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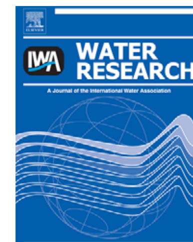

Spatial isolation favours the divergence in microcystin net production by *Microcystis* in Ugandan freshwater lakes

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ABSTRACT

It is generally agreed that the hepatotoxic microcystins (MCs) are the most abundant toxins produced by cyanobacteria in freshwater. In various freshwater lakes in East Africa MC-producing *Microcystis* has been reported to dominate the phytoplankton, however the regulation of MC production is poorly understood. From May 2007 to April 2008 the *Microcystis* abundance, the absolute and relative abundance of the *mcyB* genotype indicative of MC production and the MC concentrations were recorded monthly in five freshwater lakes in Uganda: (1) in a crater lake (Lake Saka), (2) in three shallow lakes (Lake Mburo, George, Edward), (3) in Lake Victoria (Murchison Bay, Napoleon Gulf). During the whole study period *Microcystis* was abundant or dominated the phytoplankton. In all samples mcyB-containing cells of *Microcystis* were found and on average comprised $20 \pm 2\%$ (SE) of the total population. The proportion of the mcyB genotype differed significantly between the sampling sites, and while the highest mcyB proportions were recorded in Lake Saka ($37 \pm 3\%$), the lowest proportion was recorded in Lake George ($1.4 \pm 0.2\%$). Consequently *Microcystis* from Lake George had the lowest MC cell quotas (0.03–1.24 fg MC $cell^{-1}$) and resulted in the lowest MC concentrations (0–0.5 $\mu g\,L^{-1}$) while *Microcystis* from Lake Saka consistently showed maximum MC cell quotas (14–144 fg $cell^{-1}$) and the highest MC concentrations (0.5–10.2 $\mu g\,L^{-1}$). Over the whole study period the average MC content per *Microcystis* cell depended linearly on the proportion of the mcyB genotype of *Microcystis*. It is concluded that *Microcystis* populations differ consistently and independently of the season in mcyB genotype proportion between lakes resulting in population-specific differences in the average MC content per cell.

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1. Introduction

During the last decades cyanobacteria in freshwater have been of interest due to their ability to produce various hepatotoxic and neurotoxic substances. It is generally agreed that the hepatotoxic microcystins (MCs) are the most abundant toxins produced by cyanobacteria in freshwater (WHO, 2006; Erdner et al., 2008; Hudnell, 2008). MCs are cyclic

Abbreviations: MC, microcystin; *mcy*, gene encoding the MC synthetase; HPLC, high performance liquid chromatography; DAD, diode array detection; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PC, the phycocyanin gene.

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heptapeptides that share the common structure cyclo (-D-Ala$^{(1)}$–X$^{(2)}$–D-MeAsp$^{(3)}$–Z$^{(4)}$–Adda$^{(5)}$–D-Glu$^{(6)}$–Mdha$^{(7)}$), where X and Z are variable L-amino acids (e.g. MC-LR refers to leucine and arginine in the variable positions of this peptide), D-MeAsp is D-erythro-β-iso-methyl-aspartic acid, Adda is (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdha is *N*-methyl-dehydroalanine. Structural variation has been reported most frequently in positions 2, 4, and 7 of the MC molecule resulting in over 80 structural variants that have been characterized from field samples or isolated strains (Krüger et al., 2009). In a recent paper, we could show that cyanobacteria contribute significantly to the phytoplankton of freshwater lakes in Uganda while other algal groups like diatoms, green algae, and cryptomonads are of a relatively minor importance (Okello et al., 2009). We further concluded that in Uganda the genus *Microcystis* is the only MC-producing genus which is favoured under more shallow, eutrophic conditions which is in correspondence to the general theory on how physical factors govern phytoplankton associations (Reynolds et al., 2002). In this earlier study we did not monitor *Microcystis* populations as well as the MC net production of the phytoplankton community during different seasons. This is of relevance as it is known that beside the absolute population abundance it is the proportion of MC-producing genotypes (those containing the *mcy* gene cluster encoding MC synthesis) vs. non-MC-producing genotypes (those lacking the *mcy* gene cluster) that has a decisive influence on MC net production (Sivonen and Jones, 1999; Kurmayer and Christiansen, 2009). So far research was unable to document an adaptive value of MC production, thus making it difficult to identify proximate factors triggering MC synthesis (Kaebernick and Neilan, 2001; Schatz et al., 2007). Although the transcription rate of the *mcy* gene cluster is increased from low to high irradiance conditions it is generally agreed that MC synthesis is constitutive (Kaebernick and Neilan, 2001). In the course of seasonal studies several researchers reported the occurrence of seasonal shifts in the proportion of MC-producing vs. non-MC-producing genotypes in dependence on various abiotic or biotic environmental factors (Briand et al., 2008; Hotto et al., 2008). In East Africa usually dry seasons with precipitation minima and wet seasons with maxima of precipitation have been correlated with changes in phytoplankton composition (Talling, 1986). During the rainy season (from March to May and August–November), the phytoplankton in shallow lakes will be affected directly by a reduced water temperature (2.5 °C in Kasese and 3 °C in Kampala), reduced light availability in the water column as well as increased terrestrial run-off. Only in deep lakes such as the main basin of Lake Victoria, the mixing regime will change, as a higher stability of the water column has been described during the dry season (Talling, 1986). These physical changes in the water column have a significant effect on phytoplankton community composition (Talling, 1987). Generally in shallow lakes less seasonality in phytoplankton composition is observed when compared with the main basin of Lake Victoria.

In contrast to the seasonal influence we recently emphasized that it is rather the spatial isolation of populations than the seasonal influence of biotic or abiotic factors that leads to differences in the population structure of MC-producing genotypes (Kurmayer and Gumpenberger, 2006). We further concluded that the structural variation within the position 2 of the MC molecule is selectively neutral (Kurmayer and Gumpenberger, 2006) implying that genetic drift resulting from geographic isolation has the potential to lead to new MC variants that appear unique and dominant in particular waterbodies, i.e. [Asp^3, Dhb^7]-MC-HtyY and [Asp^3, Dhb^7]-MC-HtyHty (Christiansen et al., 2008a). For lakes located in the Alps of Austria, Germany and Switzerland we hypothesized that populations diverge in their *mcy* genotype composition at least during consecutive years (Ostermaier and Kurmayer, 2009). However, we also observed that the genetic population structure changed between years, for example due to the extinction of the population during winter and a subsequent re-immigration of new genotypes (Kurmayer and Gumpenberger, 2006).

In this study we aimed to investigate the seasonal development of *Microcystis* and potential MC-producing genotypes and the resulting MC concentrations in five freshwater lakes in Uganda. *Microcystis* has been reported to dominate in shallow eutrophic lakes such as Lake George at least for decades (Ganf, 1974). While the shallow lakes close to the Ruwenzori mountain (L. George, L. Edward, L. Mburo) are naturally eutrophic, the genus *Microcystis* also has become abundant in bays of Lake Victoria as a result of human induced eutrophication (Hecky, 1993; Mugidde, 1993; Verschuren et al., 2002). It is hypothesized that if spatial isolation leads to genetic divergence in MC production then the variation in *mcy* genotype proportion between sites should significantly exceed the variation in *mcy* genotype proportion that is observed within sites during the season. *Vice versa* if spatial isolation is of minor importance, the seasonal variation in *mcy* genotype proportion as caused by unknown biotic and abiotic factors that is observed within sites should significantly exceed the between site variation.

2. Materials and methods

2.1. *Description of the study sites*

From five freshwater lakes in Uganda six sampling sites were chosen (Fig. 1): (1) A site in the center of Lake Saka (N0°41.670′, E30°14.667′), mean depth of 3.6 m. Lake Saka is a small crater lake (1.4 km^2) located at an altitude of 1520 m.a.s.l.. The other sampling sites included the shallow eutrophic lakes (2) Lake George, (3) Lake Edward, (4) Lake Mburo as well as Lake Victoria, (5) Murchison Bay near Kampala, and (6) Napoleon Gulf near Jinja, which were described previously (Okello et al., 2009).

2.2. *Field sampling and analysis of* Microcystis *abundance*

Depth-integrated water samples and plankton net samples (30 μm mesh size) were taken monthly from May 2007 until April 2008 as described (Okello et al., 2009). For DNA and MC analysis aliquots (250–2400 ml) were filtered onto GF/C filters (∅ 47 mm, Whatman, Kent, Great Britain) and the filters were dried at 50 °C. *Microcystis* were counted by the inverted

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Fig. 1 – Map of Uganda showing the six sampling sites (black circles).

microscope technique from Lugol fixed samples following standard techniques (Wetzel and Likens, 2000). The genus *Microcystis* was discriminated from other cyanobacteria according to Komárek and Anagnostidis (1999). Following the taxonomic revision for the genus *Microcystis* Kützing ex Lemmerman 1907 (Otsuka et al., 2001) all morphospecies were considered morphological varieties of individuals of *Microcystis aeruginosa* comb. nov. Kützing 1833. For each sample 400 specimens of *Microcystis* and other dominant phytoplankton genera were counted at 400-fold magnification. Only the data on *Microcystis* cell numbers and *Microcystis* biovolume are reported here while the phytoplankton community composition will be described in another publication. The average biovolume of a *Microcystis* cell was $76 \pm 12\ \mu m^3$ (1SE).

2.3. Quantification of the microcystin genotype

To estimate the abundance and the proportion of the MC-producing genotype, DNA was extracted from aliquots of the samples analyzed for *Microcystis* cell numbers and for MC as described previously (Kurmayer et al., 2003). The absolute and relative cell numbers of *Microcystis* and the *Microcystis* genotype containing the mcyB gene that is indicative of MC production were determined by means of quantitative real-time PCR as described (Kurmayer and Kutzenberger, 2003) and the same primers and probes have been used. To quantify the total population of *Microcystis* the linear regression was $y = -3.4552x + 36.229$ ($n = 6$, $R^2 = 0.998$), where y was the cycle of threshold (C_t value) at the set fluorescence threshold level obtained for the intergenic spacer region of the phycocyanin gene (PC) and x was the amount of starting DNA (given as $\log_{10}$ cell number equivalents of *Microcystis* strain HUB524). To quantify the *Microcystis* cells containing the mcyB gene only the linear regression curve was $y = -3.9759x + 40.712$ ($n = 6$, $R^2 = 0.994$), where y was the C_t value at the set fluorescence threshold level obtained for the mcyB gene and x was the amount of starting DNA aliquots used for PC (given as $\log_{10}$ cell number equivalents of *Microcystis* strain HUB524). The relative abundance of the mcyB genotype of *Microcystis* was determined by dividing the cell numbers of the mcyB genotype through the cell numbers of the total population (as estimated from the PC genotype). All measurements were done in triplicate using an Eppendorf mastercycler ep realplex system (Eppendorf, Vienna). Both gene regions that were amplified by PCR have been shown to be specific for *Microcystis* in the presence of other MC-producing cyanobacteria such as *Anabaena* or *Planktothrix* (Kurmayer and Kutzenberger, 2003). In order to include the whole study period all depth-integrated samples from all six sampling sites ($n = 72$) as well as plankton net samples from Lake George ($n = 4$) and Lake Mburo ($n = 4$) were analyzed.

2.4. Microcystin analysis

Phytoplankton collected on filter was extracted in aqueous methanol as described (Okello et al., 2009). The clear supernatants of extracts were analyzed by high performance liquid chromatography-diode array detection (HPLC-DAD) as described using a HP1100 Chemstation (Lawton et al., 1994; Kurmayer et al., 2003). MCs were quantified at 240 nm and the concentration of all MC variants was determined as concentration equivalents of [MeAsp, Mdha]-MC-LR (Cyanobiotech GmbH, Berlin, Germany). The concentration of MC-LR was calculated from the regression curve $y = 1885.3x - 6.8775$, ($R^2 = 0.99$), where y was the absorption (mAU) recorded at 240 nm and x was the μg of MC-LR injected.

HPLC fractions identified as MC were collected manually and analyzed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), (PerSeptive BioSystems, Framingham MS, USA) as described (Erhard et al., 1997). The constitution of the new MC variant [$NMeSer^7$]-MC-YR was assigned by ESI-MS and ESI-MS^2 experiments that were performed on a Q-TOF Ultima mass spectrometer (Waters, Milford, MA.) equipped with a nanospray source and operated in the positive ionization mode under the control of MassLynx 4.1.

2.5. Statistical analysis

The linear regression curves were fitted using the least square approximation and the associated statistical tests of Sigma Plot 2000 (V 6.10). The data were $\log_{10}$ transformed in order to achieve normal distribution and constant variances. The linear regressions between the total *Microcystis* cell number (as estimated from the microscope) and the abundance of the PC genotype and the mcyB genotype were compared in slope and intercept using a general factorial model of analysis of variance (ANOVA). The data were modeled as $y = \mu + \beta x + \varepsilon$, where y is the measured abundance of the PC genotype or the mcyB genotype, μ is the overall mean level, β is the effect of the PC or the mcyB genotype, x is the effect of the cell number as determined from the microscope as a covariate, and ε is the

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random deviation. To compare the mcyB proportions between sampling sites one-way ANOVA was used.

Multiple linear regression analysis was used to test the relationship between the MC concentration (in μg MC ml^{-1}) as dependent variable and the influence of *Microcystis* cell numbers as determined by the microscope or real-time PCR via PC and mcyB as independent variables. A forward stepwise analysis was employed selecting for the independent variable for inclusion that makes the most significant unique contribution to the prediction of the data. Calculations were performed using SPSS 15.0 for Windows and the F value to enter the respective model was set default ($p < 0.05$).

3. Results

3.1. *Microcystis* abundance

Microcystis appeared throughout the sampling period in all the lakes. Only on 30 September 2007 *Microcystis* abundance was below the detection limit in the depth-integrated sample obtained from Lake Victoria (Napoleon Gulf). During the study period *Microcystis* cell numbers varied between 10^4 –10^6 (Lake Saka), 10^5 –10^6 (L. George), 10^5 –10^6 (L. Edward), 10^4 –10^5 (L. Mburo), 10^4–10^5 (L. Victoria, Murchison bay), and 10^3–10^4 cells ml^{-1} (L. Victoria, Napoleon Gulf). We recorded the highest *Microcystis* biovolume in samples from Lake George (273 $mm^3 l^{-1}$) and the lowest (0.3 $mm^3 l^{-1}$) in samples from Lake Victoria (Napoleon Gulf). On average *Microcystis* constituted between 18 and 46% of the total phytoplankton biovolume (Table 1). This implied that the phytoplankton at all the sampling sites had the potential for MC production.

3.2. Quantification of the microcystin genotype

In order to test the hypothesis that populations of *Microcystis* differ in the proportion of the *mcy* genotype, the absolute abundance of both the total population as well as the subpopulation containing the mcyB gene were determined by means of real-time PCR. Overall, both the microscope as well as real-time PCR showed congruent results in estimating the total *Microcystis* population number that on average differed by one order of magnitude in lakes George and Edward, and less than an order of magnitude in the other lakes (Fig. 2). Consequently over the study period a linear relationship between cell numbers estimated via the microscope and real-time PCR of the PC genotype was found (Fig. 3A): The regression curve was $y = 0.595x + 1.661$ ($n = 80$, $R^2 = 0.62$), where y is the log_{10} cell number as determined by the real-time PCR (PC) and x is the log_{10} cell number as counted in the microscope.

In all samples mcyB-containing cells of *Microcystis* were found and on average comprised 19.9 ± 1.8 (SE) % of the total population (min = 0.5, max = 66.6%). In contrast to PC a relatively weak relationship between cell numbers as counted in the microscope and cell numbers of the mcyB genotype was found (Fig. 3B): $y = 0.3222x + 2.1935$ ($n = 80$, $R^2 = 0.13$) and the two regression curves were not parallel (ANOVA, $p < 0.001$). Particularly the measurements obtained from Lake Saka and Lake George showed a much wider scatter when compared with the measurements on the PC genotype. The proportion of the mcyB genotype differed significantly between the sampling sites (Fig. 3C): While the highest mcyB proportions were recorded in Lake Saka (min − mean ± SE − max, 24.4 − 37.0 ± 3.3 − 66.6%) and in Napoleon Gulf (12.9 − 31.6 ± 3.8 − 59.3%), the by far lowest proportion was recorded in Lake George (0.49 − 1.4 ± 0.2 − 3.6%). At the other three sites, Lake Edward (7.5 − 14.9 ± 2.8 − 36.7%), Lake Mburo (6.1 − 14.0 ± 2.2 − 32.3%), and Murchison Bay (3.3 − 16.4 ± 3.1 − 39.2%) an intermediate proportion was recorded. It is concluded that the significant differences in the proportion of the mcyB genotype between the lakes resulted in a poor correlation between microscopically determined cell numbers and the cell numbers of the mcyB genotype.

3.3. Microcystin net production

3.3.1. Composition of microcystins

The vast majority of the samples were found to contain MC. HPLC-DAD analyses documented the occurrence of eleven MC structural variants that showed an unequivocal match with the spectrum of either MC-RR, or MC-YR, or MC-LR. All fractions identified as MC by HPLC were collected and analyzed by means of MALDI-TOF for their molecular weight. The following variants could be undoubtedly identified by their retention time, their mass and by spiking using MC-RR, YR, LR standards: MC1, [Asp^3]-MC-RR ($M + H^+$ 1024), MC2, MC-RR ($M + H^+$ 1038), MC4, [Asp^3]-MC-YR ($M + H^+$ 1031), MC5, MC-YR ($M + H^+$ 1045), MC6, MC-LR ($M + H^+$ 995), MC8, [Asp^3]-MC-RY ($M + H^+$ 1031), MC9, MC-RY ($M + H^+$ 1045). [Asp^3]-MC-RY and MC-RY have been identified recently (Okello et al., 2009). The MC3 variant was determined as [$NMeSer^7$]-MC-YR by LC-MS^2 (Suppl. Table 1). The others were considered unknown: MC7 ($M + H^+$ 1013), MC10 ($M + H^+$ 1024), MC11 ($M + H^+$ 1031).

Table 1 – Cell numbers (min − mean ± SE − max, cells ml^{-1}), biovolume ($mm^3 l^{-1}$) and proportion (%) of *Microcystis* (in total phytoplankton) in depth-integrated samples from the six sampling sites from May 2007 to April 2008 (n = 12).

	Cell numbers	Biovolume	Proportion
Lake Saka	$8 \times 10^3 - 1.4 \times 10^5 \pm 3.3 \times 10^4 - 4.5 \times 10^5$	0.6 − 10.5 ± 2.6 − 34.6	1.7 − 18.3 ± 3.8 − 41.7
Lake George	$1.5 \times 10^5 - 1.0 \times 10^6 \pm 2.7 \times 10^5 - 3.6 \times 10^6$	11.6 − 81 ± 21 − 273	3.6 − 27.5 ± 5.2 − 67
Lake Edward	$4.5 \times 10^4 - 4.1 \times 10^5 \pm 1.6 \times 10^5 - 2.3 \times 10^6$	3.5 − 31 ± 12 − 177	3.8 − 20 ± 5.4 − 77
Lake Mburo	$5.5 \times 10^4 - 1.7 \times 10^5 \pm 2 \times 10^4 - 2.9 \times 10^5$	4.2 − 12.6 ± 1.5 − 22	10.5 − 21.5 ± 2.1 − 37.2
Murchison Bay	$4 \times 10^4 - 1.5 \times 10^5 \pm 2 \times 10^4 - 3 \times 10^5$	3.1 − 11.4 ± 1.5 − 23.2	21.6 − 45.6 ± 3.3 − − 59.9
Napoleon Gulf	$4.4 \times 10^3 - 2 \times 10^4 \pm 4 \times 10^3 - 4.5 \times 10^4$	0.3 − 1.6 ± 0.3 − 3.5	0 − 21.5 ± 3.7 − 44.1

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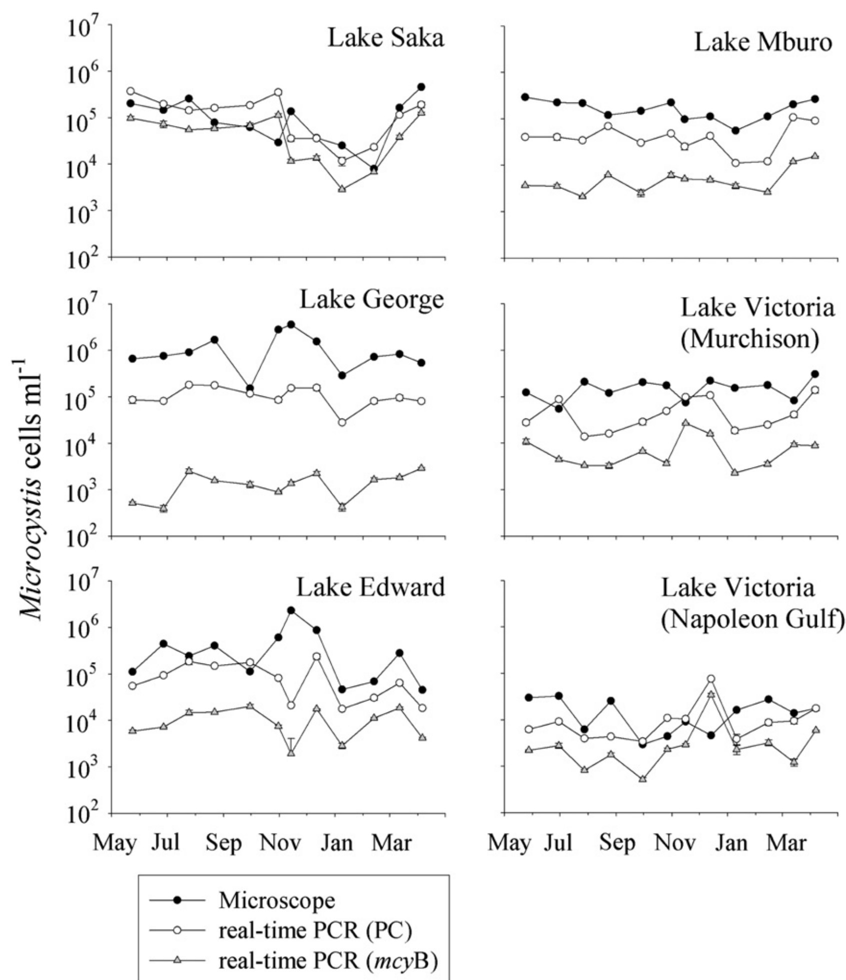

Fig. 2 – *Microcystis* cells (ml^{-1}) as estimated by the microscope (black circles), and by real-time PCR via the phycocyanin (PC) gene (white circles) and the mcyB gene (grey triangles) at the six sampling sites from May 2007 to April 2008. For the PCR estimates the mean ± SE is shown.

The sampling sites differed significantly in the relative abundance of all the MC variants (Chi square test, $p < 0.01$). For example MC-RR and [Asp^3]-MC-RR were most frequent in lakes Saka, Mburo and Edward and in Murchison Bay. In contrast MC-RY and [Asp^3]-RY were dominant at all sites except in Napoleon Gulf (Table 2). Surprisingly in the samples from Napoleon Gulf the new [NMeSer^7]-MC-YR variant occurred most frequently. Taking all sampling sites together MC-RY was most abundant, followed by MC-RR and MC-YR. In contrast MC-LR only occurred in 11% of all the samples.

3.3.2. *Concentration of microcystins*

The contribution of each MC variant to the total MC concentration (calculated as MC-LR equivalents) closely matched the frequency of occurrence. For example, MC-RR contributed on average ≥50% to the total MC in lakes Saka and Mburo. MC-RY contributed >50% to the total MC in lakes George and Edward and in Murchison Bay. All MC detected in Napoleon Gulf was dominated by the [NMeSer^7]-MC-YR variant (Table 2). The phytoplankton further differed significantly in the concentration of MC in total ($p < 0.0001$, ANOVA). On average, the MC concentrations were 28-fold higher in Lake Saka ($4.7 \pm 0.9\ \mu g\,l^{-1}$) when compared with the average ($0.2 \pm 0.1\ \mu g\,l^{-1}$) MC concentration measured in Lake George (Fig. 4). Samples from Lake Saka had the maximum MC concentration ($10\ \mu g\,l^{-1}$) in July 2007. The minimum concentrations ($0.02\ \mu g\,l^{-1}$) were recorded in Lake George in May 07, June 07, January 08 and April 08. At the sampling sites in the other three lakes intermediate MC concentrations (0.1–$2.5\ \mu g\,l^{-1}$) were recorded. Within the lakes, the total MC concentration varied seasonally from 12-fold to 30-fold.

3.4. *Genotype determined microcystin production*

3.4.1. *Relation of the PC and mcyB genotypes to microcystin concentrations*

Both PC and mcyB genotype cell numbers were highly significantly related to MC concentrations: $y = 1.33 \times 10^{-8} + 0.000195$ ($R^2 = 0.28$) for the PC genotype and $y = 6.16 \times 10^{-8} + 0.00038+$ ($R^2 = 0.62$) for the mcyB genotype. With the exception of Lake George the cells of the mcyB genotype showed an increase of the

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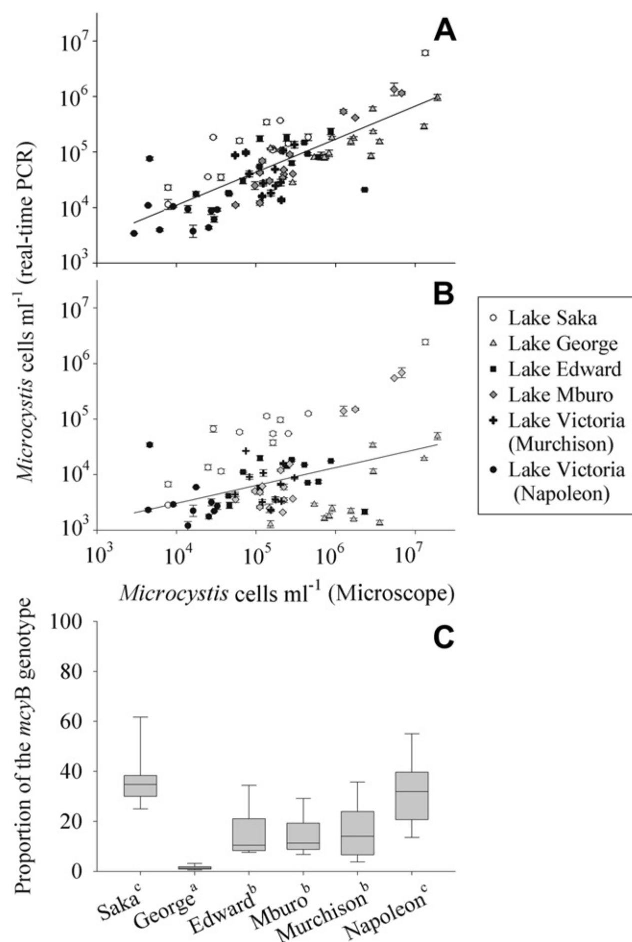

Fig. 3 – Relationship between the *Microcystis* cell numbers as estimated in the microscope and the cell numbers as estimated via real-time PCR (mean ± SE) for the (A) phycocyanin gene (indicative of the total *Microcystis* population), (B) the mcyB gene (indicative of MC production) at the six sampling sites from May 2007 to April 2008. The details on the regression curves are given in the text. (C) Proportion of the mcyB genotype at the six sampling sites for the same data set. The whiskers of each box indicate the 10th and 90th percentiles (n = 12). The differences were tested by one-way ANOVA followed by the Tukey post-hoc comparison procedure ($p < 0.001$). Superscripts indicate homogeneous subsets ($p > 0.05$).

average MC content by a factor of 2.9–7.8 when compared with the MC content of the PC genotype (Fig. 5A). Corresponding to the lowest proportion of the mcyB genotype in Lake George (Fig. 3C) the MC content of the mcyB genotype from Lake George showed a 69-fold increase when compared with the MC content of the PC genotype. Consequently the mcyB genotype occurring at the six sampling sites rather differed in absolute numbers than in the *in situ* activity or in the regulation of MC net production.

3.4.2. *Relation of* Microcystis *cells to microcystin concentrations*

For all sampling sites highly significant positive linear relationships between the total MC concentration and *Microcystis* cell numbers were obtained (Table 3). However, relating the total MC concentrations to *Microcystis* cell numbers revealed a >100-fold variation in the average MC contents per cell between lakes (Fig. 5B). Corresponding to its lowest mcyB genotype proportion *Microcystis* from Lake George consistently showed the lowest MC cell quotas (0.03–1.24 fg cell^{-1}) while *Microcystis* from Lake Saka showed maximum MC cell contents (14–144 fg cell^{-1}). While the between site variation was found reduced in plankton net samples the ranking of sampling sites by their average MC contents per *Microcystis* cell was not affected (data not shown). It is concluded that at all sites MC production was related to the occurrence of *Microcystis* as enumerated in the microscope while between sites the populations differ consistently and independently of the season in their average MC content per cell.

3.4.3. *Relation of the mcyB genotype proportion to the microcystin content*

Over the study period the proportion of the mcyB genotype was linearly related to the average cellular MC content per cell: $y = 1.2884x - 0.7835$ ($n = 77$, $R^2 = 0.58$), where x is the $\log_{10}$ proportion of the mcyB genotype and y is the $\log_{10}$ MC content in fg MC cell^{-1} (Fig. 5C). In order to explain MC concentrations the forward multiple regression analysis revealed a significant inclusion of the mcyB genotype abundance as the first and most significant predictor variable ($R^2 = 0.68$) and subsequently the microscopically determined *Microcystis* cell numbers as the second predictor variable ($R^2 = 0.73$): $y = 0.862x + 0.256z - 7.805$ ($R^2 = 0.73$, $n = 77$), where x is the $\log_{10}$ abundance of the mcyB genotype (cells ml^{-1}), z is the $\log_{10}$ cell number (ml^{-1}) determined in the microscope and y is the $\log_{10}$ MC concentration (μg ml^{-1}). It is concluded that the *Microcystis* populations differ genetically in the mcyB proportion which can indeed explain the variation in the average MC content of *Microcystis* cells observed among the lakes during the study period.

4. Discussion

4.1. *Correlation of* Microcystis *cell numbers with microcystin net production*

For all lakes the abundance of *Microcystis* cells was significantly positively related to MC production. In contrast MC production was negatively related to the abundance of *Planktothrix* in Lake Saka and to the abundance of *Anabaena* in Lake Victoria in Napoleon Gulf and Murchison Bay. Significant relationships between the total MC concentration and *Anabaena* cell numbers were observed for the sites in lakes Mburo, Murchison Bay and Napoleon Gulf (data not shown). However, as we were unable to detect genes involved in MC production of any other taxa than *Microcystis* in the same habitats (Okello et al., 2009), we consider this relationship as due to the co-occurrence of these taxa and *Microcystis* (Okello et al., 2009). In addition nine strains of *Planktothrix* sp. were isolated from Lake Saka in April 2008 and analyzed for MC production. None of the strains were found to contain MCs and/or the mcyE/mcyB gene part of the *mcy* gene cluster (Rainer Kurmayer, unpublished results).

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Table 2 – Relative frequency of occurrence (%) and proportion (mean ± SE) in HPLC chromatograms of each microcystin variant in the depth-integrated and the plankton net samples at the six sampling sites from May 2007 to April 2008 (n = 24). For each site the most abundant MC variant is marked in Bold.

	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11
$M+H^+$	1024	1038	1063	1031	1045	995	1013	1031	1045	1024	1031
Structural variant	[Asp^3]MC-RR	MC-RR	[NMeSer^7]-MC-YR	[Asp^3]MC-YR	MC-YR	MC-LR	Unknown	[Asp^3]MC-RY	MC-RY	Unknown	Unknown
Retention time (min)	14–14.5	15.1–15.7	16.0–16.4	17.0–17.7	18.0–18.9	19.0–19.9	20.9	21.0–21.8	23.0–23.9	24.0–25.0	27.8–27.9
Lake Saka											
Frequency	17	**100**	13	21	67	63	0	67	83	17	0
Proportion	0.8 ± 0.6	**58.7 ± 4.1**	3.2 ± 2	0.4 ± 0.2	17.3 ± 3.5	4.4 ± 1.3		8.0 ± 2.8	7.0 ± 1.3	0.1 ± 0.07	
Lake George											
Frequency	0	4	0	0	0	0	0	33	**92**	0	0
Proportion		1.6 ± 1.6						12.0 ± 4.4	**78.0 ± 6.6**		
Lake Edward											
Frequency	25	67	0	38	46	4	0	13	**96**	50	33
Proportion	0.6 ± 0.2	8.3 ± 1.7		0.3 ± 0.1	1.8 ± 0.5	0.03 ± 0		5.9 ± 4.3	**81.1 ± 4.5**	1.0 ± 0.2	0.3 ± 0.1
Lake Mburo											
Frequency	58	96	0	54	92	58	67	79	**100**	17	54
Proportion	2.6 ± 0.9	**46.9 ± 2.8**		0.8 ± 0.2	12.9 ± 1.5	1.6 ± 0.4	1.8 ± 0.4	2.2 ± 0.5	**27.7 ± 3.5**	2.6 ± 0.4	0.4 ± 0.1
Murchison Bay											
Frequency	0	63	29	0	25	79	0	0	88	33	0
Proportion		3.9 ± 1.9	7.8 ± 3.0		2.9 ± 1.4	12.4 ± 2			**48.8 ± 6.3**	6.7 ± 1.7	
Napoleon Gulf											
Frequency	0	17	**88**	0	17	8	0	4	4	4	0
Proportion		3.9 ± 1.9	**82.1 ± 6.9**		4.7 ± 2.4	0.5 ± 0.3		0.5 ± 0.5	2.1 ± 2.1	2 ± 2	
Total											
Frequency	17	58	22	19	41	35	11	33	77	20	15
Proportion	0.7 ± 0.2	23.0 ± 2.1	15.5 ± 2.8	0.3 ± 0.1	6.6 ± 0.9	3.3 ± 0.6	0.3 ± 0.09	4.8 ± 1.2	40.5 ± 3.2	2 ± 0.5	0.1 ± 0.02

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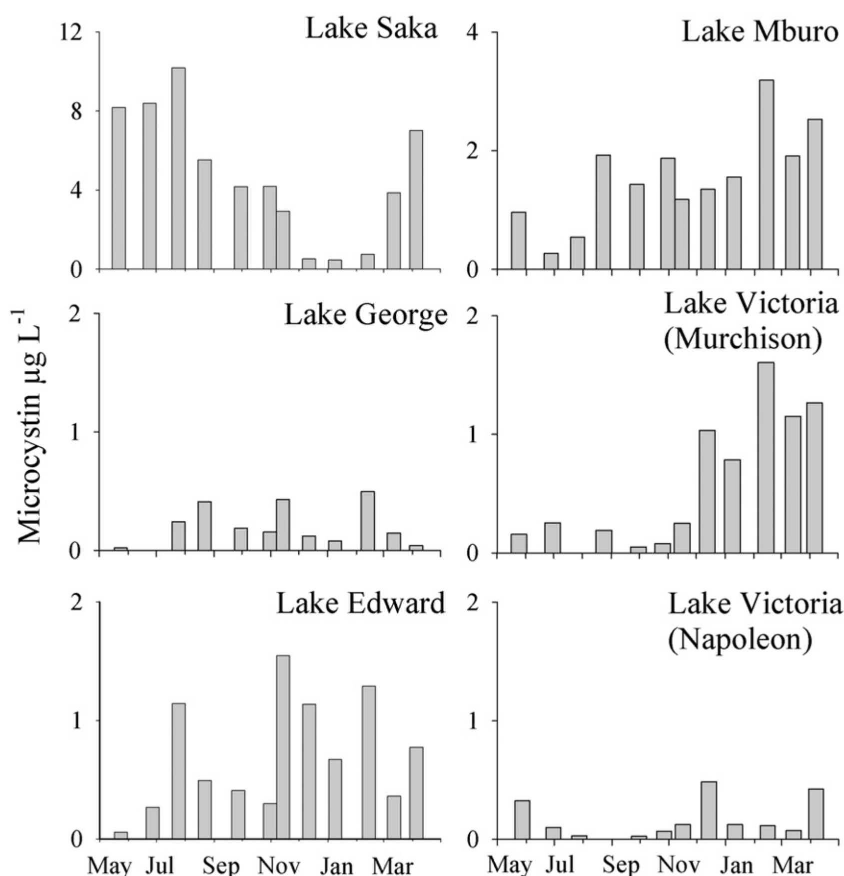

Fig. 4 – Microcystin concentrations (µg MC l^{-1}) at the six sampling sites from May 2007 to April 2008. Note that the scales on the y-axis differ.

Microcystis probably constitutes the most widely distributed MC-producing organism on earth. MC-producing strains have been isolated from all continents (Sivonen and Börner, 2008). A phylogenetic analysis of 164 *Microcystis* strains revealed the occurrence of two phylogenetic clades only that contained the *mcy* gene cluster, while the other four clades did not (Tanabe et al., 2007). The same authors concluded that those phylogenetic clades either containing or lacking the *mcy* gene cluster constitute cryptic ecotypes that are adapted to various environmental conditions. Notably these MC-producing ecotypes seem to have a wide geographic distribution, as typically MC-producing genotypes occur in waters inhabiting *Microcystis* (Kurmayer and Christiansen, 2009). In contrast *Anabaena* – although occurring frequently worldwide – shows a more restricted pattern of MC production as so far only MC-producing strains from Europe, North America and North Africa have been reported (Sivonen and Jones, 1999). When compared with *Microcystis* the genus *Anabaena* shows a much wider genetic variation, for example the taxonomic discrimination of the two morphologically distinct genera *Anabaena* and *Aphanizomenon* cannot be confirmed by 16S rDNA sequence analysis (Gugger et al., 2002). Recently, MC-producing *Anabaena* occurring even in brackish water in the Gulf of Finland in the Baltic Sea has been reported (Halinen et al., 2007) implying that the genus *Anabaena* is composed of MC-producing ecotypes showing resistance to increased salinity (5.03–6.67 practical salinity units). Consequently, although *Microcystis* cell numbers as determined in the microscope typically correlate with MC production, *Anabaena* cells cannot be used to infer MC concentrations in water.

4.2. *Differences in microcystin net production between sites*

The average MC cell quotas of *Microcystis* differed significantly between populations (Fig. 5A, B). Environmental conditions such as light availability and nitrogen availability have been shown to increase MC production in *Microcystis*. For example Wiedner et al. (2003) reported a linear increase in MC content per cell of *Microcystis* strain PCC7806 from 40 to 80 fg $cell^{-1}$ under light conditions from 10 to 100 µmol m^{-2} s^{-1}. Long et al. (2001) observed a variation in MC content per cell of *Microcystis* strain MASH 01-A19 from 0.052 to 0.116 fmol $cell^{-1}$ under nitrogen limiting and nitrogen-replete conditions. Typically, environmental factors have been shown to modulate MC production per cell up to 5-fold, while larger variation (up to 30-fold) at 30 °C vs. 12.5 °C has been reported in exceptional cases only (Sivonen and Jones, 1999). In this study the average MC contents differed between *Microcystis* populations by 16–150-fold in integrated samples and 2.5–23-fold in plankton net samples. This range of variation substantially exceeds the variation observed for single strains under variable environmental conditions in the laboratory. Consequently it is more likely that genetic differences between populations such as

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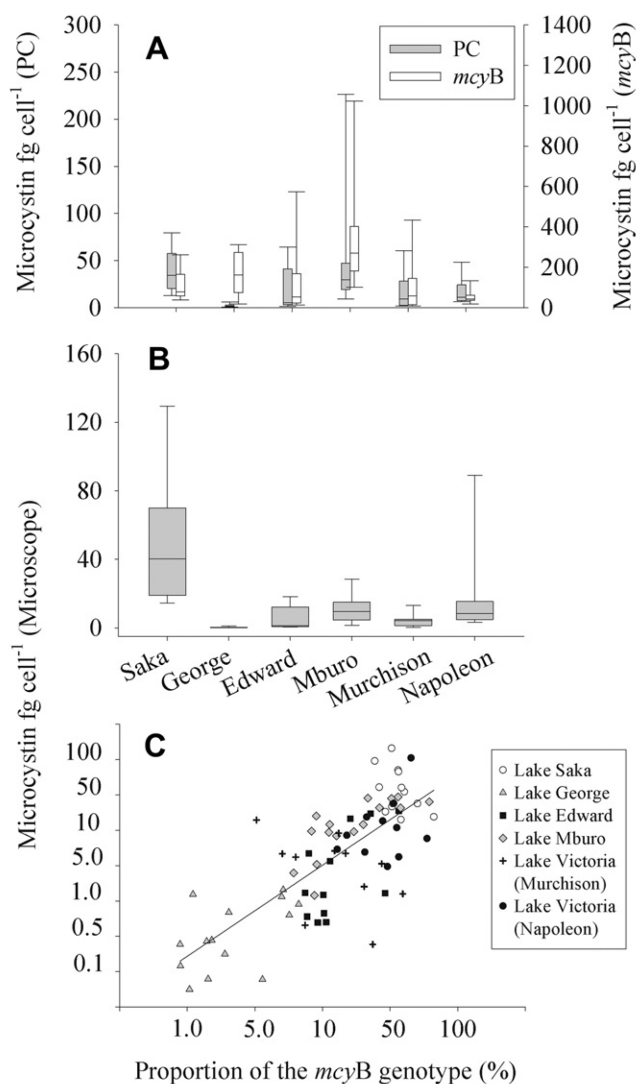

Fig. 5 – Microcystin cell quotas (fg MC cell^{-1}) of (A) the PC and the mcyB genotype of *Microcystis* and (B) of *Microcystis* cells as determined in the microscope at the six sampling sites from May 2007 to April 2008 (n = 12). (C) Dependence of the microcystin content (fg MC cell^{-1}) on the proportion of the mcyB genotype of *Microcystis* for the same data set.

the variable proportion of the *mcy* genotype contributed to the variation in MC content that is observed. Indeed by applying real-time PCR to estimate the proportion of the *mcy* genotype in the individual *Microcystis* populations it could be shown that the average proportion of the *mcy* genotype was significantly related to the average MC content per cell (Fig. 5C). As suggested by one reviewer it might be that the inclusion of an estimate of the transcriptional rate of the mcyB genotype leads to an even higher correlation coefficient as observed in this study ($R^2 > 0.58$). According to the results observed in this study, however it is unlikely that the recording of the transcriptional rate of the mcyB genotype only is able to explain the variability in the average MC content between sites. It is concluded that the differences in the *mcy* genotype proportion between sites have a major impact on MC production while possible environmental influences (such as a higher irradiance in a less densely populated water column) cannot be excluded, but are of minor importance.

Table 3 – Linear regression curves on the dependence of microcystin concentrations on *Microcystis* cell numbers as determined in the microscope in the depth-integrated and the plankton net samples at the six sampling sites from May 2007 to April 2008.

	Sample size	R^2	Linear regression curve[a]
Lake Saka	24	0.97	$y = 3.18 \times 10^{-8}x + 0.00654$
Lake George	24	0.81	$y = 1.3 \times 10^{-9}x - 0.000787$
Lake Edward	24	0.66	$y = 2.38 \times 10^{-8}x + 0.000475$
Lake Mburo	24	0.94	$y = 2.44 \times 10^{-8}x - 0.000919$
Lake Victoria (Murchison Bay)	24	0.87	$y = 2.74 \times 10^{-9}x + 0.000599$
Lake Victoria (Napoleon Gulf)	24	0.87	$y = 6.33 \times 10^{-9}x + 0.000128$
Total	144	0.40	$y = 1.41 \times 10^{-8}x - 0.00245$

a y is the microcystin concentration (μg MC ml^{-1}) and x is the *Microcystis* cell concentration (cells ml^{-1}).

4.3. Differences in microcystin genotype proportion between sites

We have shown previously that populations of cyanobacteria in lakes may diverge in *mcy* genotype composition even if they are located only a few kilometres apart due to spatial isolation (Kurmayer and Gumpenberger, 2006). While this geographical isolation may result in the evolution of MC structural variants that appear to be unique and dominant (Christiansen et al., 2008a), this study is the first that demonstrates, that in consequence MC net production may differ quantitatively between sites as well. The structural analysis of protein phosphatase 1 – MC complexes did not provide evidence that the most variable amino acid residues at positions 2 and 4 of the MC molecule are of functional consequence (Bagu et al., 1997; Maynes et al., 2005). In contrast a quantitative change in MC production might be of a selective consequence. For example it has been shown that dissolved MC affects the growth of several submersed and emersed macrophytes negatively (Wiegand and Pflugmacher, 2005) and allelopathic effects on other phytoplankton and zooplankton species have been repeatedly suggested (Gross, 2003; Leflaive and Ten-Hage, 2007; Martins and Vasconcelos, 2009). However, it has also been shown that herbivorous organisms may develop behavioural or physiological resistance to MC production (Kurmayer and Jüttner, 1999). Following the concept of co-evolutionary interactions between herbivores and plants producing defensive compounds (Futuyama, 1983; Jongsma and Bolter, 1997) one might speculate that particularly in those *Microcystis* populations showing lowest mcyB proportion the allelopathic role of MC is increasingly replaced by bioactive compounds other than MCs (Welker and von Döhren, 2006). If this conclusion is true then one might expect that MC production is becoming selectively neutral to individual *Microcystis* colonies. While it is likely that only strong selective pressure led to the evolution of the *mcy* gene cluster in cyanobacteria it is known that the *mcy* gene cluster

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probably evolved about two billion years ago (Rantala et al., 2004). According to this hypothesis the majority of the modern cyanobacterial lineages had lost the *mcy* gene cluster during their evolution. Unexpectedly within species such as *Planktothrix* the loss of the *mcy* gene cluster in strains happened on a much shorter time scale in evolution, yet has been found to be a rather rare event that happened a few million of years ago (Christiansen et al., 2008b). It was further concluded that in the meantime both the genotype retaining the *mcy* gene cluster and the genotype that lost the *mcy* gene cluster diverged and adapted to various other environmental conditions. Consequently it is impossible to compare costs and benefits of MC production between strains unless these strains have been genetically characterized in total (by comparative genome analysis) in order to elucidate potential hidden ecophysiological differences. It is likely that the *Microcystis* populations investigated in this study also diverged in other phenotypic characters not directly linked to MC production.

The results are of relevance with regard to the question of whether biogeography can influence toxin production in cyanobacteria. For example, in this study MC-LR that is most frequently found in populations of *Microcystis* in Europe (Via-Ordorika et al., 2004) could only be rarely detected in Ugandan freshwater lakes. MC-LR is known to have a ten-fold higher toxicity to vertebrates when compared with MC-RR and therefore those Ugandan water samples also should be less toxic to livestock and humans when compared with European habitats. In summary, the seasonal variation in average *mcy* proportion within each of the sites could not outweigh the between site variation in *mcy* genotype proportion, thus leading to a rather stable divergence in MC production of *Microcystis* between the spatially isolated populations. This lake-specific divergence might lead to a divergence in MC production on a wider geographic scale affecting MC production both qualitatively and quantitatively resulting in a so far unrecognised bio geographic pattern.

5. Conclusions

The finding that *Microcystis* is a consistent MC producer has important implications for water monitoring. By counting *Microcystis* cells under the microscope, *Microcystis* cell numbers can be used as a proxy to predict MC concentrations in surface water. Since for a specific sampling site a relatively minor variation in the average MC content both during dry and rainy seasons has been found, worst case MC concentrations could be calculated from cell numbers using the maxima of cellular MC quotas as reported for each sampling site. The microscopical approach is considered feasible as the microscopical enumeration technique is well established and the maintenance of technically sophisticated equipment is avoided. However, quantifying the mcyB genotype directly could make more accurate predictions of MC concentrations. In contrast the influence of the transcriptional rate of the mcyB gene on the observed variation in MC net production between sites is considered of minor importance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.watres.2010.02.018.

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