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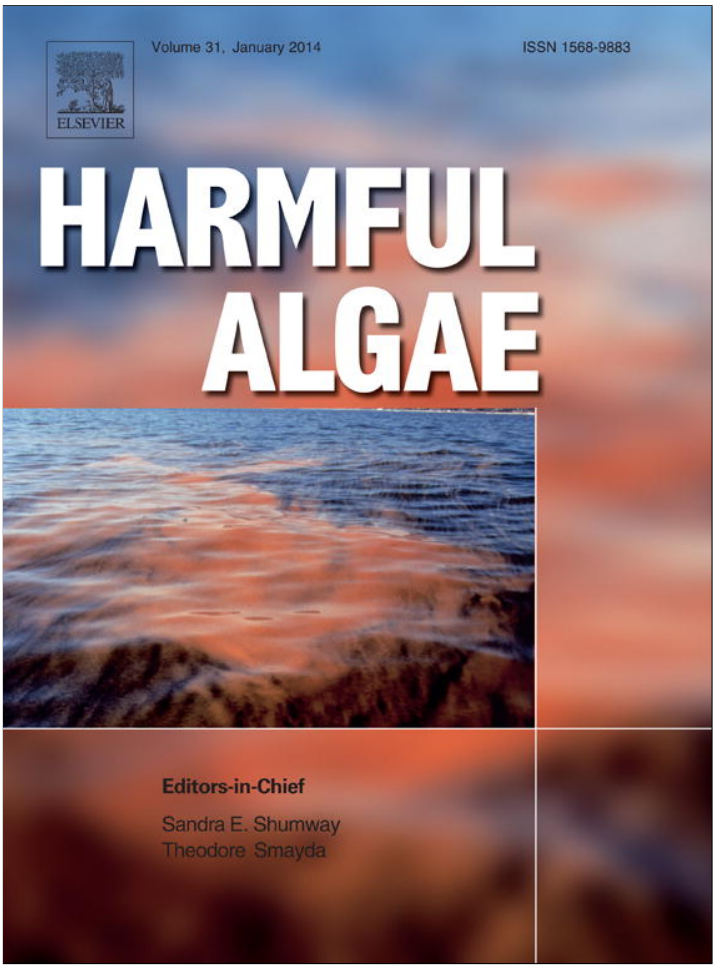

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Microcystins and two new micropeptin cyanopeptides produced by unprecedented *Microcystis aeruginosa* blooms in North Carolina's Cape Fear River

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ABSTRACT

The Cape Fear River is the largest river system in North Carolina. It is heavily used as a source of drinking water for humans and livestock as well as a source of irrigation water for crops, and production water for industry. It also serves as a major fishery for both commercial and recreational use. In recent years, possibly related to increased eutrophication of the river, massive blooms of cyanobacteria, identified as *Microcystis aeruginosa* have been observed. Bloom samples collected in 2009 and 2012 were chemically analyzed to determine if they contained cyanobacterial toxins known as microcystins. Both blooms were found to produce microcystins in high yields. Microcystins are potent hepatotoxins that can be bio-accumulated in the food chain. Recent biological studies have also shown a host of other potentially harmful effects of low level microcystin exposure. Detailed chemical analysis of these blooms led us to discover that these blooms produce an additional family of cyanobacterial peptides know as the micropeptins, including two new members named micropeptins 1106 and 1120. The biological activities of these new molecules have not yet been determined, although protease activity has been well documented for this peptide group. These data indicate a need for thorough monitoring of toxin levels especially during bloom events in addition to additional biological testing of other cyanopeptides present in blooms.

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1. Introduction

Through a variety of factors including increased nutrient loading due to agriculture, livestock production and urban development, the occurrence of dense blooms of potentially toxic cyanobacteria is becoming more common (Codd et al., 2005; Smith, 2003; Wiegand and Pflugmacher, 2005; Michalak et al., 2013). Blooms of cyanobacteria present ecological, economic and health issues to all organisms (including humans) that interact with waters in which these blooms occur. The density of the bloom can obscure light and shade macrophytes and other algae, as well as deplete dissolved oxygen levels through bloom death and decay. More troubling are the myriad negative health effects related to toxins produced by blooms of cyanobacteria. Toxicity symptoms attributed to cyanotoxins include gastroenteritis, nausea, vomiting, fever, sore throat, blistered mouth, eye and ear irritation, myalgia, severe abdominal pains, visual disturbances, kidney damage and liver damage (Burkholder, 2002).

Microcystins are potent hepatotoxins and are among the most widely investigated toxins because of their frequency in nature and their adverse health effects on humans (Falconer, 1989; Carmichael et al., 2001; de Figueiredo et al., 2004), as well as fish, birds, zooplankton and mammals (Burkholder, 2002; Vasconcelos, 1990; Demott and Moxter, 1991; Rogers et al., 2011; Garcia and Martinez, 2012). Because of these potential health threats, the World Health Organization has established provisional guidelines regarding limits of microcystins in drinking water (1 μg/L) (World Health Organization, 1999). Limits on other cyanotoxins have yet to be addressed due to insufficient data and lack of certified standards (Falconer, 1989). Chemically, microcystins are described as a family of cyclic heptapeptides with over 80 known members (Lifshits and Carmeli, 2012). Microcystins are cyclic heptapeptides (Fig. 1) and typically contain the motif of amino acids D-Ala^1-R$_1$-D-MeAsp^3-R$_2$-Adda^5-D-glutamate^6-Mdha^7. Two of these unusual amino acids N-methyldehydroalanine (Mdha) and 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda) are essential for activity. The structural variety found in the microcystin group is due to substitution of the two variable L-amino acids (R1 and R2 in Fig. 1) and this is usually denoted in the suffix of the nomenclature. Most of the microcystins are water

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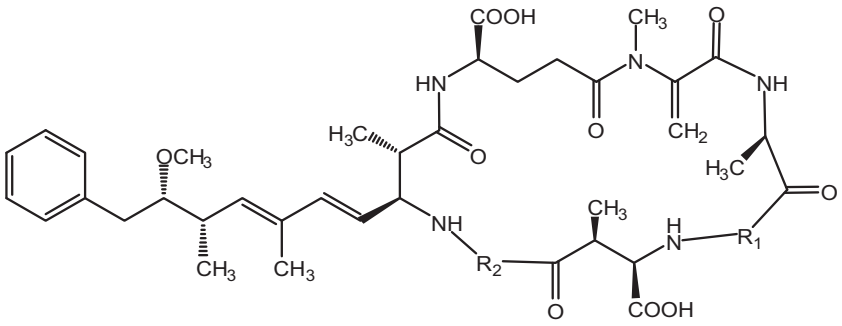

Microcystin	R_1	R_2	MW (Da)
LR	Leu	Arg	994
RR	Arg	Arg	1037

Fig. 1. Chemical structures of microcystins LR and RR found in the Cape Fear River blooms.

soluble, which aids in their dispersal in water and results in their accumulation in the liver of ingesting organisms where they disrupt the key eukaryotic protein phosphatases PPA1 and PPA2 which leads to the observed toxic effects of these compounds (Ikehara et al., 2009).

The Cape Fear River is the largest river entirely within the state of North Carolina, USA. It is used extensively for municipal drinking water, irrigation and industrial production water. The river receives significant nutrient loading from agricultural runoff and municipal and industrial wastewater treatment plant effluents (Mallin et al., 1999; NCDENR, 2005). Fortunately, due to elevated turbidity from anthropogenic land use upstream, troublesome blooms had heretofore been exceedingly rare in this river (Mallin et al., 1999; Dubbs and Whalen, 2008). However, during the fall of 2009, a dense bloom of cyanobacteria was observed in the lower Cape Fear River downstream from Lock and Dam #1. This man-made feature, roughly 40 miles north of Wilmington, North Carolina, is a popular recreation area for boating and fishing. Another large cyanobacterial bloom occurred in the same area during the summer of 2012, and in both cases the blooms were found to be comprised primarily of *Microcystis aeruginosa*. *Microcystis* blooms are widespread and have been observed in freshwater systems around the world. This prolific genus has been shown to produce a wide variety of biologically active secondary metabolites with most, but not all, strains producing microcystin hepatotoxins. Therefore, it was important to chemically analyze these blooms to determine whether they were toxin producers and consequently represented an environmental or health threat.

2. Materials and methods

2.1. Cyanobacterial bloom collection

The *Microcystis* blooms were concentrated on the water surface. In 2009 cyanobacterial samples were collected by scooping surface concentrations up with a bucket and then pouring into a 20 μm sieve. This was done until a sizable clump of algae was concentrated. This clump was then placed in a screw-capped plastic container and preserved with ethanol. In 2012 surface bloom material was collected and concentrated by repeatedly scooping surface material into 18 cm × 32 cm sand sample bags with approximately 60 μm mesh size (HUBCO, Inc., Hutchinson, KS), until a sizable clump was evident. The bag and material was then placed into a screw-capped plastic container in ethanol until processing at the laboratory.

Chlorophyll *a* samples were collected on-site in 125 mL opaque plastic containers, stored on ice until processing in the laboratory, where samples were filtered through 0.7 μm glass fiber filters. The filters were wrapped individually in aluminum foil, placed in an airtight container, and stored in a freezer. After 24 h each filter was ground using a Teflon grinder prior to extraction with 90% acetone solution (10 mL) and allowed to extract the chlorophyll from the material for 3 h. The solution containing the extracted chlorophyll was then analyzed for chlorophyll *a* concentration using a Turner AU-10 fluorometer following Welschmeyer (1994).

2.2. Cyanopeptide extraction and preliminary fractionation

The cells were collected on glass microfiber filters (VWR International, LLC Brand, Grade-691, 15 cm diameter) and extracted twice overnight with aqueous MeOH (80%). The MeOH extracts were combined and concentrated *in vacuo* to water (50 mL). MeOH (450 mL) was added to make a 90% MeOH/H_2O extract before partitioning against hexane (1:1). The resulting organic MeOH layer was dried *in vacuo* before chromatography using a Supelco® ENVI-18 SPE column eluted with 20%, 40%, 60%, 80%, and 100% aq. MeOH aliquots, and a final wash with hexane. Fractions collected at each step were analyzed by LC/UV/MS.

Fractions of interest (40%, 60%, and 80% aq. MeOH) were combined and further separated by reversed phase C_{18} column chromatography (BAKERBOND Octadecyl (C_{18}) 40 μm Prep LC Packing, J.T. Baker), and elution with a stepwise gradient (30–70% of aq. MeOH (10% steps; 50 mL each)). Two aliquots (12.5 mL) were collected at each step. The column was finally washed with 100% methanol and 11 fractions collected in total.

2.2.1. Microcystin purification

Bakerbond extracts containing microcystins were separated by reversed phase HPLC (Waters SunFire™ C_{18} 3.5 μm 4.6 mm × 150 mm column), eluted with a linear gradient of ACN/H_2O + 0.1% formic acid (23–43% ACN, 0.8 mL/min, 240 nm, 20 min). Final purification was accomplished with 35% ACN isocratic method (0.8 mL/min).

2.2.2. Micropeptin purification

The combined Bakerbond extracts containing micropeptins based on LC–MS analysis were separated by reversed phase HPLC (Waters SunFire™ C_{18} 3.5 μm 4.6 mm × 150 mm column), with a linear elution gradient of ACN/H_2O + 0.1% formic acid (23–27% ACN, 0.8 mL/min, 20 min) and monitored at 240 nm and 276 nm.

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Final purification was achieved by isocratic reversed phase HPLC (Waters SunFire™ C_{18} 3.5 μm 4.6 mm × 150 mm column, 30% ACN/70% H_2O + 0.1% formic acid).

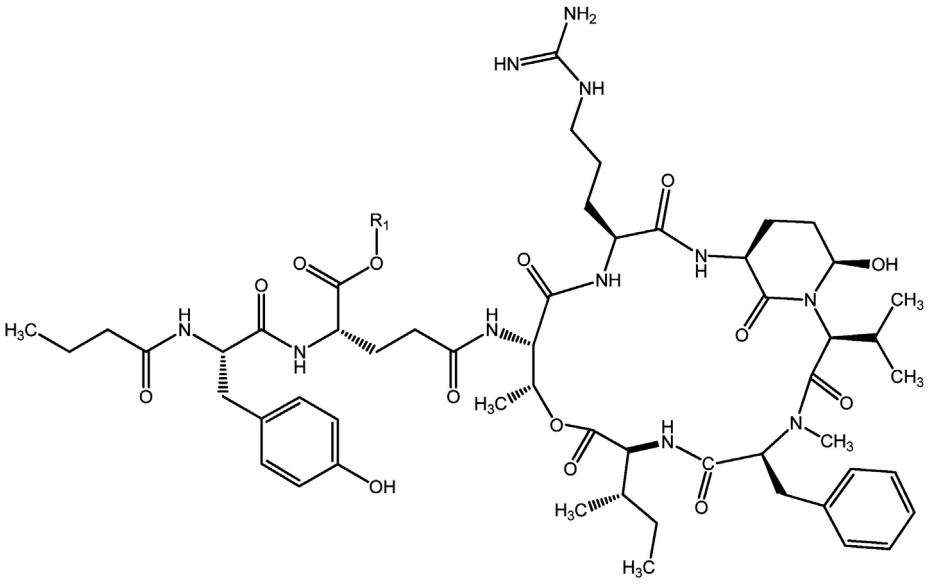
3. Results and discussion

Samples of the Cape Fear River *Microcystis aeruginosa* blooms were collected and the cellular biomass concentrated. Both blooms were relatively dense as determined by chlorophyll *a* concentration with the 2012 bloom roughly 7 times more dense than the previous collection (64 μg/L in 2009 and 441 μ/L in 2012). These collections afforded enough biomass to perform further chemical examinations. After extraction of the 2009 sample with organic solvents and initial solvent partitioning steps, the samples were analyzed by liquid chromatography coupled with mass spectrometry (LC–MS). Molecular ions of 996.8 (M+H) and 1038.7 (M+H), combined with the characteristic UV chromophore (λ_{max} 240 nm) suggested the presence of microcystins LR and RR in these samples. In order to confirm their identity, it was necessary to purify the molecules and characterize them by NMR. The semi-purified extract was subjected to a series of chromatographic purification steps including open-column chromatography and semi-preparative and analytical scale high performance liquid chromatography (HPLC) to yield the purified mycrocystins. Analysis of the pure compounds by 1D and 2D NMR, confirmed the characteristic Mdha and D-Me-Asp[3] moieties and further comparison of the data with reported literature values verified the two compounds as microcystins LR and RR, two of the most toxic members of the microcystin family of compounds (Fig. 1) (Gupta et al., 2003). Somewhat unusually, in both blooms microcystin RR was found to be the dominant component, occurring at roughly three times the concentration of the LR analog.

In addition to microcystins LR and RR, the LC–MS analytical data also indicated the presence of additional secondary metabolites from the 2009 bloom with mass ions of 1106.6 $(M+H)^+$ and 1120.7 $(M+H)^+$ and a characteristic minor UV peak at 280 nm. An extensive search of published chemical databases yielded no matches indicating that they may be new compounds. Guided by the LC–MS analytical data these target compounds were purified by a process similar to the scheme outlined above for the microcystins. Examination of the 1D and 2D NMR spectral data of the purified compounds suggested they were new congeners of the micropeptin class of cyanopeptides (Fig. 2). Key resonances consistent with the characteristic 3-amino-6-hydroxy-2-piperdone (Ahp) unit were observed (Welker and Von Döhren, 2006) as well as a low field oxymethine at 5.51 ppm corresponding to a depsipeptide link through threonine. The remaining NMR data were consistent with resonances of a butyric side chain and additional amino acids (N-N-disubst. Val, N-Me-Phe, Ile, Thr, Arg, Glu and Tyr), establishing these compounds as micropeptins (see Supplemental Data for NMR spectra and assignments).

Like the microcystins, micropeptins are also non-ribosomally produced (Nishizawa et al., 2011), though unlike microcystins, the micropeptins are classified as depsipeptides since they contain a cyclic ester linkage. Micropeptins comprise a diverse group of more than 130 members (Lifshits and Carmeli, 2012). Micropeptin 1120 was identified as the methyl ester of micropeptin 1106 based on the mass difference of 14 and a methyl singlet at 3.62 ppm assigned to the methyl ester of the Glu moiety. These compounds are most closely related to micropeptins 88A–88F with the Cape Fear analogs incorporating an arginine residue rather than tyrosine or leucine at the 5th position (Ishida et al., 1998). It is likely that micropeptin 1120 is an isolation artifact of 1106 and not naturally produced. In addition to the planar structure, the absolute configuration of the component amino acids was determined using Marfey's method (Fujii et al., 1997) (see Supplemental data for Marfey's reaction experimental and results).

Although acute toxicity has not been demonstrated for the micropeptins, they have repeatedly demonstrated *in vitro* protease inhibition activity. Depending on the nature of the amino acid occupying the fifth position from the carboxy terminus, these depsipeptides have shown selectivity for either chymotrypsin or trypsin-like serine proteases (Gademann and Portmann, 2008). In the new Cape Fear micropeptins, this position is occupied by



Micropeptin 1106 $R_1 = H$

Micropeptin 1120 $R_1 = CH_3$

Fig. 2. Structure of micropeptins 1106 and 1120.

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arginine. A recent study showed that micropeptins with basic amino acids in the 5th position such as Arg or Lys tend to be selective for trypsin-like proteases whereas micropeptins with hydrophobic amino acids such as Tyr or Leu in this position are selective for chymotrypsin-like proteases (Gademann and Portmann, 2008). Therefore, the new analogs isolated from the Cape Fear River blooms likely exhibit trypsin inhibition activity although biological testing has yet to be undertaken.

Our analysis of the Cape Fear River blooms of *Microcystis aeruginosa* highlights the need for more thorough monitoring of cyanobacterial blooms both for toxin production as well as the presence of other metabolites whose biological activities have yet to be assessed. *M. aeruginosa*, and other species of bloom-forming cyanobacteria are of concern due to their potentially detrimental effects on human health as well as livestock, birds, and fisheries. In addition to their hepatotoxic effects, sub-toxic levels of microcystins have recently been shown to have endocrine disrupting effects and to cause genetic alterations in model animal systems (Rogers et al., 2011; Garcia and Martinez, 2012). The risk of exposure expands beyond organisms that come in direct contact with the bloom and surrounding waters. A recent study showed microcystins to be present in the tissue of lettuce crops irrigated with contaminated water (Hereman et al., 2012) and another study illustrated how microcystins are bioaccumulated in the food chain (Kozlowsky-Suzuki et al., 2012). As Cape Fear River water is also used extensively in North Carolina crop irrigation and as a source of livestock drinking water, the potential exposure risks will need to be closely monitored during bloom events.

4. Conclusion

Our analysis of the unprecedented blooms of *M. aeruginosa* in the Cape Fear River in 2009 and 2012 indicate that microcystins LR and RR were present in both cases. This suggests a persistent production of toxins from these blooms, which is cause for concern. Microcystins, especially microcystin LR, have been implicated globally as the major toxins produced by blooms of *Microcystis aeruginosa*. Consequently, there has been a widespread effort to detect and regulate MCLR levels to minimize the harmful effects of toxin exposure. However, recent studies suggest that other analogs such MCRR are also quite toxic (235 μg/kg body weight) (Gupta et al., 2003). Our investigations of the Cape Fear River blooms showed that these strains produce the microcystin RR congener at levels roughly 3 times that of LR, an important fact that would have been missed by monitoring methods focused on MCLR detection and would lead to an underestimation of river water toxicity. Furthermore, our analysis showed that the Cape Fear River *M. aeruginosa* strain produced new cyanopeptides, micropeptins 1106 and 1120 at levels comparable to the microcystins in both the 2009 and 2012 blooms. Other than scant enzyme-based assays which indicated their protease activity, little is known about their full range of biological activity and potential effects on the environment. The relatively high concentrations of micropeptins found in the Cape Fear River blooms strongly suggest that they play an ecological role which needs to be further investigated. It is also important that further biological testing with this class of compounds be performed in order to determine their potential effects on human and animal health following both acute and chronic exposure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.hal.2013.09.010.

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