Spatial and temporal variation in microcystin concentrations during perennial bloom of *Planktothrix agardhii* in a hypertrophic lake

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Temporal and spatial variation in the concentrations of intra- and extra-cellular microcystins were studied in a hypertrophic lake with bloom of *Planktothrix agardhii* (Gomont) Anagnostidis et Komarek. Concomitantly with increase in water temperature (from 2 to 20 °C) abundance of *P. agardhii* increased from 1.9×10^5 to 4.3×10^7 trichomes L^{-1}. In autumn, in spite of temperature lower (14°C) than in summer it was still very high. Mass development of *P. agardhii* (to 6×10^6 L^{-1} and higher) caused a severe decrease in water transparency (to 0.5 - 0.2 m in summer/autumn). The cyanobacterium density was relatively uniform within water column; only in summer (July) it was significantly higher (by about 30%) in surface than in bottom layer. From spring to autumn microcystins (MCs) were mainly biomass-bound (up to 90 µg MC-LR equiv. L^{-1}), whereas the level of extra-cellular toxins was much lower (up to 2 µg L^{-1}) and relatively stable. Only in winter, high amounts of MCs (11.3 µg L^{-1}) were released from decaying biomass into water. The increasing concentrations of biomass-bound microcystins in the lake water positively correlated ($R^2 = 0.9863$; $y = -0.1285x^2 + 7.14x$) with the abundance of *P. agardhii* and the highest concentrations of the intracellular MC fraction were found during the exponential phase of *P. agardhii* growth. In addition, the surface-sampled biomass of *P. agardhii* contained in autumn 2-fold more MCs (2.75 µg MC-LR equiv. *per* 10^6 *P. agardhii* trichomes) than the bottom-sampled one (1.41 µg MC-LR equiv. *per* 10^6 trichomes). This is the first report showing that despite the homogenous distribution of *P. agardhii* in water column of a shallow lake, various seasonal and spatial distributions of both extra-cellular and intracellular fractions of microcystins occur.

Keywords: cyanobacterial bloom, hypertrophic lake, intra- and extra-cellular microcystins, *Planktothrix agardhii*

Introduction

In European shallow turbid lakes and reservoirs a dominance of the filamentous cyanobacterium *Planktothrix agardhii* (Gomont) Anagnostidis et Komarek has been frequently reported (Rűcker et al. 1997, Wiedner et al. 2002, Nixdorf et al. 2003, Pawlik-Skowrońska et al. 2004, Akcaalan et al. 2006). *P. agardhii* is a common hepatotoxin producer with higher microcystin (MC) content *per* dry weight than *Microcystis* spp (Fastner et al. 1999a). As a consequence of genotypic differentiation *Planktothrix* populations consist of many clonal strains with considerable variability of contents and numbers of structural microcystin variants (Fastner et al. 1999b, Laub et al. 2002, Welker et al. 2004). *P. agardhii* may pro-

duce at least 25 different isoforms of microcystins that are heptapeptides (Mbedi et al. 2005) and within one population non-producers and microcystin-producers, which are morphologically identical, usually coexist. As reported by Welker et al. (2004) 18 strains of *P. agardhii* were isolated from a single sample of lake water, but only in 7 of them microcystins were detected. As recently established, one clonal strain may produce several various MC variants of different toxicity (Tonk et al. 2005). Therefore, in natural water bodies species/strain composition of cyanobacterial communities is largely responsible for total microcystin concentration. Several laboratory studies revealed that microcystin production may also vary within a range of environmental factors like nutrient concentration, light and temperature (Watanabe & Oishi 1985, Sivonen 1990, Kaebernick et al. 2000, Wiedner et al 2003). The aim of this paper was

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to study seasonal dynamics and spatial distribution of biomass-bound (intracellular) and extra-cellular microcystins in a "phytoplankton-dominated", hypertrophic lake with perennial blooms of *P. agardhii*.

Material and methods

Study area and sampling

Shallow Syczyńskie Lake (51° 17´ 12´´ N; 23° 14´ 16´´ E) is located in Eastern Poland (Polesie Lubelskie region). Morphometric parameters of the lake (max depth 2.9 m; mean depth 0.9 m) has been previously published (Kornijów & Pęczuła 2005). Water samples were taken from the lake surface and bottom (10-15 cm above the sediment) at the depth 2.4 -2.8 m in March - October 2004. In winter, surface layer of lake sediment was additionally sampled. Aliquots of water samples were analysed for ammonia, nitrate and orthophosphates by spectrophotometric methods. Temperature, pH, conductivity, oxygen saturation and water transparency (Secchi depth) were also measured.

P. agardhii abundance

Potentially toxic cyanobacteria in the lake water were identified by light microscopy. Abundance of *P. agardhii* was determined according to Utermőhl (1958) method and has been given in terms of trichomes L^{-1}. 100μm trichome was used as a reference. Dry weight of the surface scum consisted of *P. agardhii* was determined after overnight drying at 90 °C.

Determination of total microcystins by GC/MS

Due to possible presence of numerous different variants of microcystins in *P. agardhii* biomass, their total concentrations in a filtered lake water (extra-cellular MCs) and in a phytoplankton biomass (biomass-bound fraction) were determined by gas chromatography /mass spectrometry according to Harada et al. (1996) and Kaya & Sano (1999). The method is based on oxidation of Adda (amino-acid responsible for biological activity of microcystins) to 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) and determination of MMPB as methyl ester. Phytoplankton biomass was separated from 0.5 - 1 L of the lake water by its concentration on GF/C filters (Whatman, UK) to 1 ml. Filtered water samples and phytoplankton biomass were kept at -20 °C prior extraction for microcystin analysis. Both the biomass and filtered and evaporated   (at 40 ºC) lake water samples were extracted with acidified 75% methanol . The biomass was additionally disrupted by means of ultrasonication

(3- times for 5 min, 50 W) and extracts were collected. The obtained extracts were evaporated under inert gas; sub-samples were oxidised with $NaIO_4$ and $KMnO_4$, extracted with n-hexane and derivatised with 14% BF_3-methanol (Kaya & Sano 1999; Tsuji et al. 2001). As a modification, phenylbutyric acid (PB) was used as internal standard after oxidation step. Derivatised samples were dissolved in n-hexane and 1 μl samples in organic phase were subjected to GC/MS analysis (Saturn 2000, Varian). VA-5MS capillary column (30 m x 0.25 mm; 0.25 μm) was used. In EI-MS mode the identification and quantification of MMPB methyl ester was based on ions at m/z 91, 131 and 190; for PB methyl ester 91, 104 and 146 m/z were used. Identification of MMPB and PB was confirmed by CI-MS at m/z 191 and 147, respectively. Acetonitrile was used as reagent gas. For microcystin quantification standard MC-LR (Calbiochem) was used. Total microcystin concentrations were expressed as equivalents of MC-LR. Determinations were made in triplicates.

Statistical analyses

Statistical tests were carried out using the software package STATGRAPHICS plus 5.0. The data were tested for homogeneity of variance and normality and subjected for one-factor analysis of variance (ANOVA). Differences between means were determined by Tukey's *post hoc* multiple range test at P<0.05 for this procedure. The variation about means is displayed graphically as ± the standard error of the mean (SE).

Results

Such habitat characteristics of lake Syczyńskie like shallowness and high contents of dissolved nutrients (Table 1) were the factors that supported mass development of the typical of turbid water, N_2 non-fixing cyanobacterium *P. agardhii* (Fig.1A). Throughout the year of study, the temperature, pH and conductivity did not differed between surface and bottom layers of the lake water, however, bottom layers contained higher concentrations of dissolved orthophosphates and ammonium ions and lower oxygen saturation than the surface layers (Fig.1B, Table 1). The very high density of *P. agardhii* occurred in water column throughout the year and increased from 1.9×10^5 trichomes L^{-1} in winter (March) to 4.3×10^7 trichomes L^{-1} by September (Fig.1A). Vertical distribution of *P. agardhii* in the lake was almost uniform from winter to autumn. Microscopic observations revealed that in the ice-covered lake numerous trichomes of *P. agardhii* over-wintered on the   lake sediments. Significantly higher density (by about 30 %)

Table 1. Annual average values and ranges (*) of some physico-chemical parameters in different water layers of lake Syczyńskie (2004).

| Parameter	Surface layer	Bottom layer
pH	8.3	8.3
	*(8.0 - 8.7)	*(8.0 - 8.7)
Conductivity (µS/cm)	548	527
	*(511 - 599)	*(503 - 551)
Oxygen saturation	123.7	85.8
(%)	*(111 - 133.5)	*(81.5 - 91)
NH_4-N (µM)	33.4	42.8
	*(18.1 - 87.5)	*(20.2 - 105.8)
NO_3-N (µM)	< 71.4	< 71.4
PO_4-P (µM)	2.0	3.2
	*(0.2 - 8.2)	*(0.5 - 10.8)

Fig. 1. Differences in: (A) spatial distribution of *P. agardhii* in lake Syczyńskie; (B) temperatures of surface and bottom water layers; (C) water transparency. Asterisk symbol (*) in A indicates significantly higher abundance of the cyanobacterium in surface layer (at P<0.05).

in surface than in bottom water layer was observed only in July. Water transparency and light conditions in water column decreased considerably from 0.9 m (May) to 0.2 - 0.3 m (July-October) at increase in water temperature from 16 to 20 ºC (Fig. 1C) and approximate 210 - fold increase in the density of *P. agardhii* population (Fig.1A).

Taxonomic analysis of phytoplankton in this lake revealed that population of *P. agardhii* was the main and almost only source of microcystins throughout the year, with exception of the period June /July, when several *Anabaena* spp - potential MC producers occurred. *Anabaena* spp abundance was, however, 20 - 40- fold lower than that of *P. agardhii* (data not shown) and completely disappeared already in August when *P. agardhii* abundance was still very high (Fig.1A). Monthly changes in microcystin concentrations (both extra-cellular and biomass-bound fractions) in the lake are presented in Fig. 2A. In March, at the lowest water temperature, the highest extra-cellular microcystin concentration (approx.11 µg MC-LR equiv. L^{-1}) and low levels of the biomass-bound fraction (approx.1.75 µg MC-LR equiv. L^{-1}) were found. The high level of extra-cellular MCs observed in winter decreased 5-times with increasing temperature (from 2 to 12°C) in spring and stayed relatively constant (1-2 µg MC-LR equiv. L^{-1}) in summer and autumn. At increasing densities of *P. agardhii* from May to July increasing concentrations of biomass-bound microcystins in the lake water were observed (Figs.1A, 2A). In summer and autumn the biomass-bound microcystins always prevailed over the extra-cellular MC fraction. Even in early autumn, when water temperature decreased from 20 to 14 ºC the level of the intracellular microcystins was high and relatively stable. Interestingly, during logarithmic growth of *P. agardhii* in
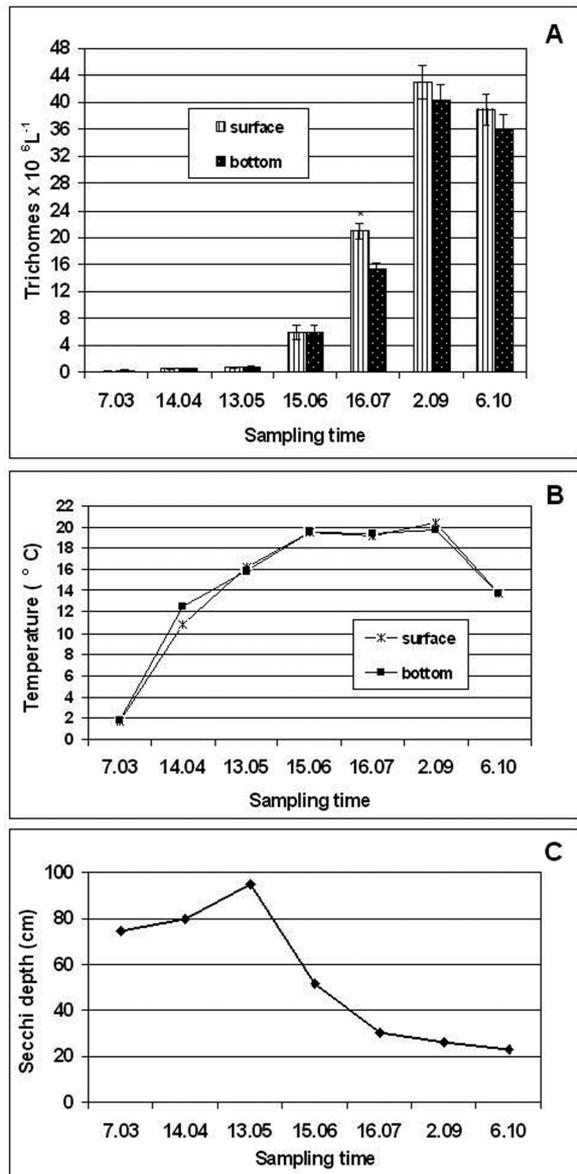
May-July, while water transparency decreased from 0.9 to 0.3 m, the cyanobacterial biomass predominated by *P. agardhii* contained more microcystins (4.7 – 7.0 µg MC-LR equiv. *per* 10^6 *P. agardhii* trichomes) than the biomass sampled in other studied seasons (Fig. 2A).
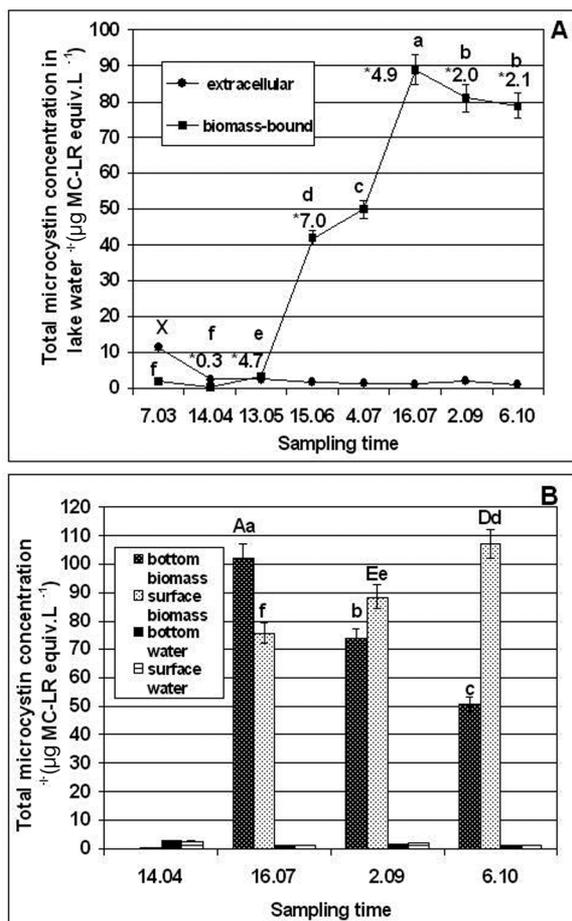
Fig. 2. Changes in the average concentrations of microcystins (biomass-bound and extra-cellular fractions) in water column of the lake (A); distribution of the biomass-bound and extra-cellular microcystins in different water layers (B). All data are expressed as means ± SE, n=3-4. 2A- asterisk symbols (*) indicate the biomass-bound MCs referred to 10^6 trichomes of *P. agardhii*; different lower case letters (abcdef) indicate significant differences between contents of the biomass-bound MCs in lake water (at P<0.05); X designates statistically higher concentration of extra-cellular MCs in winter than in other seasons (at P<0.05). 2B – letters designate significant differences in concentrations of the biomass-bound MCs found at different sampling terms (at P<0.005) in groupings: abc (bottom-sampled biomass); def (surface-sampled biomass); ADE designate significantly higher concentrations of the biomass-bound MCs in the particular water layer.

More detailed studies on spatial distribution of microcystins in water column carried out from spring to autumn (Fig. 2B) revealed non-essential differences in extra-cellular MC concentrations between bottom and surface water layers. However, in summer and autumn, at very low water transparency (≤ 0.2 m) caused by the very

dense bloom of *P. agardhii*, the cyanobacterial biomasses sampled from bottom and surface water layers differed in microcystin contents. During four months of the very dense bloom of *P. agardhii* (July - October) the intracellular MC content in bottom-sampled biomass gradually decreased but in the surface-sampled one it increased (Fig. 2B). In October, when *P. agardhii* vertical distribution in water column was almost uniform (Fig.1A), the surface-sampled biomass of the bloom contained approx. 2-fold more microcystins (2.75 µg MC-LR equiv. *per* 10^6 *P. agardhii* trichomes) than the bottom-sampled one (1.41 µg MC-LR equiv. *per* 10^6 trichomes).

Discussion

In nutrient-rich, shallow, freshwater bodies population of the microcystin producer *P. agardhii* may reach very high densities being a predominant cyanobacterial species in summer/autumn seasons (Wiedner et al. 2002). The studied lake Syczyńskie was predominated by filamentous cyanobacteria (Oscillatoriales) with *P. agardhii* reaching in summer/autumn about 80 % of the phytoplankton biomass and 96% of the abundance of all potentially toxic cyanobacteria (Wiśniewska et al. 2007). The non-toxic *Limnothrix redekei* Van Goor predominated only in early spring (Kornijów & Pęczuła 2005, Wiśniewska et al. 2007). In a shallow eutrophic French lake and in a dam reservoir in Poland densities of *P. agardhii* reached 4.5×10^6 trichomes L[-1] (Briand et al. 2002) and 8×10^6 trichomes L[-1] (Pawlik-Skowrońska et al. 2004), respectively. In the lake Syczyńskie, which is extremely rich in ammonium ions, *P. agardhii* created perennial, dense bloom of even higher density (4.3×10^7 trichomes L[-1]). This toxin-producing cyanobacterium is able to adapt to low light and low temperatures (Rücker et al. 1997) and therefore it may occur at high densities in winter both in water and sediments of the ice-covered lakes. Similar observation was reported for another potentially toxic cyanobacterium *Microcystis* (Verspagen et al. 2004).

The highest concentration of extra-cellular microcystins found in the water of the lake Syczyńskie in winter, at the lowest *P. agardhii* abundance, suggests, that the majority of the summer population of the cyanobacterium decayed in the coldest season and microcystins were released into water and lake sediments. Under field conditions microcystins are normally considered to be confined within cyanobacterial cells and enter the surrounding water after cell death, as it has been recently proved under laboratory conditions in *Microcystis aeruginosa* (Rohrlack & Hyenstrand 2007). Extra-cel-

lular MCs in the lake water were relatively persistent and even in spring (at temperatures up to $12°C$) they quantitatively dominated in the lake water over the biomass-bound fraction. The population of *P. agardhii* in Lake Syczyńskie was able to produce high amount of microcystins (approx.1.1 mg MC g $^{-1}$ DW of surface scum; Wiśniewska et al. 2007) that was however, 2-times lower than the MC content (2.8 mg MC g^{-1} DW) reported for a seston of *P. agardhii*-dominated lake in Germany (Wiedner et al. 2002). It can be explained by the fact that even in one population of *P. agardhii* several strains of different genotypes and abilities to produce microcystins occur (Kurmayer et al. 2004). Morphologically identical genotypes of *P. agardhii* may differ also in other features like ability to produce stronger or weaker gas vesicles (Beard et al., 2000), what may cause different distribution of the cyanobacterium and its toxins in water column. To the best of our knowledge, very little is known on spatial and temporal distribution of microcystins in water layers of hypertrophic, polymictic lakes (Park et al., 1998), especially, dominated by *P. agardhii* blooms. The extra-cellular MC concentrations found in the Lake Syczyńskie in summer were very low in comparison with the biomass-bound toxins and similar to those reported in the hypertrophic lake Suwa (Japan) with *Microcystis* bloom (Park et al.1998) and in the Langer See in Germany (Wiedner et al. 2002). As stated in this work, *P. agardhii* bloom did not collapse at decreased water temperatures in early autumn, and *P. agardhii* trichomes died only partially in frosty winter. Taking into account the level of the biomass-bound MC in autumn (about 80 μg MC-LR equiv. L^{-1}) and the extra-cellular MC concentration in winter (11 μg MC-LR equiv. L^{-1}), it may be supposed that only about 14 % of the intracellular toxins was released into water during partial bloom decay. However, such number may be underestimated because the released microcystins can be adsorbed on lake sediments (Rapala et al. 1994, Tsuji et al. 2001) and/or degraded by heterotrophic bacteria (Harada et al. 2004). As presented in this paper, the extra-cellular MC concentration gradually decreased in warmer spring months reaching a constant, low level, most probably, due to increased microbial activity (Harada et al. 2004). However, most MCs produced by *P. agardhii* stayed intracellular until following winter.

Vertical distribution of *P. agardhii* in the studied lake was almost homogenous, whereas MC distribution was not. Surprisingly, in September-October, at quantitatively negligible records of other potentially toxic cyanobacteria (Wiśniewska et al. 2007), *P. agardhii* biomass sampled from the surface and bottom water layers, contained different amounts of microcystins. The surface-sampled biomass of *P. agardhii*, which was better illuminated, contained 2-fold higher amounts of MCs than the self-shaded bottom-sampled one. The observed, for the first time under natural conditions, phenomenon is consistent with recent reports on dependence of MC production in the isolated strain of *P. agardhii* on light intensities (Tonk et al. 2005). It has been found that high light intensities caused increased production of some variants of microcystins, especially MC-DeLR, which is more toxic than other MC variants. Similar observation was reported in the case of *Microcystis* strain PCC 7806 and *M. aeruginosa* (Kaebernick et al. 2000, Wiedner et al. 2003, Gerbersdorf 2006) in that photosynthetically active radiation revealed a positive effect on toxin production. Competition for light is an important selective factor in phytoplankton communities in eutrophic waters and it has been recently experimentally proved between toxic and non-toxic strains of *Microcystis aeruginosa* (Kardinaal et al. 2007). The higher content of intracellular MCs found in the surface-sampled biomass of *P. agardhii* than in the bottom-sampled one may also suggest that MC can serve, to some extent, for protection against exceeded intensity of UV/Vis radiation. Such assumption is supported by reports showing that increased transcription of MC genes responsible for MC synthesis occurs not only under increasing light intensity (Kaebernick et al. 2000) but also under prolonged exposure to UV radiation (Kim et al. 2005). As reported by Van Donk et al. (2001) *Planktothrix* sp growth was not inhibited by UV radiation and was much more UV- resistant than that of picocyanobacteria and other small algae which do not produce microcystins .

The obtained results show that in shallow, polymictic lakes, despite almost uniform, vertical distribution of *P. agardhii*, the cyanobacterium-produced microcystins have different temporal and spatial distribution. The extra-cellular microcystins quantitatively dominate in colder seasons whereas the biomass-bound toxins in warmer seasons and thus they may variously influence lake biocenosis.

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