INCIDENCE OF MICROCYSTINS (HEPATOTOXINS) IN FLOATING SCUMS IN THE SWARTSPRUIT RIVER, SOUTH AFRICA

EGUZOZIE, K. U.[1*] – MAVUMENGWANA, V.[1] – NKOSI, D.[2] – KAYITESI, E.[1] – NNABUO-EGUZOZIE, E. C.[3]

[1]University of Johannesburg "Doornfontein Campus" Faculty of Science, Department of Biotechnology and Food Technology, Doornfontein 2028, Johannesburg, South Africa (tel:+27115596456)

[2]University of Johannesburg "Doornfontein Campus" Faculty of Science, Department of Applied Chemistry, Doornfontein 2028, Johannesburg, South Africa (tel: +27115596148)

[3]University of South Africa "Florida Campus" College of Agriculture and Environmental Sciences, Department of Environmental Sciences, Florida 1709, Johannesburg, South Africa (tel: +27114713296)

**Corresponding author*
e-mail:eguzoziekennedy@yahoo.co.uk
(tel: +27763173476; fax: +27-11-559-6425)

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Abstract. A study which probed the bioaccumulation, detection and quantitative variations of microcystins in floating scums of *Microcystis flos-aquae* employing optimized solid phase extraction liquid chromatography electrospray ionization mass spectrometry was undertaken. A quantitative profile and dominance of MCYST-LR over MCYST-YR and MCYST-RR congeners across sampled sites, as well as the occurrence of MCYST-(H_4)YR and (D-Asp^3, Dha^7) MCYST-RR during the summer months was observed. Analysis of maximum and minimum concentrations of quantified MCYSTs showed 270, 14.10 (µg toxin/g DW), 141.5, 1.43 (µg toxin/g DW) and 72.28, 0.15 (µg toxin/g DW) for MCYST-LR, MCYST-RR and MCYST-YR respectively. One way ANOVA showed there was significant difference between mean MCYSTs concentrations across the sampling sites ($P < 0.05$) and a marginal significant difference in mean MCYSTs concentrations among variants ($P = 0.04$). The results suggest that the most prevalent bloom forming species is *Microcystis flos-aquae*, often producing hepatotoxic microcystins, which can negatively impact aquatic animals and human health. The identification of sites with higher MCYST-YR, MCYST-LR, MCYST-RR concentrations and distributions in the pond and MCYST-LR dominance will be of particular importance to the metropolitan authorities, researchers and general public of the water safety quality of this freshwater reservoir.

Keywords: *cyanotoxins, electrospray ionization, eutrophication, hazardous cyanobacteria bloom, microextraction*

Abbreviations: Microcystins (MCYSTs), dry weight (DW), MCYST-LR (D-Ala–L-Leu–erythro-b-Me-DisoAsp–L-Arg–Adda–D-isoGlu–N-Me-dehydroAla), MCYST-YR (D-Ala–L-Tyro–erythro-b-Me-D-isoAsp–L-Arg–Adda–D-isoGlu–N-MedehydroAla), MCYST-RR (D-Ala–L-Arg–erythro-b-Me-D-isoAsp–L-Arg–Adda–D-isoGlu–N-MedehydroAla).

Introduction

Microcystins (*Figure 1a*) are naturally occurring cyclic heptapeptides with high toxicity (LD_{50} value, mouse i.p: 50-500 µg toxin/kg) (Codd et al., 2005). They are identified as hepatotoxins because they inhibit some special enzymes (PP1 and PP2A). Freshwater microcystin producing blue green algae include (*Mycrocystis spp*., *Anabena*

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spp., *Aphanizomenon spp*., *Nostoc spp*., and *Planktothrix spp*. (Codd et al., 2005). A comprehensive review of the toxins produced by diverse species of blue green algae is in literature (Chorus, 2001). The related and similar toxin referred to as nodularins (*Figure 1b*) is a cyclic pentapeptide and is also produced by toxigenic blue green algae.

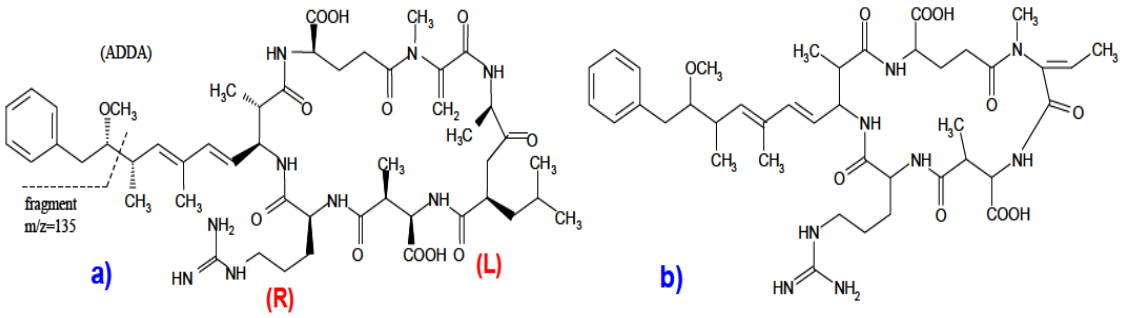

Figure 1. *Structures of cyanobacterial toxins. a) Microcystin-LR. The other microcystins are derived from this by replacing the marked amino acids (L) and (R) by the amino acids indicated in their names. b) Nodularin.*

Microcystins can be found in lakes, rivers and other drinking freshwater reservoirs under conditions which permits harmful algal blooms (HABs). They are characterized by the presence of a unique non-proteinogenic β-amino acid called ADDA (Maizels and Budde, 2004; de Figueiredo et al., 2004). A 2 letter suffix (xy) is attributed to each distinct toxin to represent the 2 variant amino acids (Chorus, 2001). X is usually leucine, arginine or tyrosine and Y arginine, alanine and methione. The toxicity of microcystins is dependent on the ADDA group, the N-methyl dehydroalamine (MeDha) group and its structural cyclic nature (Maizels and Budde, 2004; de Figueiredo et al., 2004; Msagati et al., 2006). As a result of their high chemical stability, they can outlast for long periods in a water environment. The ADDA group is favourably confined and conserved in microcystins and nodularins (Gulledge et al., 2003; Zeck et al., 2001). This makes them stable against physiological replacements by other amino acids. As a result, the ADDA group has been used in numerous analytical techniques for the selective detection and identification of microcystins and its congeners (Msagati et al., 2006). These techniques have incorporated toxicity based assays, (Kyselkova and Marsalek, 2000) enzyme linked immunosorbent assays (Metcalf et al., 2000; McElhiney and Lawton, 2005; Baier et al., 2000; Fischer et al., 2001), phosphatase inhibition assays, (Xu et al., 2000; Chen et al., 2009) HPLC/UV/PDA (Lawton and Edwards, 2008; Chen et al., 2011), HPLC/MS (Azevedo et al., 2002; McElhiney et al., 2000; Ame et al., 2010), LC/MS with various interface and ionization configurations (Mayumi et al., 2006; Spoof et al., 2003; Miles et al., 2013). The methods have provided reliable information which can be used for the extraction, detection and identification of microcystins and its analogs (Lawrence and Menard, 2001). Reports show that in positive mode LC-ESI-MS, the ADDA group in microcystins give a characteristic fragment ion m/z 135 corresponding to the [phenyl-$CH_2CH(OCH_3)$]$^+$ ion (Msagati et al., 2006). *Microcystis flos-aquae* is a typical constituent of hard water lakes and ponds, where it frequently occurs at high population levels in mid-to late summer. This paper reports incidence of cyanobacterial hepatotoxins in cultures of *Microcystis flos-aquae*

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(cyanobacteria) isolated from bloom environmental samples collected from the Swartspruit River, North West Province, South Africa where this species typically co-dominates with other algae including *Anabaena spiroides*, *Ceratium hirundinella* and *Microcystis aeruginosa*. Hence monitoring their occurrence, quantitative variations and prevalence in domestic drinking and freshwater reservoirs is of paramount interest to our research group.

Materials and Methods

Reagents, analytical standards and laboratory materials

All organic solvents (HCOOH, MeOH, MeCN, $CHCl_3$) were of high purity analytical grade (HPLC grade) and were purchased from Merck (Johannesburg, South Africa). Microcystin standards (MCYST-YR, MCYST-LR and MCYST-RR) were bought from CyanoBiotech GmbH (Berlin, Germany) and supplied by Industrial Analyical (pty) Ltd (Johannesburg, South Africa). C18 SPE cartridges (Waters Oasis^{TM} HLB cartridge: 60g, 3 mL) were bought from Waters Inc. (Milford MA, USA) and delivered by Microsep (pty) Ltd, SA (Johannesburg, South Africa). Water samples were filtered using Whatman GF/C glass-fiber filters, porosity 0.45 μm (Whatman International Ltd, Maidstone, UK). A digital pH meter with a combination electrode (Hanna, Singapore) was used for pH adjustments. The amounts of chemicals and reagents were measured using a CP225D Satorius balance (Satorius, Germany) for milligram or lower quantities. Liquid transfer measurements were performed with pipettes (Socorex, Germany). The Waters^{TM} LC instrument used contained the following components: a K-1001 single piston pump (Knauer, Germany), a Waters^{TM} 3100 mass detector coupled with an electrospray ionisation source (ESI) conditioned with the mobile phase for 10 min, an analytical C18 column (Waters Symmetry 300^{TM}, 4.6 mm, x 75 mm, 3.5μm) using the Alliance Waters^{TM} e2695 separation module). The system was connected and networked with a Hewlett Packard (USA) Laser Jet 1200 Series printer for recording chromatograms, using a 1451-1 chromatogate Data System, version 2.55 (Knauer, Germany). Solvents and mobile phase used were degassed with online degasser attached to the solvent delivery system.

Study area

The river for the study is located in Greater Hartbeespoort Metropolitan Area, North West Province, South Africa. The study sites include: (i) JKSTT (-25º74'9426"S +27º90'4568"E), (ii) RTVLS (-25º75'1059" +27º90'2755"E), (iii) KALTS (25º74'9426"S +27º90'4568"E) and (iv) MDFTS (-25º74'8338"S +27º90'6078"E). The site selection were based on; areas in the river that could easily be reached by either boat or by walking along the banks of the river, relative distance to fishing and animal conservation area, relative distance to the nearby water treatment facility and proximity to the Hartbeespoort sewage works along the Bapsfontein road.

Sample collection and analysis of physicochemical parameters

Samples were collected on a once a-month basis from November 2014 to February 2015 from four pre-determined sites. Algal samples were harvested according to standard protocol and established procedures (Hoeger et al., 2007). Triplicate of surface water (0-10cm) was collected using a polymethyl methacrylate sampler from the river.

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On-site physicochemical parameters were measured using a multifunction meter (YSITM 6-Series Sonde, Marion, Germany).

Species identification and enumeration

Identification and enumeration of cyanobacteria present in environmental samples was performed following established procedures (Mioni et al., 2011). Water samples for microscopy analysis were collected as follows: approximately 600 mL of surface water (grab) samples were fixed with 2.5% (v/v) glutaraldehyde in the field and filtered through a 1-μm pore size, 25-mm diameter, black polycarbonate filters (GE Osmonics, Monroe, NC) for morphologic taxonomic identification of cyanobacteria. The abundance of cyanobacteria cells was determined on a Zeiss Axioplan epifluorescence microscope at 400X magnification using green excitation (Zeiss Filter Set 20, excitation 546 nm bandpass, and emission 575–640 nm bandpass filters). Samples were preserved in Lugol's Iodine solution, and cyanobacterial cells were enumerated on a Nikon Eclipse TE200 inverted microscope (Fetscher et al., 2009). Morphological characteristics were determined by cell shape and changes in auto-fluorescence or cell organization.

Sample preparation for microcystin extraction

Algal cells were filtered from the scum on pre-weighed GF/C glass fiber filters (47 nm, 0.45 μm). The filters were severally flushed with autoclaved distilled water to remove any surface/superficial microcystins remaining on the cells. The filtrate was at -20^{o}C prior to freeze drying. Microcystin extraction from weighed algal cells (1.0 g pellets) using 70% MeOH. The microcystin extracts were subjected to $CHCl_3$/MeOH/H_2O liquid partitioning to remove pigments, other co-eluting compounds and any additional cartridge blocking materials (Cardozo et al., 2006) prior to extraction and pre-concentration of algal extracts by solid phase extraction techniques. Extracts were then analysed using LC-ESI-MS (Camean et al., 2004). *Figure 2* depicts the solid phase extraction protocol.

LC-ESI-MS optimized conditions

The mobile phase consisted of a mixture of 0.5% formic acid/94.5% Milli-O water (A) and 5% acetonitrile (B). The columns were operated at room temperatures (23.5^{o}C). A reverse phase system consisting of 25% acetonitrile (10 min), 25% acetonitrile (20 min), 25% acetonitrile (11.2 min), 70% acetonitrile (10 min) was used in analyte elution within a gradient window of 20 min. Sample injection volume and flow rates were set at 0.5 μL and 0.5 mL/min respectively. The ion source for the electrospray mass spectrometry was operated on both positive and negative ESI modes for all quantification experiments.

Identification of microcystin analytes

For structural identification of MCYST-LR, MCYST-YR and MCYST-RR variants in the environmental samples, the detected molecular ion masses, molecular ion fragments and retention times were analyzed relative to the observed molecular ion masses, fragments and retention times of the authentic MCs standards as well as comparison with literature values. The assigned values are as follows: MCYST-LR

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(m/z, 996.3, $[M+H]^+$, ADDA m/z, 135, RT 7.86), MCYST-YR (m/z, 1.047.3 $[M+H]^+$, 523.4 $[M+2H]^{2+}$, ADDA m/z 135, RT 7.59), MCYST-RR (m/z, 1,039, $[M+H]^+$, ADDA, m/z, 135, RT, 6.72).

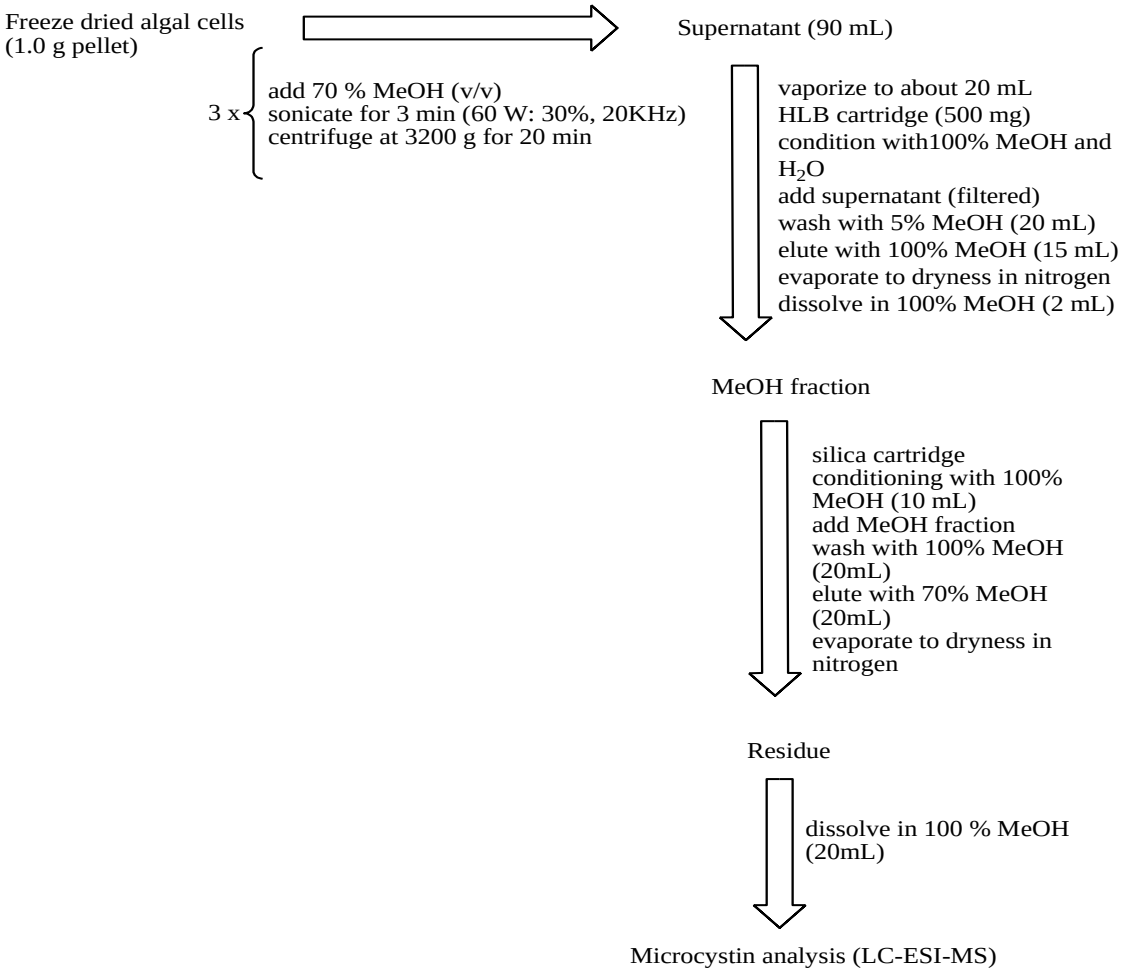

Figure 2*. Procedure for optimized solid phase microextraction of freeze dried algal cells microcystin before liquid chromatography-electrospray ionization mass spectrometry analysis.*

Quantification of cyanobacterial hepatotoxic microcystins

The quantification of each MCYSTs variant was determined using the authentic external standard calibration method. Analysis was performed in triplicates to generate mean values for each microcystin variants. Evaluation was based on the linear equation of the regression line of the individual MCYST (-LR, -YR and -RR) variants with the available authentic reference standard. Quantitative analysis as achieved using peak areas substituted on linear regression equations: $Y = 5.342x - 4.218$ ($r^2 = 0.9965$), $Y = 4.030x - 2.518$ ($r^2 = 0.9948$) and $Y = 3.098 + 1.172$ ($R^2 = 0.9950$) of standard curves for 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 μg/mL MCYST-LR, MCYST-RR and MCYST-LR variants respectively. Once-a-month (November 2014 - February 2015) mean concentrations for each variant and total concentrations of variants were calculated.

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Statistical analysis

One-way ANOVA was used to test significant differences among mean microcystin-LR variant concentrations across sampling sites and sampling periods as well as among microcystin variants. *Figures 3-5* depict One-way analysis of variance (ANOVA) representation of mean microcystin-LR variant concentrations (μg/g DW) across the sampled sites, mean microcystin-LR variant concentrations (μg/g DW) across the sampling periods and mean microcystin-LR variant concentrations (μg/g DW) among MCYST variants respectively.

Results

Physicochemical parameters of river

There were significant variability in the pond water quality across the studied sites during the assessment periods (*Table 1*). The temperature of the pond ranged from 24.4 to 24.7^{o}C and from 25.3 to 25.5^{o}C in November and December 2014 respectively and from 25.3 to 25.7^{o}C and from 24.2 to 24.5^{o}C in January and February 2015. The sites were more alkaline with a range of 8.5 to 8.9 during the November and December 2014 sampling period and slightly alkaline (7.1 to 7.7) during the January and February 2015 sampling months. There was not much variations in dissolved oxygen concentration across the sites with DNSPHKP1 (8.9 mg/L) in the month of February 2014 and UPSPHKP2 (3.4 mg/L) in November 20115. There was considerable spatial variations in PO_4^{2-} (0.48 to 0.98 mg/L) and NO_3^{2-} (1.04 to 1.70 mg/L) across the sampled sites during the assessment summer months. Sites UPSPHKP1 and DNSPHKP2 showed marked difference in physicochemical parameters compared to other sites especially for pH, dissolved oxygen and nutrients.

Microcystin identification and quantification

A total of three major microcystin congeners (MCYST-LR, MCYST-YR and MCYST-RR) and two minor microcystin variants were detected, identified and separated based on m/z signal in positive ESI mode. Structures of the two minor variants are consistent to MCYST-(H_4)YR (m/z 1053.1, $[M+H]^+$, m/z, 135, ADDA and (D-Asp^3, Dha^7) MCYST-RR (m/z 1006, $[M+H]^+$, m/z 135, ADDA) when compared to literature values. *Table 2* depicts the sampling site and specific monthly mean concentrations of each microcystin variant, total MCs concentrations (quantified only) and percentage of each variant. Each concentration is a mean value of n = 3, $\bar{x}$ = mean concentration, se = standard error of sample mean concentration and rsd = relative standard error of sample mean concentrations. One-way ANOVA was used to test significant differences among mean microcystin-LR variant concentrations across sampling sites and sampling periods as well as among microcystin variants. Figures 3-5 depict one-way analysis of variance (ANOVA) representation of mean microcystin-LR variant concentrations (μg/g DW) across the sampled sites, one-way analysis of variance representation of mean microcystin-LR variant concentrations (μg/g DW) across the sampling periods and one-way analysis of variance representation of mean microcystin-LR variant concentrations (μg/g DW) among MC congeners. The results describe high significant difference ($P < 0.05$) across the sampling sites and marginal significant difference ($P = 0.02$) across sampling periods whereas there was no significant difference ($P = 0.72$) among MC variants.

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Table 1. *On-site physicochemical parameters from each sampling site and period (November 2014 – February 2015). JKSTT = Jukskeispruit tributary, RTVLS = Rietvlei stream, KALTS = Kaalspruitfontein stream, MDFTS = Modderfontein stream, * Reinting waste water treatment plant*

Month	Sampling site	Temp (^oC)	Surface Water Temp (avg ^oC)	Conductivity (µS/cm)	Dissolved Oxygen (mg/L)	pH	PO_4^{2-}	NO_3^{2-}	$Si(OH)_4$	n	Chlorophyll-a (µg/L)
Nov 2014	JKSTT	25.2	24.95	387.3 ± 0.92	8.9 ± 0.36	8.4 ± 0.07	0.80	0.53	0.55	3	0.06 ± 0.01
	RTVLS	24.8		368.3 ± 1.55	8.5 ± 0.29	8.3 ± 0.07	0.85	0.51	0.59	3	0.08 ± 0.02
	KALTS	24.7		361.9 ± 0.56	7.9 ± 0.41	8.4 ± 0.07	0.75	0.49	0.61	3	1.49 ± 0.03
	MDFTS	25.1		378.9 ± 0.75	7.2 ± 0.20	8.5 ± 0.09	0.68	0.42	0.55	3	1.46 ± 0.27
Dec 2014	JKSTT	25.8	25.43	359.7 ± 1.27	7.3 ± 0.29	8.2 ± 0.07	0.58	0.33	0.59	3	1.89 ± 0.06
	RTVLS	25.4		362.6 ± 2.32	7.8 ± 0.25	8.0 ± 0.06	0.62	0.41	0.61	3	1.22 ± 0.02
	KALTS	25.2		359.9 ± 0.98	7.1 ± 0.25	8.4 ± 0.07	0.60	0.39	0.58	3	3.62 ± 0.02
	MDFTS	25.3		378.3 ± 1.25	7.3 ± 0.31	8.3 ± 0.06	0.67	0.36	0.66	3	5.52 ± 0.02
Jan 2015	JKSTT	25.2	25.55	355.5 ± 2.00	6.0 ± 0.35	7.8 ± 014	0.52	0.33	0.52	3	0.23 ± 0.02
	RTVLS	25.4		355.5 ± 0.63	3.4 ± 0.25	7.4 ± 0.08	0.62	0.39	0.70	3	3.49 ± 0.01
	KALTS	25.9		349.4 ± 1.62	3.9 ± 0.13	8.2 ± 0.14	0.67	0.36	0.62	3	5.58 ± 0.03
	MDFTS	25.7		365.2 ± 2.60	6.1 ± 0.45	8.3 ± 0.17	0.72	0.37	0.69	3	0.02 ± 0.01
Feb 2015	JKSTT	24.7	24.55	364.3 ± 0.66	6.2 ± 0.21	8.1 ± 0.14	0.62	0.42	0.62	3	0.02 ± 0.01
	RTVLS	24.2		359.2 ± 1.45	3.3 ± 0.22	8.2 ± 0.14	0.64	0.51	0.59	3	0.04 ± 0.01
	KALTS	24.9		360.5 ± 0.98	3.4 ± 0.22	8.5 ± 0.07	0.66	0.52	0.57	3	1.89 ± 0.02
	MDFTS	24.2		360.7 ± 1.13	3.5 ± 0.14	8.7 ± 0.20	0.52	0.53	0.64	3	1.39 ± 0.01

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Table 2. *Sampling site and specific monthly mean concentrations of each MCYST variant, total MCYST concentrations (quantified only) and percentage of each variant. (Each concentration is a mean value of n = 3, $\bar{x}$ = mean concentration, se = standard error of sample mean concentration and rsd = relative standard error of sample mean concentrations.*

MCs VARIANTS		JSKTT				RTVLS				KALTS				MDFTS			
		Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb	Nov	Dec	Jan	Feb
	min	22.0	46.4	50.20	50.05	90.00	215.85	270.2	199.0	90.09	215.90	270.9	199.50	17.74	18.0	16.20	14.0
	max	22.08	46.5	50.50	50.10	90.04	215.90	270.3	199.10	90.05	215.85	270.5	199.20	17.80	18.10	16.25	14.18
MCYST-	$\bar{x}$	22.04	46.37	50.77	50.08	90.02	215.8	270.23	199.05	90.07	215.89	270.7	199.35	17.78	18.06	16.22	14.10
LR	se	0.04	0.15	0.15	0.03	0.02	0.03	0.06	0.05	0.03	0.06	0.05	0.06	0.03	0.05	0.03	0.09
	rsd	0.18	0.33	0.30	0.06	0.02	0.01	0.02	0.03	0.49	0.54	0.30	0.39	0.25	0.28	0.16	0.65
	min	7.3	8.0	14.15	9.90	15.80	48.10	72.08	70.0	15.90	50.01	72.25	70.22	0.14	1.50	1.70	1.32
	max	7.4	8.05	14.20	9.98	15.88	48.20	72.20	70.20	15.88	50.02	72.30	70.24	0.15	1.52	1.72	1.34
MCYST-	$\bar{x}$	7.36	8.02	14.18	9.84	15.86	48.15	72.13	70.10	15.89	50.02	72.28	70.23	0.15	1.51	1.71	1.33
YR	se	0.05	0.03	0.03	0.04	0.05	0.06	0.06	0.1	0.03	0.02	0.01	0.02	0.03	0.05	0.03	0.09
	rsd	0.72	0.31	0.18	0.41	0.33	0.12	0.09	0.14	13.32	2.78	0.30	0.33	0.25	0.29	0.16	0.65
	min	6.46	4.20	17.30	13.42	57.60	141.20	141.50	70.80	57.60	141.20	141.50	70.80	4.0	6.28	5.82	1.42
	max	6.50	4.22	17.18	13.50	57.80	141.30	141.50	70.82	57.75	141.25	141.50	70.82	4.02	6.31	5.83	1.43
MCYST-	$\bar{x}$	6.48	4.32	17.40	13.46	57.67	141.23	141.50	70.81	57.68	141.23	141.50	70.81	4.01	6.30	5.82	1.43
RR	se	0.02	0.01	0.1	0.04	0.12	0.06	0.06	0.06	0.06	0.06	0.06	0.03	0.01	0.02	0.01	0.01
	rsd	0.36	0.27	0.57	0.30	0.20	0.04	0.04	0.05	1.08	0.5	0.61	0.42	0.25	0.24	0.10	1.36
	$\Sigma\,\bar{x}$	35.88	58.71	82.35	73.38	163.55	405.18	483.86	339.96	163.64	407.14	484.48	340.39	21.94	25.87	23.75	16.86
	% -LR	61.43	78.98	61.65	68.25	55.04	53.18	55.85	58.55	55.04	53.03	55.87	58.57	81.04	69.81	68.29	83.63
	% -YR	20.51	13.66	17.22	13.41	9.70	11.88	14.94	20.62	9.71	12.29	14.92	20.63	0.68	5.84	7.2	49.27
	% -RR	18.05	7.36	21.08	18.34	35.26	34.86	29.24	20.83	35.25	34.69	29.21	20.80	18.28	24.35	24.51	1.48

JKSTT = Jukskeispruit tributary, RTVLS = Rietvlei stream, KALTS = Kaalspruitfontein stream, MDFTS = Modderfontein stream.

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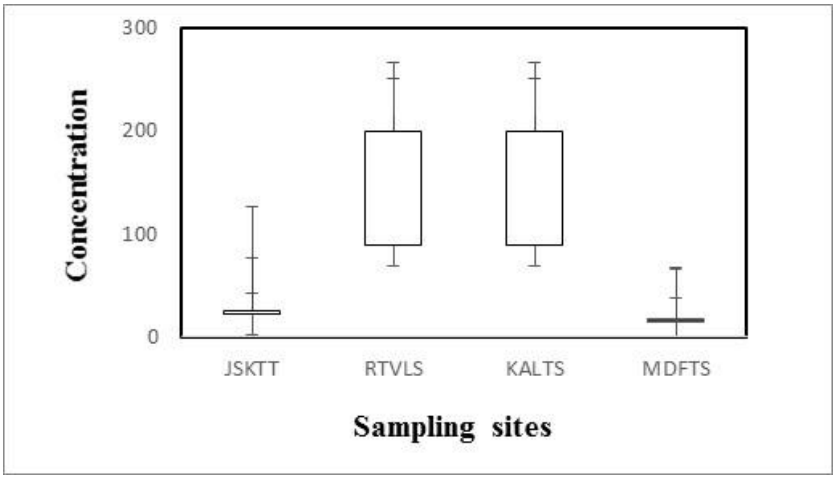

Figure 3. *One way ANOVA statistical difference of mean MCs concentrations (µg/g DW) across sampling sites JSKTT, RTVLS, KALTS and MDFTS. (P < 0.05, 95 % Confidence Interval of Mean)*

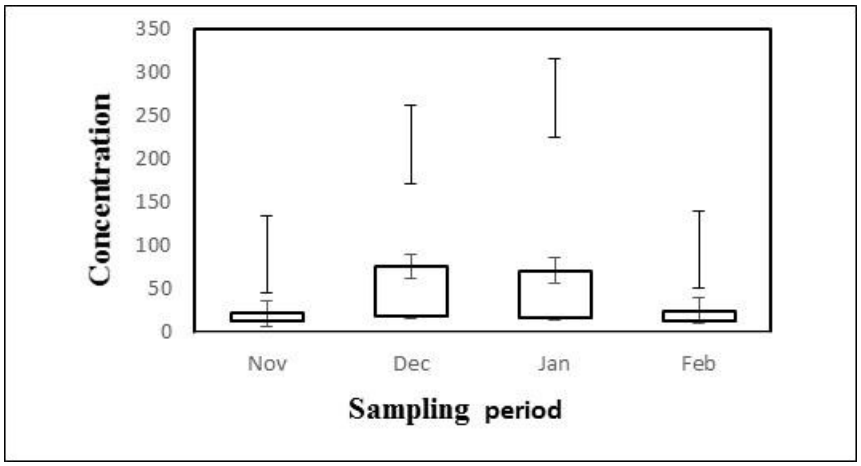

Figure 4. *One way ANOVA statistical difference of mean MCs concentrations (µg/g DW) across sampling periods November 2014 to February 2015. (P = 0.02)*

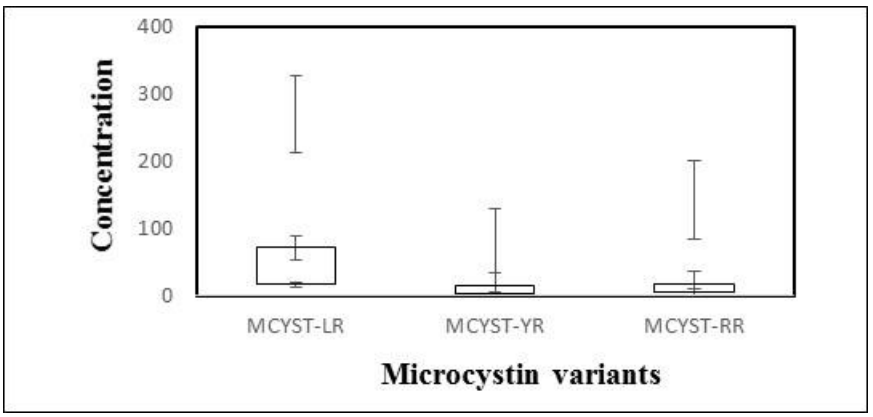

Figure 5. *One way ANOVA statistical difference of mean MCs concentrations (µg/g DW) among variants (P = 0.72).*

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Discussion

The Swartspruit River is a freshwater reservoir used for agricultural, domestic supply of portable drinking water as well recreational activities in the Greater Hartbeespoort Metropolitan Area, South Africa. Eutrophication and harmful cyanobacteria blooms have been the cause of water quality problems in this pond. *Table 1* depicts the physiological parameters that prevailed in the pond during the assessment periods. The prevalence and proliferation of microcystis species as well as the production of hepatotoxic microcystins have been reported to depend on a number of physicochemical parameters prevailing in a freshwater ecosystem (Wicks and Thiels, 1990; Sivonen and Jones, 1999; Jacoby et al., 2000; Dziallas and Grossart, 2012). Parameters like optimal pH and temperature (Dziallas and Grossart, 2012) as well as light intensity (Oberholster and Botha, 2010; Dziallas and Grossart, 2012) are important parameters known to favour algal growth. Algal growths were noticeable throughout the sampling periods during which the average temperature in November 2014 to February 2015 ranged between 24.2^{o}C and 25.9^{o}C. The temperature range (24.2 and 25.9) is typical for summer months in the region and has been reported to favouring the growth of microcystis species (Oberholster and Bother, 2010; Sivonen, 1990; Rapala et al., 1997; Msango, 2007). The pH levels in the pond ranged between 7.4 ± 0.08 and 8.7 ± 0.02. This range is known to be appropriate for the desolvation of phosphates in a water column (Greenwald, 2012). The availability of soluble phosphates in water increases as the pH shifts towards alkaline conditions of up to 8.9 Greenwald (2012). The optimal pH of between 7.4 and 8.7 observed in the pond is characteristic of most eutrophied freshwater bodies presumably because this pH range is suitable for metabolic activities and growth of toxic algae (Harding and Paxton, 2001; Owuor et al., 2007). Additionally, the observed pH range during the assessment period during which floating algal cells were conspicuous on the surface of the pond corroborates other reports from studies (Oberholster and Botha, 2010; Harding and Paxton, 2001; Owuor et al., 2007) in the region which suggest that this pH range is favourable for growth and distribution of microcystis species in a water body. An increase in the mean concentrations of all MCYSTs extracted particularly in December 2014 during which the water surface in the pond was completely green probably was due to higher concentrations of chlorophyll-a measured. Reports suggest that algal cells multiplication could result in an increased algal biomass paralleled by an increased chlorophyll-a production, thus resulting in elevated microcystin production. Our results (compare *Table 2*, *Figures 3 & 4*) clearly suggest that higher concentrations of microcystins were recorded in sites RTVLS and KALTS in the sampling month of December 2014 and January 2015 where chlorophyll-a concentration was highest compared to sites JSKTT and MDTTS. This observation corroborates reports (Oberholster and Botha, 2010; Msango, 2007) which has attributed increased total microcystin concentrations expressed as MCYST-LR eq/L to higher chlorophyll-a concentrations for algal cells collected from a freshwater dam in the region. Areas around site RTVLS and KALTS are known as some of the historical algal concentration sites in the pond. Hence the occurrence of harmful algal bloom in these sites could be attributed to their locations and to that conditions that favour massive algal cell growth and aggregation are prevalent in these sites. The observed decrease in the dissolved oxygen concentrations in these sites especially towards the end of the summer months could be attributed to microbial activities taken place (Dziallas and Grossart, 2012). Our presumption is that the decrease in dissolved oxygen concentrations is an indication of higher microbial activities, probably due to bacteria

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feeding on dying algal cells. The decrease in microcystin concentrations extracted from freeze dried algal samples from all the sampled sites in February 2015 supports this assumption. Decrease in the concentration of microcystin towards the end of the summer months is of importance concerning public health since higher concentrations of microcystins are expected presumably due to cell deaths. The observed decrease in numbers of visible algal cells and clear water towards the end of summer in the pond could be a very deceiving water safety indicator to users of this freshwater pond, especially should there be no post bloom monitoring strategy to ascertain hepatotoxic microcystin concentrations particularly after summer. Sites RTVLS and KALTS experienced visible algal cell bloom accumulation all through the sampling season. This probably is due to wind direction and effects of river currents caused by the inflow of water upstream from the Jukskeispruit tributary and Kaalspruit River. In addition, these site are located within a few meters from a water treatment facility and near an animal conservation area. The sites were also characterized by quiet current and turbulence effects. Reports suggest that algal blooms naturally occur in calm, nutrient rich water reservoirs (Falconer and Humpage, 2005). Site JSKTT was relatively calm. This seemingly is because it is protected from strong winds by bushes in the vicinity of the animal conservation area. The water mass around site MDFTS is largely from the Modderfontein stream inflow and Swartspriut River outflow into the Hartbeespoort dam. No significant quantities of algal cell blooms were observed in this sampling site.

The ADDA fragment ion $[PhCH_2CH(OCH_3)]^+$ (m/z 135) have been used for the detection and identification of microcystins and nodularins (Zhang et al., 2012; Chang et al., 2014; Msagati et al., 2006) and results from the α-cleavage of the methoxy group of the ADDA residue in microcystins (del Campo and Ouahid, 2010; Jurczak et al., 2004). The observed molecular ion masses, fragments and retention times were analysed relative to those of the authentic reference standards used in the study and compared to literature values. The assigned values: MCYST-LR (m/z, 996.3, $[M+H]^+$, ADDA m/z, 135, RT 7.86), MCYST-YR (m/z, 1.047.3 $[M+H]^+$, 523.4 $[M+2H]^{2+}$, ADDA m/z 135, RT 7.59) and MCYST-RR (m/z, 1,039, $[M+H]^+$, ADDA, m/z, 135, RT, 6.72) depict the presence of microcystins in the water samples corroborating similar reports from freshwater reservoirs in the region (Msagati et al., 2006; Oberholster et al., 2009). Further analysis of the data revealed two other variants from the algal extracts from all the sampled sites. Our presumption is that the cyanobacterial species producing the hepatotoxic microcystin, dominated and was well distributed in the river. Structures of the two minor congeners is consistent to MCYST-(H_4)YR (m/z 1053.1, $[M+H]^+$, m/z, 135, ADDA, (Mazur-Marzec et al., 2008) and (D-Asp^3, Dha^7)MCYST-RR (m/z 1006, $[M+H]^+$, m/z 135, ADDA) when compared to literature values (Hoger et al., 2007; Wood et al., 2010) isolated from *Microcystis.aeruginosa* in other locations (Blom et al., 2001; Messineo et al., 2009; Vareli et al., 2009). *Figure 5* showed that the observed minimum and maximum MCYST-LR concentration were around 16.22 μg toxin/g and 270.23 μg toxin/g DW, across the sampling sites. The concentration range of 270.23 μg toxin/g is more than the toxicity levels of cyanobacteria toxins (Cazenave et al., 2006). Mouse assay toxicity levels of MCYST-LR (LD_{50} 25 μg toxin/g to 50 μg toxin/kg DW is more than that of MCYST-RR (LD_{50} 200 μg toxin/g to 800 μg toxin/kg DW. Ito et al. (2002) has reported that both cyanobacterial MCYST-LR and MCYST-RR equally inhibit the activities of PP1 and PP2A enzymes. The inhibition of the activities of these enzymes is known to result in metabolic dysfunction in animals, and sometimes death in cases of higher dosages (Butler et al., 2009). Furthermore, MC-LR is known to exhibit

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inhibitory effects against antioxidants and detoxification activities on fish gills (Cazenave et al., 2006) at dosages around 10 μg/L. Our results (*Figure 5*) clearly implies that MCYST-LR is the dominant variant in all the sampling sites. The fact that its dominance in all the sampled sites would present a major health hazard to the public may be arguably ignored. This is because the uptake rate (Klitzke et al., 2011), synergistic effects of other toxigenic moieties (Osswald et al., 2011) and the quantity of toxin ingested (Blom and Jüttner, 2005) that are physiologically more important in relation to animal poisoning than toxicity values of any microcystin variant (Osswald et al., 2011). The bioaccumulation of MCYST-LR in fish, animals and plant tissues resulting from elevated extracellular microcystin concentrations in water is of importance. This is because toxicological effects pose a serious health risk to the public consuming food products resource as well as other users of a contaminated water resource for domestic, recreational and agricultural activities (Blom and Jüttner, 2005). Even though there are no guidelines for MCYST-LR concentrations in recreational waters due to its low toxicity, the World Health Organization (WHO) has set a limit of 25 μg MCYST-LR eq/L for total MCYSTs concentrations in recreational waters (WHO 2003). The authors are of the view that upon algal cell lysis and death towards the end of the summer season, MCYST-LR would have added the largest proportion to the total MCYSTs concentration, exceeding the WHO threshold level for recreational waters. Consequently, it could be argued that elevated concentrations of MCYST-LR alone could potentially present a health hazard to consumers of products or services of these freshwater reservoir. Blom et al. (2001) are of the opinion that the toxic activity of (D-Asp^3, Dha^7) MCYST-RR was greater than that of the parent MCYST-RR in algal grazers. Although further studies are still required to fully characterise the structure of (D-Asp^3, Dha^7) MCYST-RR isolated from the algal extracts studied, its occurrence in the Swartspruit River is of particular interest concerning what could be its synergistic effects (if any) to the toxicity of the dominant MCYST-LR variant. Our findings reasonably suggest that a stratified freshwater impoundments like the Swartspruit River could be over-enriched and contain excess nutrients given rise to massive algal blooms. Optimal penetration of light during the summer months uniquely determines the depth to which photosynthesis is practical and algae can flourish. (Hely-Hutchinson and Schumann, 1997). Our assumption is that *Microcystis flos-aquae* and *Microcystis aeruginosa* blooms started in temperatures of 24.2°C and peaked at temperatures of between 25°C and 27°C. The dissolved oxygen concentration in the upper layers of the impoundment was periodically higher during the bloom sampling periods than off bloom periods complementing reports describing the seasonal and temporal bloom variations of toxigenic cyanobacteria population predominantly *Microcystis spp* in other locations (Downing and van Ginkel, 2004; Prakash et al., 2009; Arzate-Cardenas et al., 2010). These findings are in harmony with other reports which have indicated that microcystin seasonal variations are a familiar occurrence in many eutrophic water reservoirs due to changing environmental factors, species succession and dynamics (Kardinal and Visser, 2005). The observed quantitative variations in microcystins mean concentration across the sampled sites and sampling periods is not an uncommon characteristics considering the size of the river. Moreover, this investigation was carried out during the summer months in South Africa close to the onset of the winter season when *Microcystis spp* have been reported to undergo overwintering (Fallon and Brock, 1981; Tsujimura et al., 2000). The findings portrays typical trends in microcystin profiles representing the rivers water situation during the summer season. Monitoring

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the distribution of microcystins across all the sampling sites studied could reasonably imply that they were produced by the dominant and well distributed cyanobacteria species *Microcystis flos-aquae*. This assumption corroborates findings from previous studies in the region which revealed that *Microcystis spp* are the prevalent MCYSTs-producing species (Scott, 1991; Oberholster and Botha, 2010). *Microcystis flos-aquae* was the most common species of this genus detected in the pond during our study. Maximum bloom development was observed during mid-summer seasons (November 2014 and February 2015) when the water column was thermally stratified. The cell count of 3.2×10^5 cells L^{-1} for the *Microcystis* taxa was remarkable high. This could be attributed to our sampling close to the shoreline. The presence of hepatotoxic microcystins in the filtered fraction probably is an underestimation of the total microcystin concentration present in the environmental samples. Even though no significant extracellular toxin was detectable, this fraction is characteristically a small percentage of the total microcystin pool (e.g. < 9%) during the early stages of toxigenic population development. Reports indicate they can become the majority fraction in ageing and lysing cyanobacterial populations (Codd et al., 2005). Whether the microcystins detected in the particulate material are attributable to all of the cyanobacterial species found, is not clear at present. The assignation of microcystin variants detected to individual cyanobacteria strains awaits ongoing phylogenetic molecular sequencing investigation in our research group.

To the best of our knowledge, little or no relevant information currently exist that have reported the occurrence, analytical identification and quantitative variations of hepatotoxic microcystins (MCYST-LR, MCYST-YR, MCYST-RR) from toxigenic hyper-bloom samples collected from the Swartspruit River employing optimized solid phase microextraction-liquid chromatography-electrospray ionization-mass spectrometry. The authors are of the opinion that the formulation of any monitoring and strategic remediation interventions to minimize potential health risk to the public and environment caused by the bioaccumulation of harmful cyanobacterial blooms and their cyanotoxins in such as fresh water reservoir could be favourably dependent on information presented here, especially where high risk sites have been identified (Falconer and Humpage, 2005). Our assumption is that the information, data, figures and findings presented in this report would provide a better insight of the dangers posed by the bioaccumulation of cyanobacterial hepatotoxins in a fresh water reservoir like the Swartspruit River. Our goal is to monitor and assess the state of water quality and safety of the Swartspruit River as a portable drinking freshwater resource.

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