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First report of saxitoxin production by a species of the freshwater benthic cyanobacterium, *Scytonema* Agardh

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ABSTRACT

Saxitoxins or paralytic shellfish poisons (PSP) are neurotoxins produced by some species of freshwater cyanobacteria and marine dinoflagellates. Samples collected from the metaphyton of a drinking-water supply's pre-treatment reservoir and a small eutrophic lake in New Zealand returned positive results when screened using a Jellett PSP Rapid Test Kit. The dominant species in the sample was identified as *Scytonema* cf. *crispum*. A non-axenic clonal culture (UCFS10) was isolated from the lake. The partial 16S rRNA gene sequence shared only a 91% or less sequence similarity with other *Scytonema* species, indicating that it is unlikely that this genus is monophyletic and that further in-depth phylogenetic re-evaluation is required. The *sxtA* gene, which is known to be involved in saxitoxin production, was detected in UCFS10. Saxitoxin concentrations were determined from the lake samples and from UCFS10 using pre-column oxidation high performance liquid chromatography with fluorescence detection. Saxitoxin was the only variant detected and this was found at concentrations of 65.6 μg g^{-1} dry weight in the lake sample and 119.4 μg g^{-1} dry weight or 1.3 pg $cell^{-1}$ in UCFS10. This is the first confirmation of a saxitoxin-producing species in New Zealand and the first report of saxitoxin production by a species of *Scytonema*.

1. Introduction

Saxitoxins are fast-acting neurotoxins that inhibit nerve conduction by blocking sodium channels. They are produced by freshwater cyanobacteria (Sivonen and Jones, 1999) and marine dinoflagellates. In the latter, they are known as paralytic shellfish poisons (Llewellyn, 2006). The human health effects of saxitoxins are well-described in numerous reports of human toxicity associated with the consumption of contaminated shellfish (Etheridge, 2010; Llewellyn, 2006). Over 30 saxitoxin variants have been isolated and characterised (Llewellyn, 2006). Saxitoxin is the most potent variant with an intraperitoneal LD_{50} of 10 μg kg^{-1} body weight (mouse) (Wilberg and Stephenson, 1960). Saxitoxin variants recorded in cyanobacteria include decarbamoyl derivatives (dc), gonyautoxins (GTX); neosaxitoxin (neoSTX), N-sulphonocarbamoyl toxins (C-toxins), saxitoxin (STX) and a class of toxins produced by *Lyngbya wollei* (Humpage et al., 2010). In Australia, saxitoxins produced by cyanobacteria have caused sheep mortalities (Negri et al., 1995). Saxitoxin and analogues were the only neurotoxins identified in *Anabaena circinalis* from the Murray Darling River (Humpage et al., 1994) where an extensive *A. circinalis* bloom in 1991 resulted in the death of over 1600 stock (Blue-Green Algae Task Force, 1992; Bowling and Baker, 1996; Steffensen et al., 1999).

Cyanobacteria known to produce saxitoxins are *A. circinalis* in Australia (Humpage et al., 1994), *Cylindrospermopsis raciborskii* in Brazil (Lagos et al., 1999), *L. wollei* in North America (Yin et al., 1997), *Planktothrix* in Italy (Pomati et al., 2000), and *Aphanizomenon* spp. in Europe (Ballot et al.,

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2010; Ferreira et al., 2001; Pereira et al., 2000, 2004), North America (Mahmood and Carmichael, 1986) and China (Liu et al., 2006). Rapala et al. (2005) postulated that *A. lemmermannii* also produces saxitoxins based on toxins detected in field samples with high (95–100%) dominance of this species. In New Zealand, using an ELISA assay, Wood et al. (2006) detected low levels of saxitoxins in 38 different water bodies. A neuroblastoma assay provided additional evidence to support the identification of saxitoxins at some of these sites (Wood et al., 2006). In 2003 a cyanobacterial bloom (predominantly *Anabaena* spp.) in the WaikatoRiver caused taste and odour problems in the drinking-water supplied to the city of Hamilton and satellite towns along the length of the river. Extremely low levels of saxitoxins were detected (via ELISA assay) in water samples taken from the water treatment intake and throughout the water treatment process (Kouzminov et al., 2007). The cyanobacterial species responsible for these records of saxitoxin production in New Zealand were not identified.

We report on the isolation, culture and preservation of *Scytonema* cf. *crispum* (C. Agardh) Bornet, and the screening for potential cyanotoxins in environmental and cultured samples. Phylogenetic analyses based on a region of the 16S rRNA gene and the *sxtA* gene were undertaken to in order to compare this isolate with sequences from other *Scytonema* species. This report is the first confirmation of a freshwater saxitoxin-producing cyanobacterium in New Zealand and the first report of saxitoxin production by a species of *Scytonema*.

2. Materials and methods

2.1. Site description and sample collection

During routine monitoring, water and benthic algal samples (100 mL) were collected from the shoreline of a drinking-water pre-treatment reservoir in the south-east of the South Island, New Zealand. The reservoir is mesotrophic (<0.1 mg L^{-1} total nitrogen; <0.02 mg L^{-1} total phosphorus; Burns et al., 2000), ca. 3.7 ha in area, with a maximum depth of 10 m and annual water temperatures ranging between 2.0 and 25.0 °C.

The Groynes (43°27′02″ S, 172°36′18″ E) is a recreational reserve on the outskirts of Christchurch, South Island, New Zealand. The reserve contains four interconnected, eutrophic lakes (10-year average; 0.28 mg L^{-1} total nitrogen; 0.044 mg L^{-1} total phosphorus), with water temperatures ranging between 4.5 and 20.0 °C (Environment Canterbury, unpublished data). Water samples and algal mats were collected from the metaphyton at the shoreline of the largest lake (ca. 1.7 ha in area, with a maximum depth of ca. 1.8 m), and stored in 60 mL sterile containers.

2.2. Culture, isolation and identification

Filaments of the dominant cyanobacteria in field samples from both locations were streaked onto agarised $BG11_0$ medium (1% agar, BG11 without $NaNO_3$; Rippka et al., 1979). After approximately four weeks incubation (25 ± 0.5 °C; 16:8 h light:dark; 26 ± 10 μ mol photons m^{-2} s^{-1}) single filaments were pulled through fresh agarised $BG11_0$ using forceps in order to remove epiphytic bacteria and algae. A single non-axenic clonal culture (UCFS10) from the lake was successfully isolated and maintained in 40 mL $BG11_0$ aliquots in 60 mL polycarbonate containers.

Field material from the lake and reservoir, and UCFS10 were identified using Olympus light microscopes (BX50 and BX51) at 800–1000× magnification. Morphological features, including size and shape of vegetative cells, heterocytes and filaments (trichome plus sheath) and the presence of false-branching, were used for identification with reference to Geitler (1932). Filament width, and length and width of vegetative cells and heterocytes were recorded ($n = 50$) in the field specimen from the lake and in strain UCFS10.

Strain UCFS10 was cryopreserved using the rack and controlled freezing methods described by Wood et al. (2008).

2.3. Genetic analysis

Sub-samples for genetic analysis were taken from UCFS10 using sterile forceps. DNA was extracted using a MoBio Power Soil™ kit (Carlsbad, CA, USA). PCR amplification of the 16S rRNA gene and intergenic spacer region (ITS) were performed in a 50 μL reaction tube containing approximately 30 ng of DNA, 480 nM of 27F (Jungblut et al., 2005) and 23S30R (Rueckert et al., 2007) primers (Geneworks, Australia), 200 μM dNTPs (Invitrogen), 1 × *Taq* PCR buffer (Invitrogen), 0.2 μL of Platinum *Taq* DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen) and 1.2 μg non-acetylated bovine serum albumin (Sigma). Thermal cycling conditions were; 94 °C for 2 min followed by 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, repeated for 30 cycles with a final extension at 72 °C for 7 min. Screening for genes putatively involved in the synthesis of microcystins, nodularins, anatoxin-a, homoanatoxin-a and saxitoxins used the protocols in Jungblut and Neilan (2006), Cadel-Six et al. (2009), and Ballot et al. (2010).

All PCR reactions were undertaken on an iCycler thermal cycler (Biorad, USA). PCR products were visualised by 1% agarose gel electrophoresis with ethidium bromide staining and UV illumination. Amplicons of the correct size were purified using a High Pure PCR product purification kit (Roche Diagnostics) and sequenced bi-directionally using an Applied Biosystems 3130xl Genetic Analyser and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The primers 27F/809R (Jungblut and Neilan, 2006) and 740F/1494R (Neilan et al., 1997); 23S/30R (Rueckert et al., 2007); and sxtaf and sxtar (Ballot et al., 2010) were used for sequencing the 16S rRNA, ITS and *sxtA* genes respectively.

The 16S rRNA, ITS and *sxtA* gene sequences from UCFS10 were compared with sequences from the NCBI Genbank database (Benson et al., 2008). Sequences were aligned and phylogenetic trees created using the Neighbour-Joining (NJ) algorithm with 1000 bootstrap replicates (MEGA version 4.1; Tamura et al., 2007). The partial 16S rRNA and *sxtA* gene sequences generated during this work were deposited in NCBI Genbank database under accession numbers HM629428 and HM629429.

2.4. Cyanotoxin extraction and analysis

A 5 g wet weight sub-sample of cyanobacterial mat from the reservoir was sonicated (10 min) in acetonitrile (5 mL)