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ASSESSMENT OF *MICROCYSTIS* BLOOM TOXICITY ASSOCIATED WITH WILDLIFE MORTALITY IN THE KRUGER NATIONAL PARK, SOUTH AFRICA

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ABSTRACT: Based on previous necropsy results, *Microcystis* blooms in constructed water impoundments in the Kruger National Park (KNP) have been identified as a cause of wildlife mortality. In response to wildlife mortality during 2007, water samples, containing algal bloom material, were collected during February 2007 and July 2007 from four dams (Nhlanganzwani, Mpanamana, Makhohlola, and Sunset) in the southeastern part of the KNP as part of the follow-up investigation. The toxicity of the *Microcystis* blooms was determined using the enzyme-linked immunosorbent assay (ELISA), protein phosphatase inhibition (PPI) assay, mouse bioassay, and African sharptooth catfish (*Clarias gariepinus*) primary hepatocytes. Both the ELISA and PPI assays indicated that the water sample collected during February 2007 from the Nhlanganzwani Dam, and samples collected from the Nhlanganzwani and Sunset dams in June 2007, were toxic. These dams, exhibiting the toxic *Microcystis* blooms, were also associated with the wildlife mortality. Mice injected intraperitoneally with water samples from Nhlanganzwani Dam (February 2007) induced hepatotoxicity and mortality within 1 hr. Primary hepatocytes from the sharptooth catfish exposed to samples from these dams gave similar results. This laboratory investigation and results strongly incriminate the toxic *Microcystis* blooms as the cause of the wildlife mortality. Eutrophication and bloom formation appear to have been the consequence of the high numbers of hippopotami (*Hippopotamus amphibius*) in specific dams.

Key words: ELISA, microcystins, *Microcystis* spp., sharptooth catfish hepatocytes, wildlife mortality.

INTRODUCTION

Cyanobacteria (blue-green algae) are known to produce toxins that are directly implicated in livestock and wildlife mortality worldwide (Soll and Williams, 1985; Kellerman et al., 2005). The most toxic freshwater cyanobacteria are *Microcystis* spp., *Anabaena* spp., and *Planktothrix* spp., and the most common toxins produced are microcystins, especially microcystin-LR (Hitzfeld et al., 2000).

Available published data indicate that large numbers of livestock die every year in southern Africa from ingestion of cyanobacteria and their toxins (Steyn, 1945; Kellerman et al., 2005). Most poisonings of terrestrial animals occur after drinking *Microcystis* bloom material; however, mortality usually occurs with exposure to water where there is a heavy bloom that forms a dense surface scum. Animals drinking such concentrated scum can easily consume a fatal dose (Carmichael, 1995).

Wildlife mortality from cyanobacteria poisoning has increased in southern Africa during the past few years (Soll and Williams, 1985; Bengis et al., 2006), but unfortunately, published data on wildlife mortality are limited. Bengis and coworkers (2006) reported that during the autumn and early winter of 2005, large numbers of wildlife carcasses were observed near Mpanamana and Nhlanganzwani constructed water impoundments (dams) of the Kruger National Park (KNP). Most carcasses found were decomposed or partially consumed by scavengers; however, a complete necropsy performed on a fresh zebra (*Equus burchelli*) carcass found during that peri-

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od, confirmed the diagnosis of cyanobacteria poisoning. The necropsy revealed acute hepatic swelling with mottled orange and brown discoloration, general petechiation and icterus, and acute pulmonary edema. Diffuse hepatic necrosis was observed microscopically, and the histopathology lesions were compatible with a *Microcystis* bloom intoxication. A heavy *Microcystis* bloom was visible in both Nhlanganzwani and Mpanamana dams, and water samples from these two dams showed a dominant presence of *Microcystis* spp. The mouse bioassay and follow-up histopathology done on the mice livers also confirmed the earlier diagnosis of *Microcystis* poisoning. A total of 54 carcasses were discovered, most of which were clustered around these two dams. Carcasses included those of ruminants, hind-gut digesters, and even predators (Bengis et al., 2006).

In this study, we investigated the toxicity of cyanobacteria blooms in four dams (Nhlanganzwani, Mpanamana, Makhohlola, and Sunset dams) from the southeastern part of the KNP to explain the wildlife mortality that occurred between February 2007 and July 2007 in that area of the park. Carcasses of several different species, including white rhino (*Ceratotherium simum*), Burchell's zebra (*Equus burchelli*), blue wildebeest (*Connochaetes taurinus*), and impala (*Aepyceros melampus*) were found in a typical point-source pattern around the dams. Toxicity of the cyanobacteria blooms was investigated using the enzyme-linked immunosorbent assay (ELISA), protein phosphatase inhibition (PPI) assay, mouse bioassay, and African sharptooth catfish primary hepatocytes.

MATERIALS AND METHODS

Water samples and associated algae were collected by KNP personnel during February 2007 and July 2007 from four dams (Nhlanganzwani, Mpanamana, Makhohlola, and Sunset dams) in the southeastern part of the park (Fig. 1). Samples used for the identification of the cyanobacteria were collected in clean, 250-ml, plastic containers and preserved with Lugol's iodine. Water samples used for toxin analysis were collected in clean, 250-ml, plastic containers, placed in a cool and dark environment, and transported to the Onderstepoort Veterinary Institute (OVI) Toxicology Laboratory for analysis. Samples for microcystin toxin analysis were frozen until they were tested. Preserved water samples were examined for cyanobacteria using an XDS-1B optical microscopy (a product of Chongqing Optical and Electrical Instrument Company, Chengdu, China; supplied by Lasec SA, Cape Town, South Africa) at a magnification of 200–1,000×.

Cyanobacteria toxins were extracted from the frozen water samples following the modified method described by Falconer (1993) using a VirTis Benchtop SLC freeze-dryer (SP Industries, Warminster, Pennsylvania, USA). Freeze-dried samples were then weighed and resuspended in distilled water at 200 mg in 10 ml. Extracted samples were stored at −20 C until analyzed.

Microcystin levels present in the extracted samples from the KNP dams were determined using a commercial ABRAXIS-Microcystin ELISA kit according to the manufacturer's instructions (Aqualytic Environmental and Laboratory Services, Gauteng, South Africa) and a commercial RediPlate 96 EnzChek Serine/Threonine Phosphatase assay kit (Scientific Group, Gauteng, South Africa). For both the ELISA and PPI assays, a standard curve was constructed using microcystin-LR (MC-LR) standards, and concentrations of the extracted samples were determined from these standard curves (Prism software, GraphPad Software Inc., San Diego, California, USA). Six tests were performed for each quantitative assay, and samples showing concentrations higher than 5 µg/l were diluted to obtain more accurate results. A microplate ELISA spectrophotometer (BioTek Instruments, Inc., Winooski, Vermont, USA; Supplied by Analytical & Diagnostic Products, Gauteng, South Africa) and Fluoroskan Ascent FL fluorescent microplate reader (Thermo Electron Corporation, Waltham, Massachusetts; supplied by AEC-Amersham, Gauteng, South Africa) were used to measure absorbance (450 nm) and fluorescence (355 of 460 nm) for the ELISA and PPI assays, respectively.

Adult male mice (CD-1 SPF strain, Onderstepoort Biological Products [OBP], Gauteng, South Africa) weighing 25–45 g were housed individually in the Toxicology Laboratory Animal Facility with vermiculite as bedding material. They were provided with pellet food

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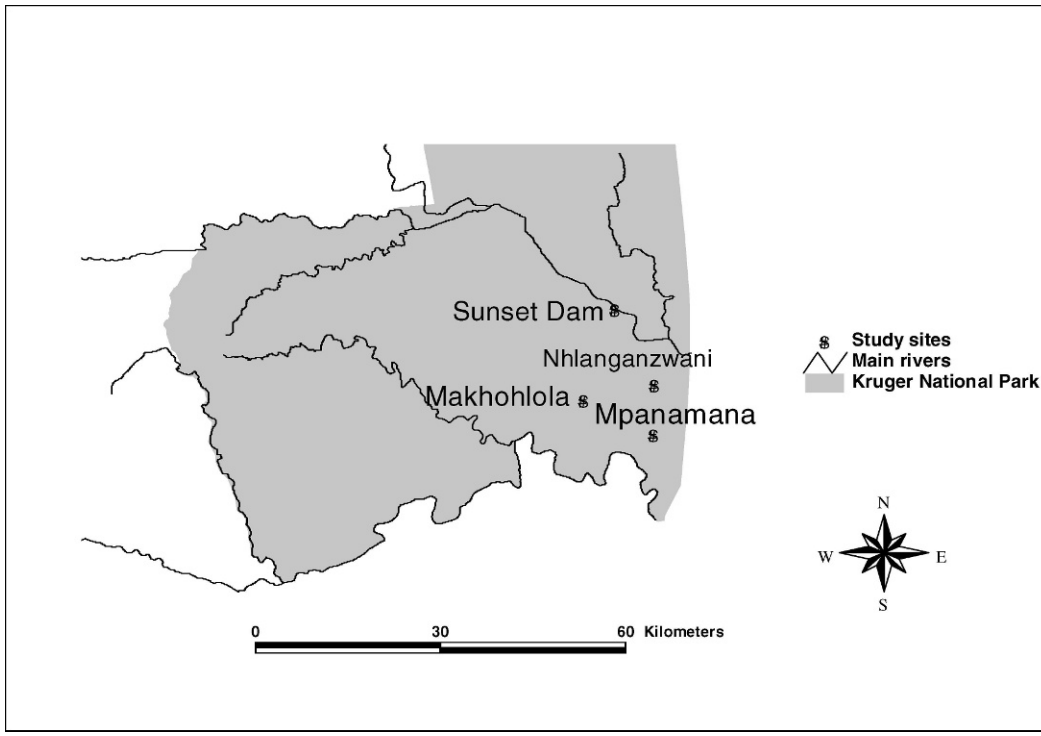

FIGURE 1. Location of the four constructed water impoundments, the Nhlanganzwani, Mpanamana, Makhohlola, and Sunset dams, in the Kruger National Park (KNP). Water samples containing blue-green algae (cyanobacteria) were collected from the dams between February 2007 and July 2007.

(OBP) and water ad libitum. Preweighed mice were injected intraperitoneally (ip) with 1 ml of the extracted cyanobacteria samples. Control mice were also injected ip with 1 ml saline solution. Two mice were used for each sample tested. All mice were observed for 24 hr after ip injection, during which, signs of poisoning and survival times were recorded. All surviving animals were sacrificed at the end of the observation period. Mortality, with the characteristic enlarged liver (i.e., liver weight/body mass>7%), was used as the measure of hepatotoxicity (Heresztyn and Nicholson, 2001). Mice liver tissue samples were also examined by a veterinary pathologist for histopathologic changes.

Primary hepatocytes were isolated from the African sharptooth catfish (*Clarias gariepinus*) using a modified two-step collagenase in situ perfusion method (Naicker et al., 2007). Briefly, the first perfusion was performed with a HEPES buffer, pH 7.5, 20 mM (Highveld Biologicals, Lyndhurst, South Africa) at a flow rate of 0.5 ml/min. Final perfusion was performed for 12 min at a flow rate of 5 ml/min with a perfusion solution containing collagenase type IV (Sigma-Aldrich, Midrand, South Africa). The yield of cells was about 3×10^6 cells/ml. Cells were plated in MatriGel (BD Biosciences, San Jose, California, USA) based membrane matrix coated 96-well plates and maintained at 16 C in a 5% CO_2 humidified atmosphere for 24 h to allow cells to attach to the wells (50,000 cells/well).

Assessment of cell viability was carried out using a modified method of Mosmann (1983) based on methyl-thiazol-tetrazolium (MTT; Sigma-Aldrich). After attaching to the wells, the catfish hepatocytes were exposed to serially diluted extracted samples from KNP (Nhlanganzwani, Mpanamana, Makhohlola, and Sunset dams; 100 µl of extracts added to 100 µl of M199 culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone). Control wells were prepared by adding 200 µl of culture medium. Three tests were performed on each extracted sample from KNP. Cells were incubated at 16 C for 72 hr in a 5% CO_2 humidified atmosphere. At the end of the incubation period, 10 µl of MTT (5 mg/ml in PBS) was added into each well, and cells were further incubated for 4 hr. The formation of color was

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TABLE 1. Microcystin concentrations, liver weight/body mass %, EC_{50}, and wildlife deaths associated with extracted samples from the Kruger National Park (KNP) (February 2007).

KNP samples[a]	Microcystin concentrations[b] ELISA (mg/l)	Microcystin concentrations[b] PPI (mg/l)	Mouse bioassay[c] Liver weight/body mass %	EC_{50} (μM)[d]	Wildlife deaths
Nhlanganzwani Dam[e]	49.41±0.98	10.95±1.58	9.47±0.78	54.9±8.06	2 white rhinos
Mpanamana Dam	1.56±0.07	0.03±0.01	NT[f]	84.7±6.14	No deaths
Makhohlola Dam	0.10±0.004	0.006±0.004	NT	95.8±4.06	No deaths
Sunset Dam	0.25±0.003	0.05±0.005	NT	93.8±3.24	No deaths
Control[g]			6.65±0.92		

[a] Duplicate samples were collected from each dam.

[b] Six tests were performed for each quantitative assay (enzyme-linked immunosorbent assay [ELISA] and protein phosphatase inhibition [PPI]). Microcystin concentration values are mean±SD.

[c] Two mice were used for each water sample tested. Liver weight/body mass>7% was used as a measure of hepatotoxicity in the mouse bioassay.

[d] Exposure concentration of an algal material expected to cause cytotoxicity in 50% of the cell population (EC_{50}) values were obtained after exposure of catfish primary hepatocytes for 72 hr to extracted samples from KNP. EC_{50} values are mean±SD for triplicate tests.

[e] KNP dam associated with wildlife deaths.

[f] NT = Not tested.

[g] Control = Saline solution constituted control in the mouse bioassay.

measured with a microtiter plate spectrophotometer (BioTek) at 570 nm. Cell viability was estimated as the percentage of absorbance of the sample relative to control.

The cytotoxic response of the catfish primary hepatocytes after exposure to the four extracted samples from KNP (Nhlanganzwani, Mpanamana, Makhohlola, and Sunset dams) was expressed as EC_{50} (the exposure concentration of an algal material that is expected to cause cytotoxicity in 50% of the cell population).

RESULTS

During February 2007, Nhlanganzwani Dam had the highest microcystin concentration of 49.41 mg/l when compared with the other three KNP dams (Mpanamana, Makhohlola, and Sunset dams) based on the ELISA (Table 1). Microcystin concentration of the other three KNP dams ranged between 0.10 mg/l and 1.56 mg/l. During this period, two white rhino carcasses were found near Nhlanganzwani Dam (Table 1). Four months later (June 2007), the microcystin concentrations had increased to 103.16 mg/l in the Nhlanganzwani Dam and 1.11 mg/l in the Sunset Dam (Table 2). At the same time, a gradual decrease in microcystin concentration to below 0.1 mg/l was observed in the Mpanamana and Makhohlola dams. Within a period of about 4 wk (June–July 2007), a sharp increase in microcystin concentration was observed in the Sunset Dam, where the toxin levels reached 124.46 mg/l (Table 2). During this period, several wildlife deaths were reported in the area around Nhlanganzwani Dam (15 white rhinos, eight zebras, and five blue wildebeest) and Sunset Dam (one white rhino and six impala; Table 2). The microcystin concentrations reported in this study (49.41–124.46 mg/l) are very high compared with other reports (Lehman, 2007; Masango et al., 2008). The risk of acute mortality is extremely high if animals are exposed to these high toxin concentrations.

The PPI assay was done only on samples collected during February 2007 to confirm the ELISA results. Even though the concentrations were reduced, a similar trend in the microcystin concentrations was observed with the PPI assay when compared with the ELISA results (Table 1). Because ELISA is a chemical test, it reacts more equally with different

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TABLE 2. Microcystin concentrations and wildlife deaths associated with the extracted samples from Kruger National Park (KNP) (June to July 2007) determined by enzyme-linked immunosorbent assay (mg/l).

KNP Samples	Microcystin concentrations (mg/l)[a]	Wildlife deaths
Nhlanganzwani Dam[b]	103.16±13.06	15 white rhinos, 8 zebras, 5 blue wildebeest
Mpanamana Dam[c]	0.001±0.00025	No deaths
Makhohlola Dam[c]	0.0002±0.0001	No deaths
Sunset Dam[c]	1.11±0.33	No deaths
Sunset Dam[c]	124.46±9.39	1 white rhino, 6 impala

[a] Tests were done in triplicate. Microcystin concentration values are mean±SD.

[b] Samples collected in June 2007.

[c] Samples collected in July 2007 soon after wildlife deaths near the Sunset Dam.

microcystin congeners than the PPI assay, which is an activity (toxicity) assay, and the less-toxic congeners give less of a response than the more toxic ones, such as microcystin-LR.

To confirm microcystin toxicity in vivo and to minimize the use of animals in the current study, only the toxicity of extracted samples from Nhlanganzwani Dam (February 2007) was investigated using the mouse bioassay because these extracted samples had microcystin concentrations above 6 mg/l, the minimum concentration of microcystin-LR that can induce hepatotoxicity and mortality in mice (Table 1). Mice dosed with the extracted samples from Nhlanganzwani Dam demonstrated symptoms typical for microcystin poisoning. Macroscopically, the livers of these mice were enlarged and appeared dark-red because of the abnormal accumulation of blood (Figs. 2, 3).

The EC_{50} values obtained after exposure of the catfish primary hepatocytes for 72 hr to the four extracted samples from KNP collected in February 2007 ranged between 54.9 μM and 95.8 μM (Table 1).

During this investigation, complete necropsies were performed on zebra, blue wildebeest, white rhino, and impala carcasses found near Nhlanganzwani and Sunset dams. The histopathology recorded and reported in these cases were all compatible with a diagnosis of cyanobacteria poisoning. Other carcasses found during this investigation were unsuitable for histopathologic confirmation because the carcasses either were too decomposed or were partly consumed by scavengers.

DISCUSSION

Microcystins, produced by several freshwater cyanobacteria, are responsible for intermittent, but repeated, outbreaks of animal and human poisoning from exposure to these toxins in surface waters (Ott and Carmichael, 2006). This study reports on the toxicity of *Microcystis* blooms that were investigated during episodes of wildlife mortality that occurred between February 2007 and July 2007 in the southeastern part of the KNP.

The wildlife mortality event in the KNP during 2007 was suspected to have been caused by cyanobacteria toxins (microcystins). Soon after the detection of a cyanobacteria bloom in Nhlanganzwani Dam (February 2007), water and algal samples were collected for toxin analysis. Mpanamana, Makhohlola, and Sunset dams were also sampled during this period (February 2007) because they are also located in the southeastern part of the Park and have been previously suspected of being involved in *Microcystis* poisoning of wildlife. At the time of the bloom in February 2007, *Microcystis aeruginosa* was found to be the dominant cyanobacteria in the water samples collected from the four KNP dams. Nhlanganzwani Dam had the highest microcystin concentration

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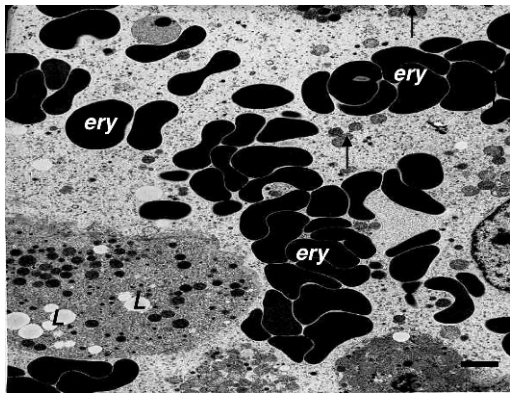

FIGURE 2. Mouse liver tissue after intraperitoneal injection with extracted water sample containing cyanobacteria from Nhlanganzwani Dam (February 2007). Hepatic hemorrhaging indicated by presence of a large number of erythrocytes (ery) in the tissue. Mitochondria (arrows) and lipid droplets (L). Bar=1 μm.

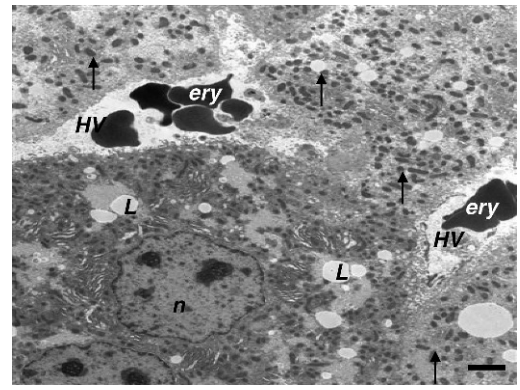

FIGURE 3. Control mouse liver tissue after intraperitoneal injection with saline solution characterized by presence of few erythrocytes (ery) contained within the hepatic vessel (HV). Large numbers of mitochondria (arrows) indicating a normal functioning liver. Nucleus (n) and lipid droplets (L). Bar=1 μm.

of 49.41 mg/l, whereas the other three KNP dams had concentrations ranging between 0.10 mg/l and 1.56 mg/l.

In June 2007, a cluster of wildlife mortality, involving white rhinos, zebras, and blue wildebeest was observed near the Nhlanganzwani Dam. At the time of this incident, *Microcystis aeruginosa* was once again the dominant species, and the microcystin levels had reached 103.16 mg/l. No wildlife mortality was reported for the other three KNP dams (Mpanamana, Makhohlola, and Sunset dams). The microcystin concentrations for these dams were below 0.10 mg/l, with the exception of Sunset Dam, which had reached a concentration of 1.11 mg/l. Within a period of about 4 wk (June–July 2007) a rapid increase in microcystin concentration was observed in the Sunset Dam, where the toxin levels increased to 124.46 mg/l. This *Microcystis* bloom was accompanied by the third incident of wildlife mortality, which included white rhino and impala.

Environmental factors, such as temperature, pH, organic nutrients, and age of cyanobacteria cells, have been shown to influence *Microcystis* toxicity (Wicks and Thiel, 1990; Downing et al., 2005; Lehman, 2007). Lehman (2007) reported that *Microcystis* blooms and increased microcystin concentrations were associated with higher nitrogen:phosphorous ratios. The presence of *Microcystis* blooms and high microcystin levels in the Nhlanganzwani and Sunset dams were most probably due to the presence of high numbers of hippopotami (*Hippopotamus amphibious*) in the dams, and the seasonal decrease in water levels (April to September in southern Africa) during the investigation period. Organic matter excreted (feces) by the hippopotami in the water, together with the animals stirring up the nutrient-rich sediment of these earthen dams, appear to have caused an increase in eutrophication, resulting in conditions favorable for the formation of toxic *Microcystis* blooms in the specific dams.

The PPI assay was used in this study to confirm the ELISA results; hence, it was only done on the KNP February 2007 samples. Microcystin concentrations obtained from the PPI assay were lower than those of the ELISA were. This is consistent with results obtained by Fischer et al. (2001) who found that the ELISA gave higher values than the PPI assay but had a fivefold better sensitivity.

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Only the Nhlanganzwani Dam sample (from all the samples collected during February 2007) had a microcystin concentration above 6 mg/l. We have previously shown that the minimum concentration of microcystin-LR that induces acute poisoning in mice (CD-1 SPF strain) is ≥6 mg/l (171 μg MC-LR/kg body weight; Masango et al., 2008). Therefore, the in vivo bioassay was only used for the February 2007 Nhlanganzwani Dam sample.

Microcystins have been reported to induce disruption of the cytoskeletal network in the liver, resulting in massive pooling of blood, followed by sinusoid destruction, and eventually, death by hepatic hemorrhaging (Theiss et al., 1988; Ott and Carmichael, 2006). This explains the enlarged, dark-red colored livers that were observed after dosing with the Nhlanganzwani sample extracts in the current study.

Hepatotoxicity of the extracted samples from KNP (February 2007) was further investigated by using sharptooth catfish primary hepatocytes. The viability of the catfish primary hepatocytes was affected after exposure for 72 hr to these extracts. The EC_{50} values obtained were in the range of 54.9–95.8 μM, with Nhlanganzwani Dam showing the lowest EC_{50} values. Because of their sensitivity and their ability to function similar to the liver in vivo (Guillouzo, 1998), isolated primary hepatocytes provide an attractive alternative to the mouse bioassay in toxicity testing for cyanotoxins (Masango et al., 2008).

Susceptibility of wildlife to microcystin poisoning appears to vary widely, with white rhinos and zebras being the most affected in the KNP. Soll and Williams (1985) reported the death of three white rhinos in the Barakologadi Game Reserve caused by suspected *Microcystis* poisoning. It is, however, not very easy to diagnose microcystin-related deaths in wildlife; carcasses of poisoned animals are seldom found, and in cases where they are found, they are usually decomposed or have been partially consumed by scavengers.

In conclusion, the laboratory results obtained in this study confirm that the incidents of wildlife mortality that occurred in the KNP during 2007 were most probably due to the toxic *Microcystis* blooms and high microcystin concentrations in the drinking water. The cyanobacteria blooms in individual dams were directly associated with the number of hippopotami making use of a specific dam at that moment. This study highlights the need for further investigation of wildlife poisonings from microcystins.

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