Water Research 124 (2017) 454–464

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

# Benthic cyanobacteria: A source of cylindrospermopsin and microcystin in Australian drinking water reservoirs

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ARTICLE INFO

*Article history:*
Received 24 March 2017
Received in revised form
21 July 2017
Accepted 29 July 2017
Available online 2 August 2017

*Keywords:*
Benthic cyanobacteria
Microcystin
Cylindrospermopsin
Drinking water

ABSTRACT

Cyanobacteria represent a health hazard worldwide due to their production of a range of highly potent toxins in diverse aquatic environments. While planktonic species have been the subject of many investigations in terms of risk assessment, little is known about benthic forms and their impact on water quality or human and animal health. This study aimed to purify isolates from environmental benthic biofilms sampled from three different drinking water reservoirs and to assess their toxin production by using the following methods: Enzyme-Linked Immunosorbent Assay (ELISA), High-Performance Liquid Chromatography (HPLC), Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) and quantitative PCR (qPCR). Microscopic observation of the isolates allowed the identification of various filamentous cyanobacterial genera: *Anabaena* (benthic form), *Calothrix* and *Nostoc* from the Nostocales and *Geitlerinema, Leptolyngbya*, *Limnothrix, Lyngbya, Oxynema, Phormidium* and *Pseudanabaena* representing non-heterocystous filamentous cyanobacteria. The *Phormidium ambiguum* strain AWQC-PHO021 was found to produce 739 ng/mg of dry weight (d/w) of cylindrospermopsin and 107 ng/mg (d/w) of deoxy-cylindrospermopsin. The *Nostoc linckia* strain AWQC-NOS001 produced 400 ng/mg (d/w) of a microcystin analogue. This is the first report of hepatotoxin production by benthic cyanobacteria in temperate Australian drinking water reservoirs. These findings indicate that water quality monitoring programs need to consider benthic cyanobacteria as a potential source of toxins.



## 1. Introduction

Planktonic cyanobacteria can challenge drinking water management and treatment processes by growing exponentially and forming a large biomass also called a bloom. In addition, these phototrophic bacteria have the ability to produce toxic secondary metabolites that are released into the water after cell death or during water treatment (Falconer and Humpage, 2005, 2006). Over the past twenty-five years, knowledge of toxigenic cyanobacteria has developed to the stage that they are now recognised as a major risk factor for drinking water quality in Australia and worldwide (Falconer and Humpage, 2005, 2006; Hudnell, 2008).

While planktonic cyanobacteria have been the subject of many investigations in terms of risk assessment, little is known about benthic species and their impact on water quality or human and animal health. Benthic cyanobacteria are a part of biofilms growing on the surface of the sediment in water bodies. They are organised in mats or clusters, forming, with the help of other autotrophic microbes, the photosynthetic layer of epilithic biofilms (Quiblier et al., 2013). In these mats, thin filamentous micro-algae are woven into several juxtaposed layers creating a complex matrix. During periods of increased photosynthetic activity, air bubbles form within the biofilm, causing some segments to detach and float. The accumulation of floating mats close to banks has led to the consumption of high levels of toxins by animals, causing death in many cases (Gugger et al., 2005; Mez et al., 1997). The presence of these toxins at high concentrations in floating mats also represents a threat to human health (i.e. through cutaneous exposure and accidental ingestion) as the water sources investigated were mainly used for recreational purposes.

Studies worldwide established the production of known cyanotoxins by benthic species. In New Zealand and Europe, the highly

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http://dx.doi.org/10.1016/j.watres.2017.07.073
0043-1354/

potent neurotoxin anatoxin-a was detected in cyanobacterial mats (Cadel-Six et al., 2007; Edwards et al., 1992; Gugger et al., 2005; Wood et al., 2011).

Similarly, hepatotoxins have been detected in benthic cyanobacteria worldwide (Mez et al., 1997; Mohamed et al., 2006; Izaguirre et al., 2007; Seifert et al., 2007; Bormans et al., 2014; Fetscher et al., 2015). Microcystin-LR and -YR were identified in benthic mats in the Nile River and connected irrigation canals, demonstrating potential toxic issues for the use of this water as a source for irrigation and drinking water (Mohamed et al., 2006). In one instance, the production of microcystin (MC) by benthic Oscillatoriales was associated with cattle death in eleven separate alpine pasture sites in Switzerland (Mez et al., 1997). Occasionally MCs were detected in benthic mats in drinking water reservoirs (Izaguirre et al., 2007). Benthic cyanobacterial taxa also produce cylindrospermopsin (CYN) (Seifert et al., 2007; Bormans et al., 2014). *Lyngbya wollei*, a benthic cyanobacterial species, was established as the producer of CYN and deoxy-cylindrospermopsin (deoCYN) in Queensland (Australia) freshwater ecosystems (Seifert et al., 2007). While MCs are considered to be mostly retained inside the cell (Rapala et al., 1997), CYNs are excreted by cyanobacterial cells more extensively and extracellular concentrations often exceed intracellular content (Bormans et al., 2014). This makes the potential production of CYN by benthic species of particular interest. Indeed, the routine monitoring of the risk associated with cyanotoxins is primarily achieved by enumeration of problematic planktonic species in water samples. In the commonly applied alert level framework monitoring program, toxin analyses are conducted only if cell densities are above the Alert Level (i.e. 6500 cells/mL for *Microcystis* spp. and 15,000 cells/mL for *Cylindrospermopsis raciborskii* (Chorus, 2012)). In the absence of potential toxigenic culprits in the water column the presence of toxins is not routinely investigated. Therefore, in the absence of extensive toxicological data and a regular monitoring program tailored for toxigenic benthic cyanobacteria, the risk they represent to human and animal health is unknown and needs to be evaluated.

For the purpose of evaluating the risk associated with the production of highly potent hepatotoxins by benthic cyanobacteria in three Australian drinking water reservoirs, this study aimed to purify isolates from environmental benthic biofilms sampled from three different water bodies and to assess their production of known cyanobacterial hepatotoxins by using the following methods: Enzyme-Linked Immunosorbent Assay (ELISA), High-Performance Liquid Chromatography (HPLC), Liquid Chromatography–Mass Spectrometry (LC-MS/MS) and quantitative PCR (qPCR).

## 2. Materials and methods

### 2.1. Strain isolation, culturing and morphological identification

Sediment core samples were collected from a total of twenty locations within three Australian drinking-water reservoirs: Location 1 (SA-L1) and Location 2 (SA-L2) in South Australia and Location 3 (NSW-L3) in New South Wales, using a custom-made core sampler (50 mm diameter). The phototrophic (top) layer of the core samples, most likely to contain benthic algae, was scraped off and preserved in a container for transportation. A mass of approximately 500 mg of sediment was resuspended in 10 mL of filtered reservoir water. Drops of the suspension were placed on 1% agar media: ASM-1, BG11, WC and MLA. The isolation of species was conducted as previously described (Rippka et al., 1988; Gaget et al., 2017a). All cultures placed on agar were maintained at 21 °C under white light (Osram Universal White) with a photosynthetic photon flux density (PPFD) of 30 μmol quanta $m^{-2}$ $sec^{-1}$. The rest of the suspension was preserved by adding 10% v/v Lugol and observed by light microscopy (Nikon Eclipse 50i microscope) in order to identify the species present in the collected mats.

The level of purity of the cultures and their identification were determined by observation using light microscopy with phase contrast (Nikon Eclipse 50i microscope, with images captured using a Nikon Digital Sight camera, Nikon, Shinagawa, Tokyo, Japan). The Cyanoprokaryota part 2 and 3 taxonomic keys were used for identification (Komárek and Anagnostidis, 2005a; 2005b) and names were modified if needed using references available on the algaebase website (http://www.algaebase.org/) in order to fit to the most recent taxonomy available. Five of the isolates purified were entered into the AWQC culture collection as follows: I013-0013 (AWQC-GEIT001), I013-0028 (AWQC-PHO021), I013-00033 (AWQC-NOS001), I013-0034 (AWQC-LIM001) and I013-0038 (AWQC-LYN001). From microscopic observation, the isolation process was successful as it allowed the culture of 80% of the diversity originally observed in the environmental samples.

### 2.2. Preparation of samples for toxin analysis

A total volume of 1.5 L of culture was centrifuged at 1614 g for 15 min. The supernatant was discarded and pellets were placed at −20 °C and subsequently freeze dried over 24 h (−100 kPa, −30 °C). Each freeze dried sample was then resuspended using MilliQ water to obtain a final concentration of 10 mg dry weight (d/w)/mL and sonicated (2–4 min: 10 s pulses at intensity 6 and 30 s rest (Branson Digital Sonifier Model 450, Branson ultrasonics/Emerson, Danbury, CT, USA). Extracts were then centrifuged at 1121 g for 5 min. In order to discard cell debris, the supernatant was filtered through a membrane (Acrodisc® 25 mm PF syringe filters 0.8/0.2 μm pore size, PN4187, Pall Life Sciences, Port Washington, New York, USA and 10 cc/mL sterile Terumo Luer syringes, Terumo, Hatagaya, Tokyo, Japan). Filtered extracts were kept at −20 °C prior to analysis.

Two cost-friendly techniques, the ELISA and qPCR assays, were used as screening tools for the detection of hepatotoxins in cell extracts. The ELISA allows the direct detection of the molecule, while the qPCR confirms the presence of the genes involved in the synthesis of these toxins. Considering that the qPCR assays used in this study were initially developed for planktonic species, it was of great interest to seek for potential gene sequence variability between planktonic and benthic strains, which could affect the efficiency of these tests.

Upon confirmation of toxin production, the extracts, which tested positive were further analysed and the concentration and congener identification were done by HPLC for MC and by LC-MS for CYN.

### 2.3. Detection of toxins by ELISA

The assays used are indirect competitive ELISAs (Abraxis, Warminster, PA, USA): Microcystin/Nodularins (ADDA), ELISA Kit # 520011; Cylindrospermopsin Plate Kit Part # 522011; Saxitoxin, PST Plate Kit Part # 52255B. Extracts were diluted to reach a final concentration of 1 mg/mL (d/w) using the diluent provided in each kit. The assay was conducted according to the kit instructions. The intensity of the blue colour obtained after reaction is inversely proportional to the concentration of the toxin present in the sample. Colour was quantified using a microplate reader (Perkin Elmer VICTOR3 plate reader; Wellesley, MA, USA). Concentrations of toxins in the samples were determined by interpolation using the toxin standard curve generated with each run. Each sample was tested in either duplicate or triplicate in each experiment, depending on the kit recommendations. Three independent