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Temporal variation in microcystin production by *Planktothrix agardhii* (Gomont) Anagnostidis and Komárek (Cyanobacteria, Oscillatoriales) in a temperate lake

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Abstract – Eutrophication of freshwater lakes has led to blooms formed by cyanobacteria often associated with toxins harmful to livestock and humans. Environmental conditions that favor toxin production during cyanobacterial blooms are, however, not well understood. Moreover, the ability to use cyanobacteria quantity to assess the level of threat associated with toxin production is a topic of discussion. The purpose for this study was to examine *Planktothrix agardhii* dynamics in a shallow, temperate hypertrophic lake and to determine the factors that affect microcystin production. In addition, the relationship between *P. agardhii* morphology and microcystin production was examined. The study spanned 2 years, and we documented a perennial *P. agardhii* bloom that contributed up to 99% of the total biomass. Intracellular microcystins were primarily detected throughout the study, with the highest concentration in October. Microcystin concentrations ranged from 3.4 to 71.2 $\mu g.L^{-1}$, and they had a strong, positive correlation with *P. agardhii* biomass. In contrast, the levels of weight-specific microcystin were relatively stable throughout the entire study, ranging from 0.23 to 1.18 $\mu g.mg^{-1}$. We also found that environmental factors, such as water temperature, phosphate level, ammonium nitrogen and transparency, were the most related to microcystin production. Furthermore, a significant relationship between filament morphology and toxin concentration suggested that there were different morphotypes within the toxic and non-toxic populations of *P. agardhii*. Our study showed that *P. agardhii* biomass and filament morphology may be useful characteristics for the identification of threats associated with cyanotoxins.

Key words: Cyanobacteria / filament morphology / hepatotoxins / *Planktothrix agardhii* / shallow lakes

Introduction

Eutrophication of freshwater lakes has led to frequent cyanobacteria blooms often associated with toxins that are harmful to livestock and humans (Carmichael and Falconer, 1993). Among the cyanobacteria, filamentous *Planktothrix agardhii* (Gomont) Anagnostidis and Komárek frequently occupy shallow, turbid lakes in temperate zones (Nixdorf, 1994; Rücker *et al.*, 1997). *P. agardhii* blooms are typical in lakes across Europe, including deep alpine lakes (Scheffer *et al.*, 1997; Humbert and Le Berre, 2001; Nixdorf *et al.*, 2003; Stefaniak *et al.*, 2005). As these blooms can produce cyanotoxins, they are a growing source of concern.

The cyanotoxins produced by *P. agardhii* include microcystin, the most common hepatotoxin. *P. agardhii* produces at least 25 different isoforms of microcystin (Mbedi *et al.*, 2005), with a higher production of microcystin per dry weight than *Microcystis* spp. observed in previous field studies (*e.g.*, Fastner *et al.*, 1999). Of the many microcystin variants, microcystin-LR, -YR and -RR are the most common among freshwater ecosystems

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worldwide (Chorus, 2001). Investigations regarding which variants are present in the water and in what quantities are important, as these variants differ in bioactivity. The most toxic are microcystin variants with hydrophobic L-amino acids (*e.g.*, microcystin-LR), whereas the least toxic are those with more hydrophilic amino acids (*e.g.*, microcystin-RR) (Falconer, 2005). The many microcystin variants are associated with substrate specificity of adenylation domains in the mcyS NRPS modules (Mikalsen *et al.*, 2003) and genetic differences in the microcystin synthetase gene (Neilan *et al.*, 2008). Moreover, *P. agardhii* produces additional bioactive secondary metabolites that are toxic to fish and crustaceans (Ernst *et al.*, 2001; Keil *et al.*, 2002).

Previous studies have shown that certain environmental conditions (such as high temperature, high light intensity and high nutrient supply) that accelerate the growth rate of *P. agardhii* and other producers also increase the production of microcystin (Sivonen, 1990; Tonk *et al.*, 2005). *P. agardhii* blooms primarily occur during the summer; however, perennial populations have also been documented (Briand *et al.*, 2002; Yéprémian *et al.*, 2007; Pawlik-Skowrońska *et al.*, 2008). Investigations into *P. agardhii* perennial population dynamics suggest that *P. agardhii* tolerates relatively low light intensities and is often a dominant species in steady-state plankton assemblages within hypertrophic shallow lakes during summer (Scheffer *et al.*, 1997; Nixdorf *et al.*, 2003).

Certain studies have also indicated that there are annual and diurnal differences in *P. agardhii* filament morphology (Romo, 1994; Hašler and Pouličkova, 2003; Pouličkova *et al.*, 2004). However, whether this filament morphology variation is related to the *P. agardhii* variation in toxin production is unknown. In natural *Microcystis* sp. populations, microcystin cell quotas and the relative proportions of microcystin-producing and non-microcystin-producing genotypes have been associated with colony size (Kurmayer *et al.*, 2003). A recent study by Akcaalan *et al.* (2006) also showed a positive relationship between *P. agardhii* filament length and microcystin content. Morphological variation could thus be a potentially useful tool in estimating microcystin concentrations during *P. agardhii* dominance in lakes.

The aims of this study were two-fold. Firstly, over 2 years, we examined microcystin production dynamics and *P. agardhii* biomass in a highly eutrophic lake. Secondly, we investigated whether microcystin production is either related to filament morphology or primarily mediated via *P. agardhii* biomass and prevailing environmental conditions.

Material and methods

Study lake

The study was conducted in Lake Lubosińskie (52°31.40'N, 16°22.57'E) with a surface area of 0.23 km^2. This is a shallow lake (average depth of 2.6 m) located near the city of Poznań in the Wielkopolska region, western Poland. The lake is polymictic, highly eutrophic and surrounded by agricultural catchments.

Sampling

Considering the small size of the lake and the absence of thermal stratification, phytoplankton samples were collected from one sampling station located in the middle of the lake. Sampling was conducted from July 2006 to March 2008 (except for May and June 2007, when access to the lake was closed) twice in a month during summer/autumn season (July–October) and once a month during the rest of the year using a "Limnos sampler" (volume 5 L). On each sampling date, samples were taken from 0.5 m below the water surface layer. The phytoplankton samples were preserved using acid Lugol's solution and formaldehyde immediately after sampling, and they were stored under cool and dark conditions until they were counted. Before counting, the samples were left undisturbed for at least 48 h, after which, the upper layer of water was gently decanted off, and the lower layer (40 mL) contained the settled phytoplankton species used in the phytoplankton analysis.

Phytoplankton analysis

Phytoplankton identification and counting were conducted using a light microscope (magnification 400 ×). All individuals, including single cells, colonies and filaments, were counted over at least 160 fields in a Fuchs–Rosenthal chamber, which ensures that at least 400 specimens were counted to reduce the counting error to less than 10% ($P < 0.05$; Javornický, 1958). Phytoplankton biomass was estimated from volumetric analysis of cells using geometric approximation (Hindak, 1978; Wetzel and Likens, 2000) and expressed as wet weight. The mean diameter of *P. agardhii* cells was determined by measuring the first 50 filaments in each sample using a Zeiss microscope Axioskop 2 MOT and the image analysis program KS300.

Chemical and physical analysis

Water samples for chemical analyses were simultaneously collected with the phytoplankton samples. The water samples were analyzed for ammonium, nitrate, nitrite, total nitrogen, orthophosphate and total phosphorus using the DR 2010 HACH Spectrophotometer following standard methods from HACH (1997). For chlorophyll-*a* analyses, 200 mL of water was filtered through a GF/C Whatman filter. The concentration was spectrophotometrically determined after 90% acetone extraction and calculated using Lorenzen's formula (Wetzel and Likens, 2000). During the field sampling, water temperature, pH and conductivity were determined using a multiparameter Elmetron CPC-401 probe. Water

transparency was measured for each sample using a Secchi disk (SD).

Microcystin analysis and quantification by high-performance liquid chromatography (HPLC)

For HPLC analyses, 1 L water samples containing cyanobacterial material were filtered immediately after sampling through GF/C filters. Microcystins in the suspended material were extracted in 75% aqueous methanol. The samples were sonicated for 30 s in a Misonix (Farmingdale, NY, USA) ultrasonicator equipped with an ultrasonic probe (100 W, diameter 19 mm with "spike") and the liquid processor XL. The extracts were then centrifuged twice at $11\,000 \times g$ for 10 min at 4 °C in an Eppendorf 5804 centrifuge (Hamburg, Germany). The supernatants were collected and evaporated in an SC110A Speedvac® Plus, ThermoSavant (Holbrook, NY, USA). Before HPLC analysis, the samples were redissolved in 1 mL of 75% aqueous methanol and filtered through Gelman GHP Acrodisc 13 mm syringe filter with 0.45 μm GHP membrane and minispike outlet (East Hills, NY, USA).

For dissolved microcystins, 1 L samples of filtered water were concentrated on Baker (Deventer, Netherlands) C_{18} solid-phase extraction (SPE) cartridges (sorbent mass: 500 mg). Microcystins were eluted from the C_{18} cartridges by 3 mL of 90% aqueous methanol containing 0.1% trifluoroacetic acid (TFA). The eluates were evaporated to dryness and the samples were redissolved in 1 mL of 75% aqueous methanol before HPLC analysis (Jurczak *et al.*, 2005). Microcystin concentration was determined using a gradient mobile phase including $H_2O + 0.05\%$ TFA (eluent A) and acetonitrile (ACN) + 0.05% TFA (eluent B) as well as diode-array detection at 200–300 nm. Sample volume was 20 mL, flow rate was 1 mL.min^{-1} and column temperature was 40 °C. Intracellular microcystins in the cyanobacterial extracts were identified using the characteristic absorption spectra and retention times for microcystin standards microcystin-LR, MC-RR and MC-YR. HPLC-enabled microcystin detection at the 0.01 μg.L^{-1} level after concentrating the samples. To examine the weight-specific microcystin content (μg.mg^{-1} fresh weight), the ratio for the concentration of intracellular microcystin (the microcystin concentrations within cyanobacterial cells per liter of lake water (μg.L^{-1}) and *P. agardhii* biomass per liter of lake water (mg.L^{-1}) was calculated).

Data analysis

We first examined whether the microcystin concentration and the weight-specific microcystin content demonstrated temporal autocorrelation using SPSS (SPSS, 1999). The microcystin concentration refers to the microcystin level within cyanobacterial cells per liter of lake water, and the weight-specific microcystin content is the microcystin cellular concentration per mg of *P. agardhii* biomass.The latter thus reflects the variation in microcystin production per unit of *P. agardhii* biomass. Correlograms indicated that the microcystin concentration showed significant temporal autocorrelation, while the weight-specific microcystin content was not autocorrelated.

To model the microcystin concentrations, we thus used the generalized linear mixed model (GLMM), and the autocorrelation structure was modeled via a time covariate that indicated the sampling day. We also examined whether microcystin concentration was correlated with the measured environmental variables, season (included as a categorical variable) and filament morphology (length and width) for *P. agardhii*. We defined four seasons as follows: summer: July–September; autumn: October–November; winter: December–February; spring: March–April. We identified the most parsimonious model using Akaike's information criterion (Burnham and Anderson 1998). Analysis was conducted using the "lme" function with the autoregressive correlation structure (function "corAR1") in the nlme R-package (R Development Core Team, 2005).

To analyze the weight-specific microcystin content, we followed Soininen *et al.* (2007) and Korhonen *et al.* (2010), we also used the general linear model (GLM) in the R software package (R Development Core Team, 2005). We also analyzed whether the microcystin content was correlated with environmental factors, season and *P. agardhii* filament morphology.

Before the regression analyses, we tested whether the measured environmental variables showed strong intercorrelations. As all pairwise correlations between the variables were < 0.7, we initially incorporated all the variables into the analyses as they may have had independent effects on the models. The possible among-season variation in *P. agardhii* filament size was tested using the Kruskal–Wallis analysis of variance (ANOVA). Finally, Spearman's rank-order correlation analysis was used to examine the correlation between *P. agardhii* biomass, microcystin concentration and weight-specific microcystin content via the Statistica 7.1 software.

Results

Water chemistry and physical parameters

Based on the concentrations of total phosphorus (mean [TP] = 0.40 mg.L^{-1}), total nitrogen (mean [TN] = 5.2 mg.L^{-1}), water transparency (mean Secchi depth = 0.20 m), conductivity (mean cond. = 683.4 μS) and chlorophyll-*a* levels in the water (mean [chl-*a*] = 189.8 μg.L^{-1}), the lake was categorized as highly eutrophic.

Phytoplankton composition

Cyanobacteria dominated phytoplankton communities throughout the study. *P. agardhii* was the most abundant

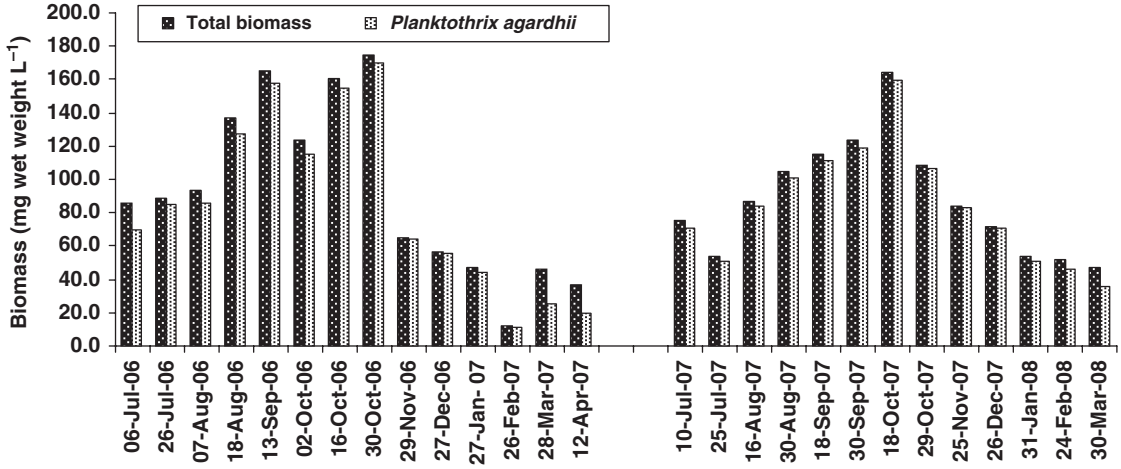

Fig. 1. Dynamics of the *Planktothrix agardhii* biomass (mg wet weight.L^{-1}) against the total phytoplankton biomass (mg wet weight.L^{-1}).

species in the community and accounted for 53.8–98.8% of the total phytoplankton biomass, with the highest and lowest values occurring during October and February, respectively, for both years (Fig. 1). In addition to *P. agardhii, Aphanizomenon gracile* Lemmermann, *Jaaginema subtillissimum* (Kütz. ex De Toni) Anagnostidis & Komárek, *Limnothrix redekei* (Van Goor) Meffert and *Pseudoanabaena limnetica* (Lemmermann) Komárek were also common, though their abundances were notably lower. Other groups of algae were less abundant. Chlorophyta were present primarily during summer and spring, while Bacillariophyceae, Cryptophyta and Dinophyta were more characteristic in winter and autumn.

Filament morphology

According to ANOVA, there were significant ($P < 0.001$) differences in filament length and width among the seasons studied. The filaments were widest in spring and longest in winter, but they were the shortest and narrowest during summer (Figs. 2(a) and (b)).

Microcystin concentration

The extracellular microcystin concentration was undetectable in most of our samples, except for the small concentrations of microcystin-LR ranging from 0.38 to 0.60 μg.L^{-1} which were detected in four samples during summer 2006. Therefore, further analyses and results herein relate to the intracellular concentrations.

Three different variants of intracellular microcystin (microcystin-RR, -YR and -LR) were identified. Microcystin-RR concentrations were consistently the highest, whereas concentrations of microcystin-YR and -LR were significantly lower (Fig. 3).

The total intracellular microcystin concentration was the highest in the middle of August 2006 and in the middle of September 2007. Even during winter, the microcystin

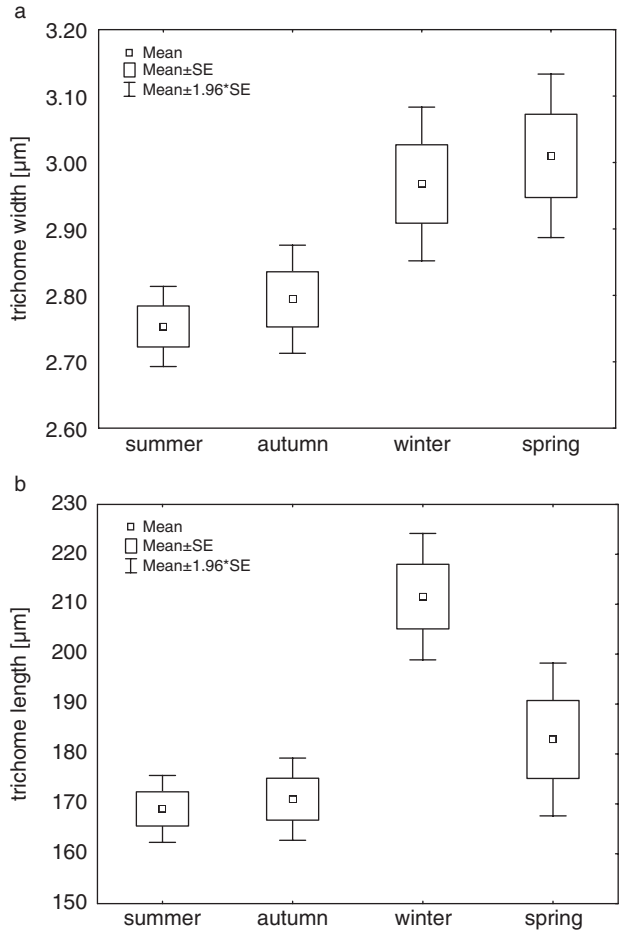

Fig. 2. Variation in the size of *P. agardhii* filaments: (a) filament width ($n = 1350$ and $P = 0.000$) and (b) filament length ($n = 1350$ and $P = 0.001$) among the seasons.

concentration remained at a relatively high level in both the years, compared with earlier studies (Fig. 4). The microcystin concentration correlated closely with the *P. agardhii* biomass ($r = 0.83$, $P < 0.05$ and $n = 27$) (Fig. 3).

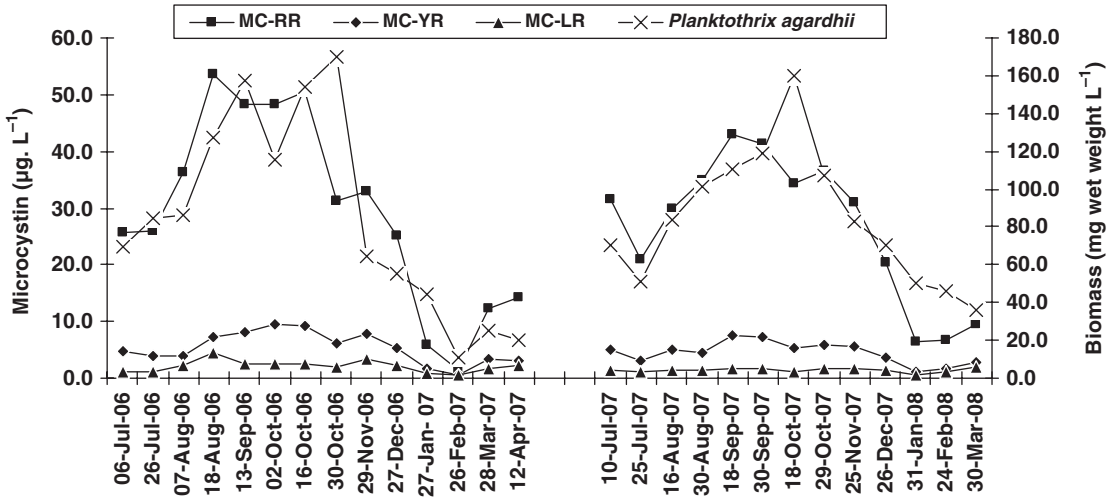

Fig. 3. Temporal changes in the concentrations of different microcystin variants (µg.L^{-1}) against the *P. agardhii* biomass (mg wet weight.L^{-1}).

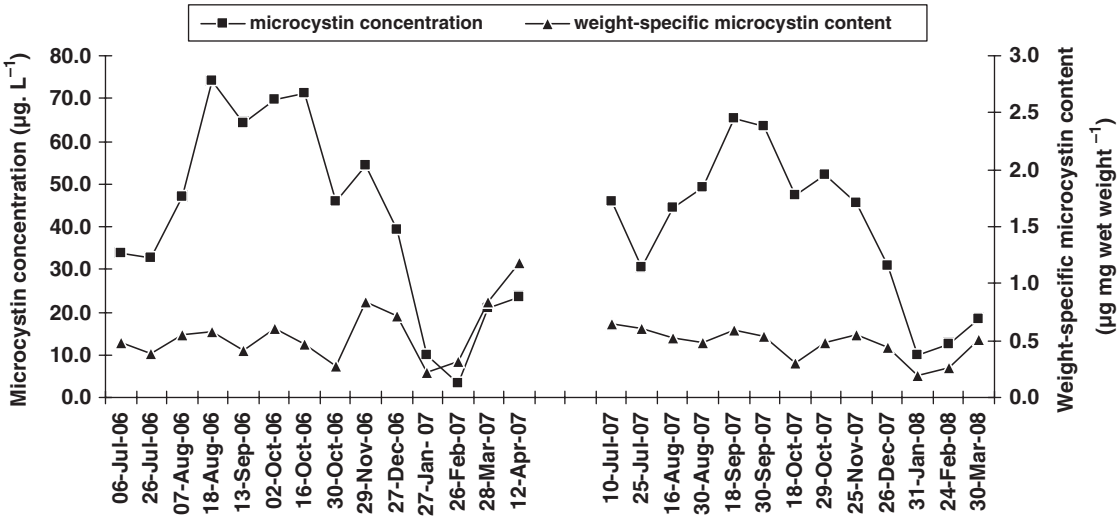

Fig. 4. Temporal changes in the total microcystin concentrations (µg.L^{-1}) and weight-specific microcystin content (µgmg wet weight.L^{-1}).

Weight-specific microcystin content

The weight-specific microcystin content was relatively constant over the study period, with small peaks during autumn 2006 and spring 2007, as well as small declines in winter (Fig. 4). Weight-specific microcystin content correlated only very weakly and negatively with the *P. agardhii* biomass ($r = 0.14$, $P < 0.05$ and $n = 27$).

The GLMM and GLM analysis

From the GLMM analysis with AIC, the microcystin concentration in the water correlated positively with temperature, ammonium nitrogen and total phosphorus, and it correlated negatively with water transparency and nitrate nitrogen (Table 1). GLM analysis, in turn, showed that weight-specific microcystin content correlated positively with pH and filament width, and it correlated negatively with phosphate concentration and temperature (Table 2).

Discussion

Throughout the entire study period, *P. agardhii* dominated the phytoplankton community. According to Scheffer (1998), such a long dominance of cyanobacterium from the Oscillatoriales group is an indication of the "third stable state" in the algal community. To our knowledge, we showed for the first time that microcystins can constantly be produced during such an ecological equilibrium. As additional microcystin producers were absent, we believe that *P. agardhii* was the only source of toxin in this lake. We emphasize that intracellular microcystins were present throughout the entire study period, while among

Table 1. Results of the GLMM analysis for analyzing microcystin concentrations ($\mu g.L^{-1}$) in the water and correlating environmental factors (bold indicates significance at $P < 0.05$). The variables were selected using Akaike's information criterion. Temporal autocorrelation in concentrations was considered using a time covariate in the mixed model.

Parameters	*B*	*F*	*P*
Intercept	− 45.78	509.2	**<0.0001**
Temp.	1.47	30.00	**0.0001**
pH	9.42	0.10	0.761
SD	− 140.55	16.73	**0.001**
P-PO4	− 54.51	2.38	0.145
$N\text{-}NH_4$	7.82	22.28	**0.0003**
$N\text{-}NO_3$	− 14.09	15.48	**0.002**
TP	64.18	6.32	**0.025**
TN	2.41	0.68	0.424

*SD, Secchi depth; Temp., temperature; TP, total phosphorus; TN, total nitrogen; $P\text{-}PO_4$, phosphates; $N\text{-}NH_4$, ammonium nitrogen; $N\text{-}NO_3$, nitrate nitrogen; B, regression coefficient.

Table 2. Results of GLM analysis for weight-specific microcystin content ($\mu g.mg^{-1}$) and explaining environmental factors (bold indicates significance at $P < 0.05$). The variables were selected using Akaike's information criterion. R^2-values refer to the amount of variation jointly explained by the selected variables.

Parameters	*B*	Std. Error	*T*	*P*
$R^2 = 0.759$				
Intercept	0.34	1.10	0.31	0.758
Temp.	− 0.02	0.01	− 2.90	**0.009**
pH	0.10	0.04	2.63	**0.016**
Cond.	− 0.002	0.001	− 1.60	0.125
$P\text{-}PO_4$	− 1.76	0.34	− 5.16	**0.001**
Filament width	0.51	0.13	3.97	**0.001**

*Cond., conductivity; Temp., temperature; $P\text{-}PO_4$, phosphates; *B*, regression coefficient; Std._Error, standard error; *T*, test variable; *P*, significance.

the extracellular microcystins, only microcystin-LR was detected during a short period in 2006. Fewer microcystins in the water of temperate lakes were previously observed (Chorus, 2001). However, in contrast to other studies, we have not observed extracellular microcystin peaks from, for example, cyanobacteria loss and decomposition at the end of the bloom (Chorus, 2001).

Nonetheless, the toxin level in the water was far from constant. Total intracellular microcystin concentrations were highest in autumn, which is consistent with recent studies by Yépréman *et al.* (2007) and Briand *et al.* (2008a). We found that microcystin concentrations closely followed the *P. agardhii* biomass. Our findings concur with studies that have documented a strong positive relationship between the microcystin concentration and the *P. agardhii* biomass (Wiedner *et al.*, 2002; Catherine *et al.*, 2008). Such a strong positive relationship can be explained by the stable coexistence of microcystin-producing genotypes, which were also detected in an earlier study by Mankiewicz-Boczek *et al.* (2011). However, it should be noted that there were microcystins even in winter, when the *P. agardhii* biomass was low. During the coldest months (January and February), intracellular microcystin concentrations were lowest, but relatively high (ranging from 3.39 to 12.43 $\mu g.L^{-1}$) compared to previously published winter microcystin concentrations (see Briand *et al.*, 2002; Janse *et al.*, 2005). This indicates that *P. agardhii* can produce toxins even during unfavorable conditions.

Different hepatotoxin variant concentrations fluctuated widely over the study period; desmethyl microcystin-RR was the most concentrated. This is consistent with earlier studies (Briand *et al.*, 2002; Kurmayer *et al.*, 2004; Halstvedt *et al.*, 2008). Thus, the microcystin composition was primarily populated by the microcystin-RR variant, while co-existence of different microcystin variants is a common phenomenon (Yépréman *et al.*, 2007; Halstvedt *et al.*, 2008). Our study showed, however, stable proportions among the detected microcystin variants throughout the study. These toxins, especially the most toxic variant, microcystin-LR (Falconer, 2005; Tonk *et al.*, 2005), may severely alter ecosystem function throughout the entire year. Such alterations include allelopathic effects on other phytoplankton (Suikkanen *et al.*, 2004); suppression of zooplankton grazing, which can lead to changes in phytoplankton assemblages (Gilbert, 1990; Ghadouani *et al.*, 2003); hepatotoxic effects on fish (Anderson *et al.*, 1993); and toxin accumulation in the tissues of invertebrates and fish (Liras *et al.*, 1998; Lehtiniemi *et al.*, 2002). However, as intracellular microcystin was primarily detected, we suppose that its influence on living organisms in this lake was limited and largely restricted to organisms feeding on *P. agardhii*.

Overall, GLMM analysis showed that the microcystin concentration simultaneously correlated with a variety of environmental factors. The strongest correlations were detected between the microcystin concentration and temperature, visibility and ammonium nitrogen. These factors could be related to microcystin concentrations by regulating the population densities and metabolism of *P. agardhii* (Sivonen and Jones 1999; Briand *et al.*, 2008b). Previous studies also indicate that populations of *P. agardhii* develop optimally in warm waters, especially during late summer and autumn (Van Liere and Mur, 1980), and they prefer water rich in ammonium nitrogen. Furthermore, a negative correlation between *P. agardhii* biomass and Secchi depth is unsurprising, as *P. agardhii* is known to adapt well to low-light conditions, and self-shading even favors its development (Halstvedt *et al.*, 2007).

However, the *P. agardhii* biomass and the weight-specific microcystin content were not positively related, supporting the notion that microcystin production per unit of *P. agardhii* biomass was relatively stable throughout the entire study. Small declines in weight-specific microcystin content could be associated with a lower contribution from microcystin-producing genotypes, as suggested earlier by Kardinaal and Visser (2005) and

Kardinaal *et al.* (2007). Furthermore, GLM revealed that the microcystin content was negatively related to phosphate levels and water temperature. This relationship seems to be partially consistent with Briand *et al.* (2008a, b), who suggested that a negative correlation between cell abundance and the proportion of microcystin-producing genotypes in a natural *P. agardhii* population can be explained by dominance of microcystin-producing genotypes under limiting conditions. Sivonen and Jones (1999) and Mikalsen *et al.* (2003) also stated that the variation in toxin content per cell may depend on changes in environmental conditions and succession of certain strains differing in toxicity.

Toxin production was not, however, solely determined by either environmental conditions or *P. agardhii* biomass. We found that there was also a significant correlation between filament width and toxin production. The effect of filament morphology was independent of any other factor, given the fact that filament width was incorporated in the best GLM model identified using AIC. We showed that when weight-specific microcystin content was the highest during spring, filaments were widest. Overall, we observed predictable seasonal patterns for microcystin concentration and filament width, as both increased from summer to autumn and from winter to spring. These results are consistent with our previous molecular studies (Mankiewicz *et al.*, 2011), indicating a highest density for the mcy*E* gene, also observed in spring. Differences in filament morphology and microcystin production have been related to a patchy distribution of *mcy* genes in *P. agardhii* populations (Kurmayer *et al.*, 2004). Until now, little was known about the regulation of these genes and their relationship with microcystin pools and filament morphology. Common mechanisms explaining filament variations in morphology and toxicity may be related to environmental conditions. It has already been shown that the same strain grown under different conditions will differ remarkably in cell dimensions (Laamanen *et al.*, 2001). Thus, it is very likely that the same environmental conditions favorable for toxic strain development also have a pronounced effect on filament dimensions. Further experimental studies are, however, necessary to examine whether filament morphology is related to toxin production, and whether there is a physiological mechanism that could explain this relationship. Nonetheless, wider *P. agardhii* filaments may indicate higher microcystin production.

In conclusion, we showed that microcystin concentrations were positively related to the biomass of *P. agardhii*, a dominant species in phytoplankton communities. However, the weight-specific microcystin content and the *P. agardhii* biomass were not related. These findings support the notion that microcystin production per unit of *P. agardhii* biomass was relatively stable throughout the entire study, except for the unfavorable conditions during late autumn and early spring, when microcystin-producing genotypes likely dominated. Among the environmental factors, temperature, visibility, phosphates and ammonium nitrogen were most strongly related to microcystin production via regulating the population densities, metabolism and strain selection for *P. agardhii*.

In addition, the positive correlation between microcystin production and filament width suggests that there might be different morphotypes within toxic and non-toxic *P. agardhii* populations. However, more studies are necessary to discern whether filament morphology is a useful indicator of toxicity in *P. agardhii* populations.

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