1 Could the presence of larger fractions of non-cyanobacterial species be used as a predictor of
2 microcystin production under variable nutrient regimes?

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13

14 **Abstract**

15 The occurrence of cyanobacteria and microcystin is highly dynamic in natural environments and

16 poses one of the biggest challenges to water resources management. While a number of drivers

17 are known to be responsible for the occurrence of cyanobacterial blooms, the drivers of

18 microcystins production are not adequately known. This study aims to quantify the effects of the

19 changes in the structures of phytoplankton and cyanobacterial communities on the dynamics of

20 microcystin production under highly variable nutrient concentration. In our study, nutrient

21 variability could explain 64% of the variability in microcystin production. When changes in the

22 fractions of non-cyanobacteria versus cyanobacteria genera were additionally included, 80% of

23 the variability in microcystin production could be explained; under high nutrient concentrations,

24 non-cyanobacterial phytoplankton groups were dominant over cyanobacteria and cyanobacteria

25 produced more toxins. In contrast, changes in the cyanobacterial community structures could

26 only explain a further 4% of the dynamics of microcystin production. As such, the dominance of

27 non-cyanobacterial groups appears to be a useful factor to explain microcystin occurrence in

28 addition to traditionally used factors such as absolute cyanobacterial cell numbers, especially
29 when the nutrient regime is taken into account. This information could help to further refine the
30 risk assessment frameworks which are currently used to manage the risk posed by cyanobacterial
31 blooms.

32
33 **Keywords** Phytoplankton; cyanobacteria; microcystin production; allelopathic interaction;
34 nutrients; public health

36 **Introduction**

37 An adequate understanding on the dynamics of microcystin production will lead to a better
38 management of cyanobacterial blooms and reduce their impact on water resources. While the
39 occurrence of algal blooms is considered to be a significant threat to water safety and security around
40 the world, the presence of high level of cyanotoxins, such as microcystins, significantly increase the
41 risk to the environment and the public. As such, it is critical to understand the key drivers behind the
42 toxin production during a bloom situation.

43 The dynamics of microcystin production in a bloom depends on the toxin production on a cellular
44 level, caused by changes in environmental factors such as nutrients and light (Chorus and Bartram
45 1999, Van de Waal et al. 2009, Zurawell et al. 2005), and has been shown to be associated with the
46 changes in the planktonic communities' structure, in particular phytoplankton populations, including
47 cyanobacterial as well as non-cyanobacterial species (Holland and Kinnear 2013, Joung et al. 2011,
48 Pflugmacher 2002, Yang et al. 2014), and with changes in the ratio of toxic to non-toxic genotypes
49 (Janse et al. 2005, Kardinaal et al. 2007). To date, many causation factors leading to changes in the
50 community structure of phytoplankton populations and the development of cyanobacterial blooms
51 have been proposed and debated (Dadheech et al. 2014, Macías et al. 2008, O'Neil et al. 2012, Paerl
52 and Otten 2013).

Allelopathy has been suggested as a key mechanism in determining the community structure and the dynamics of phytoplankton populations (Holland and Kinnear 2013, Macías et al. 2008). The theory of allelopathy suggests that the ability of certain phytoplankton groups to dominate the community may be enhanced by the production of toxic or non-toxic compounds (Camacho 2008, Graneli and Hansen 2006, Li and Li 2011, O'Neil et al. 2012). This theory has been used in an attempt to explain the ecological role of microcystin production in cyanobacteria; however, this has not been extensively supported by experimental data (Babica et al. 2006, Berry et al. 2008, Jang et al. 2006). As such, cyanobacteria species could increase their competitive advantage through the production of microcystin (Huisman and Hulot 2005), especially under certain conditions of nutrient limitation (Graneli et al. 2008). Phytoplankton exposure to microcystin has been suggested to be associated with growth inhibition and deterioration of photosynthesis and enzymatic activities in some non-cyanobacteria species (Babica et al. 2006, O'Neil et al. 2012). Consequently, it can be hypothesized that the dynamics of microcystin production is related to the inter-species interaction between cyanobacteria and other phytoplankton species, interaction within cyanobacterial communities, as well as the direct effects of environmental factors on phytoplankton. While the concept of allelopathy is hard to prove *in situ*, as the effect of the chemical on the receiving organism is alleviated by the effects of other environmental factors, an increase in the production of toxins by cyanobacteria in the presence of a larger fraction of non-cyanobacterial genera might be an indirect indicator for allelopathy. Many studies have been focusing on the effect of nutrients on microcystin production (Chorus and Bartram 1999, Orihel et al. 2012, Zurawell et al. 2005); while a limited number of studies have also investigated the combined effects of phytoplankton community structure and cyanobacterial species composition on microcystin production (Engström-Öst et al. 2011, Kardinaal et al. 2007, Millie et al. 2009), none has studied this in relation to changes in nutrient levels. This information is essential and would enable managers to assess the potential toxicity of cyanobacterial blooms from the structure of the phytoplankton community and cyanobacterial composition under different environmental conditions. The present study aims to quantify the effects of the changes in the structure of

79 phytoplankton communities, including cyanobacterial species composition, on the dynamics of
80 microcystin production under highly variable nutrient regimes. It is anticipated that nutrient dynamics,
81 specifically phosphorus, nitrogen and iron, alone are not good predictors of cyanotoxin production and
82 that key changes in the phytoplankton communities, especially a dominance of non-toxin producing
83 classes, such as chlorophytes or diatoms, may be responsible for triggering an allopathetic response
84 leading to a higher toxin production.

85

86 **Materials and Methods**

87 *Study area*

88 This study was carried out in Jackadder Lake (31°54'30"S, 115°47'36"E), Bibra Lake (32°5'25"S,
89 115°49'16"E) and Yangebup Lake (32°6'56"S, 115°49'33"E) located on the Swan Coastal Plain,
90 Western Australia. These lakes are shallow with mean depth of 2.1 m, 1.1 m, and 2.5 m in Jackadder
91 Lake, Bibra Lake and Yangebup Lake, respectively. Jackadder Lake is surrounded by 6.6 ha of
92 parkland (Arnold 1990, Woodward 2008), while Bibra Lake is surrounded by urban areas and a golf
93 course. Yangebup Lake is surrounded by residential, agriculture and industrial areas. Jackadder Lake
94 and Yangebup Lake are permanent lakes, while Bibra Lake is subject to seasonal drying. Prolonged
95 stable thermal stratification is usually prevented in these lakes during summer due to continuous or
96 intermittent wind mixing that creates a homogeneous environment throughout the water column
97 (Arnold and Oldham 1997, Davis et al. 1993). These lakes were selected due to levels of
98 cyanobacterial biomass and microcystin concentration reported in earlier extensive sampling
99 (Reichwaldt et al. 2013, Sinang et al. 2013). These lakes represent systems with low, medium and high
100 cyanobacterial biomass and microcystin concentration. Mean cyanobacterial biomass in an earlier
101 study was reported as 28 $\mu\text{g chl-}a\text{ L}^{-1}$ in Jackadder Lake, 108 $\mu\text{g chl-}a\text{ L}^{-1}$ in Bibra Lake, and 80 μg
102 $\text{chl-}a\text{ L}^{-1}$ in Yangebup Lake. Mean cellular microcystin concentrations (mg g^{-1} cyanobacterial dry
103 mass) was 4.8 mg g^{-1} in Jackadder Lake, 35 mg g^{-1} in Bibra Lake and 1.7 mg g^{-1} in Yangebup Lake
104 (Sinang et al. 2013).

105

106 *Sampling*

107 The study lakes were sampled twice a month between January and March 2010. Samples were
108 collected from three points on each lake on every sampling occasion. Samples were taken from waist-
109 deep water at the same point on every sampling occasion. Bibra Lake dried up in late February;
110 therefore no samples were taken from this lake in March.

111 Grab water samples were taken from 15 cm below the surface and stored immediately in a cool
112 container. Parameters analysed from these samples were total phosphorus (TP), total dissolved
113 phosphorus (TDP), total iron (TFe), total dissolved iron (TDFe), total nitrogen (TN), total dissolved
114 nitrogen (TDN), ammonium (NH_4^+), cyanobacterial biomass, total phytoplankton biomass,
115 intracellular and extracellular microcystin fractions, and cellular microcystin concentration. Samples
116 for dissolved nutrients analyses were pre-filtered with a 0.45 μm syringe filter (Acrodisc, HT Tuffryn)
117 before storing at -20°C. Water samples for phytoplankton enumeration were preserved with Lugol's
118 iodine (1 % final concentration).

119

120 *Nutrient analyses and phytoplankton biomass*

121 TP and TDP concentrations were analyzed using the ascorbic acid method, while TFe and TDFe
122 concentrations were analyzed with the Phenanthroline method, according to standard methods (APHA
123 1998). TN, TDN, and NH_4^+ were analyzed at the South Coast Nutrients Analysis Laboratory (Albany,
124 Western Australia) with the standard colorimetric methods on a segmented flow auto-analyser
125 (Alpkem, Wilsonville, OR, USA). Cyanobacterial and total phytoplankton chlorophyll-*a* were
126 measured with a benchtop version of a FluoroProbe (bbe Moldaenke, Germany), which is capable of
127 identifying up to four spectral algal groups based on their specific excitation spectrum of chlorophyll
128 fluorescence (Beutler et al. 2002, Ghadouani and Smith 2005). The biomass of each group is given in
129 equivalent $\mu\text{g chl-}a \text{ L}^{-1}$. Fluoroprobe measurements of total chlorophyll-*a* gave a good correlation

130 against samples extracted according to standard methods (APHA 1998) (linear regression analysis: R^2
131 = 0.94, $N = 32$, $P < 0.05$) (Sinang, S.C., unpublished data).

132 Water samples were filtered through pre-combusted and pre-weighed 47mm GF/C filter papers to
133 collect the phytoplankton biomass and to separate intracellular from the dissolved microcystin fraction.
134 Filter papers containing particulate organic matter and phytoplankton biomass were dried at 60 °C and
135 re-weighed after 24 hours to obtain the dry biomass (Chorus and Bartram 1999). These filter papers
136 were then moistened with Milli-Q water and stored at -20°C until intracellular microcystin extraction.
137 As we were interested in the actual toxic potential of cyanobacteria, cyanobacterial dry mass was
138 calculated from the total dry mass by adjusting it to the percentage of cyanobacterial biomass
139 measured with the FluoroProbe.

140 Cyanobacteria were identified and enumerated to the taxonomic level of genera with 10-50 mL water
141 samples fixed in Lugol's solution using an inverse microscope according to Utermöhl (Utermöhl
142 1958). A minimum of 200 cells or colonies of the most abundant cyanobacteria were counted, and the
143 size of 20 individual cells or colonies of each occurring genera was measured by using an inverse
144 microscope. Cell densities were converted to biovolume by means of geometric figures that best
145 approximated the shape of individual taxa (Hillebrand et al. 1999). The biovolume of each
146 cyanobacterial genera was then used to calculate the biovolume fraction of the respective
147 cyanobacterial genera in the total cyanobacterial community.

148

149 *Microcystin extraction and quantification*

150 Filters were freeze-thawed twice to break the cells prior to methanol extraction (Lawton et al. 1994).
151 Each filter was placed into a separate centrifuge tube and 5mL 75 % methanol-water (v/v) was added.
152 Filters were sonicated on ice for 25 minutes, followed by gentle shaking for another 25 minutes. The
153 extracts were then centrifuged at 3750 rpm for 10 minutes at room temperature. Extracts were
154 carefully transferred into conical flasks, and two more extractions were done per filter. All three
155 extracts (75 % methanol) were pooled and diluted with Milli-Q to 20 % methanol. Intracellular

156 microcystin extracts and the pre-filtered water containing dissolved microcystin were subjected to
157 solid phase extraction (SPE) (Waters Oasis HLB) as a clean-up step and for concentrating the
158 microcystins. Sample loading speed to the SPE was adjusted to <10 mL per minute. After the samples
159 had passed through, each cartridge was rinsed with 10 mL 10, 20 and 30 % methanol-water (v/v),
160 before microcystin was eluted with 100 % methanol + 0.1 % trifluoroacetic acid (TFA) into 6mL glass
161 vials and samples were evaporated to dryness under a mild nitrogen stream in a 40°C water bath.
162 Finally, samples were re-dissolved in 30 % acetonitrile and analysed with HPLC-PDA systems
163 (Waters, Alliance 2695).
164 HPLC analysis was carried out by using the Alliance 2695 (Waters, Australia) with a PDA detector
165 (1.2nm resolution) and an Atlantis T3 3µm column (4.6 x 150mm i.d). Mobile phases used were
166 acetonitrile + 0.05 % v/v trifluoroacetic acid (TFA) and Milli-Q water + 0.05 % v/v TFA. Microcystin
167 peak separation was achieved by using a linear gradient a flow rate of 0.6 mL min⁻¹ (ISO 2005).
168 Column temperature was maintained at 37.5 ± 2.5 °C. The limit of detection per microcystin peak was
169 1.12ng. Microcystin was identified by PDA spectra at 238nm. Commercially available microcystin-LR
170 standard (Sapphire Bioscience, Australia; purity ≥ 95 %) was used to quantify microcystin
171 concentrations in all samples.

172

173 *Statistical analyses*

174 Redundancy analysis (RDA) (Legendre and Legendre 1998) was carried out in R language to quantify
175 the effects of nutrients, and the structure of phytoplankton and cyanobacterial community on
176 microcystin production. The RDA analysis was carried out using the combined data from three lakes
177 as we emphasize on finding the general trend rather than individual lake pattern. Explanatory variables
178 used in the RDA analysis were nutrient concentrations in the water column, the fraction of different
179 phytoplankton groups of total phytoplankton biomass, and the fraction of different cyanobacterial
180 genera of total cyanobacterial community. Response variables used in the RDA analysis were cellular
181 microcystin concentration and total microcystin concentration in the water column. The canonical

relationship was computed with standardized explanatory and response variables and tested for the level of significant after 999 permutations. In all analyses, results were considered significant at $P < 0.05$.

Results

Nutrient profiles of studied lakes

The nutrient profiles in Jackadder, Bibra and Yangebup Lakes during the study period are shown in Fig. 1A-H. Nutrient concentrations varied on a temporal basis within lakes and spatially between lakes. Phosphorus concentrations were higher in Bibra Lake than in Jackadder and Yangebup Lakes throughout the sampling period. Mean TP concentrations (Fig. 1A) ranged from 22 up to $\sim 92 \mu\text{gL}^{-1}$, 230 to $>1000 \mu\text{gL}^{-1}$, and 28 to $>150 \mu\text{gL}^{-1}$ in Jackadder, Bibra and Yangebup Lakes, respectively. Meanwhile, mean TDP concentrations (Fig. 1B) ranged from 12 to $24 \mu\text{gL}^{-1}$, 17 to $142 \mu\text{gL}^{-1}$, and 14 to $37 \mu\text{gL}^{-1}$ in Jackadder, Bibra and Yangebup Lakes, respectively. TN (Fig. 1C) and TDN (Fig. 1D) concentrations in Bibra Lake were higher by up to one order of magnitude than concentrations in Jackadder and Yangebup Lakes. In contrast, mean molar TN:TP in Bibra Lake was lower than the ratios in Jackadder and Yangebup Lakes (Fig. 1E). Mean TN:TP ranged from 40 to 132, 36 to 84, and 65 to 255 in Jackadder, Bibra and Yangebup Lakes, respectively. NH_4^+ decreased over time in Jackadder and Yangebup Lakes (Fig. 1F). Mean NH_4^+ concentrations ranged from 43 to $170 \mu\text{gL}^{-1}$, 157 to 239, and 40 to 143 in Jackadder, Bibra and Yangebup Lakes, respectively. Mean TFe (Fig. 1G) and TDFe (Fig. 1H) concentrations were higher in Bibra Lake than in the other two lakes during the beginning of the study. Mean TFe ranged from 77 to $247 \mu\text{gL}^{-1}$, 147 to $220 \mu\text{gL}^{-1}$, and 51 to $110 \mu\text{gL}^{-1}$ in Jackadder, Bibra and Yangebup Lakes, respectively. Mean TDFe ranged from 24 to $174 \mu\text{gL}^{-1}$, 61 to $117 \mu\text{gL}^{-1}$, and 21 to $89 \mu\text{gL}^{-1}$ in Jackadder, Bibra and Yangebup Lakes, respectively.

208

209 *Dynamics of phytoplankton community, cyanobacterial composition and microcystin production*

210

211 The phytoplankton composition in Jackadder, Bibra and Yangebup Lakes changed during the study
212 period (Fig. 2). Phytoplankton composition varied on a temporal basis within lakes and spatially
213 between lakes. In Jackadder Lake, cyanobacteria were dominant (68%) from January to early
214 February. In late February, the phytoplankton community shifted to mainly chlorophytes and diatoms,
215 and cyanobacteria only accounted for 7% of the community. Then in early March, cyanobacteria
216 started to increase and contributed to 67% of the total phytoplankton biomass by the end of March. In
217 Bibra Lake, chlorophytes dominated with the biomass up to 66%, while cyanobacteria only contributed
218 27% to the total phytoplankton biomass in January. Cyanobacteria contributed up to 49% of the total
219 phytoplankton biomass in early February, and the dominant phytoplankton changed to chlorophytes in
220 late February. In Yangebup Lake, cyanobacteria were dominant in January and cyanobacteria
221 contributed up to 67 % to the total phytoplankton biomass. In February, cyanobacterial fraction in the
222 total phytoplankton biomass decreased from 64 to 17%, and chlorophytes took over the major fraction
223 of total phytoplankton biomass in late February. In early March, cyanobacteria contributed 48% to the
224 total phytoplankton biomass in Yangebup Lake, and the fraction decreased to 32% in late March.
225 At the cyanobacterial genus level, cyanobacterial composition was highly variable between lakes, and,
226 within a lake, in time. The cyanobacterial community in Jackadder Lake was mainly composed of
227 three potential microcystin producing genera, namely *Microcystis* spp., *Planktothrix* spp. and
228 *Anabaena* spp. (Fig. 3A). There was a shift of dominance from *Microcystis* spp. to *Planktothrix* spp.
229 within a 7 day period in January, after which *Microcystis* spp. was again dominant (Fig. 3A).
230 *Planktothrix* spp. biovolume reached $2.208 \times 10^3 \text{ mm}^3 \text{ mL}^{-1}$ in late January and decreased continuously
231 towards the end of the study period (Fig. 4A). From February until the end of the study period,
232 *Microcystis* spp. was the major fraction in the cyanobacterial community with biovolumes ranging
233 from 0.318×10^3 to $1.896 \times 10^3 \text{ mm}^3 \text{ mL}^{-1}$ (Fig. 4A). *Anabaena* spp. was present as a small fraction in

234 January and February and increased to 50 % of the total cyanobacterial community in March (Fig. 3A).
 235 In Bibra Lake, *Anabaenopsis* spp., *Anabaena* spp., *Planktothrix* spp., *Nodularia* spp. and *Microcystis*
 236 spp. were the cyanobacteria detected, and their percentage biomass varied over time (Fig. 3B). In
 237 January, there was a change in dominance from *Anabaenopsis* spp. to *Microcystis* spp. within a 7 day
 238 period (Fig. 3B). During its dominance in early January, *Anabaenopsis* spp. reached a biovolume of
 239 $4.884 \times 10^3 \text{ mm}^3 \text{ mL}^{-1}$, but it was no longer detected after the end of February (Fig. 4B).
 240 Simultaneously with the decrease of *Anabaenopsis* spp, the fraction of *Microcystis* spp. increased from
 241 early January and remained the dominant cyanobacterial genera throughout the study period (Fig. 3B).
 242 *Microcystis* spp. biovolumes ranged from 1.865×10^3 to $111.34 \times 10^3 \text{ mm}^3 \text{ mL}^{-1}$ (Fig. 4B). *Anabaena*
 243 spp. also coexisted, but the total biovolume was always less than 50% of total cyanobacteria biomass
 244 (Fig. 3B). The highest *Anabaena* spp. biovolume was detected at $80.2 \times 10^3 \text{ mm}^3 \text{ mL}^{-1}$ in early
 245 February (Fig. 4B). *Planktothrix* spp. and *Nodularia* spp. contributed less than 5% of the total
 246 cyanobacterial biomass in Bibra Lake (Fig. 3B). In Yangebup Lake, *Microcystis* spp. was the dominant
 247 genera throughout the study period (Fig. 3C). *Microcystis* spp. biovolumes ranged from 2.766×10^3 to
 248 $9.839 \times 10^3 \text{ mm}^3 \text{ mL}^{-1}$ (Fig. 4C). *Planktothrix* spp. and *Nodularia* spp. contributed less than 20 and 1%
 249 to the total cyanobacterial biomass, respectively (Fig. 3C).
 250 Cellular microcystin concentration ($\text{mg MC-LR}_{\text{equiv}} \text{ g}^{-1}$ cyanobacterial dry mass) and total microcystin
 251 concentration in the water were highly variable over time in all lakes (Fig. 5). Cellular microcystin
 252 concentration ranged in three orders of magnitude in Jackadder Lake, and two orders of magnitude in
 253 Yangebup and Bibra Lakes. In all lakes, cellular microcystin concentration increased from January to
 254 February and decreased in March. The highest mean cellular microcystin concentration occurred in
 255 late February in all lakes. The highest mean cellular microcystin concentration was 1.4 mg g^{-1} dry
 256 mass in Jackadder Lake, 0.6 mg g^{-1} dry mass in Bibra Lake, and 0.5 mg g^{-1} dry mass in Yangebup
 257 Lake. Total microcystin concentration in the water varied by an order of magnitude in all lakes. In
 258 Bibra Lake, total microcystin concentration increased significantly from January to February, and
 259 mean concentrations ranged between $0.7 \mu\text{g L}^{-1}$ in January to $6.9 \mu\text{g L}^{-1}$ in February. In Jackadder

260 Lake, mean total microcystin concentration ranged between 0.2 and 1.3 $\mu\text{g L}^{-1}$, and the highest
261 concentration occurred in early March. In Yangebup Lake, mean total microcystin varied between 0.4
262 and 2.1 $\mu\text{g L}^{-1}$, and the highest mean concentration was also detected in early March.

263

264 *Correlations between nutrients, phytoplankton and cyanobacterial community structure and*
265 *microcystin production*

266

267 RDA analysis showed that the dynamics of microcystin production in the studied lakes were
268 significantly correlated to nutrient concentrations in the water column. Nutrients explained 64% of
269 variation in cellular microcystin concentration (Fig. 6). Cellular microcystin concentration was closely
270 correlated to iron concentration, while total microcystin concentration in the water was closely
271 correlated to phosphorus and nitrogen availability in the systems. Inclusion of cyanobacterial
272 composition as an explanatory variable in addition to nutrients increased the percentage explained
273 from 64% to 68% ($P < 0.05$), suggesting that nutrients were more important as a factor influencing the
274 dynamics of cellular microcystin production than the cyanobacterial composition (data not shown). In
275 contrast, including the phytoplankton community as an explanatory variable in addition to nutrients,
276 80% of variability in microcystin production could be explained ($P < 0.05$) (Fig. 7). The combination
277 of the phytoplankton community and nutrients as explanatory variables increased the ability to predict
278 the dynamics of microcystin production by 16%, indicating the significance of both variables on
279 microcystin production. As shown in the RDA biplot (Fig. 7), high microcystin production correlated
280 to high nutrient concentrations in the presence of a high fraction of chlorophytes and diatoms and a
281 low fraction of cyanobacteria in the phytoplankton community. In addition, higher cellular and total
282 microcystin concentrations were associated with lower fractions of cyanobacteria present in the
283 systems (Fig. 8). There was a significant, but very weak correlation between total cyanobacterial
284 biomass and total microcystin concentration in the water ($R^2 = 0.175$; $P < 0.05$; $y = 0.99 + 0.05 \cdot x$).

285

286 **Discussion**

287 Changes in water levels during dry months can have strong effects on nutrient dynamics, which
288 subsequently could affect the structure of phytoplankton communities in lakes (Coops et al. 2003,
289 Jeppesen et al. 2015). As such, this study has taken into account both nutrient and phytoplankton
290 dynamics to understand the dynamics of microcystin production. The results of our study suggested
291 that high microcystin production in the studied lakes was significantly correlated to high nutrient
292 concentration and low TN:TP. Our results agree with previous studies, which have reported that
293 increased phosphorus, nitrogen and iron concentrations could enhance microcystin production (Albay
294 et al. 2005, Jiang et al. 2008, Scott et al. 2014), and that higher microcystin concentrations can be
295 found at lower TN:TP ratios (Orihel et al. 2012). The observed close positive relationships between
296 microcystin production with phosphorus and nitrogen are potentially due to the high cyanobacterial
297 cellular requirement of phosphorus for microcystin biosynthesis and biomass production (Vezie et al.
298 2002). In addition, a positive correlation between iron and microcystin production may be related to an
299 earlier suggestion that microcystin production involves enzyme synthetase, in which the enzyme's
300 activity is controlled by the amount of iron present (Utkilen and Gjølme 1995).

301 In addition to the possible direct effect of nutrients on the dynamic of microcystin production, previous
302 studies have also suggested that nutrients might indirectly regulate microcystin production through
303 their effects on cyanobacterial abundance and its distribution (Dai et al. 2008, Millie et al. 2009, Wu et
304 al. 2014). Unlike chlorophytes and diatoms, a high cyanobacterial fraction was pointing in the opposite
305 direction to high nutrient availability (Fig. 7). This result indicates that high nutrient concentrations in
306 our systems did not favor the dominance of cyanobacterial genera. Instead, high phosphorus, nitrogen
307 and iron concentrations in the water column may have triggered the dominance of chlorophytes and
308 diatoms, due to their ability to grow faster than cyanobacteria (Huisman et al. 2005, Jensen et al. 1994,
309 Reynolds et al. 2006).

310 Microcystin production is complex and various environmental factors including nutrients have been
311 shown to have direct effects on the dynamics of microcystin production (Rinta-Kanto et al. 2009). Our

study suggests that the dominance of non-cyanobacterial groups is an additional factor to be considered and that changes in the structure of the phytoplankton community may have a large effect on microcystin production. Changes in the structure of the phytoplankton community explained up to 16% of the variability in microcystin production. Moreover, cyanobacteria tend to increase microcystin production in the presence of a high fraction of other phytoplankton groups. This result supports the theory of allelopathic interactions in cyanobacteria through the higher production of toxin microcystin with an increasing number of competitors (Holland and Kinnear 2013, Huisman and Hulot 2005, Rzymski et al. 2014). Earlier studies on allelopathy suggest that cyanobacteria may be capable of react to the presence of competitors by increasing microcystin production stimulated by the presence of extracellular products released by competitors such as chlorophytes (Bittencourt-Oliveira et al. 2014, Kearns and Hunter 2000, Pinheiro et al. 2013). In addition to the effects of changes in the structure of phytoplankton community, the dynamics of microcystin production could also be related to the succession of toxic and non-toxic cyanobacterial genotypes (Janse et al. 2005, Kardinaal et al. 2007, O'Neil et al. 2012), which was not analyzed in this study.

Based on the available cyanobacterial genera composition data, our results suggest that the changes in the structure of the cyanobacterial community did not affect the dynamics of microcystin production to a large extent. This result indicates that the succession of *Planktothrix* spp., *Anabaena* spp., *Nodularia* sp., *Anabaenopsis* spp., and *Microcystis* spp. in our lakes may not be related to changes in microcystin production in response to cyanobacterial competitors, and, as such would not support the theory of allelopathic interaction between different cyanobacteria genera (Paerl and Otten 2013, Švercel 2013). Rather, the succession of different cyanobacteria genera may be related to different cyanobacterial genera benefitting from different concentrations of phosphorus, nitrogen and iron species (Chorus and Bartram 1999, Huisman and Hulot 2005). Drastic changes to the structure of cyanobacterial communities are possible even with only slight changes in the cyanobacterial total biomass (Soares et al. 2009). It was suggested that this could be due to the short doubling times of cyanobacteria in

addition to different cyanobacterial genera occupying different niches, which develop due to temporal changes in the water's physicochemical properties (Reynolds 1998). As an example, *Microcystis* spp. are known to be capable to store high amounts of phosphorus and to use phosphorus very efficiently (Baldia et al. 2007), while *Planktothrix* spp. has a lower affinity towards phosphorus (Istvánovics et al. 2002). In addition, Nagai *et al.* (2007), has suggested that *Planktothrix agardhii* requires an iron concentration 30 times higher compared to *Microcystis aeruginosa* to achieve the same growth rate. This indicates that cyanobacteria may react differently to environmental conditions created by other genera, which could lead to drastic changes in the structure of the cyanobacterial community over short periods of time (Beverdors 2013). As an example, the higher iron requirement of *Planktothrix* spp. might reduce the iron availability in the water column, and it would thus become favorable for *Microcystis* spp. in the system. Therefore, the succession of different cyanobacteria genera is driven by a complex interaction of environmental conditions, including the availability of nutrients, and allelopathic interactions through cyanotoxins or other metabolites (Švercel 2013).

351

352 **Conclusions**

Our results indicate that the changes in the structure of the phytoplankton community and changes in nutrient concentrations are important factors that determine the dynamics of microcystin production. Under high nutrient concentration, non-cyanobacterial phytoplankton groups were dominant over cyanobacteria, and cyanobacteria produced more microcystin. Our results might therefore support the theory of allelopathic interactions between cyanobacteria and non-cyanobacterial competitors. We conclude that eutrophication could lead to the occurrence of more toxic cyanobacterial blooms directly through increased nutrient concentrations, and indirectly through changes in the phytoplankton community leading to increased toxin production within the cyanobacterial cells. Our results also suggest that changes in cyanobacterial community composition may not be of major importance for the dynamics of microcystin production. In relation to the microcystin risk assessment

during toxic cyanobacterial blooms, our results therefore suggest to consider the fraction of total cyanobacteria in addition to the absolute cyanobacterial biomass, which is currently the basis of existing WHO alert levels for microcystin risk assessment (Churro et al. 2012). Our conclusion is drawn based on the quantification of total biomass for each cyanobacterial genera without differentiating between microcystin producing strains and non-producing strains. The quantification of toxic and non-toxic cyanobacteria strains would have allowed for a deeper understanding of the microcystin dynamics and the dominance within the cyanobacteria community and we therefore suggest to include molecular characterisation of cyanobacterial communities in future studies to get a clearer picture of the role of strain dynamics on microcystin production, which would help to develop a more assertive microcystin risk assessment.

Additionally, a direct relationship between the structure of the phytoplankton/cyanobacteria community and microcystin production is likely to be established. Such a relationship illustrates the possibility of using the cyanobacterial fraction in the phytoplankton community as an ecological indicator to infer the level of microcystin production or risk of microcystin contamination in shallow water bodies. The relationship observed in this study was in agreement with the theory of allelopathic interaction between cyanobacteria and non-cyanobacteria. As direct microcystin quantification is not regularly conducted in water quality monitoring, the observation of high nutrient concentrations in the presence of potentially toxic cyanobacterial species, together with the dominance of non-cyanobacterial phytoplankton groups over cyanobacteria could be used as an indication that toxin production per cell is high and should prompt direct microcystin quantification. Even so, we propose further testing of this relationship with a larger number of lakes, including verification for single lakes, to prove its application for lake management.

Acknowledgments

This project was funded by the Australian Research Council's Linkage Project funding scheme (LP0776571) and the Water Corporation of Western Australia. We wish to thank the City of Cockburn

389 and the City of Stirling for permission to sample the lakes, Professor Pierre Legendre, Laura Firth and
390 Kevin Murray for their valuable statistical advice, and Liah Coggins for her help in the editing of the
391 manuscript. During the study, Sinang, S.C was supported by a scholarship from Universiti Pendidikan
392 Sultan Idris (UPSI) and Malaysia Government.

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562

563 **Fig. 1** Mean nutrient concentrations (A: TP; B: TDP; C: TN; D: TDN; E: TN:TP; F: NH_4^+ ; G:
564 TFe; H: TDFe) in Jackadder, Bibra and Yangebup Lakes from January to March 2010.

565

566 **Fig. 2** Phytoplankton community composition (% of total) in (A) Jackadder, (B) Bibra and (C)
567 Yangebup Lakes from January to March 2010. Bibra Lake dried up in early March; therefore no
568 samples were taken from this lake in March.

569

570 **Fig. 3** Cyanobacterial community composition (% of total) in (A) Jackadder, (B) Bibra and (C)
571 Yangebup Lakes from January to March 2010. Bibra Lake dried up in early March; therefore no
572 samples were taken from this lake in March.

573

574 **Fig. 4** Biovolume concentrations ($\text{mm}^3 \text{mL}^{-1}$) of cyanobacterial genera over time in (A)
575 Jackadder, (B) Bibra and (C) Yangebup Lakes from January to March 2010. Bibra Lake dried up
576 in early March; therefore no samples were taken from this lake in March.

577

578 **Fig. 5** Mean cellular microcystin concentration (mg g^{-1} cyanobacterial dry mass) and mean total
579 microcystin concentration in the water ($\mu\text{g L}^{-1}$) in Jackadder, Bibra and Yangebup Lakes from
580 January to March 2010; error bars represent standard errors.

581

582 **Fig. 6** RDA biplot of cellular microcystin (cMC) and total microcystin (tMC) concentration with
583 nutrients; solid lines = environmental variables; short dashed lines = response variables.
584 Canonical axis 1 and 2 represent a linear combination of the environmental variables.

585

586 **Fig. 7** RDA biplot of cellular microcystin (cMC) and total microcystin (tMC) with nutrients and
587 phytoplankton community (*Chloro* = chlorophytes; *Crypto* = cryptophytes; *Diatom*; and *Cyano* =

588 cyanobacteria); solid lines = environmental variables; short dashed lines = response variables.

589 Canonical axis 1 and 2 represent a linear combination of the environmental variables.

590

591 **Fig. 8** Correlation between cyanobacterial fraction and cellular microcystin concentration (mg g^{-1}

592 cyanobacterial dry mass) (solid line; $R^2 = 0.542$; $P < 0.05$; $y = 2.85 - 1.78 \cdot x$) or total microcystin

593 concentration ($\mu\text{g L}^{-1}$) (dashed line; $R^2 = 0.105$, $P < 0.05$, $y = 2.96 - 3.08 \cdot x$).

594 Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 6

Fig. 7

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