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# Toxic *Microcystis aeruginosa* in Cyanobacterial blooms Collected in Botswana

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## Abstract

*Cyanobacterial blooms are prevalent in surface water bodies in Botswana during summer months. Microscopic examination of cyanobacterial blooms from the Gaborone oxidation ponds showed the predominance of toxic Microcystis aeruginosa and contained up to 1.7 mg/g DW microcystin-LR. Cyanobacterial cells were raptured by freeze-thawing. The resulting cell suspension inhibited seed germination and seedling growth of two plants, Brassica nigra (cultivated) and Bidens pilosa (weed), in a concentration dependent manner. Complete inhibition of germination of B. pilosa seeds was observed with cell suspension containing 5 mg DW/ml. Pollen grains from Setcreasea pallida were also highly sensitive to the presence of toxic M. aeruginosa cells with complete inhibition observed when 0.3 mg DW/ml was incorporated into the growth media. Purified microcystin-LR was less toxic to the seeds of B. nigra and B. pilosa and to the pollen grains from S. pallida, suggesting that other factors from the freeze-thawed cells may have contributed to the toxicity of the cyanobacteria cells. However, these plants can be used to screen environmental samples containing M. aeruginosa as an initial assessment tool to test for toxicity of the cyanobacterial blooms.*

**Keywords**: Cyanobacteria, Botswana, *microcystis aeruginosa*, microcystins, *brassica nigra*, *bidens pilosa.*

## Introduction

Botswana is a landlocked country located in Southern Africa and lies between latitudes 17° and 27°S and longitudes 20° and 30°E. About 70% of the land mass is semi-arid and is dominated by the Kalahari Desert. Most of the country receives less than 450 mm of rainfall during the rainy season that stretches between December and April. Even, during the rainy season, the country often suffers prolonged periods of drought. Surface water is therefore scarce and great value is attached to the few pans, dams, ephemeral rivers and watering holes that provide water for irrigation, human and animal consumption[1,2]. Water conservation and reuse of wastewater are vigorously promoted by the government[3]. Wastewater contains high levels of nutrients such as nitrates and phosphates, which support algal blooms and phytoplankton proliferation[3,4].

Toxic cyanobacteria bloomshave been found in freshwater bodies in many parts of the world[5]. Toxic cyanobacteria blooms in water amenities has led to poisoning incidents of wildlife, domesticated animals and humans[6,7].Algal blooms have also been observed in water bodies in Botswana during summer months and may consist of potentially toxic cyanobacteria[2,8].

The major toxic cyanobacteria found in freshwater blooms include the genera *Microcystis, Anabaena, Nostoc* and *Oscillatoria*[9]. These organisms produce toxins such as microcystins, hepta- or penta-cyclic peptides,which have strong affinity for liver cells and are called hepatotoxins. The cyclic peptides in the microcystin structure consist of conserved and variable amino acids and the name is often derived from the two variable amino acids in the structure. The most common microcystins are microcystin-LR (with leucine and arginine) and microcystin-RR (with two arginine molecules)[10]. Microcystins are strong inhibitorsof serine phosphatases in eukaryotic cells. This inhibition negatively affects intracellular signalling, cell differentiation and carbon dioxide fixation, photosynthesis, and starch storage[11]. Microcystin-LR has been shown to cause oxidative damage and to depress the glutathione content of *Arabidopsis thaliana* while at the same time increasing superoxide dismutase and catalase activity[12]. There is evidence to show that the presence of algal blooms can also reduce the abundance and diversity of aquatic plants[13].

Several different methods have been applied to extract and detect cyanobacterial toxins from test samples. The methods used include, immunoassays, enzyme inhibition assays, and high performance liquid chromatography (HPLC)[14,15]. Some of these methods are expensive, time consuming and require expertise. Many developing countries including Botswana, lack the necessary infrastructure and manpower to carry out these analysis on a routine basis. Biological assays on the other hand do not require sophisticated equipment and have been used to screen for different toxic compounds in environmental samples. The mouse bioassay isa widely used standard method for regular cyanobacterial toxin monitoring procedures and is the reference method accepted worldwide for paralytic shellfish poisoning (PSP) testing[16]. Other organisms such as *Daphnia* and *Artemia* can detect lower toxin concentrations and may eventually replace the mouse bioassay[17].

In this study, algal blooms were collected from wastewater holding ponds in Gaborone, Botswana, examined for the dominant cyanobacteria species and analyzed for the presence of microcystins by HPLC and HPLC-MS. Algal blooms were also tested for toxicity using the mouse bioassay. Additionally, the blooms were also screened for toxicity using plant-based bioassays involving seedlings of *Bidens pilosa*and *Brassica nigra*, and pollen grains from *Setcreaseapallida*.

## Material and Methods

**Sample collection:** Algal bloomsamples were obtained from holding ponds that receive wastewater effluents from the municipal activated sludge treatment plant in Gaborone, the capital city of Botswana. The samples were collected between January 2000 and December 2004. The water from these ponds is discharged into the Notwane River which serves as a water source for livestock and wildlife downstream. Samples were also collected downstream along the Notwane River. The algal bloom samples (4-6 L) were collected by skimming the algal scum off the water surface using small buckets. The samples were coded BGA-0-12, depending on the location and date of collection. The collected samples were placed in cooler boxes and transported to the lab for microscopic examination and toxin analysis.

**Sample clean up and concentration:** Each algal bloom sample was first filtered through four layers of cheesecloth and then through a 72 µm sieve to remove the larger particles. The filtrate was transferred to a 2 L separatory funnel and left to stand for 2 hours to allow the cells to float. Most of the water was drained off and the concentrated cell suspension (200-300 ml) collected. A potion (50 ml) was stored at 4$^{o}$C for microscopic examination and dry weight determination. A second potion of the cell suspension (50 ml) was stored at -20$^{o}$C and tested for toxicity. A known volume of the cells was dried to constant weight in an oven at 60˚C in order to determine the dry weight (DW). The rest of the cells (100-200 ml) were lyophilized and stored at -84$^{o}$C for further analysis.

**Microscopy:** Samples were examined under a Zeiss compound microscope to determine the type of dominant algae in the blooms. Cyanobacteria were identified according to the morphological keys published by Canter-Lund and Lund[18].

**Toxin Analysis:** The method by Lawton et al[19] was used with modifications to extract the algal toxins. Freeze-dried cells (1 g) were mixed with acid washed sand in a mortar and 50 ml of ice-cold water: methanol: n-butanol (75:20:5, v/v/v) added. The mixture was homogenized to a fine paste and then centrifuged at 20000xg for 35 minutes in a refrigerated centrifuge at 4°C. Solvents from the supernatant were evaporated on a Rotary Evaporator at 40°C and residue re-dissolved in20% methanol/water (30 ml). The sample was divided into 3x10 ml portions and each portion applied to a conditioned 5 g Sep Pak C-18 cartridge (Waters, Milford, MA). Cartridges were conditioned by washing with methanol (10 ml), water (10 ml) and 20% methanol/water (10 ml) in succession. After loading the sample, the Sep Pak cartridges were sequentially washed with water (15 ml) and 20% methanol (10 ml) before eluting the toxins with 80% methanol/water (10 ml). The toxin fractions from the three cartridges were pooled and the solvents evaporated on a rotary evaporator at 40$^{o}$C before taking up the toxins in 1 ml methanol.

The sample from the Se-Pak cartridges were further separated by preparative HPLC and the toxin fractions corresponding to microcystin-LRor microcystin-RR were pooled. Preparative HPLC was done on a Waters HPLC System with a Model 610 pump and Model 486 Variable Wavelength Detector (Waters, Milford, MA) set at 238nm. The toxins were separated on a 30x300 mm µ Bondapak $C_{18}$ column (15-20 µm) at 30°C, with a mixture of acetonitrile and ammonium acetate (20 mM) (35:65, v/v) at a flow rate of 10 ml/min. Solvents from the pooled samples were evaporated and the residue re-dissolved in methanol for toxicity tests and mass spectral analysis.

Analytical HPLC separations were performed on a 4.6x100 mm Hypersil column (MOS, 3 µm) with a column temperature of 30°C and eluted with a mixture of acetonitrile and ammonium acetate (20 mM). Samples were eluted at a flow rate of 2 ml/min. Microcystin peak areas were calculated by comparing them to standard microcystin-LR and microcystin-RRfrom Sigma (St Louis, MO) using an Apex Chromatography Workstation (Milford, MA). Stock solutions containing HPLC purified microcystin-LR (0.1 mg/ml) and microcystin-RR (0.5 mg/ml) were prepared and used in subsequent toxicity tests.

**Confirmation of identity of toxins:** The identities of the toxins were confirmed using a Liquid Chromatography - Mass Spectrometry (LC-MS) system (Thermo Quest, Berlin, Germany). LC Separation was done on a 3.0x150 mm XTerra C-18 column (3.5 µm) (Waters, Milford, MA, USA). The toxins were separated and eluted with a mixture of acetonitrile and ammonium acetate (20 mM) at a flow rate of 0.5 ml/minute. The mass analyses were carried out on an MS fitted with an electrospray ion source in positive ion mode and a 4 kV capillary voltage.

**Freeze-thawed cells of *M. aeruginosa*:** The concentrated cell suspension was freeze-thawed as follows: the frozen sample was thawed in a water bath at room temperature and then rapidly frozen in acetone-dry ice mixture. The freeze-thawing process was repeated three times to rapture the cells. The raptured cell suspension was then used in toxicity testing along with purified microcystin-LR and microcystin-RR.

**Mouse Bioassay:** The toxicity of *Microcystis aeruginosa* was determined using male mice (average weight of 15-20 g). Mice were obtained from the Botswana Animal Care Facility and used according to approved protocols. Three mice were used per treatment and were given food and water *ad libitum*. The freeze-

thawed cell suspension was diluted with sterile water and 0.1 ml of cell suspensions injected intraperitoneal into mice. Mice were injected with 0-0.6 mg of cells per 10 g body weight. The mice were observed for signs of toxicity and euthanized with carbon dioxide after 3 hrs. Dead mice were dissected to observe any damage to internal organs.

**Seed germination and seedling growth bioassays:** Seed germination bioassays were performed as described by Kos et al[20]. Two types of seeds were selected based on cultural differences; commercially available and performance tested *Brassica nigra* (exotic to Botswana) and *Bidens pilosa* (a common weed in Botswana). Seeds were surface sterilized by immersing in 5% sodium hypochlorite solution for thirty minutes and rinsed with sterile distilled water before use in germination and growth assays. Freeze-thawed cells (0-6 mg/ml), purified microcystin-LR (0-5 µg/ml) and purified microcystin-RR (0-0.5 µg/ml) were transferred to sterile 15 ml culture tubes (Methanol was evaporated off the microcystin samples). Sterile Hoagland's medium (5 ml) containing 1.2% bacteriological agar at 45°C was transferred to the culture tubes and mixed well, and the media allowed solidifying. Three surface sterilized test seeds were placed, in triplicates, on the media surface and incubated in the dark at 25°C. The seeds were observed for germination (appearance of radicle) after 24 hours.The tubes were then transferred to a growth chamber at 25°C for 7 days with 12 h light/12 h dark photoperiod. Controls with 1 ml sterile water were also prepared and observed as above. Seedlings were harvested after 7 days and the shoot and radicle lengths recorded. The shoot and radical lengths (mean of triplicates) were expressed as a percentage of the control seedlings.

**Pollen germination and tube elongation bioassay:** Pollens from *Setcreaseapallida*, a common ornamental plant that flowers throughout the year, were used in this study. The plant has large pollen grains that germinate easily in a simple water-based medium containing sucrose (10%), calcium nitrate (0.03%), andboric acid (0.01%). Freeze-thawed cells (0-6 mg/ml), purified microcystin-LR (0-5 µg/ml and microcystin-RR (0-5 µg/ml) were incorporated into the germination medium and mixed well. A drop of each germination medium was placed on a microscope slide and pollen grains introduced by touching the media with the open anthers of freshly picked flowers. The slide was placed on glass rods in a petri dish containing 1 ml of sterile water (to create a humid environment). Controls with only the germination medium were also prepared. The petri dishes (triplicates) were incubated at 25°C for 2 hrs. After 20 minutes of incubation, the slides were examined under a Zeiss compound microscope and the number of germinated and non-germinated pollen grains from ten randomly selected fields of view was recorded under the 10x objective. Pollen germination was expressed as a percentage relative to the control experiments. The pollen tube lengths of 10 randomly selected pollen grains in each of the 10 viewing areas were measured after 2 hours of incubation. The measurements were made using an eyepiece graticule and a stage micrometer. The average lengths of pollen tubes were calculated and expressed as a percentage of the control experiment.

## Results and Discussion

**Cyanobacteria species and microcystins:** The cyanobacteria samples collected from sewage holding ponds were dominated by *Microcystis aeruginosa*. Other cyanobacteria species were detected, but to a lesser extent, and included *Microcystis flosaquae, Oscillatoria brevis, Scenedesmus quadricauda, Spirulina subsala* and *Spirulina princeps* as shown in table-1. The dry weight (DW) of the algal cells ranged between 6-12 mg DW per ml of cell suspension. The bloom in the BGA-12 sample was almost entirely made up of *Microcystis aeruginosa* and contained 10.9 mg DW of algal cells per ml of cell suspension and was further analyzed for microcystins. Although cyanobacteria blooms are common throughout the year in Botswana, they were more prevalent during the summer months which are characterized by periods of sunny skies and high temperatures (25-35°C). Occurrence of cyanobacteria, including *Microcystis aeruginosa,* in water bodies is highly variable and is affected by both physical and chemical factors (light, temperature, pH, salinity, phosphates and nitrates)[21]. Microcystin-LR and microcystin-RR were detected in the algal samples by HPLC and the identities were confirmed by LC-MS. The most abundant mass ions $(M+H)^+$ observed were 995.5 (microcystin-LR) and 1138.0 (microcystin-RR). The concentrations of microcystin-LR in the algal blooms ranged from 0.2 to1.7 mg/g dry weight (DW) as shown in table-1. BGA-12 contained both microcystin-LR (0.40 mg/g DW) and microcystin-RR (0.13 mg/g DW).The co-occurrence of microcystin-LR with microcystin-RR can lead to synergistic toxic action by the two microcystins[22].

**Mouse Bioassay:** The *Microcystis aeruginosa* blooms were found to be highly toxic to mice. Table-1 shows that mice injected with freeze-thawed cell suspensions of 75 mg/kg body weight or higher were lethal to mice, with 100% mortalities being recorded for concentrations at or above 166 mg/kg body weight. All fatalities came within 3 hrs of injection. The $LD_{50}$, based on the lyophilized cell suspension, was estimated to be between 75-100 mg/kg body weight (between 30-40 µg microcystin-LR/kg body weights. Wolf and Frank[23] reported $LD_{50}$ between 25-150 µg/kg body weights for microcystin-LR and between 500-1000 µg/kg body weights for microcystin-RR.

**Seed germination tests:** Although the standard mouse bioassay has been used to screen for toxic cyanobacteria blooms, there is need to minimize the use of animals in toxicity testing. Seed germination assays can serve as quick and reliable methods for screening large numbers of toxic cyanobacteria samples. Seeds of many vascular plants undergo a series of physiological changes that make them more sensitive to environmental signals[24]. Freeze-thawed BGA-12, containing both microcystin-

LR and microcystin-RR, was tested for its effect on the germination and growth of *B. nigra* and *B. pilosa* seeds. Figure-1 shows that the presence of *M. aeruginosa* (BGA-12) inhibited the germination of *B. nigra* and *B. pilosa* seeds in a concentration dependent manner. *B. pilosa* seeds were more sensitive and were completely inhibited by 5 mg DW/ml of BGA-12 (equivalent to 2.0 µg microcystin-LR/ml). The growth of shoots and radicles of *B. nigra* and *B. pilosa*, lengths measured after 7 days of growth, were also found to be inhibited in a concentration dependent manner as shown in figure-2 and figure-3. *B. pilosa* seedlings were more sensitive to the presence of BGA-12 cells than the *B. nigra* seedlings. In *B. pilosa*, the radicles were also more sensitive to *M. aeruginosa* than the shoots; shoot and radicle formation were completely inhibited when 5 mgDW/ml of *M. aeruginosa* cells were incorporated into the media. Most physiological effects are related to the inhibition of protein phosphatases by cyanobacterial toxins. Differences can also be attributed to seed size and adaptation to environmental cues. *B. pilosa* seeds are smaller but are a hardy weed that is well adapted to hash environments[25]. It has been shown that different plant species respond differently to the presence of toxins in the growth environment and significant decreases in root lengths have also been observed in other plants[26,27].

**Table-1**
**Predominant cyanobacteria species found in algal bloom samples collected from the Gaborone Oxidation Ponds in Botswana**

| Sample ID | Types of cyanobacteria | Microcystin-LR (mg/g) |
|---|---|---|
| BGA-0 | *Microcystis aeruginosa, Oscillatoria brevis, Spirulina subsala and Anabaena* spp. | 1.10 |
| BGA-1 | *Microcystis aeruginosa, Spirulina subsala and Anabaena* spp. | 1.70 |
| BGA-2 | *Microcystis aeruginosa*, *Gomphosphaeria* spp. | 0.63 |
| BGA-3 | *Microcystis aeruginosa*, *Spirulina subsala*, *Oscillatoria brevis*, *Anabaena* spp. | 0.55 |
| BGA-9 | *Microcystis aeruginosa, Microcystis flosaquae, Oscillatoria brevis, Oscillatoria rubescence, Scenedesmus quadricauda, Spirulina subsala* and *Spirulina princeps.* | 0.20 |
| BGA-12 | *Microcystis aeruginosa* | 0.40 |

**Table-2**
**Effects of freeze-thawed cells of *Microcystis aeruginosa*on mice, Fatalities were recorded after 3 hrs**

| Algal cells (mg/kg body weight) | No. of surviving mice | Dead mice (%) |
|---|---|---|
| 0 | 3 | 0 |
| 25 | 3 | 0 |
| 50 | 3 | 0 |
| 75 | 2 | 33.3 |
| 100 | 1 | 66.7 |
| 250 | 0 | 100 |
| 300 | 0 | 100 |

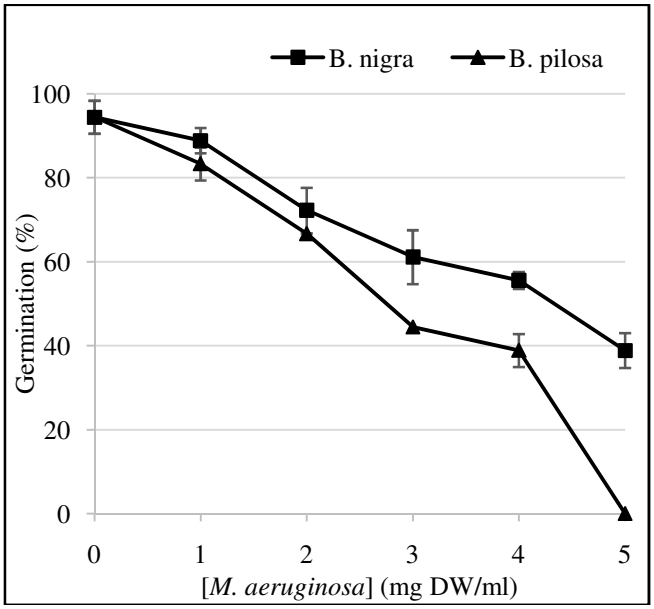


**Figure-1**
**Effect of freeze-thawed *Microcystis aeruginosa* cells (BGA-12) on the germination of *Brassica nigra* and *Bidens pilosa* seeds after 24 hrs of incubation**

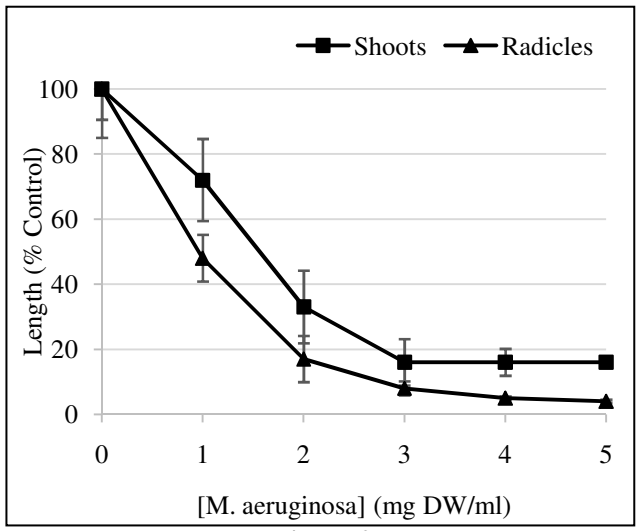


**Figure-2**
**Effect of freeze-thawed *Microcystis aeruginosa* cells on the growth of *B. nigra*shoots and radicles after 7 days of growth**

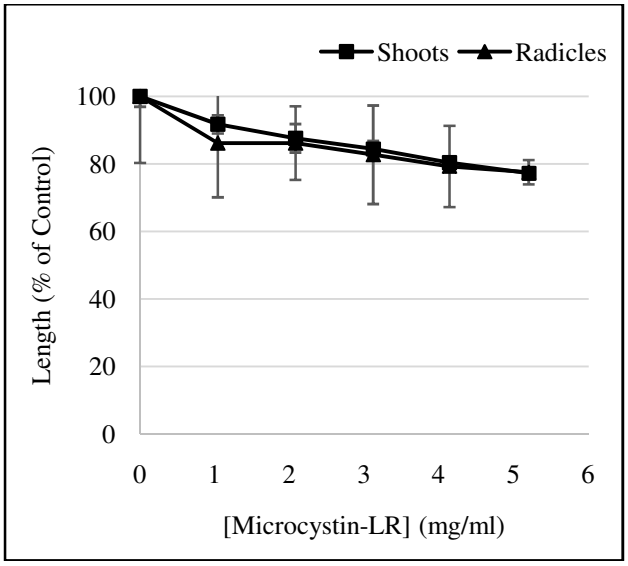


**Figure-4**
**Microcystin-LR inhibited the growth of *B. nigra* seedlings**

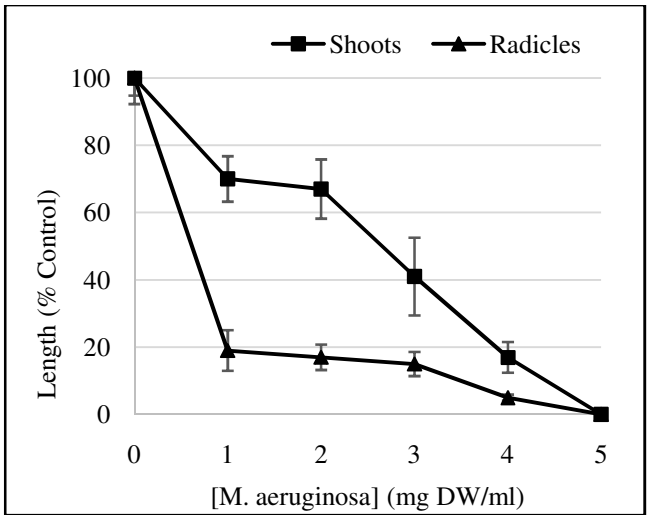


**Figure-3**
**Effect of freeze-thawed *Microcystis aeruginosa* cells on the growth of *B. pilosa* shoots and radicles after 7 days of growth**

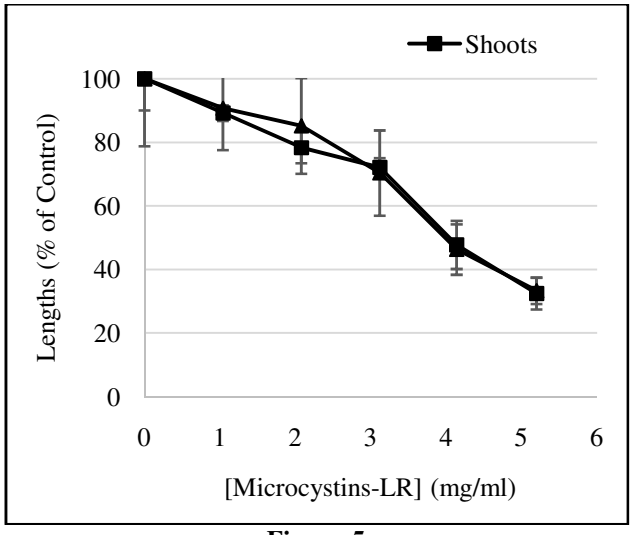


**Figure-5**
**Microcystin-LR inhibited the growth of *B. pilosa* seedling**

When purified microcystin-LR was tested on *B. nigra* and *B. pilosa* seeds at concentrations similar to those found in the freeze-thawed *M. aeruginosa* cells, the shoots and radicles were also inhibited in a concentration dependent manner as shown in figure-4 and figure-5. *B. pilosa*seedlings were more sensitiveto microcystin-LR than *B. nigra*seedlings. Much lower concentrations ofmicrocystin-LR(4 µg/ml) were required to bring about 50% inhibition of growth (relative to controls) in *B. pilosa* shoots and radicles than in *B. nigra* seedlings (which required > 10 µg/ml to give similar effects). Pflugmacher[27] also showed that crude toxin extractshad greater effects on maize seedling growth than the purified microcystins. These results suggest the involvement of other compounds, in addition to microcystins, in toxicity.

**Pollen germination and pollen tube growth tests:** Freeze-thawed BGA-12 cells and purified microcystin-LR were also tested on germinating *Setcreaseapallida* pollen grains. The number of pollen grains germinating after 20 minutes of incubation was counted and the lengths of the pollen tubes were measured after 2 hrs of incubation. Figure-6 shows that the effects of cyanobacterial cells on pollen germination and pollen tube growth were similar, 50% inhibition (relative to controls) was observed when tested with media containing about 0.05 mg DW/ml of *M. aeruginosa*. Complete inhibition was observed when *M. aeruginosa* concentrations rose above 0.3 mg DW/ml. Purified microcystin-LR and microcystin-RR, at concentrations similar to those found in the freeze-thawed cells, also inhibited pollen germination and pollen tube growth as shown in figure-7. However, the concentrations required to bring about 50%

inhibitions were much higher than those required for freeze-thawed cells. Pollen germination was reduced to less than 50% of the controls at microcystin-LR concentration of 2.5 µg/ml, almost 6 times higher than that observed in BGA-12 cells (0.02 µg/ml). Figure-8 shows that the amount of microcystin-RR (4 µg/ml) required to bring about 50% inhibition of germination were even higher than that required for microcystin-LR. These effects on pollen germination and pollen tube growth may be related to inhibition of protein phosphatases in pollen grains. Similarly, calyculin A and okadaic acidwere shown to inhibit pollen germination and pollen tube growth in *Lilium longiflorum*[26]. Another cyanobacteria toxin, cylindrospermopsin, was found to inhibit tobacco pollengermination at concentrations ranging from 5 to 1000 µg/ml[28].

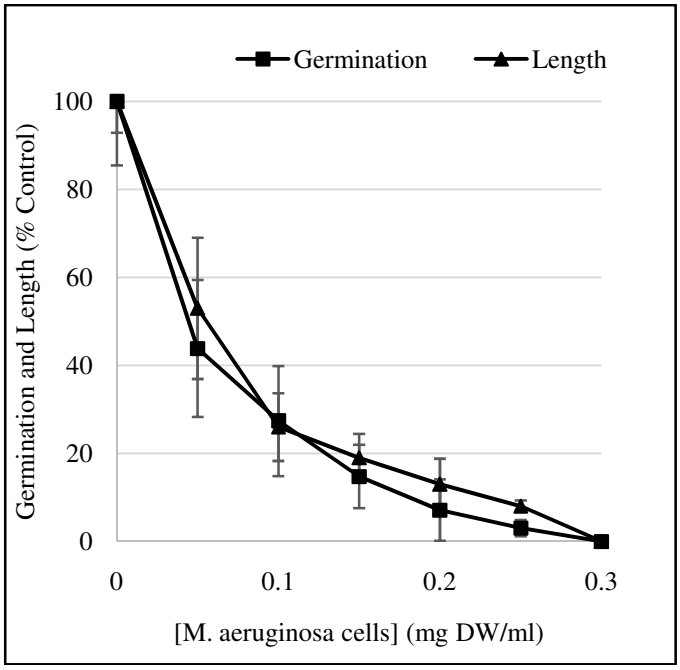


**Figure-6**
**Effect of freeze-thawed *Microcystis aeruginosa* cells (BGA-12) on the germination and pollen tube growth of freshly harvested pollens from *Setcreaseapallida***

## Conclusion

In this study we found that the algal blooms from the Gaborone oxidation ponds were dominated by highly toxic *M. aeruginosa* that contained high levels of microcystin-LR. We also demonstrated that plants such as *B. nigra* (cultivated*)* and *B. pilosa (*weed*)*, and pollen grains from *S.pallida*, were highly sensitive to the presence of toxic *M. aeruginosa* cells and to microcystin-LR in the growth media. These plants can be used to screen environmental samples containing *M. aeruginosa* for toxicity and can be used as an initial assessment tool before applying more sophisticated equipment such as HPLC, or LC-MS.

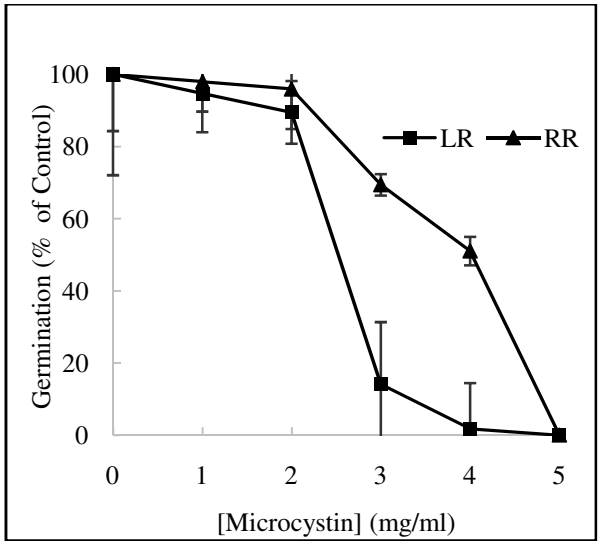


**Figure-7**
**Effect of purified microcystin-LR and microcystin-RR on the germination of fresh pollens grains from *Setcreaseapallida***

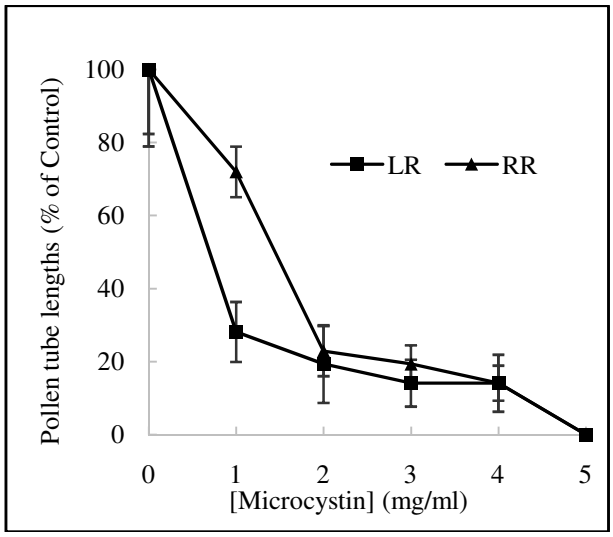


**Figure-8**
**Effect of purified microcystin-LRand microcystin-RRon the pollen tubes offresh pollens grains from *Setcreaseapallida***

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