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Toxicity of the cyanobacterium *Limnothrix* AC0243 to male Balb/c mice

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ABSTRACT

A growing list of freshwater cyanobacteria are known to produce toxic agents, a fact which makes these organisms of concern to water authorities. A cultured strain of *Limnothrix* (AC0243) was recently shown to have toxic effects in *in vitro* bioassays. It did not produce any of the known cyanobacterial toxins. The intrapertoneal toxicity of aqueous extracts of the material was therefore tested in mice to determine whether the observed effects might be of public health relevance to drinking water supplies. The results indicate that *Limnothrix* AC0243 is acutely toxic to mice, causing widespread cellular necrosis in the liver, kidneys and gastrointestinal tract within 24 h of exposure. Sub-lethal effects lasted at least 7 d. These results suggest that *Limnothrix* AC0243 produces a novel toxin ("Limnothrixin") and that further work is therefore urgently required to quantify the potential public health implications.

1. Introduction

Cyanobacteria are known to produce a range of bioactive compounds including some that are toxic to animals and humans (Humpage, 2008). Because these organisms sometimes proliferate to very high numbers in our increasingly eutrophic drinking water sources, they and the toxins they produce are of concern to the water industry (Falconer, 2005a; 2005b; Falconer and Humpage, 2005; 2006).

Limnothrix is a commonly occurring cyanobacterial genus with only a recent history of possible toxicity (Bernard et al., 2011; Torres-Ariño and Mora-Heredia, 2010). *Limnothrix redekei* (Van Goor) Meffert may dominate in temperate waters (Nõges et al., 2010) whereas various morphotypes related to *Limnothrix planctonica* (Woloszyńska) Meffert are more common in the tropics (Komárek and Anagnostidis, 2005). This genus may be found in samples containing *Cylindrospermopsis raciborskii* (Bormans et al., 2005). Consequently, in incidences where animal deaths have been reported, the presence of toxins (cylindrospermopsins) produced by the latter has been suspected.

In a search for toxin producing algae in Central Queensland, Australia, an extract from a *Limnothrix* isolate was found to inhibit protein synthesis in a cell free system used to screen for cylindrospermopsin (Bernard et al., 2011). These authors also found that exposure of Vero cells to this extract produced a reduction in cellular ATP levels. This isolate (AC0243) had the morphological characteristics of *Geitlerinema unigranulatum* (R.N. Singh) Komárek and Azevedo but was found to closely align genetically with *L. redekei* (Bernard et al., 2011).

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This paper reports on the initial *in vivo* study of the effects of extracts of *Limnothrix* AC0243 on mammalian tissues using the mouse bioassay (intraperitoneal administration). This limited study was primarily aimed at demonstrating whether the toxic effects observed *in vitro* had relevance for the whole animal. A further aim was to show toxicity of aqueous extracts because previous experience with a toxic strain of *Phormidium* (Baker et al., 2001) had shown, in that case, that the toxin could not be extracted from the cell material into aqueous solvent, and so was largely irrelevant to drinking water quality.

2. Materials and methods

2.1. Cyanobacterial culture and test extracts

Cultures of *Limnothrix* AC0243 were grown axenically in ASM1 medium (Gorham et al., 1964). The cultures were grown in 1 L Erlenmeyer flasks according to the methods outlined in Bernard et al. (2011). Mats of the culture were selected from the vessel, freeze-dried and weighed. A total of 900 mg freeze-dried *Limnothrix* material was obtained. This material was used to make Extract 1 (see below). Extracts were made by adding 5 ml of sterile injectable saline (Pfizer) to the pooled material. This was sonicated for 15 min on high in a Soniclean Water Bath then centrifuged at 16060 × g for 5 min (Heraeus Biofuge Pico). The supernatant was filtered through a 0.2 μm syringe filter (Sarsdedt Filtopur S 0.2) yielding sterile extract. A further 79 mg of freeze-dried material from the same cultures was later added to the pellet from Extract 1 to make Extract 2, which was processed as for Extract 1. Extract 1 (1.5 ml, equivalent to 180 mg freeze-dried material/ml) was used in Experiments 1 and 2 as indicated, while Extract 2 (2.3 ml, equivalent to 195 mg freeze-dried material/ml) was used in Experiment 2.

2.2. Animals

Thirteen male 6 week old Balb/c specific pathogen free mice (average weight 21 g) were obtained from the Animal Resource Centre, Perth. They were housed in plastic boxes with white softwood sawdust bedding and allowed access to filtered water and feed *ad libitum*. The mice were acclimatised for a period of 4 days prior to the start of the experiments.

2.3. Mouse bioassays

The research was conducted under Central Queensland University Animal Ethics Approval A0811-237. Approval was given to use only 13 mice due to the likelihood that the mice would suffer pain and possibly die from the treatments.

2.3.1. Experiment 1

The purpose of this experiment was to determine the time-course of the toxicosis over one week. Two mice were injected intraperitoneally with 300 μl of Extract 1, and a third mouse was injected with the same amount of sterile injectable saline. Signs of the toxicosis were noted at regular intervals. All 3 mice were killed after 7 d, autopsied and their organs preserved for histology. Mouse body weights were recorded before injection and on subsequent days. Weights of the liver, kidneys and spleen were recorded at autopsy, as were observations of gross changes to organ appearance.

2.3.2. Experiment 2

Following observation of the timecourse in Experiment 1, a second experiment was undertaken to provide organs for detailed histology at predefined time-points (2 h, 4 h, 6 h and 24 h). As in Experiment 1, each mouse was injected with 300 μl of extract. The mice killed at the 2 h, 4 h and 24 h time-points were injected with Extract 2, while the 2 mice killed at 6 h were injected with Extract 1. In addition, 2 mice were treated with 300 μl of a solution containing the *Limnothrix* cell debris (that is, Extract 2 after sonication and before the centrifugation stage). Finally, 2 mice were treated with saline and killed at 24 h to provide control tissues. Lack of mice and extract prevented duplication of all time points.

2.4. Histology

At the conclusion of both experiments, body and selected organ weights were recorded. Organs were preserved in phosphate-buffered 4% formol saline (except testes, for which Bouin's Solution was used) and prepared for histology. Tissues prepared included liver, kidneys, spleen, pancreas, duodenal, ileal, and colonic sections of intestine, stomach, heart, lungs, thymus, brain, testes. The carcass was also preserved. Organs were processed and embedded in paraffin wax (Paraplast Plus). Histological preparation and staining with hematoxylin and eosin was performed by Sullivan Nicolaides Pathology, Rockhampton, Australia.

2.5. Statistics

The body and organ weights from animals in Experiment 2 were analysed for significant difference from the concurrent controls using the one-sided *t*-test (GraphPad Prism).

3. Results

3.1. Toxicity of the test material in the protein synthesis inhibition assay

The potency of the freeze-dried material was determined using the cell-free protein synthesis assay as previously described (Froscio et al., 2009). An extract of 10 mg of this material in 1 ml MQ-water completely inhibited protein synthesis, whereas 100 μg/ml caused about 40% inhibition, and 10 μg/ml was not inhibitory at all, which indicated that the material was of similar potency to that described in Bernard et al. (2011).

3.2. Time-course over 7 days (Experiment 1)

3.2.1. Behavioural observations

Within 1 h the treated mice were prostrate. By this time, their hind sections were extremely sensitive to a light touch, and they had lost coordination and strength in the hind limbs.