Microcystin-RR Like Toxin Identified in the Cyanobacterium *Anabaena flos-aquae* Strain CCAP 1403/13B Culture

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Abstract

Cyanobacteria abound in freshwaters in Ghana, including those used for the supply of drinking water. However, there have been no studies on their toxicity, the toxins they produce and their attending public health effects. As part of research activities to identify cyanobacteria and cyanotoxins associated with four reservoirs (Weija, Kpong, Owabi and Barekese), used for the production of drinking water in Ghana, *Anabaena flos-aquae,* a toxic cyanobacterium, was cultured in the laboratory with the objective of identifying potential cyanotoxins that may be associated with some of the cyanobacteria commonly found in Ghanaian waters. Cultures were kept in a growth chamber with continuous illumination at 20 µmol photon/m^2/s and constant aeration at a temperature of 25 ^{o}C. High pressure liquid chromatography (HPLC) analysis of extract from the culture of *Anabaena flos-aquae* strain CCAP 1403/13B produced a toxin with a retention time similar to microcystin-RR external standard. The concentration of microcystin-RR quantified from *Anabaena flos-aquae* was 10.6 µg/g DW. The biomass of lyophilized cells extracted was 52 mg. *Anabaena flos-aquae* is mainly known to produce neurotoxins, notably anatoxin-a and anatoxin-a(s). *Anabaena* and *Microcystis* were reported to be responsible for the lethal poisoning of over 2000 people in Bahia, Brazil through drinking water which resulted in the death of 88 children from gastro-enteritis over a period of 42 days.

Introduction

Microcystins (cyclic peptide hepatotoxins) have been described and detected in several cyanobacteria genera including *Anabaena* (Krishnamurthy *et al*., 1986), *Microcystis* (Botes *et al*., 1984), *Oscillatoria* (Brittain *et al*., 2000) and *Planktothrix* (Meriluoto *et al*., 1989). Globally, the most frequently occurring cyanotoxin encountered in fresh and brackish waters are the cyclic peptides known as microcystin (McElhiney & Lawton, 2005). Microcystins are hepatotoxic and have been described as potent liver toxins (Falconer *et al.*, 1994), and in chronic doses known to promote and initiate the growth of tumours. It has also been found to inhibit protein phosphatase activity (Yohizawa *et al*., 1990). Microcystins are known to be synthesised non-ribosomally and by thiotemplate mechanisms. In Ghana, there have been no studies on cyanobacteria, cyanotoxins and possible adverse public health effects on humans. This is despite the established knowledge that cyanobacteria have been implicated in several poisoning episodes of humans and animals world wide through drinking water.

In Bahia, Brazil, *Anabaena* and *Microcystis* were responsible for a lethal outbreak attributed to cyanobacterial toxins from drinking water which resulted in the

death of 88 children from over 2000 cases of gastro-enteritis over a period of 42 days (Teixera *et al*., 1993). Another human mortality occurred in Brazil, at a haemodialysis clinic where patients were treated with drinking water contaminated with cyanotoxins (Jochimsen *et al*., 1998)). Over 50 people died in this incident. Examination of the phytoplankton in the reservoir showed the dominance of the cyanobacteria *Microcystis*, *Anabaena* and *Anabaenopsis*. Recent studies on cyanobacteria samples from the Kenyan alkaline lakes, Bogoria and Nakuru, have shown the presence of the cyanotoxins, microcystins and anatoxin-a (Krienitz *et al*., 2003). Lake Bogoria was mainly dominated by *Anabaena fusiformis* while Lake Nakuru was dominated by *Anabaena fusiformis* and *Anabaenopsis* sp. *Anabaena* species are mainly known to produce neurotoxins mainly anatoxin-a and anatoxin-a(s) and other hepatotoxins (Sivonen & Jones, 1999).

Sivonen *et al*. (1990) reported a statistical association between neurotoxicity and *Anabaena lemmermanni*, *Anabaena flos-aquae* and *Gomphosphaeria naegeliana*. The same study also reported that *Anabaena* was the most common bloom-forming genus in toxic samples and was suspected to be the causative organism responsible in all cases of cattle poisoning during the study period. *Anabaena flos-aquae* had been described as one of the most toxic strains of cyanobacteria (ARNAT). Neurotoxins are a broad group of heterocyclic nitrogenous compounds which cause death by respiratory arrest in mouse bioassay (Sivonen & Jones, 1999).

In Ghana *Anabaena flos-aquae* and *Anabaena spirodes* are the dominant species of this genera found in most brackish, estuarine and fresh water bodies (Addico & Frempong, 2004; Addico *et al*., 2009). As part of research activities into cyanobacteria and cyanotoxins associated with four reservoirs used for the production of drinking water in Ghana, a culture of *Anabaena flos-aquae*, a toxic cyanobacterium commonly found in Ghanaian water bodies, was purchased and cultured in the laboratory with the objective of identifying potential cyanotoxins that may be associated with cyanobacteria commonly found in Ghanaian waters.

Materials and methods

The cultures were purchased from the Culture Collection of Algae and Protozoa (CCAP), Windermere Laboratory, United Kingdom. *Anabaena flos-aquae* strain CCAP 1403/13B was cultured in Jaworski medium (JM) (Thompson *et. al*., 1988). The cultures were cultivated in duplicates in 2-litre conical flasks in a growth chamber with continuous irradiance from a cool white florescence tube with a photon flux density of 20 µmol photons/m^2/s and constant aeration. Temperature was kept at 25 ^{o}C and humidity below 60% (Senogles-Derham *et al*., 2003). *Anabaena flos-aquae* strain CCAP 1403/13B cells were harvested at the late exponential phase by centrifugation and cell material lyophilized by freeze drying and stored at - 20 ^{o}C.

Extraction and analysis of microcystin

Extraction of microcystins from *Anabaena flos-aquae* cells and analysis using the high pressure liquid chromatography (HPLC) method was done as described in Harada *et. al*., (1999) and used in Addico *et al*. (2006). Microcystins

were identified by comparison of UV spectra (200-300 nm) and retention time with standards of microcystin-LR, -LW, -RR, -YR, -LF (Table 1). Unidentified microcystin peaks and all other peaks possessing characteristic microcystin UV spectrum were quantified as MC-LR (Fig. 1) (McElhiney & Lawton, 2005) using extrapolation of HPLC peak areas at 238 nm to a linear calibration curve of microcystin-LR standard ($n = 5$, $r^2 = 0.999$).

Analysis of the *Anabaena flos-aquae* strain CCAP 1403/13B extract in this study showed a peak at the retention time of 12.642 min (Fig. 1) similar with the retention time of 12.607 min obtained from the external microcystin-RR standard used in the analysis (Fig. 3). Quantification of this

TABLE 1
Microcystin standard solution used to analyse Anabaena flos-aquae *extract*

Microcystin standards	*Retention time (min)*	*Area (mAU)*	*Source of standards*
MC-RR	12.607	31.9	Purified and identified by MS
Nodularia	14.424	13.3	DHI
MC-YR	15.925	5.9	Purified and identified by MS
MC-LR	16.813	18.0	Purified and identified by MS, Alexis
MC-LW	27.337	10.5	Biochemicals
MC-LF	27.763	14.9	Biochemicals

Results and discussion

Anabaena flos-aquae (Fig. 2) is a widely studied strain of cyanobacteria (Sivonen *et al*., 1992; Namikoshi *et al*., 1992). All these authors identified neurotoxins from *Anabaena flos aquae* as a major toxin. Harada *et al*. (1991) reported the production of a very potent neurotoxin, anatoxin-a from *Anabaeana flos-aquae* strain NRC 525–17 and three other toxic compounds with hepatotoxicity, one of which was identified as 3-desmethylmicrocystin LR. *Anabaeana flos-aquae* is known to be very toxic and had been implicated in numerous animal and human poisoning episodes (Ballot *et al*., 2002). Drinking water treatment processes in Ghana are very basic comprising mainly of alum flocculation, sedimentation, rapid sand filtration and chlorination. Such conventional treatment plants have been described as inadequate for the removal of cyanotoxins (Keijola *et al*., 1988; Lawton & Robertson, 1999).

Analysis of the *Anabaena flos-aquae* strain CCAP 1403/13B extract in this study showed a peak at the retention time of 12.642 min (Fig. 1) similar with the retention time of 12.607 min obtained from the external microcystin-RR standard used in the analysis (Fig. 3). Quantification of this fraction showed the production of 10.6 µg/g DW. The high similarity (over 99.9%) of retention time in the analysis to that of the standard suggests that the toxin produced by *Anabaena flos-aquae* strain CCAP 1460/13 is microcystin-RR. *Anabaena flos-aquae* are mainly known to produce microcystin-LR and neurotoxins, mainly anatoxin-a and anatoxin-a(s) and not microcystin-RR.

Recent studies by Ballot *et al*. (2005) have identified microcystin-RR from Lake Sonachi and Simbi, Kenya, dominated by *Anabaena fusiformis* (over 94%) and *Anabaena abijate,* respectively. Even though the quantity of microcystin-RR identified in this study is low, the mere fact that this toxin is not known to be commonly produced by *Anabaena flos-aquae* makes the results an interesting contribution to the knowledge of

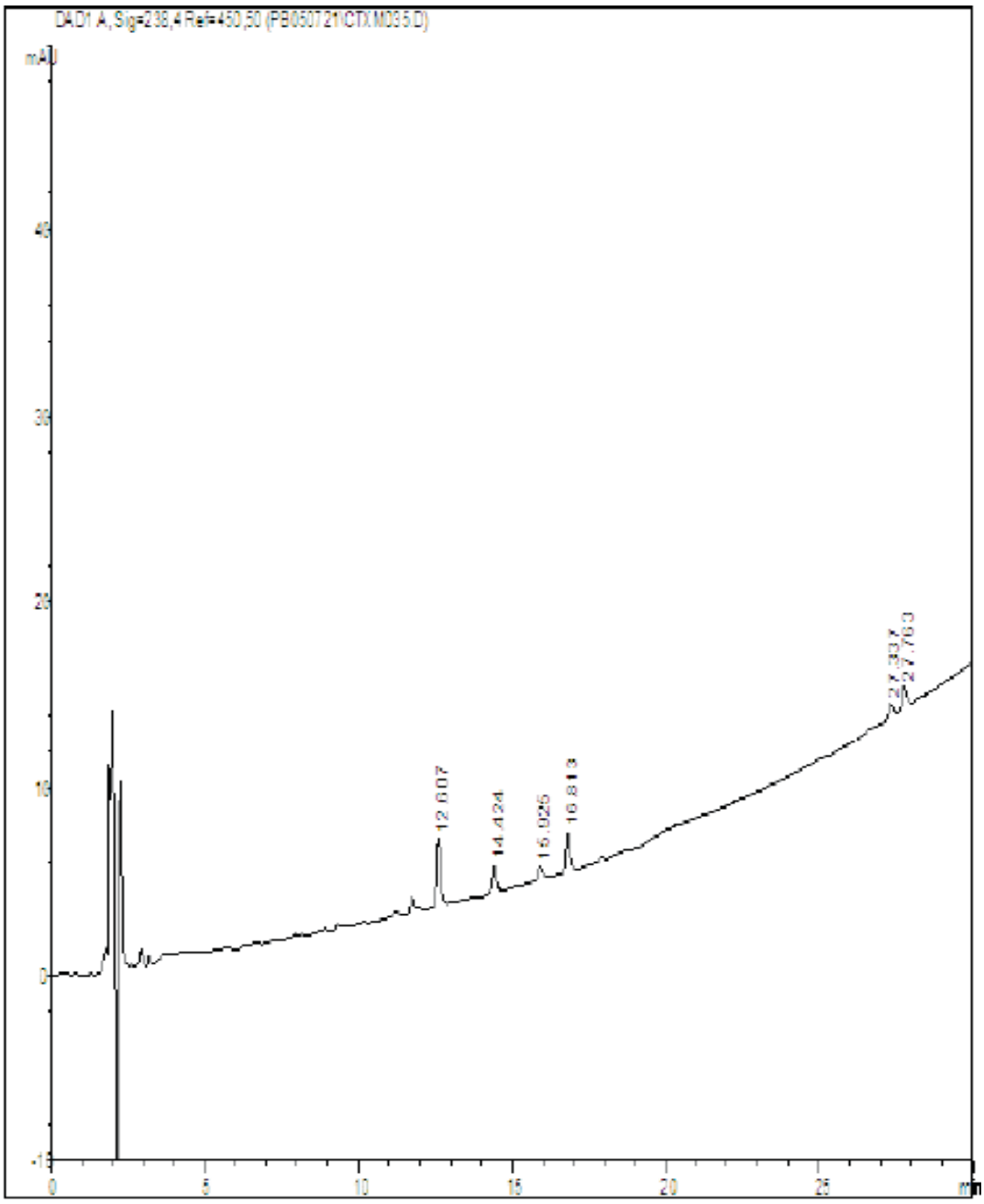

Fig. 3. HPLC-UV chromatogram of external standards of microcystins and nodularin used in analysis of *Anabaena flos-aquae* strain 1403/13B showing retention time of MC-RR at 12.607 min

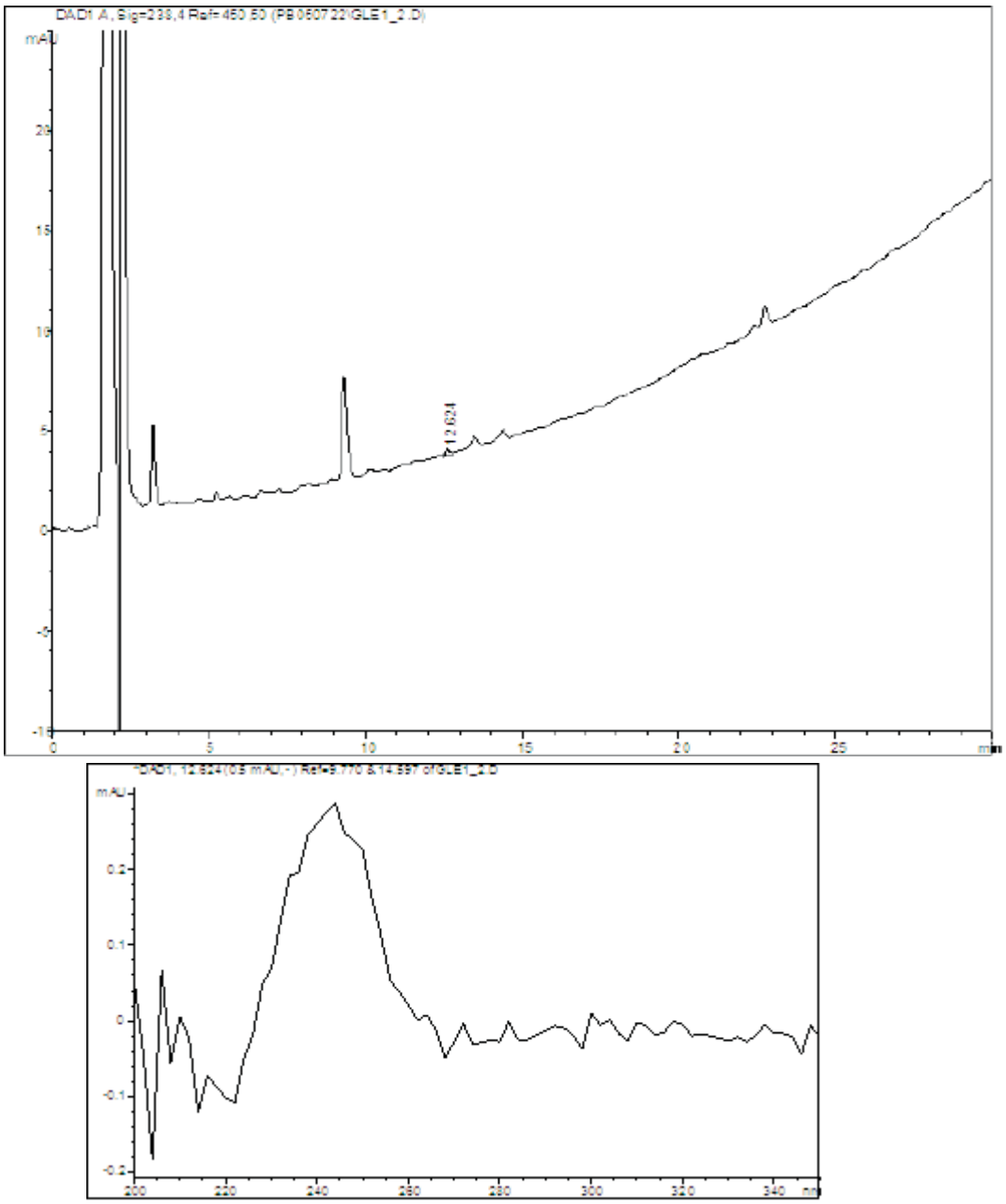

Fig. 1. Chromatogram (HPLC) of the extract of *Anabaena flos-aquae* strain CCAP 1403/13B (biomass: 0.052 g) - Ref: 12.642 min, with maximum absorbance of 238-240 nm. Below: Spectrum of microcystin-RR toxin

Fig.2. *Anabaena flos-aquae* species found in Ghanaian freshwater bodies

cyanotoxins productivity of cyanobacteria. It is important that the experience obtained in this study be applied to algal (phytoplankton) biomass and water samples collected from drinking water reservoirs in Ghana to determine the presence or otherwise of microcystin, and the efficiency of microcystin removal from different drinking water treatment plants in comparison with World Health Organization provisional guideline value of 1 µg/l microcystin-LR (WHO, 1998).

Conclusion

The presence of *Anabaena flos-aquae* especially in reservoirs sourced for the production of drinking water is a potential health risk and this experiment will go a long way to help determine whether Ghanaian drinking water from these reservoirs are being poisoned by toxins from these algae, so that actions are taken to stem this.

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