DEATH OF A DOG ATTRIBUTED TO THE CYANOBACTERIAL (BLUE-GREEN ALGAL) HEPATOXOTOXIN NODULARIN IN SOUTH AFRICA

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ABSTRACT
A bull terrier died after drinking water at the margin of Zeekoevlei near Cape Town. At that time, Zeekoevlei, a hypertrophic coastal lake, contained a bloom of the cyanobacteria *Nodularia spumigena* and *Microcystis aeruginosa*. The circumstances of the incident, clinical signs of poisoning and histopathology, which mainly revealed extensive liver damage, were consistent with cyanobacterial poisoning. The cyanobacterial bloom material contained 3.47 µg mg⁻¹ dry weight of the pentapeptide hepatotoxin nodularin. It is inferred that the dog died of cyanobacterial hepatotoxicosis due to the ingestion of nodularin.

Key words: Dog poisoning, cyanobacterial (blue-green algal) hepatotoxicosis, *Nodularia*, nodularin.

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INTRODUCTION
The common genera of cyanobacteria (blue-green algae) which form blooms and scums in nutrient-enriched water bodies include species capable of producing potent toxins. These include neurotoxins, hepatotoxins and inflammatory agents. Deaths of wild animals, farm livestock and domestic pets, birds and fish after contact with, or ingestion of, cyanobacterial scums or blooms have been reported in several countries over many years. On a global basis, most poisoning incidents have been associated with blooms of the genus *Microcystis*. In South Africa, most of the incidents of toxic cyanobacteria were reported from the former Transvaal and Orange Free State, although *Microcystis aeruginosa* blooms are widely found throughout the country. The known hepatotoxins of *Microcystis* spp., a wide range of potent hepatapeptides (microcystins, originally termed cyanoginosins), were first fully identified in South African material. Animal deaths thought to be due to another cyanobacterial hepatotoxin, nodularin, have been reported from New Zealand, Scandinavia and Germany.

It is likely that the first animal mortalities linked to the ingestion of cyanobacterial blooms and scums in the scientific literature by Francis in 1878 were not due to microcystins, but to the related hepatotoxin, nodularin. The hepatotoxic scums described by Francis (1878) in investigations into large numbers of animal deaths around the shores of Lake Alexandrina, South Australia were *Nodularia spumigena*. Strains of this organism, isolated from New Zealand, the Baltic Sea and Australia produce the cyclic pentapeptide hepatotoxin, nodularin, and smaller amounts of nodularin variants.

Reports of sporadic mortalities in ducks, young cattle, and particularly dogs along the Danish, German, Swedish and Finnish coasts of the Baltic Sea and at a brackish north German lake, after ingesting *Nodularia spumigena*, suggest the involvement of nodularin(s) in the intoxications.

This report describes the presence of the cyanobacterial hepatotoxic pentapeptide, nodularin, for the first time in South African waters. This toxin appears to have been responsible for the death of a dog at a coastal lake.

CASE HISTORY
On 16 March 1994 (Day 1) a 3-year old female Bull Terrier was presented at the St Francis Veterinary Hospital, Bergvliet, Cape Town, with a history of lethargy, vomiting and inappetence after drinking water from Zeekoevlei the previous day (Day 0). The owners of this dog were lakeside residents at Zeekoevlei, and the animal habitually played in, or drank water from, the lake. Zeekoevlei (34°04’S, 18°31’E), a shallow, freshwater coastal lake (area 256 ha, mean depth 1.9m) is situated near Cape Town. The lake is surrounded by urban development and has been subjected to massive anthropogenic perturbation. As a consequence, the lake contains elevated concentrations of the plant nutrients nitrogen and phosphorus, with annual mean total nitrogen and phosphorus concentrations of 2.9 and 0.54 mg L⁻¹ respectively. Nutrient abundance, in combination with climatic factors (Harding, unpublished observations 1992-1994), supports the perennial dominance of Zeekoevlei by cyanobacteria (mean annual chlorophyll a = 220 µg L⁻¹). The phytoplankton

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assemblage of Zeekoevlei has previously been dominated by *Microcystis aeruginosa* for as long as available records indicate.

Clinical examination of the dog revealed normal rectal temperature and a blood smear was negative for the presence of *Babesia canis* trophozoites. The animal was admitted and an intravenous fluid and electrolyte infusion (Ringers lactate, Intramed) was started. Subcutaneous injections of procaine penicillin (Depocillin, Intervet S.A.), prednisolone (Deltacortril, Pfizer Laboratories), clonbutin sodium (Bykahepar, Byk Gulden S.A.), prochlorperazine (Stemetil, Rhone-Poulenc Rorer S.A.) and a Vitamin B complex (Vitamin B Complex Injection, Centaur Laboratories, Premier Pharmaceuticals) were administered. Blood samples were taken for alanine transaminase (ALT), alkaline phosphatase (ALP) and serum creatinine determination. The animal's condition improved overnight (Day 1/Day 2) and it ate well. Despite this apparent onset of recovery there were signs of icterus and the treatment, including intravenous fluid therapy, was continued. The results of the serum analysis performed on blood samples taken on Day 1 revealed a massively elevated serum ALT activity of 12000 U/L (normal range 1 to 40 U/L). The serum activity levels of ALP and creatinine concentration were within normal limits. The dog's condition deteriorated rapidly on Day 3, but it continued to eat and respond to its owner when visited. The dog's condition appeared to stabilise again on Day 4, and the treatment was maintained with the addition of intravenous esmolol, Innovar (Essentiale, Rhone-Poulenc Rorer S.A.). The animal died suddenly in the early hours of Day 5, before further blood samples could be taken to establish the liver enzyme activities. No post mortem was performed, but because poisoning due to cyanobacterial toxins was suspected, liver samples were collected and preserved in 10% buffered formalin. Liver tissue was processed routinely and stained with haematoxylin and eosin (HE) prior to microscopic examination.

Toxicological investigation

Water samples for chemical, algological and cyanobacterial toxin analysis were collected on Day 2 from the location where the animal had been observed to drink. The water adjacent to the shoreline contained a very dense layer of buoyant cyanobacterial cells. Microscopic examination of fresh and preserved (Lugol's iodine) phytoplankton was performed using a Zeiss Axiosvert 35 inverted photomicroscope and a magnification range of 100–400x. Freshly collected cyanobacterial scum material was sent to the University of Dundee, Scotland, followed by further lyophilised material for toxicity assessment and toxin analysis.

Toxicity assessment of the Zeekoevlei bloom material was performed by intraperitoneal mouse bioassay using lyophilised Zeekoevlei bloom material resuspended in distilled water and disrupted by ultrasonification (MSE Soniprep, 4 × 10 sec bursts at full power in an ice-bath).

Toxin identification and quantification was by photodiode-array high-performance liquid chromatography (PDA-HPLC). Methanol andacetonitrile (Rathburn Walkern, Scotland); trifluoroacetic acid (TFA) (Fisons, Loughborough, England) and Milli-Q grade water (Millipore, Milford, Maryland, USA) was used throughout. All reagents were HPLC grade. Twenty five mg of lyophilised Zeekoevlei bloom material was resuspended in 1 ml methanol containing 0.1% (v/v) TFA. After standing at room temperature for 30 min, the material was centrifuged at 12 000 × g in a microcentrifuge for 2 min and the supernatant decanted for analysis. Fifteen µl of supernatant was analysed by PDA-HPLC using equipment consisting of a model 600E solvent delivery system, a Model 717 WISP autosampler and a Model 991 PDA detector at 200–300 nm with 3 nm resolution (Waters, Millipore, Milford, Maryland, USA). A Waters µBondapak C18 column (300 × 3.9 mm i.d.) with a temperature control module at 40°C was used. The eluents were water - 0.05% (v/v) TFA and acetonitrile - 0.05% TFA in a linear gradient at a flow rate of 1 ml min⁻¹. A nodularin standard was prepared by dissolving 17 µg of the toxin in 250 µl of methanol, 15 µl being injected for PDA-HPLC.

RESULTS

Microscopic pathology

Histopathological examination of the liver revealed pericellular fibrosis with duplication of the central veins. Hydropic to fatty degeneration of hepatocytes and the distention of the bile canaliculi with bile pigment were also observed.

Toxicology

A dense population of the filamentous cyanobacterium, *Nodularia spumigena*, accounted for about 95% of the bloom and scum material, the remainder being colonies of *Microcystis aeruginosa*. Monitoring of the water body during the week of the poisoning incident indicated a water temperature of 21.7°C, pH 9.5, concentrations of total nitrogen and phosphorus of 9900 and 510 µg l⁻¹, respectively, and a chlorophyll a concentration of 450 µg l⁻¹.

The cyanobacterial scum material was toxic in intraperitoneal mouse bioassays, with signs of liver damage being consistent with cyanobacterial hepatotoxins. This was confirmed with a second batch of lyophilised material, for which an MLD₉₀ (25 g mice, intraperitoneal route) of 10 mg dry weight kg⁻¹ was obtained.

PDA-HPLC of methanol extracts of the lyophilised bloom material showed two peaks (Fig 1A). The major peak had the same elution time (13.3 min) as nodularin standard. When methanol extracts were spiked with standard, the size of the major peak increased (Fig 1B). Figure 1C shows the matching ultraviolet absorption spectra of nodularin standard and the bloom 13.3 min peak, further indicating the latter to be nodularin. A second component, with an elution time of 12.2 min (Fig 1A) did not have an absorption spectrum characteristic of nodularin. The amount of nodularin present in the scum, quantified by reference to nodularin standard in PDA-HPLC was 3.47 mg g⁻¹ dry weight of the scum sample.

DISCUSSION

No reports of animal poisonings attributed to cyanobacterial intoxication appear to exist for the south-western region of South Africa prior to 1994. Subsequently, 2 cases of cattle deaths and 1 of sheep were recorded during the first five months of 1994. Early reports of farm livestock and wild animal deaths associated with cyanobacterial blooms and scums indicated a substantial and widespread health hazard to animals drinking from
lakes, dams and farm ponds containing such blooms and scums. With the application of improved methods for cyanobacterial toxin identification and quantification and knowledge of the potency and pathology of the toxins in animals, it is becoming increasingly possible to ascribe animal intoxications to particular toxins.

This report appears to be the first recorded death of an animal after drinking from a South African lake dominated by *Nodularia spumigena* and where the presence of nodularin has been confirmed. The microscopical changes observed in the dog's liver were indicative of toxic liver injury, although cardiac fibrosis could not be ruled out. In the absence of live axenic monocyano-bacterial cultures from Zeekoevlei, it can only be assumed that the nodularin was produced by the *Nodularia spumigena* bloom. However, this would appear to be a reasonable assumption, as *Nodularia spumigena* is the only cyanobacterium shown to produce nodularins. The small numbers of *Microcystis aeruginosa* colonies, accounting for only approximately 5% of the cyanobacterial bloom at the time of the dog's suspected intoxication, may have been the source of the second compound detected by PDA-HPLC (Fig 1A and B). However, the absorption spectrum of the 12,2 min-eluting compound was not characteristic of nodularin or microcystins. The concentration of nodularin found in the scum material, 3.47 μg mg⁻¹ dry weight, is similar to that found in laboratory cultures of *Nodularia spumigena* and indicates the high potential for hepatotoxicity of the Zeekoevlei scum at the time of this incident.

In addition to the toxicity reported here, *Nodularia spumigena* was implicated in 2 cases of cattle death in the south-western Cape between December 1993 and December 1994. Sheep mortalities that occurred during April 1994 were associated with *Microcystis aeruginosa*. The combined effect of these outbreaks created an enhanced awareness of the potential threat posed by cyanobacterial toxins amongst the veterinary fraternity, water engineers, farmers and the public at large in the Western Cape Province. Relevant information has been disseminated through public meetings, radio broadcasts, the erection of warning

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**Fig. 1:** Photodiode array high-performance liquid chromatography (PDA-HPLC) of Zeekoevlei *Nodularia spumigena* scum collected at the site of, and shortly after, the suspected poisoning of the dog. **A:** PDA-HPLC of methanol extract of scum material; **B:** PDA-HPLC of same quantity of methanol extract of scum, spiked with nodularin standard; **C:** ultraviolet absorption spectra of nodularin standard (broken line) and 13,3-min scum peak from A (solid line).
signs at affected water bodies and the distribution of information pamphlets. To monitor water quality, regular examination of the City of Cape Town’s raw potable- and recreational surface waters, using PDA-HPLC\(^1\), was instituted in July 1994, in addition to existing monitoring of the phytoplankton assemblages for the presence of cyanobacteria.

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