{-1}). Physical disruption of the cyanobacterial cells resulted in H_2O_2 concentrations just under 2.0 pmol H_2O_2 cell^{-1}. All stressors (aside from the use of UF 96UV) resulted in a significant production of H_2O_2 relative to control cells ($p < 0.001$). There was a significant positive correlation between toxin release and H_2O_2 production ($p < 0.001$, $r^2 = 0.59$).

3.4. Programmed cell death by oxidative stress

Exogenous addition of H_2O_2 elicited caspase activity in vitro (Fig. 5). Caspase activity was initially detected by 6 h post-introduction when *M. aeruginosa* cells were incubated in 10 μM H_2O_2. By 18 h post-incubation, caspase activity reached 9 μmol rhodamine 110 mg^{-1} protein. The addition of 100 μM H_2O_2 elicited a more rapid response in caspase activity (initial detection by 1 h post introduction of 100 μM H_2O_2). By 24 h-post introduction caspase activity was detected at 50 μmol rhodamine 110 mg^{-1} protein. Control cells that were not treated with exogenous amounts of H_2O_2 did not show any significant level of caspase activity (Fig. 5). When cells were incubated with 100 μM H_2O_2 in addition to 50 U catalase, caspase activity was suppressed for up to 10 h. After this time point caspase activity slowly increased to 9 μmol rhodamine 110 mg^{-1} protein by 24 h. When cells were preincubated with the reversible caspase inhibitor Ac-DEVD-CHO prior to the addition of 100 μM H_2O_2, capase activity was drastically reduced (Fig. 5).

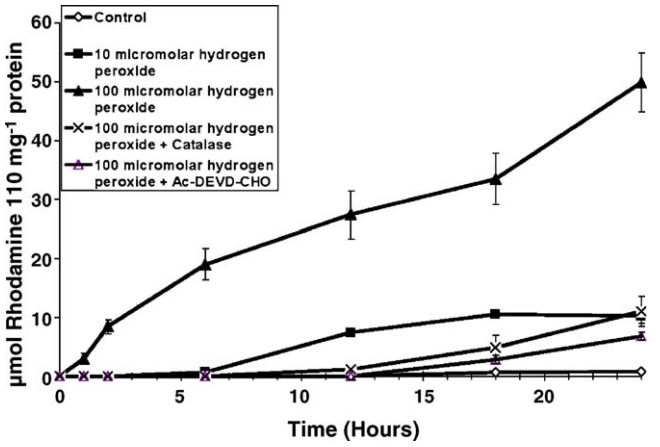

Fig. 5. Time course of the onset of programmed cell death as measured by caspase activity (DEVD cleavage) in *M. aeruginosa*. Cells were incubated with selected concentrations of H_2O_2 ($n = 3$ for each experiment). Caspase activity was quantified by the cleavage of Z-DEVD-R110 resulting in the production of the fluorescent product rhodamine 110 (Ex/Em: 496/520 nm). Catalase (1 U/ml) and the reversible aldehyde caspase inhibitor Ac-DEVD-CHO (100 μM) were used as negative controls. Units are expressed in μmol rhodamine 110 mg^{-1} protein.

4. Discussion

M. aeruginosa is a ubiquitous cyanobacterium that has often been linked to toxic blooms world-wide (Watanabe and Oishi, 1984; Jewel et al., 2003; Silva, 2003). A series of morphological and physiological characteristics may account for its great success amongst the phytoplankton community. Primarily, *M. aeruginosa* requires little energetic input to sustain cellular balance and is capable of persisting in nutrient deplete areas. Cyanobacteria can outcompete other planktonic organisms due to their high affinity for phosphorus and nitrogen. In addition, *M. aeruginosa* contains numerous gas vacuoles allowing this organism to modify its buoyancy, again giving it a distinct advantage over other phytoplankton in the community (Smith, 1950; Whitford and Schumacher, 1984).

High concentrations of *Microcystis* do not necessarily correlate with high levels of microcystins in the water column. Many cyanobacteria retain cyanotoxins within their cell structure, and upon cell lysis release these toxins into the surrounding water (White et al., 2005). The early stages of a toxic bloom are characteristically associated mostly with intracellular toxins. As the bloom ages, cell death ensues and the concentration of extracellular toxins increases (Lahti et al., 1997, White et al., 2005). Our collection of water from a dense bloom (surface scum as shown in Fig. 1A) displayed surprisingly low toxin values (3.5 μg l^{-1}). Upon exposure to selected stressors, cell bound toxins were capable of being released into the immediate vicinity at levels over 90% above what was normally secreted by dense assemblages of *M. aeruginosa*.

In photosynthetic organisms H_2O_2 is produced in response to a variety of exogenous factors including irradiation, pesticides, xenobiotics, or pathogens (Xue et al., 2005). In several species of cyanobacteria and unicellular algae, the production of H_2O_2 can coordinate a series of cellular responses including the induction of programmed cell death or apoptosis (Korsmeyer et al., 1995; Levine et al., 1996; Vardi et al., 1999). For example, ultraviolet radiation (particularly UV-B: 280–320 nm) has a detrimental effect on the development and general metabolism of cyanobacteria (Tyagi et al., 1992; Sinha and Hader, 1998, 2002). Aside from the direct damage to key proteins, enzymes and DNA, the leakage of electrons from the photosynthetic electron transport chain to oxygen enhances the formation of reactive oxygen species (ROS). The indirect damage by ROS includes oxidative lipid peroxidation, inhibition of photosynthesis, and the oxidation of photosynthetic pigments such as chlorophylls and phycobilins (He et al., 2002). A recent study demonstrated that *M. aeruginosa* released H_2O_2 into the surrounding media when specimens were irradiated with an ultraviolet source (Alam et al., 2001). Aside from the observation that cell death ensued there was no mention of toxin release into the environment. Our results indicate that when cultures of *M. aeruginosa* were irradiated by full sunlight, the UV absorbing material UF 96UV prevented any elevation in H_2O_2 release. Conversely, *M. aeruginosa* placed under UV transmitting material significantly increased production of H_2O_2. This observation supports the hypothesis that ultraviolet radiation triggers the formation of ROS in *M. aeruginosa*. More importantly, elevated levels of H_2O_2 were accompanied with a 40% increase in toxin release into the surrounding media, whereas *M. aeruginosa* protected by UV-blocking material showed no sign of H_2O_2 increase or toxin elevation.

In an attempt to use salinity alterations as a bloom elimination strategy, specimens of *M. aeruginosa* were found to be viable at salt concentrations up to 9.8‰ (Atkins et al., 2000). Salinity values surpassing this critical mark were accompanied with a reduction in total viable cell concentration and an increase in cell lysis (Atkins et al., 2000; Orr et al., 2003). Our data show nearly an 80% increase in toxin release when cells were transferred to water with a salinity of 32‰. This increase was concomitant with an increase in secreted H_2O_2, showing oxidative stress correlates with toxin release.

Since cyanotoxins are stored intracellularly, the application of algicides should be used with caution to avoid toxin release. Studies have demonstrated that microcystins are released when blooms of *M. aeruginosa* are treated with algicides such as copper sulfate or sodium carbonate peroxyhydrate (Pak-27^{TM}; Jones and Orr, 1994; Touchette et al., 2005). Post lysis, free toxin was detected at concentrations reaching 1.8 $\mu g\,l^{-1}$ in mesocosm studies. This release can be quite rapid and occurs within 3–24 h of algicide application depending on the administered dose (Jones and Orr, 1994; Kenefick et al., 1993). Paraquat (methyl viologen) is a well known herbicide/algicide that elicits toxicity in a catalytic manner requiring the presence of molecular oxygen and chloroplast photo-activation. Ultimately free radicals are produced that compromise the photosynthetic integrity of the organism. Our studies with paraquat showed a 90% increase in toxin release compared to control specimens.

Exposure of cells to low levels of physical and chemical stressors have been known to trigger apoptosis-like conditions in a variety of photosynthetic organisms including vascular plants (Lam and del Pozo, 2000; Lam et al., 2001), unicellular chlorophytes (Berges and Falkowski, 1998; Segovia et al., 2003), dinoflagellates (Vardi et al., 1999; Franklin et al., 2004; Franklin and Berges, 2004), and cyanobacteria (Berman-Frank et al., 2004). Characteristics of cells undergoing PCD include chromatin condensation, shrinkage of the nucleus and cytoplasm (Danon et al., 2000), and ordered DNA fragmentation (Hoeberichts and Woitering, 2003). These changes are believed to be directed by caspases (a family of cysteine proteases that function as a regulatory switch for many forms of PCD in animals) (Thornberry and Lazebnik, 1998).

It appears that toxin release in stressed *M. aeruginosa* was directly correlated with an H_2O_2-elicited signal for PCD. The production of H_2O_2 can trigger the initiation of PCD by direct oxidative damage to DNA, or by the indirect oxidation of regulatory molecules that ultimately provide an entrance into PCD (Slater et al., 1995). This process is termed oxidative cell stress (Van Camp et al., 1998). Aside from what has been documented in *Trichodesmium* (Berman-Frank et al., 2004), the involvement of oxidative stress on PCD has not been previously shown in other cyanobacterium.

Caspase activity was initially thought to be limited to metazoans since direct sequence homologies have not been identified in photosynthetic organisms. However, a family of caspase-related proteases has now been recognized in higher plants via iterative homology matches (Del Pozo and Lam, 1998). Caspase or "caspase-like" proteolytic activity is currently known in fungi, multiple bacterial species (Uren et al., 2000), unicellular chlorophytes (Segovia et al., 2003; Segovia and Berges, 2005) and plants during the hypersensitive response (Del Pozo and Lam, 1998, Chichkova et al., 2004). The most recent findings of caspase activity in the marine cyanobacterium *Trichodesmium* spp. (Berman-Frank et al., 2004) prompted our investigation to utilize caspase activity as a proxy for PCD in *M. aeruginosa* (Hug et al., 1999, Liu et al., 1999). We demonstrated that caspase activity in stressed *M. aeruginosa* cells might be responsible for the onset of PCD when induced with H_2O_2 as a result of oxidative stress (Fig. 4). This conclusion is supported by the fact that when cells were preincubated with the caspase inhibitor Ac-DEVD-CHO, caspase activity was essentially eliminated. These findings are in agreement with recent reports demonstrating that caspase activity is a common constituent not only in mammalian systems but in higher plants and lower algae as well (Korthout et al., 2000, Elbaz et al., 2002, Segovia et al., 2003). Our results, in conjunction with the recent caspase-like activity found in *Trichodesmium* (Berman-Frank et al., 2004), support the notion that PCD has an earlier evolutionary origin and broader significance than previously thought.

In conclusion, this is the first incidence of a large bloom of *M. aeruginosa* in the St. Lucie River, Estuary. We have demonstrated, through the use of immunodetection and molecular methodologies, that this assemblage has the genetic potential to biosynthesize microcystins and release them at concentrations ranging from 3.5 to 6.8 $\mu g\,l^{-1}$. Considering the World

Health Organization (WHO) placed a provisional guideline on microcystin concentrations of $1.0\,\mu g\,l^{-1}$ for potable water and $10\,\mu g\,l^{-1}$ for recreational use, it is critical that cyanobacterial remediation strategies be approached with caution. Guidelines that are based upon cell concentrations for calculating human risks are not adequate. The utilization of algicides, physical removal of surface scum using oil spill equipment, and increasing the salinity of water reservoirs are all currently used as bloom remediation strategies that are capable of resulting in cyanobacterial stress and subsequently the release of toxins into the surrounding water column. Residence times for these released toxins are subject to water composition and bacterial assemblages yet could persist in the water column for up to 3 weeks (Jones and Orr, 1994; Lahti et al., 1997; Chriswell et al., 1999). The key management plan for minimizing cyanobacterial blooms is to intervene at the source of the problem. Nutrient inputs and hydrologic sources are two such examples that have been identified as bloom targets by watershed management facilities (Chorus and Mur, 1999; Paerl et al., 2001; Piehler, 2005). Remediation strategies further downstream may potentially augment the problem rather than improve the quality of the water.

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