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

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Received 13 January 2011; Accepted 30 May 2011

**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 μg.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 μg.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.
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 adenyl-ation 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; Vépřémian 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 Pouličková, 2003; Pouličková 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 Lubośskie (52°31.40′N, 16°22.57′E) with a surface area of 0.23 km². 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-α 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 sus-
pended 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 11000 × 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) C18 solid-phase extraction (SPE) cartridges
(sorbent mass: 500 mg). Microcystins were eluted from the
C18 cartridges by 3 mL of 90% aqueous methanol con-
taining 0.1% trifluoroacetic acid (TFA). The eluates
were evaporated to dryness and the samples were redis-
solved in 1 mL of 75% aqueous methanol before
HPLC analysis (Jurczak et al., 2005). Microcystin con-
centration was determined using a gradient mobile phase
including H2O + 0.05% TFA (elucent A) and acetonitrile
(ACN) + 0.05% TFA (elucent 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 concen-
tration of intracellular microcystin (the microcystin con-
centrations within cyanobacterial cells per liter of lake
water (μg.L⁻¹)) and *P. agardhii* biomass per liter of lake
water (μg.L⁻¹) was calculated.

**Data analysis**

We first examined whether the microcystin concentra-
tion and the weight-specific microcystin content demon-
strated 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 cor-
related with environmental factors, season and *P. agardhii*
filament morphology.

Before the regression analyses, we tested whether the
measured environmental variables showed strong inter-
correlations. As all pairwise correlations between the vari-
able 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 vari-
ation 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 (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
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 \(\mu\text{g.L}^{-1}\) 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 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).
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 *Planktothrix 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, *Planktothrix 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 *Planktothrix agardhii* was the only source of toxin in this lake. We emphasize that intracellular microcystins were present throughout the entire study period, while among...
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 Ye´ pre´ man 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 μg 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 (Yépre´ man 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

Table 1. Results of the GLMM analysis for analyzing microcystin concentrations (μg 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.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>B</th>
<th>Std. Error</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>309.2</td>
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<td>Temp.</td>
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<td>0.0001</td>
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<tr>
<td>pH</td>
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<tr>
<td>Cond.</td>
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<td>22.28</td>
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<tr>
<td>N-NO3</td>
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<td>15.48</td>
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<tr>
<td>TP</td>
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<td>TN</td>
<td>2.41</td>
<td>0.68</td>
<td>0.424</td>
<td></td>
</tr>
</tbody>
</table>

*SD, Secchi depth; Temp., temperature; TP, total phosphorus; TN, total nitrogen; P-PO4, phosphates; N-NH4, ammonium nitrogen; N-NO3, nitrate nitrogen; B, regression coefficient.

Table 2. Results of GLM analysis for weight-specific microcystin content (μg mg⁻¹) and explaining environmental factors (bold indicates significance at P < 0.05). The variables were selected using Akaike’s information criterion. R²-values refer to the amount of variation jointly explained by the selected variables.

<table>
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<th>Parameters</th>
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<th>Std. Error</th>
<th>T</th>
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</tr>
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<td>-5.16</td>
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<tr>
<td>Filament width</td>
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<td>0.13</td>
<td>3.97</td>
<td>0.001</td>
</tr>
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</table>

*Cond., conductivity; Temp., temperature; P-PO4, phosphates; B, regression coefficient; Std._Error, standard error; T, test variable; P, significance.
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 mcyE 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.

Acknowledgements. This work was supported by the Polish Ministry of Science and Higher Education through research grant no. N 304 051 31/1855. The comments of the anonymous reviewers are greatly appreciated.

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