1 **Temporal dynamics of intra-and extra-cellular microcystins concentrations in Koka**
2 **Reservoir (Ethiopia): Implications for public health risk**

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22 Abstract

23 This study was carried out with a view of understanding the temporal dynamics of microcystin
24 concentrations in both algal seston and water samples and the associated public health risk. All
25 the major MC variants, namely MC-LR, MC-YR, and MC-RR, were detected in both the algal
26 seston and water samples. In the majority of the samples, the most potent variant, MC-LR,
27 constituted the greatest proportion of the total MC concentration suggesting extremely high
28 potential public health risk. The exceptionally high concentrations ($\mu g\ L^{-1}$) of all the variants,
29 MC-LR (815), MC-YR (466.6) and MC-RR (265.68), were observed in May. Although the
30 extracellular MCs were relatively less concentrated and less frequently detected, concentrations
31 ($\mu g\ L^{-1}$) of up to 20 of MC-LR, 6.13 of MC-YR, and 1.27 MC-RR were encountered. The
32 strong and significant association between *Microcystis* abundance and concentration of nitrate
33 (Spearman Rank Order Correlation $r = 0.793$, $p<0.001$) may suggest that nitrate is the key
34 dictating factor in the dynamics of *Microcystis,* and may have consequently influenced the MC
35 levels in the reservoir

36

37 **Keywords** Cyanobacteria,intra-cellular, extra-cellular, eutrophication, *Microcystis,* Microcystin
38 nitrogen, tropical,

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43 ## 1. Introduction

44 The emergence and expansion of harmful algal blooms and their associated toxins in fresh water
45 system is becoming a serious threat to public health of global concern (Davis et al., 2009; Zhang
46 et al., 2012). Several members of cyanobacteria including *Microcystis* are known to produce
47 potent toxins, which are of high public health risk (Falconer and Humpage, 2005). Cyanotoxins
48 associated human illness and fatal poisoning in aquatic, wild and domestic animals have been
49 reported by several authors (Backer, 2002; Carmichael et al., 1997). Several attempts have been
50 made to remove cyanotoxin from drinking water supply to minimize potential public health risk.
51 Conventional methods such as flocculation, sand filtration, and sedimentation were reported as
52 possible approaches for the removal of cyanotoxins (Jurczak et al., 2005). However, although
53 cell-bound toxins can be removed using these methods, it is required to ensure that the
54 procedures do not cause any cell disruptions thereby resulting in the eventual release of toxins
55 into the aqueous phase (de Figueiredo et al., 2004; Fan et al., 2014), which may even exacerbate
56 the condition (Hawkins et al., 1985). Furthermore, these approaches may not always be
57 effective as the toxins from the aqueous phase, for instance, cannot be removed by these
58 methods (de Figueiredo et al., 2004). They may not also be helpful for protecting the public
59 from the potential risk associated with ingesting food animals like fishes caught from lakes
60 supporting cyanobacterial blooms (Ibelings and Chorus, 2007). This situation makes the
61 potential public health risk associated with cyanobacteria and their toxins a complicated issue to
62 address. As it is the case with water bodies found in other parts of the world, the dominance of
63 potentially toxic cyanobacteria has been reported for many lakes of the Ethiopian Rift Valley
64 (Kebede and Willén, 1998; Mesfin et al., 1988; Willén et al., 2011). As most of these water
65 bodies are currently serving as sources of freshwater used for drinking water supply, watering

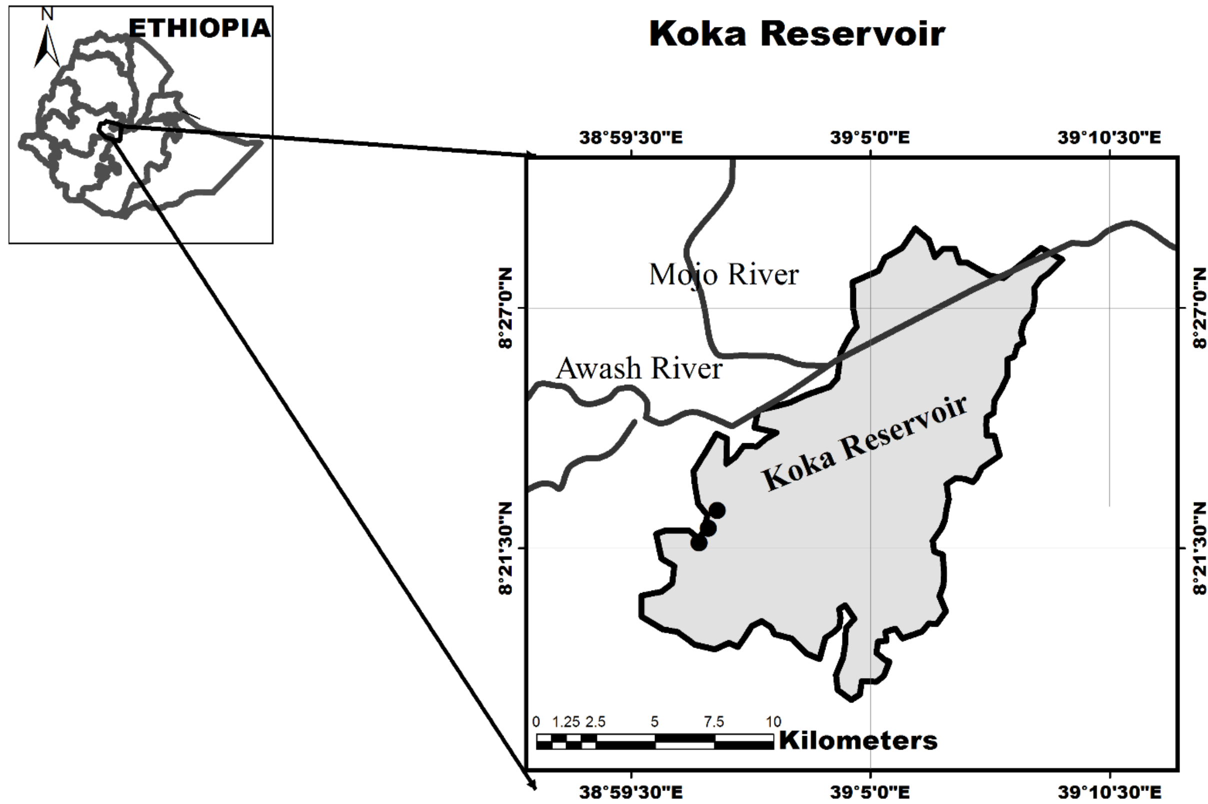
66 livestock, irrigation, fisheries, sanitation and recreation, the potential public health hazard and
67 economic loss is immense. For instance, incidence of massive fish kills in Lake Chamo, one of
68 the rift valley lakes in Ethiopia, and deaths of terrestrial wild animals around this lake in the year
69 1978 were linked to cyanotoxins (Amha and Wood, 1982). A cross sectional survey carried out
70 on seven of the Ethiopian rift valley lakes has also demonstrated the dominance of toxic
71 cyanobacteria and presence of associated toxins at concentrations much higher than the
72 permissible level in most of the water bodies (Willén et al., 2011). This suggests the potential
73 public health hazard associated with the use of these water bodies. According to Willén et al.
74 (2011), Koka Reservoir, the focus of the present study, was reported to be in the worst situation,
75 with *Microcystis aeruginosa,* overwhelmingly dominating the cyanobacterial assemblage and
76 with the level of total microcystins (MCs, 45–54 μg L−1) greatly surpassing the permissible
77 level (1 μg/l) set by WHO (WHO, 1998). Such reports from cross sectional study, may provide
78 an alarming signal inviting a detailed and more comprehensive study on the occurrence of cyano-
79 toxins. Willén et al. (2011) also suggested the need for long-term sampling at different times of
80 the year and identification of the major cyanobacterial genera related to toxin production. This
81 will also help understand the link between the recurrence of cyanobacterial blooms and
82 environmental variables to develop appropriate intervention strategies to mitigate blooms and
83 their toxins. Protection of public health through establishing an early warning system also calls
84 for such comprehensive study.

85 The present study, therefore, aimed at investigating temporal changes in *Microcystis* and the
86 concentrations of microcystin in both water and algal seston through a long–term sampling
87 program covering all the seasons. The study also addresses the physico-chemical parameters
88 associated with the dynamics of *Microcystis* bloom and their toxins.

89 ## 2. Material and methods

90 ### 2.1.Study area

91 Koka Reservoir (Fig. 1), also known as Lake Galilea, is located downstream of the upper Awash
92 catchment some 100 km south east of Addis Ababa at an altitude of 1590 m a.s.l. and at a
93 geographical position of 8°23'38.4"N and 39°04'51.6"E. The reservoir was initially constructed
94 across Awash River for the purpose of hydroelectric power generation and flood control
95 (Halcrow and Pattern, 1989). However, it is also currently serving as a source of fresh water
96 used for drinking water supply for thousands of local inhabitants, watering livestock, fisheries,
97 and irrigation. The reservoir's life has been threatened by huge siltation originating from the
98 highly degraded upper Awash catchment (Shahin, 1993). Expectedly, the huge sediment
99 transport is accompanied by a concomitant nutrient flux from this catchment, which is dominated
100 by agricultural land use that has been a major source of nutrient input to the reservoir. The huge
101 nutrient input to the reservoir might be responsible for inducing the development of
102 cyanobacterial blooms in the reservoir. The recurrent cyanobacterial blooms, almost exclusively
103 dominated by *Microcystis* species and manifested in blue- green surface scum, have been evident
104 over the last several years (Kebede and Willén, 1998; Mesfin et al., 1988; Willén et al., 2011) .
105 Toxicity of the *Microcystis* bloom has been implicated in human and cattle illness, which have
106 been reported by the local inhabitants since early 1980s (Willén et al., 2011).



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108 **Figure 1 Koka Reservoir, sampling sites and its surrounding.**

109 **2.2. Climatic conditions**

110 According to the National Meteorological Agency (NME) of Ethiopia, the reservoir area is
111 characterized by a bimodal rainfall pattern with a short minor rainy season (March-May), and a
112 long major rainy season (June to September), with a mean annual precipitation of 1012 mm
113 (Abebe, 2001). The mean maximum air temperature (ºC) was found to range from 28.9 to 33.9,
114 while the mean minimum air temperature varied between 9.1 and 15.5 (Halcrow and Pattern,
115 1989).

116

117

118 **Sampling protocol and analytical methods**

119 Thousands of the local inhabitants use the reservoir as a source of fresh water supply and make
120 their living through subsistence commercial fisheries. Sites along near-shore lines were
121 considered as high risk areas as these sites are points where local people fetch water for
122 household purposes **(Fig 1)**, wash clothes, play, swim and provide water to the livestock **(Fig. 2).**
123 Sampling was carried during seven months during the different seasons: rainy season (June-
124 September), long dry season (October-February), and short rainy season (March-May) at three
125 points along the shore. Accordingly, samples were collected during the months of May, August,
126 October, February, March, and April. The reservoir is highly turbulent exhibiting frequent
127 vertical and horizontal mixing. Samples were therefore, collected from the surface using clean
128 plastic containers following the recommendations of (APHA, 1999). Samples collected from the
129 three sampling points were mixed in equal proportion to obtain composite samples representing
130 the designated high-risk area (**Figs 1 and 2**). Samples used for microscopic identification of
131 major cyanobacterial genera and determinations of their respective cell abundance were fixed
132 with Lugol's iodine (0.01% v/v). All samples including those used for laboratory analysis of
133 other parameters were transported in iceboxes and processed and analyzed within a few hours of
134 collection.

135

136 Figure.2 Livestock drinking water at the near-shore site of the reservoir (top right), Fetching
137 water for house hold purposes (top left); bloom of *Microcystis*; patches and remnants of
138 *Microcystis* scum at the near-shore site after the collapse of the bloom (bottom left).

139 Photos by Samson Tilahun.

140 ***On site*** **measurements of physico-chemical parameters**

141 Physicochemical parameters such as Dissolved oxygen (DO), pH, water temperature (Temp),
142 salinity (Sal) and turbidity (Turb.) were measured on site using field meters. Temperature and
143 dissolved oxygen were measured at the same time using a portable digital oxygen meter (model
144 DO300, EUTECH instruments). Portable digital pH meter (model HI 9024, HANNA
145 instruments) was used for measuring pH. Salinity was measured with cond/TDS/sal/Res meter
146 (model SX713). Turbidity (NTU) was measured using a turbidity meter (model HI 93 703-11).
147 For samples with high turbidity exceeding the measurement range of the instrument; dilution was

148 carried out with turbidity free water to bring them to the measurement range of the instrument.
149 The actual turbidity of the original samples was then calculated as per the instruction manual of
150 the turbidity meter.

151 2.3.2. **Analysis of inorganic nutrients**

152 Spectrophotometric analysis of inorganic nutrients was carried out following standard procedures
153 outlined in (APHA, 1999; (Wetzel and Likens, 2000). Samples for inorganic nutrients analysis
154 were filtered on GF/F immediately upon arrival at the laboratory prior to analysis. Sodium
155 salicylate method was used for analysis of nitrate (NO_3-N). Soluble reactive phosphate-
156 phosphorus (SRP) was analyzed using the Ascorbic acid method (APHA, 1999). The Phenate
157 method was used for the analysis of ammonia ($NH_3 + NH_4^+$-N) (Wetzel and Likens, 2000).

158 **2.2.1. Identification and counting of major cyanobacterial taxa**

159 Lugol`s fixed sample (100ml) was carefully mixed to homogenize it and immediately transferred
160 to a 100 ml measuring cylinder, which served as a sedimentation chamber. The preparation was
161 then kept in the dark for 24 hours for sedimentation. The supernatant was carefully siphoned off
162 until the last 10 ml remains. Homogenization of the 10 ml sedimented sample was carried out by
163 carefully mixing the sample. Identification and enumeration of major cyanobacterial taxa was
164 carried out using a 1 mL Sedgewick-Rafter counter chamber under an inverted microscope, after
165 allowing cells to settle for 30 minutes. Literatures including (Cronberg and Komárek, 2004;
166 Komárek and Anagnostidis, 2005; Komárek and Kling, 1991) were used for identification to the
167 genus/species level. Cell abundance of the major cyanobacterial genera was carried out randomly
168 in 40-50 grids, following the equation of Hötzel and Croome (1999):.

169 $$Cell\ mL^{-1} = \frac{N * 1000mm^3}{A * D * F}$$

170 Where:

171 N is the number of cell or units counted

172 A is area of field (area of each grid, 1 mm^2)

173 D: depth of Sedgwick-Rafter chamber (1 mm)

174 2.3. Analysis of microcystien

175 Analysis of microcystins in water and algal seston was carried out following the standard
176 procedures outlined below.

177 2.3.1. Separation of intra-and extra-cellular microcystin

178 Lake water was filtered onto GF/F (pore size 0.7μm) using a vacuum pump under mild pressure
179 to avoid cell breakage and separate the intracellular cyanotoxins from the extra-cellular toxin as
180 described in (Park et al., 1998). The intracellular toxins were extracted from the cyanobacterial
181 cells retained on the GF/F filter papers (Whatman, UK). The filter papers with algal seston (for
182 cell-bound toxins) as well as particle bound toxin were dried at 50 °**C** overnight and immediately
183 transferred to -20 °**C** for long-term storage until analysis. Similarly, the filtrate (the aqueous
184 phase) was kept frozen at -20 °**C** until analysis for extra-cellular toxins.

185 2.3.2. Extractions and Analyses of Microcystins

186 All reagents used were of high-performance liquid chromatographic grade. Methanol was
187 obtained from sigma Aldrich. Solid phase extraction (SPE) system used for concentration and
188 cleanup of the water sample was purchased from Waters Corporation (USA). Microcystin-LR

189 (5µg/L), -RR (5µg/L), and YR (5µg/L) standards were purchased from Sigma Aldrich
190 (Germany).

191 **2.3.3. Sample preparation**

192 **2.3.3.1. Water sample**

193 Methanol of volume 0.5 ml was added to 50 ml water sample which is previously filtered by 0.7
194 µm pore size filter paper and then the mixture was sonicated for 10 minutes for degassing. The
195 extraction (SPE) column was conditioned by rinsing with 10 mL methanol followed by 10 ml
196 deionized water. The water sample was introduced into and passed through the conditioned SPE
197 column under vacuum at a flow rate of 1 drop/sec. After being rinsed with 10 ml of 5%
198 methanol, the column was dried for 20 min. The target analytes were eluted with 10 ml of 50%
199 methanol. The elute was then dried up by a nitrogen stream and then re-dissolved by 300 µl of 50
200 % methanol and analyzed by liquid chromatography electro spray ionization high resonance
201 mass spectrometry (LC-ESI-HRMS) method.

202 **2.3.3.2.Microcystins in algal seston and on particulate matter**

203 The extraction method was adapted from method by Lawton (Lawton et al., 1994) with a slight
204 modification as we have followed for a study on a different lake (Zewde et al., 2018a). Prior to
205 extraction, the filter paper, which contains the seston, was placed in a suitable container and
206 freeze-thawed. Filter papers were placed in glass beakers containing 20 ml of methanol and
207 allowed to extract for 1 h at room temperature. The liquor was then decanted into a pear-shaped
208 rotary evaporation flask (50 ml) and the filter was gently squeezed with a spatula to ensure
209 maximum transfer of the liquid. The extraction procedure was repeated twice. The sample was
210 rotary evaporated at 40ºC *in vacuo* until dry. Then, the liquor from the second, and subsequently

211 the third, extraction was added to the flask and dried as before. The residue was re-suspended in
212 500 µl of 50% aqueous methanol prior to analysis by the same LC-ESI-HRMS as the water
213 samples.

214 **2.3.3.3.LC-ESI-HRMS method**

215 For the separation of toxins and background, 10.0 µl of each sample and standard was injected
216 with an Accela auto sampler (Thermo Fisher Scientific) in no waste mode into a HPLC column
217 Supelco Ascentis Express C18 , 5 cm x 2.1 mm, 2,7µm (Sigma-Aldrich/Merck). The column was
218 maintained at 30 °C. Two solvents were used as mobile phase, A: acetonitrile with 0.1% formic
219 acid LC-MS chromasolv (Fluka) and B: Milli-Q water from a Simplicity system (Millipore) with
220 0.1% formic acid for LC/MS (Fluka). The solvents were pumped through the column using an
221 Accela pump (Thermo Fisher Scientific). The column was equilibrated with 80% A and 20% B
222 at 500 µl/min before the first injection and eluted with the following procedure: 20% B for 30
223 seconds, then a linear gradient up to 60% B over 6 minutes 30 seconds, and finally 95% B for 1
224 minute. The column was then re-equilibrated at 20% B for 2 minutes. The flow rate was 500
225 µl/min for all steps. The eluted components from the column were detected using electro spray
226 ionization and high-resolution MS on a Thermo Orbi trap XL (Thermo Fisher Scientific). The
227 electro spray setting was as follows: sheath gas flow rate: 70, aux gas flow rate: 10, sweep gas
228 flow rate: 10, spray voltage: 4.5 kV, capillary temp: 330 °C, capillary voltage: 37V and tube
229 lens: 80V. Detection parameters for the MS-analyzer was as follows: resolution: 30000 scan
230 type: full, polarity: positive, mass range: 330-1200, micro scans 1 and max. Inject time: 250ms.
231 Lock mass was enabled for correction of background ions from di butyl phthalate (m/z
232 279.159086), di-isooctyl phthalate (m/z 391.284286 , m/z 413.266231 and 803.543240) and
233 irganox (m/z 553.459115).(Audrey Roy-Lachapelle et al., 2015; Semyalo et al., 2011) Ion

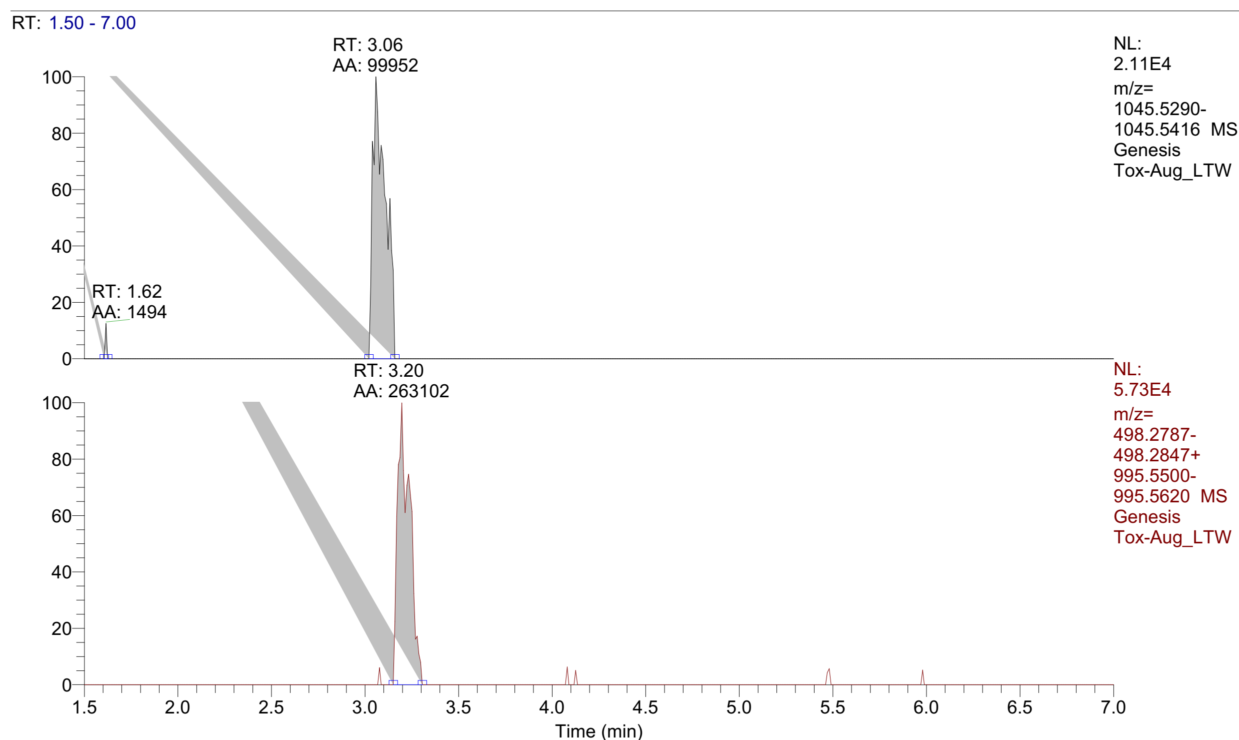
234 chromatograms where extracted for the analytes at the following masses: MC-YR (m/z
235 523.1817, m/z 1045.5353), MC-LR (m/z 498.2817, m/z 995.5560), MC-RR (m/z 519.7902, m/z
236 1038.5731) using Thermo X calibur 2.1. The mass tolerance was set to 6 ppm. A calibration
237 curve was constructed using standard concentrations produced by diluting the standards (Sigma-
238 Aldrich) in Milli-Q water. The standard concentrations were as follows (µg L^{-1}): 0.5, 1, 2, 5, 10,
239 25 and 50. The limit of detection (LoD) and limit of quantitation (LoQ) where determined based
240 on signal to noise of 3 and 9, respectively. LoD for all components was determined to be 0.5 µg
241 $L^{-1}$and LoQ to be 2 µg L^{-1}. Standards below 2 µg L^{-1} were excluded from the calibration curve.
242 Calibration curves and regression coefficients were as follows: MC-YR: Y = -
243 20995.2+35179.2X , R^2 = 0.9981, MC-LR: Y = -33075.4+73869.2X, R^2 = 0.9991 and MC-RR:
244 Y = -115052+139433X, R^2 = 0.9938. Quantitation of the unknown samples was done using
245 these calibration curves in Thermo X calibur Quan browser. Note that we have previously
246 applied these procedures for measuring concentrations of microcystins in fish species collected
247 from the same reservoir (Zewde et al., 2018b) as well as extra- and intracellular MCs
248 concentrations in samples collected from Lake Hora-Arsedi, Ethiopia (Zewde et al., 2018a).

249 **2.4. Statistical analysis**

250 Several of the variables including *Microcystis* cell abundance , major MC variants and some
251 environmental parameters have sets of data, which are highly skewed and hence fail to conform
252 to the assumption of normality (Shapiro-Wilk, $p<0.05$). Therefore, non-parametric statistics,
253 Spearman Rank Order Correlation, was used to determine the possible associations among the
254 variables. Test result was considered significant at $p<0.05$. SPSS ver. 20 was used for all
255 statistical analyses. Graphs were plotted with Sigma ver. 10.

256 **3. Results and Discussion**

257 All major MC variants, MC-LR, MC-YR, and MC-RR, were detected in algal seston samples 258 during all months except August (**Figs. 3 and 4**). The concentrations of these toxins were higher 259 than the permissible level established for drinking water (1 μg L^{-1}) on the majority of the 260 samples (**Fig.5**).



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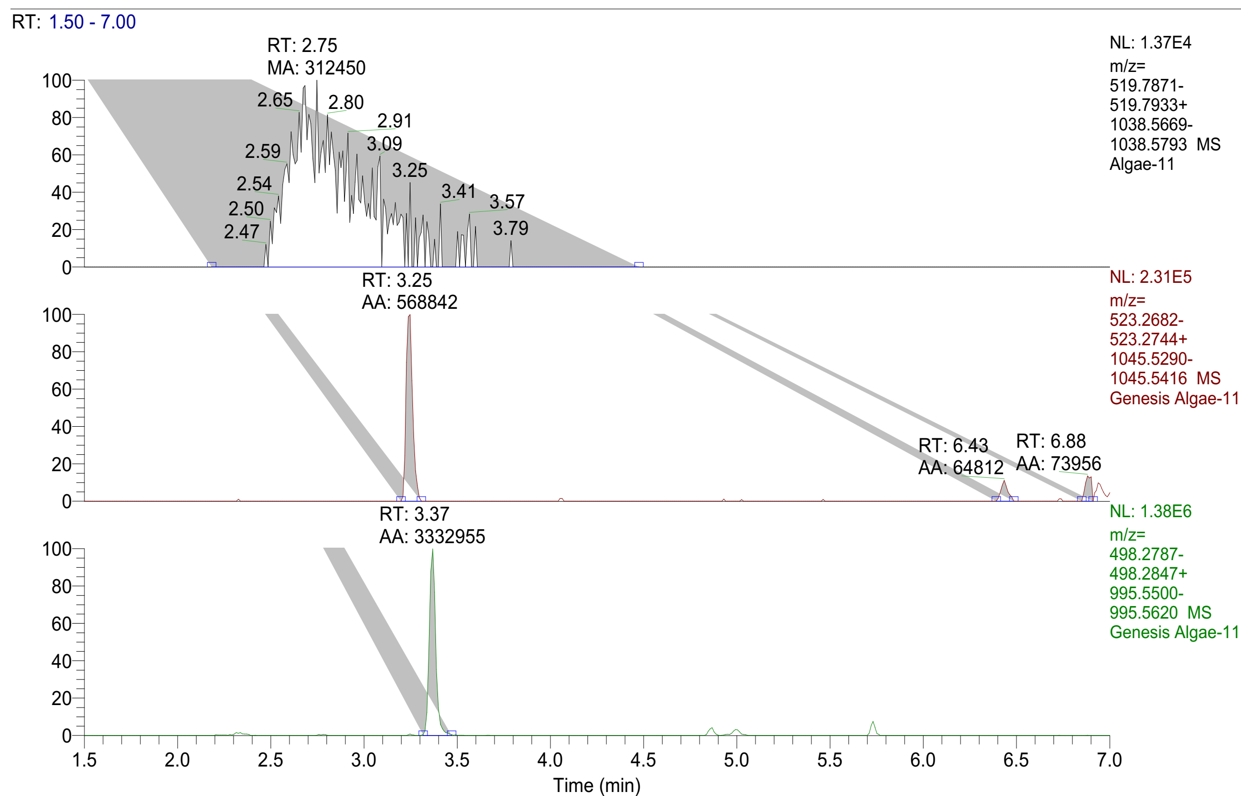
262 Figure 3. Chromatograms showing the presence of MC-YR (top) and -LR (bottom) in water 263 samples collected during August (2015) from the Koka reservoir.



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265 Figure 4: Chromatograms showing the presence of MC-RR (top), -YR (middle), and -LR
266 (bottom) in algal samples collected during March (2016) from the Koka reservoir

267

268 Exceptionally high concentrations (µg L^{-1}) of all the variants, MC-LR (815) MC-YR (466.6) and
269 MC-RR (265 .68), were observed in May **(Fig 5),** which were respectively about 815, 467, and
270 266 times the permissible level. These concentrations also exceed the maximum permissible
271 level set for water bodies intended for recreational purposes (20 µg L^{-1}) (WHO, 1998). A
272 decrease in their concentrations occurred in October, December, February, and April before their
273 increase in March was observed.

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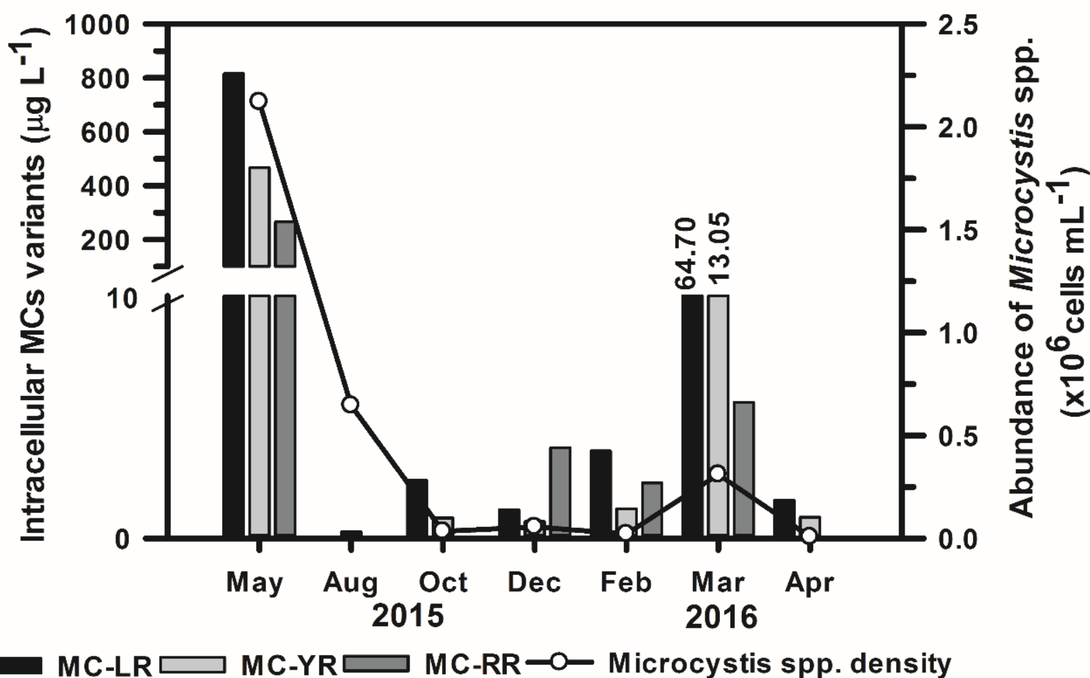
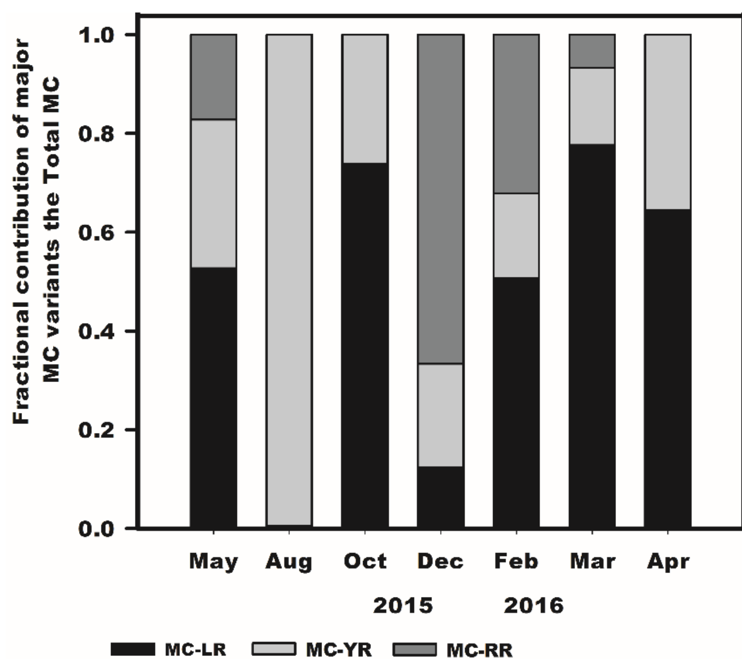


279 Figure 5. Temporal variations in the concentrations of major microcystin variants in relation to
280 the total abundance of *Microcystis* spp. at a near-shore site on Koka reservoir . Figures at the top
281 of the bars refer to the maximum levels measured for the corresponding variants

282 However, their concentrations were still higher than the WHO's safety limit of (1 µg L^{-1}) and
283 those reported for Lake Chivro in Zimbabwe (0-2.855 µg L^{-1}; (Mwaura et al., 2004). Among the
284 major MC variants, MC-LR constituted the largest proportion of the total MC concentrations in
285 all sampling months except December, when the concentration of MC-RR was the highest, while
286 in August sample, only MC-YR was detected (**Fig. 6).** Willén et al. (2011) also reported MC-LR
287 as the variant constituting the greatest proportion of total MCs in the reservoir. Such incidence of
288 occurrence of high levels of MC-LR was also reported for several other water bodies (Kotak and
289 Zurawell, 2007). Among the different MC variants, MC-LR is known to be the most potent
290 toxin, the exposure to this toxin may lead to severe liver damage (Chorus et al., 2000), massive

291 intra-hepatic hemorrhage, liver swelling and death (Weng et al., 2007), genotoxicity and
292 carcinogenicity (Dittmann and Wiegand, 2006). The high levels of MC-LR detected in the
293 majority of the samples collected from Koka Reservoir in this study, therefore, suggest
294 extremely high potential public health risk associated with the use of the reservoir as a source of
295 fresh water supply.



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297 Figure 6 Temporal variations in the relative contributions of the major MC-variants to the total
298 intra-cellular MC (May 2015- April 2016)

299 Microcystins are normally cell-bound toxins, which are released to the extra-cellular
300 environment during cell lyses (Li et al., 2010; Tsuji et al., 2001) and this contention corroborates
301 the findings of the present study in Koka Reservoir. The extra-cellular toxins were relatively less
302 concentrated and less frequently detected in samples obtained from Koka Reservoir in this study
303 compared to the cell-bound ones (**Figs 5 &7**). It should be noted, however, that the fractionation
304 procedure for intra-cellular and extra-cellular MCs does not allow us to accurately determine the
305 true proportion of the intra/extra cellular MC fractions. MCs that are bound to algal surfaces and

306 other particulate matter (with particle size larger than 0.7 μm) are retained by the filter paper and
307 are not, therefore, included in the measurement of MCs in the filtrate. These particle bound MCs
308 are actually part of the extra-cellular MCs. In this way, the procedure may overestimate the intra-
309 cellular MCs and underestimate the extra-cellular MCs.

310 Table 1. Temporal changes in the relative contribution of the total intra-cellular and extra-
311 cellular MCs in Koka reservoir during (May-2015-April 2016).
312

Month	Total MCs (in algal seston)	Total MCs(in water)	Ratio (Total MCs (water):Total MCs(in algal seston)
May	1548.13825		
Aug	0.265	11.456	43.23018868
Oct	3.2215	47.057	14.60717057
Feb	7.1075	BD*	BD*
Mar	83.3405	BD*	BD*
Apr	2.4445	1.934	0.791163837

313 *BD = Below Detection, Limit of detection (LOD) for all variants was 0.5μg/l

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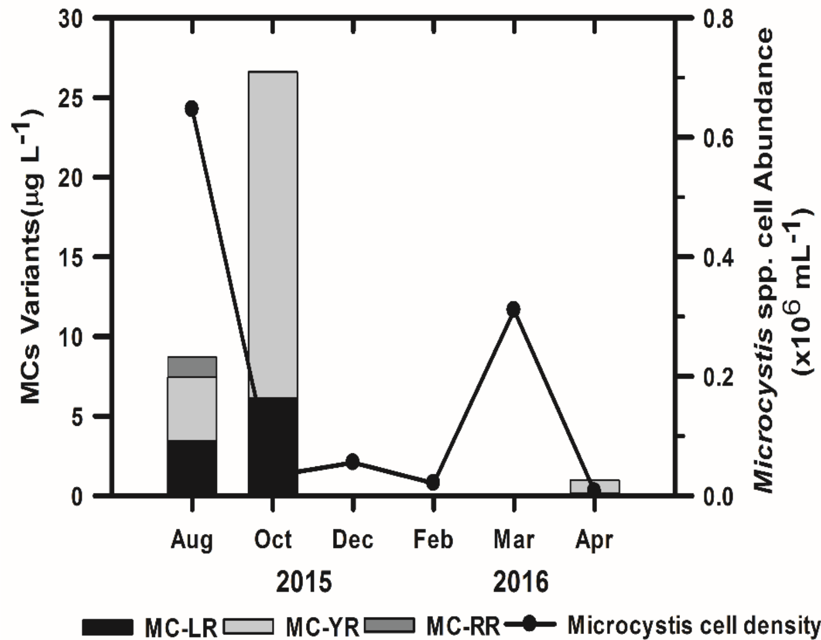


316 Figure 7 Temporal variations in the concentrations of extracellular microcystin (MCs) variants
317 in relation to cell densities of *Microcystis* spp. at near-shore site of the present study on Koka
318 reservoir during Aug. 2015-April 2016.

319 The relatively high concentrations of these extra-cellular MCs observed in August and October
320 could, therefore, be partly attributed to the most likely release of MCs due to cell lysis (Park et
321 al., 1998; Tsuji et al., 1994) following the collapse of the bloom after May, 2015, which
322 corresponded to lower *Microcystis* cell abundance during these months. The release of
323 cyanotoxins to the extra-cellular environment due to cell lysis during the collapse of a bloom has
324 also been reported previously for other freshwaters (Fromme et al., 2000; Park et al., 1998). The
325 strong, significant and positive correlation (**Table 2**) among the extra-cellular MC variants and
326 total microcystins (MC-LR, MC-YR, and Total MC) may also suggest the involvement of a
327 common and non-specific release mechanism, which could possibly be cell lyses, a condition by
328 which all cellular contents are released at the same time. Concentrations (μg L^{-1}) of up to 20 of
329 MC-LR, 6.13 of MC-YR, and 1.27 of MC-RR **(Fig.7)** were found in the water samples, with all
330 values greatly surpassing the WHO's (1996) safety limit for drinking water supply. The extra-

331 cellular fraction of total MC was >43 and >14 times the total intracellular MC concentration

332 measured in August and October, respectively **(Table 1).**

333 **Table-2 Spearman Rank Order Correlation among extra-cellular MC variants, Total**

334 **MC and *Microcystis* cell abundance**

335

	Microcystis	YR water	LR water	Total MC
Microcystis	1			
YR water	0.112	1		
LR water	0.030	**0.919***	1	
Total MC	0.030	**0.963****	**0.991****	1

336

337 ***Correlation significant at the 0.05 level (2-tailed),**Correlation significant at the 0.001 level**
338 **(2-tailed)**

339 Re-dissolution of the loosely bound MCs and their re-distribution during mixing has also been

340 reported as a possible mechanism for their high concentrations in the water column long after the

341 bloom had collapsed (Song et al., 2015). This is also possible in Koka Reservoir, which is a

342 shallow and frequently mixed water body (Kebede and Willén, 1998; Mesfin et al., 1988; Willén

343 et al., 2011). According to (Song et al., 2015; Verspagen et al., 2005), the sediment may serve

344 as a source for *Microcystis* and MCs and determine their fate (Wörmer et al., 2011; Wu et al.,

345 2012). Persistent *Microcystis* cells in the sediment, for instance, have been reported to serve as

346 inoculums for the next blooming season (Verspagen et al., 2005). Sediment of Koka reservoir

347 might have also partly contributed to the total MC concentration in the water samples as

348 sediment re-suspension due to frequent mixing is a common phenomenon in the reservoir. Re-

349 suspension of bottom sediment in Koka Reservoir in the present study was reflected by the high

350 values of turbidity (**Table-3**). The sediment may also serve as a sink for the extra-cellular MCs

351 as they tend to get adsorbed onto particulate materials (Song et al., 2015; Verspagen et al., 2005).

352 As suggested earlier, the sediment bound MCs cannot be recovered in the filtrate following the

353 procedure used in this study. This could partly explain the relatively low concentration of extra-
354 cellular MCs and their less frequent occurrence in the water samples of the present study (**Fig 7**)
355 compared the cell bound ones. However, provision of definitive conclusion on the role of the
356 sediment in the dynamics of *Microcystis* and MCs in the water/sediment interface of Koka
357 Reservoir requires further study involving sediment sampling. Similar to the intracellular MCs,
358 the total extra-cellular MC was constituted largely by MC-LR followed by MC-YR. MC-RR
359 was detected in the filtrates of August sample only, constituting only a small fraction of the total
360 extra-cellular toxins. The total intracellular MC concentration was higher than the concentration
361 of total extra-cellular MC during all sampling months except August and October. The highest
362 total MC concentration 83 (μg L^{-1}) was observed in March sample constituting MC- LR 64.7 (μg
363 L^{-1}), MC- RR 5.6 (μg L^{-1})) and MC-YR 13.03, (μg L^{-1}) next to that of May sample. It coincided
364 with the highest *Microcystis* abundance possibly favored by the relatively better availability of
365 nitrogen due to the rainfall event that occurred in March. It is possible that the toxins were
366 associated with the new *Microcystis* cells formed during the warmer months though at
367 expectedly low rate of growth associated with nitrogen-limitation compared to the diazotrophic
368 cyanobacteria. All extra-cellular MC variants were below the detection limit of the analytical
369 method used (**Table-1**) in December, February and March (**Fig. 7**). Photolysis and
370 biodegradation of the extra-cellular toxins (Tsuji et al., 1994), owing to the intense solar
371 radiation and high water temperature prevailing in semi-arid tropical region where Koka
372 Reservoir is located, might have also been partly responsible for the presence of low and
373 undetectable levels of extra-cellular toxins during the majority of the months. Several field-based
374 and experimental studies (Ballot et al., 2003; Davis et al., 2009; Jähnichen et al., 2011; Watanabe
375 and Oishi, 1985) have been carried out to study the environmental factors dictating the dynamics

376 of cyanobacterial bloom and toxin production. Water temperature, pH, nutrient availability,
377 and transparency are among the several environmental factors regulating the spatio-temporal
378 dynamics of cyanobacteria and their toxins. The majority of the limmnological parameters
379 measured in Koka reservoir during the present study period were within the ranges reported to be
380 optimal for the proliferation of *Microcystis* and toxin production. For instance, temperature
381 exceeding 20 °c generally promotes the proliferation of cyanobacteria due to its direct effect on
382 their metabolic activity and changes in the physical characteristics of the aquatic environment
383 (O'Neil et al., 2012). According to Watanabe and Oishi (1985), growth of *Microcystis*
384 *aeruginosa* increased considerably at a temperature of 32 °C under culture conditions. The water
385 temperature of Koka reservoir during the study period ranged from 22.4 to 31.1°C **(Table-3),** a
386 range of water temperature that is within the reported optimal range for *Microcystis* growth. The
387 relatively higher water temperature of the present study period could be due to the higher air
388 temperature associated with the drought.

389 Table 3. Mean, standard error, minimum and maximum monthly values of temperature, turbidity,
390 SRP (Soluble Reactive Phosphate), nitrate recorded for Koka reservoir (May 2015-April 2016)
391

Parameters	Range of values	Mean ±Std. Error
Temp. (ºC)	22.40 (Aug)-31.1 (Mar)	25.691±0.851
Turbidity (NTU)	48.29 (Jul)-2970 (Mar)	1099.763±287.311
SRP (μg L^{-1})	29.10 (Jun)-69.8 (Jan)	27.1±3.55
Nitrate (μg L^{-1})	9.69 (Apr) -250 (May)	51.4±20.6

392

393 The direct role of temperature in MC production was also reported by (Davis et al., 2009).
394 According to Davis et al. (2009), two- to threefold increase in MC levels with the increase in
395 temperature from 26.9± 1.7 to 30.6 ±1.4 was observed. The other limnological condition, which
396 is believed to favor *Microcystis* growth and MC production, is a long water residence time. Long

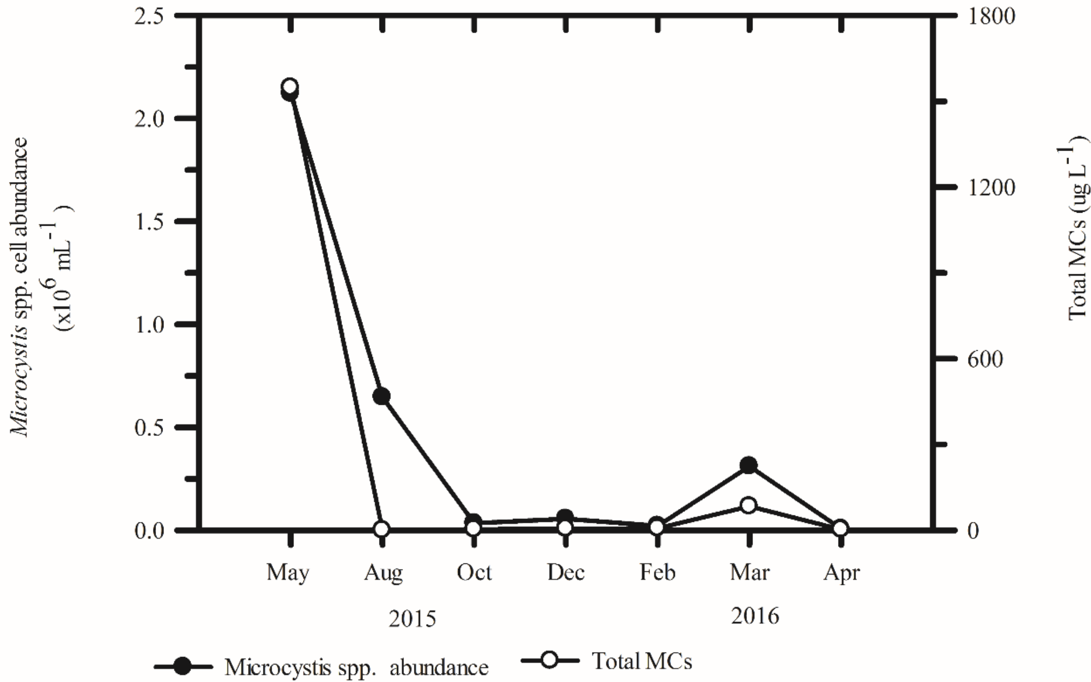
397 water residence time was reported to favor both the proliferation of *Microcystis* and MC
398 production. Extended dry condition generally increases water residence time by up to 45 %
399 (Romo et al., 2013), which was particularly true for Koka Reservoir of the present period. The
400 estimated mean water residence time of the reservoir during our sampling period was 395±24.5
401 days, which was 35% longer than that of the preceding year (258± 16.1) (Tilahun and Kifle in
402 review process). According to Romo et al. (2013), as a result of the increase in water residence
403 time due to drought, the size of *Microcystis aeruginosa* population and MCs concentration
404 increased more than twice. The results of the present study, however, show to the contrary that
405 *Microcystis* abundance was much lower than those of earlier reports (Kebede and Willén, 1996;
406 Willén et al., 2011) despite the longer water residence time of the present study period.

407 **Table 4: Spearman Rank Order correlations among *Microcystis* cell abundance, major**
408 **intracellular MC-variants, Total MC and some environmental variables.**

	Temp	**SRP**	**Nitrate**	***Microcys* cell abundance**	**MC-YR**	**MC-LR**	**MC-RR**	**Total MC**	**Sal**
Temp	1.000								
SRP	**.857***	1.000							
Nitrate	-.090	.090	1.000						
***Microcys* cell abundance**	-.143	.214	**.793***	1.000					
MC-YR	.429	.143	.270	.143	1.000				
MC-LR	.321	.036	.306	.214	**.964****	1.000			
MC-RR	.408	.445	.243	.556	.704	.704	1.000		
Total MC	.357	.214	.162	.357	**.857***	**.893****	**.927****	1.000	
Sal	**.829***	**.811***	-.291	0.000	.342	.252	.654	.523	1.000

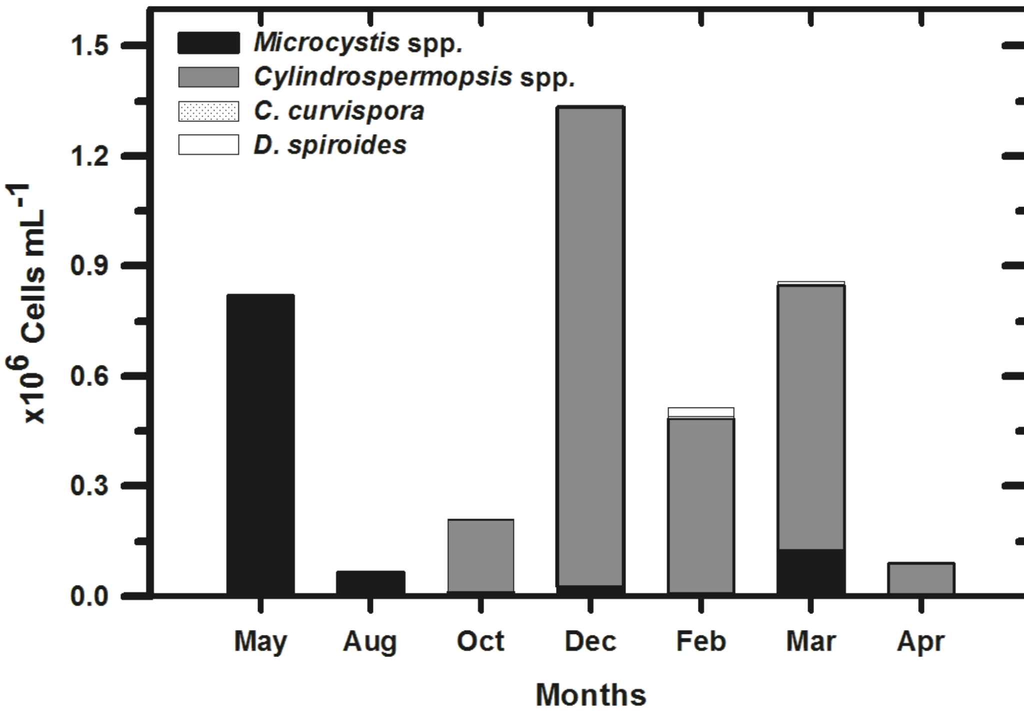
409 **Correlation significant at the 0.05 level (2-tailed), **Correlation significant at the 0.001 level*
410 *(2-tailed)*

411



412 Figure 8 Temporal variations in total intra-cellular microcystins (MCs) concentrations in relation
413 to total density of *Microcystis* spp. at near-shore site of the present study on Koka reservoir.

414



415 Figure 9 Temporal variations in the relative abundance of cyanobacterial taxa at the near-shore
416 sites of the present study on Koka reservoir.

417 The majority of the observed limnological conditions are within the optimal range reported for
418 the proliferation of *Microcystis* spp except for the low availability of combined inorganic
419 nitrogen. The unusual disappearance of dense *Microcystis* bloom and *Microcystis* spp.
420 occurrence at relatively low abundance during the present study may be associated with severe
421 nitrogen limitation (Tilahun and Kifle, 2019). According to Tilahun and Kifle (2019), the
422 observed low level of nitrogen could most probably be associated with a reduction in riverine
423 input of nutrients due to the recent incidence of El Niño induced drought in Ethiopia. The
424 nutrient limitation, which was more pronounced in nitrogen than phosphorus. Tilahun and Kifle
425 (2019), suggested that, while the available nitrogen is lost through de nitrification process owing
426 to the high water temperatures associated with the drought, phosphorus was possibly being
427 replenished through internal recycling from the huge phosphorus reserve in the sediment. The
428 significant positive association of *Microcystis* cell abundance and nitrate concentration
429 (Spearman Rank Order Correlation r =0.793, $p<0.05$) (Table-4) may suggest that nitrogen might
430 have played a key role in *Microcystis* spp dynamics, which in turn influenced the MC level in
431 the reservoir in the present study. According to Wang et al. (2010), the effect of nitrogen on MC
432 production is through its influence on *Microcystis* cell abundance. Furthermore, there is no
433 evidence of association of intra-cellular MCs with *Microcystis* spp. cell abundance and other
434 environmental variables (**Table 4**), which may suggest that nitrogen limitation was of overriding
435 importance in influencing the proliferation of *Microcystis* spp. thereby possibly affecting toxin
436 production. This is possibly because, unlike diazotrophic cyanobacteria such as
437 *Cylindrospermopsis*, the proliferation of bloom forming non-diazotrophic cyanobacteria such as
438 *Microcystis* may be seriously impacted by severe nitrogen limitation. Members of this
439 ecological group do not have an alternative source of nitrogen to survive nitrogen stress that

440 prevails over a long period such as the one observed in Koka reservoir. The role of nitrogen in
441 influencing *Microcystis* growth and toxin production was reported by (Park et al., 1993).
442 According to their report, an increase in NO_3-N concentration favored the growth of *Microcystis*
443 in Lake Sow, Japan. This is in agreement with our observation that nitrogen concentration was
444 positively and significantly associated with nitrogen concentration (Spearman Rank Order
445 Correlation r =0.793*,* $p<0.05$) as indicated earlier (**Table -4**). According to a review by (O'Neil
446 et al., 2012), MC+ strains are not competent under low-nitrogen stress because of the additional
447 nitrogen requirement for the synthesis of microcystin, which is a nitrogen-rich compound, and
448 the enzymes involved in the synthesis of the MCs. This may influence the relative abundance of
449 toxic and nontoxic (MCY- and MCY+) strains of *Microcystis* thereby influencing MC
450 concentration (Davis et al., 2009). For instance, (Dai et al., 2008), reported substantial decrease
451 in cellular MCs concentration immediately following the depletion of nitrogen suggesting the
452 importance of nitrogen for the production of MCs. In the present study however, there is no
453 direct evidence for the association of intra-cellular MC with nitrogen (Spearman Rank Order
454 Correlation r= 0.162, $p>0.05$,) (**Table-4**). This could be primarily due to the inherent problem of
455 the procedure followed that does not allow accurate determination of the true intracellular MCs,
456 which may significantly affect the possible association MCs with nitrogen levels and other
457 environmental variables. Furthermore, positive correlation between *Microcystis* biomass and
458 total intracellular MCs concentration was reported by several authors including (Wang et al.,
459 2010). Although *Microcystis* cell abundance and total intracellular MC concentration followed
460 similar temporal pattern (**Fig. 8**), Spearman Rank Order Correlation analysis showed no evidence
461 of significant association (r=,0.357 $p>0.05$). Lack of significant association between
462 *Microcrystis* cell abundance and intra-and extra cellular MC in the present study (**Table 4 and**

463 **5**) is plausible and may substantiate our claim regarding the inherent problem associated with
464 determining the intra/and extra cellular MCs fractions using the conventional procedure. We,
465 therefore, suggest understanding the dynamics of intra-/extra cellular MCs in relation to
466 environmental factors requires an accurate and exclusive determination of the intra- and extra-
467 cellular MCs. Furthermore, *Dolichospermum,* a diazotrophic cyanobacterial genus, which is
468 capable of producing MCs, was also encountered in the present study although with relatively
469 lower abundance compared to *Cylindrospermopsis* (**Fig.9)**. Occurrence of *Dolichospermum* in
470 Koka reservoir and other Ethiopian rift valley lakes was also reported by (Willén et al., 2011)
471 and also elsewhere in Africa by Krienitz et al. (2002). It is a ubiquitous fresh water genus found
472 throughout the world particularly in lentic water bodies such as lakes and reservoirs (O'Neil et
473 al., 2012). The genus was reported to possess the MCY encoding genes (Rouhiainen et al.,
474 2004). A direct evidence for MC production by this genus was also reported in some water
475 bodies (Halinen et al., 2007). However*, Microcystis* spps was exclusively attributed to the
476 observed MCs in a reservoir where *Dolichospermum spiroides* and *Microcystis* spp. co-existed
477 (Li et al., 2010). Li et al. (2010) argued that MCY gene responsible for MC production was
478 detected in *Microcystis* spp. Only. The contribution of *D. spiroides* to the observed MCs levels
479 in Koka Reservoir in the present study cannot, however, be ruled out until molecular analysis is
480 carried out. Therefore, the co-occurring *Dolichospermum* might have been partly responsible for
481 the observed MC concentration of the present study.

482 **3.1.*Cylindrspermopsis* and Cylindrospermopsin**

483 As indicated above, *Cylindrospermopsis* was the most dominant cyanobacterial genus during the
484 latter months of sampling (November to April) (**Fig. 9**). As a diazotrophic cyanobacterial genus,
485 it could be favored by the prevailing severe nitrogen limitation during those months (Tilahun and

486 Kifle, 2019). Rukuer et al. (2007) also reported lack of correlation between levels of
487 cylindrospermopsin (CYN) and nitrogen availability, while a significant correlation between
488 total phosphorus and CYN was observed. This may suggest that for members of the diazotrophic
489 cyanobacteria, nitrogen is less likely to be limiting, while phosphorus plays a key role in
490 influencing their dynamics and toxin production. The unusual dominance of *Cylidrospermopsis*
491 in Koka reservoir reported by Tilahun and Kifle. (2019) corroborates our argument. In addition
492 to producing a potent hepatotoxin like microcystin, cylindrospermopsin produced by this genus
493 has been reported to cause damage to other organs and cells such as kidneys, and lymphoid
494 cells (Hawkins et al., 1985). Signals that could possibly be of cylindrospermopsin were observed
495 during the dry months corresponding to the dominance of *Cylindrospermopsis*. This, however,
496 must be confirmed with further analysis. If future analysis confirms the presence of
497 cylindrospermopsin, this will obviously, further exacerbate the already high public health risk
498 associated with the MCs produced by *Microcystis*.

499 **Conclusions**

500 The study reveals extremely high potential public health risk associated with the use of Koka
501 Reservoir as a source of water intended for drinking water supply. The reservoir is also unsafe
502 also for recreational purposes due to the high levels of MCs, and possibly CYN, found in both
503 water and algal seston considering the abundance of *Cylindrospermopsis* spp. The fact that
504 several of the algal samples and some of the water samples contained MCs above the permissible
505 level set by WHO, the water of Koka reservoir is unsafe for house hold purposes throughout the
506 year. The result of the study may suggest that nitrogen might be the key regulating factor for the
507 dynamics of *Microcystis* and possibly MCs production in Koka reservoir during the study period.
508 The high level of MCs despite the low abundance of *Microcystis* also implies that the MCs could

509 have been much higher had it not been due to nitrogen-depletion that limited the proliferation of
510 this genus. *Dolichospermum,* the co-occurring cyanobacterium, might have also been responsible
511 for the observed level of MCs although its confirmation requires further study.

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