

**Morphological,
genetic, chemical
and ecophysiological
characterisation of two
Microcystis aeruginosa
isolates from the Vistula
Lagoon, southern Baltic***

OCEANOLOGIA, 52 (1), 2010.
pp. 127–146.

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Oceanology PAS.

KEYWORDS

Microcystis aeruginosa
Cyanobacteria
Microcystin,
mcy,
Vistula Lagoon

HANNA MAZUR-MARZEC*
GRAŻYNA BROWARCZYK-MATUSIAK
KAROLINA FORYCKA
JUSTYNA KOBOS
MARCIN PLIŃSKI

Department of Marine Biology and Ecology,
Institute of Oceanography,
University of Gdańsk,
al. Marszałka Piłsudskiego 46, PL-81-378 Gdynia, Poland;

e-mail: biohm@univ.gda.pl

*corresponding author

Received 19 November 2009, revised 24 February 2010, accepted 4 March 2010.

Abstract

The Vistula Lagoon (southern Baltic Sea) is a shallow and highly eutrophic water body, with frequent blooms of cyanobacteria dominated by *Microcystis* and *Anabaena* species. Two *Microcystis* strains, MK10.10 and MAKR0205, isolated from the lagoon were characterised in this work. The morphology of the isolates differed significantly with respect to cell size and their ability to form aggregates. Based on the 16S rRNA sequence and 16S-23S internal transcribed spacer (ITS) sequence, both isolates were classified as *Microcystis aeruginosa*. However, only one isolate, MK10.10, possessed the *mcy* genes responsible for microcystin biosynthesis and only this strain produced microcystins. The effects of environmental factors, such as light, temperature and salinity, on toxin production turned out to be minor. Under

* This work was supported by the Office of the Marshal of the Pomeranian Voivodship in Poland.

the culture conditions used in the experiments, the biomass of the toxic MK10.10 was always lower. Hybrid quadrupole-time-of-flight liquid chromatography/tandem mass spectrometry (QTOF-LC/MS/MS) was used to elucidate the structure of the microcystin (MC) variants produced by MK10.10. Based on molecular ion and fragmentation spectra, the toxins were identified as MC-LR, MC-VR and MC-HLR. Our study confirmed that some morphological criteria could be useful in preliminarily assessing the potential toxicity of a *Microcystis* bloom.

1. Introduction

Cyanobacteria of the genus *Microcystis* (Chroococcales) belong to the most common bloom-forming microorganisms in many freshwater bodies worldwide. They also occur in coastal areas of the brackish Baltic Sea (Mazur et al. 2003, Rybicka 2005, Paldavičienė et al. 2009). In eutrophic waters, with high nutrient loading and low water exchange, the mass development of *Microcystis* is promoted by warmer temperatures (Paerl & Huisman 2008, Davis et al. 2009). The *Microcystis* genus is characterised by spherical cells (2-8 μm) containing gas vesicles. In the natural environment *Microcystis* always forms colonies whose size can reach up to a thousand cells. In laboratory cultures these cyanobacteria usually disaggregate and exist as single cells. Based on cell size, colony shape, sheath characteristics and the number of cell division planes, several morphospecies have been distinguished: *M. aeruginosa* (Kützing) Kützing, *M. botrys* Teiling, *M. flos-aquae* (Wittrock) Kirchner ex Forti, *M. ichthyoblabe* Kützing, *M. viridis* (A. Braun in Rabenhorst) Lemmermann and *M. wesenbergii* (Komárek) Komárek in Kondratieva (Komárek & Anagnostidis 1999, Komárek & Komárková 2002). As some of the morphological features of the species show great variability and can overlap, this subgeneric classification has turned out to be rather troublesome and unreliable. Based on sequences of the phycocyanin operon (intergenic spacer between *cpcB* and *cpcA*, IGS) and the 16S-23S-rRNA-DNA internal transcribed spacer (ITS), Otsuka et al. (1999, 2001) postulated the unification of *Microcystis* in a single *Microcystis aeruginosa* complex, within which both toxin-producing and non-toxin producing strains can be found (Rohrlack et al. 2001, Fastner et al. 2001, Kurmayer et al. 2002; Via-Ordorika et al. 2004). *Microcystis*, like several other cyanobacterial genera, produces cyclic peptide hepatotoxins called microcystins (MCs). The toxins contain unusual, non-proteinogenic amino acids and have a general structure of cyclo(-D-Ala¹-L-X²-D-MeAsp³-L-Z⁴-Adda⁵-D-Glu⁶-Mdha⁷), where X and Z represent two variable amino acids, Adda is a C-20 β -amino acid unique to cyanobacteria (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, D-MeAsp is D-erythro- β -methylaspartic acid, and Mdha is N-methyldehydroalanine. Additionally, microcystin variants with demethylated residues at positions 3

and 7 are produced. About 80 analogues of the toxins, with minor structural differences, have been identified (Sivonen & Börner 2008). In *Microcystis* blooms, it is the non-demethylated microcystin forms that are mostly detected (MC-LR, MC-RR and MC-YR). The toxins are strong inhibitors of the eukaryotic protein phosphatases 1 and 2A, and can cause gastroenteritis, liver damage and liver tumours (MacKintosh et al. 1990). Depending on the structure of microcystins, their LD₅₀ values range from 50 µg kg⁻¹ to non-active (Zurawell et al. 2005). The presence of these toxins in drinking and recreational waters has been responsible for many incidents of poisoning in humans and animals (Kuiper-Goodman et al. 1999, Funari & Testari 2008).

MCs are synthesised by a non-ribosomal enzyme complex containing non-ribosomal peptide synthetase and polyketide synthase modules (Tillett et al. 2000). The gene cluster (*mcy*) encoding these enzymes was present in all toxic *Microcystis* isolates analysed thus far by different research groups (e.g. Kurmayer et al. 2002, Yoshida et al. 2003, Via-Ordorika et al. 2004), whereas non-microcystin producing genotypes either lack the genes altogether or possess them in incomplete and inactive form. The frequency of toxic chemotypes differs between species. It was estimated that over 70% of *M. aeruginosa* colonies contained the *mcy* genes involved in microcystin production, while in European strains of *M. wesenbergii* neither MC nor *mcy* were detected (Kurmayer et al. 2002, Via-Ordorika et al. 2004).

The toxicity of a *Microcystis* bloom depends on its biomass, the proportion of toxic and non-toxic genotypes, and the rate of toxin production by individual strains. Environmental factors such as irradiation, temperature, concentration of nutrients and water dynamics can affect the toxicity of a bloom by modifying the population structure and, to a lesser extent, by changing the rate of microcystin biosynthesis (Orr & Jones 1998, Yoshida et al. 2007, Kurmayer & Christiansen 2009).

In this work, two *Microcystis* strains were isolated from the Vistula Lagoon, southern Baltic Sea. The aim of the study was to compare the morphological, genetic and ecophysiological features of the strains. The presence of microcystin synthetase genes and the production of microcystins by the isolates were examined. Mass spectrometry was used to elucidate the structure of the microcystin variants produced by MK10.10.

2. Material and methods

2.1. Sampling and isolation of *Microcystis aeruginosa*

The Vistula Lagoon (southern Baltic Sea) is a highly eutrophic, shallow water basin with an average depth of 2.7 m and maximum depth of 5.2 m. It covers an area of 838 km² and is connected with the open waters of the

Baltic through the narrow Baltyisk Strait. The salinity of the Lagoon ranges from 1 to 4 PSU, so freshwater organisms are prevalent. From June till late September the phytoplankton is dominated by cyanobacteria, mainly of the genera *Anabaena*, *Microcystis* and *Aphanizomenon* (Rybicka 2005).

The two cyanobacterial strains, MAKR0205 and MK10.10, were isolated from the same water sample collected in the Vistula Lagoon off Kały Rybackie (54°20'22"N; 19°13'47"E) on 28 September 2005. The sample had a salinity of 2.1 PSU and a temperature of 16.4°C; the respective cell-bound concentrations of MC-LR and MC-RR in water were 19.4 $\mu\text{g dm}^{-3}$ and 173.5 $\mu\text{g dm}^{-3}$. Using a micropipette, single colonies of *Microcystis* were picked from the phytoplankton sample diluted in sterile BG11 medium (Rippka et al. 1988). These were then rinsed repeatedly in droplets of sterile medium and transferred to culture tubes containing 1 cm^3 of the medium. The isolates were incubated at 20°C and a downwelling irradiance of ca 15 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ using cool white fluorescent light with a photoperiod of 12 h dark:12 h light. To prevent the growth of eukaryotes, 1 cm^3 of cycloheximide (1 mg cm^{-3}) was added to the 50 cm^3 culture. No bacterial contamination was detected during microscopic inspections of the culture. Mean cell diameters were measured using a NIKON ECLIPSE E600 microscope and a Digital Sight DS-V1 camera with a calibrated micrometer scale. The results are based on the measurements of 115 cells from each isolate.

2.2. Culture experiments

Cyanobacterial batch cultures were grown in 250 cm^3 Erlenmeyer flasks containing 100 cm^3 of modified BG-11 medium based on MilliQ water with different amounts of NaCl added to produce final salinities of 0, 3.5, 7 and 14 PSU. These cultures were grown at an irradiance of 30 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ and a temperature of 22°C. Additionally, the effect of light and temperature on growth and toxin production was determined. Three different irradiances (10, 30, 80 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$, measured with a flat sensor at the culture surface) and two temperatures (22°C and 30°C) were used. The cultures were grown at 3.5 PSU. The experiments were initiated by the addition of an inoculum (10 cm^3) that had been acclimated for 21 days at the relevant light, temperature and salinity. The cultures were grown in duplicates for 3 weeks in culture chambers. Dry weight and toxin concentration were determined at the beginning of the experiment and every 7 days thereafter. For this purpose, *M. aeruginosa* cultures (1–10 cm^3) were filtered through pre-weighed Whatman GF/C filters. The filters and the cell pellets were dried overnight at 90°C and then re-weighed. For toxin analysis, the filters with cyanobacterial material were placed in

Eppendorf tubes and stored at -20°C . After thawing, a 2 cm^3 portion of 90% methanol was added. The extracts were prepared by 15 min bath sonication followed by 1 min probe sonication with an ultrasonic disrupter (HD 2070 Sonopuls-Bandeline, Berlin, Germany). After centrifugation at $10\,000 \times g$ for 15 min, the supernatant was transferred to a glass vial and evaporated under a stream of nitrogen gas at 50°C . The residuals were redissolved in 1 cm^3 of 30% methanol and analysed using high-performance liquid chromatography (HPLC) and liquid chromatography combined with tandem mass spectrometry (LC-MS/MS).

2.3. Analysis of MCs produced by *M. aeruginosa* strain MK10.10

2.3.1. HPLC

A Waters HPLC system (Waters, Milford, MA, USA) equipped with a model 996 photodiode-array detector was used. Samples (0.02 cm^3) were injected using a Waters 917plus autosampler. Components of the sample were separated on a Waters Symmetry RP-18 column ($5\ \mu\text{m}$; $150\text{ mm} \times 3.9\text{ mm}$ I.D.). Gradient elution with the mobile phase delivered at $1\text{ cm}^3\text{ min}^{-1}$ and consisting of 97.5% water (eluent A) and 100% acetonitrile (eluent B), both containing 0.05% TFA, was used. The initial condition was 78% eluent A for 1 min; then the proportion of eluent B was increased linearly during 15 min to 70% and held for 1 min. After that, the proportion of B was increased to 100% and held for 2 min. Then, the initial mobile phase composition was reached within 5 min. MCs were tentatively identified by comparison of their retention times and UV spectra (λ_{max} at 238 nm) with standards of MC-LR, MC-RR and MC-YR purchased from Alexis Biochemicals (San Diego, USA).

2.3.2. LC-MS/MS

The structure of MCs produced in the culture by the toxic *M. aeruginosa* strain MK10.10 was characterised by LC-MS/MS. The analytical system consisted of a QStar Elite hybrid quadrupole-time-of-flight (Q-TOF) MS/MS with turbo ion spray (Applied Biosystems MDS Sciex, Concord, ON, Canada) and an Agilent 1200 HPLC (Agilent Technologies, Waldbronn, Germany). Separation was performed on a Synergy Fusion-RP18 column ($5\ \mu\text{m}$; $50\text{ mm} \times 2.0\text{ mm}$) (Phenomenex, Torrance, CA, USA). Gradient elution with a mixture of mobile phase A (5% acetonitrile containing 0.1% formic acid) and B (100% acetonitrile containing 0.1% formic acid) was used. Phase B was linearly increased from 0% to 100% during 7 min, held for 3 min, then brought back to 0% B during 1 min. The column oven temperature was 35°C , the flow rate was $0.3\text{ cm}^3\text{ min}^{-1}$ and the injection

volume was 0.005 cm³. Mass spectra were acquired over the 100–1100 Da range with a scan time of 1.0 s. The Q-TOF instrument was operated in positive ion mode. The turbo-ion spray (400°C) voltage was 5.5 kV, with the nebuliser and curtain gas pressures set at 25 and 30 psi (1 psi = 6894.76 Pa) respectively. Structural elucidation was achieved using collision-induced dissociation (CID) with a collision energy of 50 eV and a nitrogen collision gas pressure of 6 psi. Data were acquired and processed using Analyst QS 2.0 software.

2.4. Genetic analysis

The cultures of MAKR0205 and MK10.10 were centrifuged at 10 000 × *g* for 20 min. After removal of the supernatant, the cells were frozen at –20°C and then thawed. Freezing and thawing were repeated three times. After that, the samples were sonicated in an ultrasonic bath for 30 min, then frozen and stored at –20°C till further analysis. Total nucleic acids were extracted from the cells of both isolates using the protocol described in the Genomic Mini AX Plant isolation kit (A&A Biotechnology, Gdynia, Poland). The 16S rRNA fragments (about 1200 bp) were amplified using the universal bacterial primers 16S27F and the cyanospecific reverse primer

Table 1. Primers used in this study

Gene region and primer	Sequence	References
16S rRNA		
16S27F	AGAGTTTGATCCTGGCTCAG	Wilmotte et al. 1994
23S30R	CTTCGCCTCTGTGTGCCTAGGT	Taton et al. 2003
ITS		
322Fa	TGTACACACCGCCCGTC	Iteman et al. 2000
340Ra	CTCTGTGTGCCTAGGTATCC	
<i>mcyA</i>		
MSF	ATCCAGCAGTTGAGCAAGC	Tillett et al. 2001
MSR	TGCAGATAACTCCGCAAGTTG	
<i>mcyB</i>		
2156-F	ATCACTTCAATCTAACGACT	Mikalsen et al. 2003
3111-R	AGTTGCTGCTGTAAGAAA	
<i>mcyC</i>		
PSCF1	GCAACATCCCAAGAGCAAAG	Ouahid et al. 2005
PSCR1	CCGACAACATCACAAAGGC	
<i>mcyE</i>		
mcyE-F2	AGAGTTTGATCCTGGCTCAG	Iteman et al. 2000
MicmcyE-R8	CTTCGCCTCTGTGTGCCTAGGT	

23S30R. Amplification of the 16S-23S rRNA (ITS) gene (348 bp) was performed with primers as described previously by Itean et al. (2000). The presence and variability of the four following regions of the *mcy* gene cluster were studied: *mcyA* (291 bp), *mcyB* (758 bp), *mcyC* (674 bp) and *mcyE* (812 bp). Primers to amplify these fragments were designed based on sequence information of the gene cluster. The primers and their sequences, as well as the references describing the conditions of amplification reactions that were applied in this study, are given in Table 1. The DNA amplification reactions were conducted in the Mastercycler personal thermocycler (Eppendorf, Hamburg, Germany).

The PCR mixture contained 0.01 cm³ of 2 × PCR mix (0.1 U/10⁻³ cm³ Taq DNA polymerase, 4 mM, MgCl₂, 0.5 mM dNTPs; Fermentas, Germany and A&A Biotechnology, Poland, Sigma-Aldrich), 0.001 cm³ of each primer, 0.007 cm³ of MilliQ water and 0.001 cm³ of the *Microcystis* DNA to give a final volume of 0.02 cm³. The PCR products were analysed by electrophoresis on 1.0% agarose gel in 1 × TBE (Tris-borate-EDTA) buffer. The bands were stained with ethidium bromide and documented with a Nikon camera. The lengths of DNA fragments were estimated by comparison with two markers: 'Ideal' (DNA, Gdańsk, Poland) and M100-1000 (A&A Biotechnology, Gdynia, Poland). The slices of agarose gel containing the stained PCR products were cut out. DNA was isolated and purified using Gel-Out and Clean-Up kits, according to the protocols supplied by the manufacturer (A&A Biotechnology, Gdynia, Poland). The purified DNA was sent for sequencing to the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, IBB PAS, Warsaw, Poland. Ten microlitres of purified DNA were sequenced with a Big DyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) using an ABI3730/xl Genetic Analyser (Applied Biosystems, Darmstadt, Germany). DNA sequences were aligned using MEGA software, version 4 (Tamura et al. 2007). We compared the 16S rRNA and ITS sequences of the two isolated strains and of five other *Microcystis* strains available in GenBank (<http://www.ebi.ac.uk/clustal/index.html>): *Microcystis aeruginosa* PCC9701 (AM773534), *Microcystis* sp. clone 197 (FJ495667), *Microcystis aeruginosa* NIES-44 (AB254437) (16S rRNA sequences) and *Microcystis* sp. clone 34 (FJ495731), *Microcystis* sp. clone 99 (FJ495802) (ITS sequences). The sequences at the level of nucleotides were analysed using the ClustalW and Gendoc programs. The percentage identity of the 16S rRNA gene and ITS fragments of the *Microcystis* isolates (MAKR0205 and MK10.10) with the cyanobacterial sequences from the GenBank were calculated with the NCBI BLASTn program version 2.2.10.

Nucleotide sequence accession numbers. The nucleotide sequences produced in this study were deposited at EMBL. They were assigned accession numbers FN678904 (16 rRNA) and FN687183 (16S-23S ITS) for sequences from MAKR0205, and FN678905 (16 rRNA) and FN687184 (16S-23S ITS) for sequences from MK10.10.

3. Results

3.1. Morphological features of the isolates

Regardless of the culture conditions, the MAKR0205 strain grew mainly as double (58.5%) and single (23.0%) cells; only a few cells formed aggregates consisting of three (8.8%) or four (9.7%) cells (Figure 1a). The cell diameter ranged from 3.3 μm to 5.5 μm (average $4.4 \pm 0.5 \mu\text{m}$), which classified the strain as small-type (S) (Bittencourt-Oliveira et al. 2001). MAKR0205 cells were surrounded with a mucilaginous envelope approximately 2.6 μm in thickness. Under the same culture conditions, the strain MK10.10 formed elongated colonies, irregular in outline, with various cell densities (Figure 1b). Based on the cell diameter of MK10.10, which ranged from 3.9 μm to 8.6 μm (average $5.9 \pm 1.0 \mu\text{m}$), this strain was classified as large-type (L). The mucilaginous envelope of the colonies was approximately 1.6 μm thick.

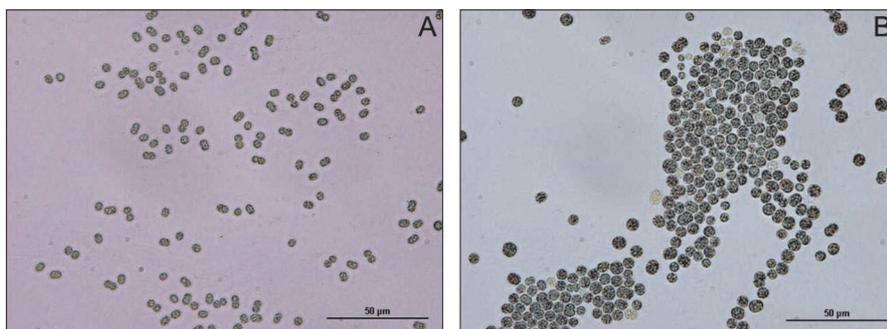


Figure 1. *Microcystis aeruginosa* isolates from the Vistula Lagoon: MAKR0205 (a) and MK10.10 (b)

3.2. Effect of light, temperature and salinity on *Microcystis* isolates

The biomass of the isolates was determined on the basis of dry weight (d.w.) per culture volume measurements. On days 14 and 21 of the experiment conducted in BG-11 medium of different salinity, the highest biomass (2.35 mg cm^{-3}) was achieved by MAKR0205 grown at

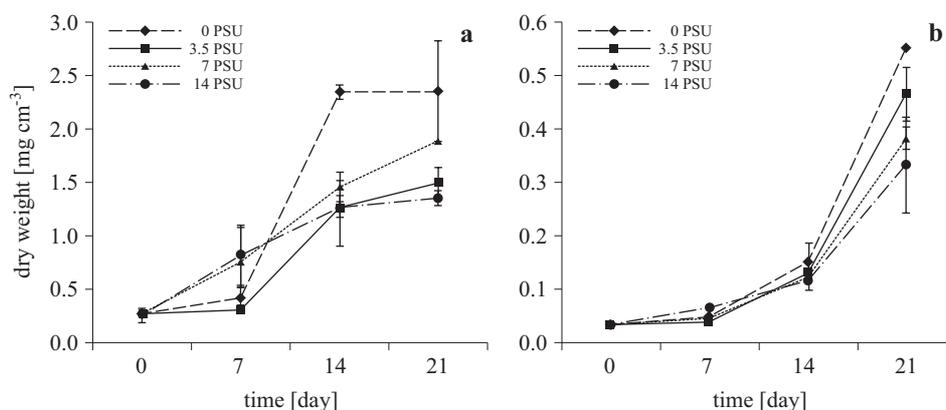


Figure 2. Effect of salinity on the growth of *Microcystis aeruginosa* isolates: MAKR0205 (a) and MK10.10 (b) (cultures incubated at $30 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ and 22°C). Vertical bars indicate standard errors

0 PSU (Figure 2a). In the media of higher salinities, the strain reached maximum d.w. values on the last day of the experiment. They ranged from $1.35 \pm 0.07 \text{ mg cm}^{-3}$ at 14 PSU to $1.90 \pm 0.00 \text{ mg cm}^{-3}$ at 7 PSU. The highest values of MK10.10 d.w. were also measured on the last day of the experiment (Figure 2b): they ranged from $0.33 \pm 0.09 \text{ mg cm}^{-3}$ at 14 PSU to $0.55 \pm 0.00 \text{ mg cm}^{-3}$ at 0 PSU. The effect of salinity on biomass was not significant (at $p > 0.05$; ANOVA test).

The differences in biomass of both *Microcystis* cultures grown under various temperature and light regimes were observed only on the last day (26) of the experiment (Figure 3a). On that day, the d.w. of the

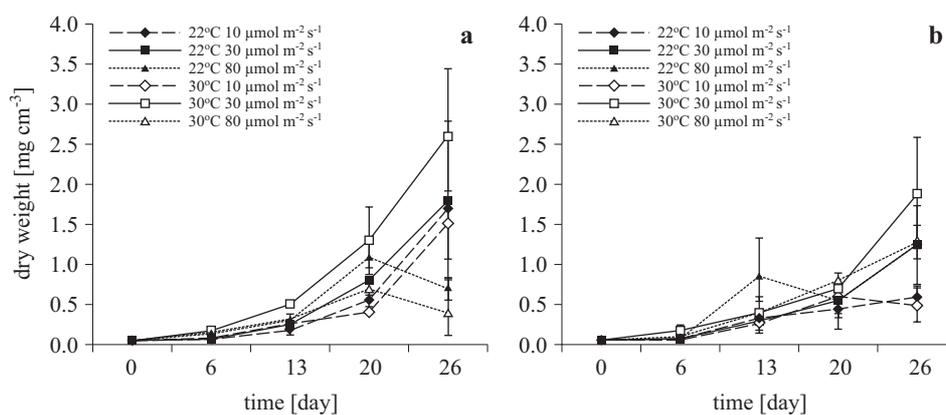


Figure 3. Effects of light and temperature on the growth of *Microcystis aeruginosa* isolates: MAKR0205 (a) and MK10.10 (b) (cultures grown at 0 PSU). Vertical bars indicate standard errors

MAKR0205 strain was the highest ($2.60 \pm 0.85 \text{ mg cm}^{-3}$) at $30 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ and temperature 30°C . On the same day and at the same photon flux density, but at a lower temperature (22°C), the d.w. of MAKR0205 was slightly lower ($1.80 \pm 1.98 \text{ mg cm}^{-3}$). At the highest irradiance, $80 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, the d.w. of MAKR0205 was the lowest. On day 26, it was $0.40 \pm 0.28 \text{ mg cm}^{-3}$ and $0.70 \pm 0.14 \text{ mg cm}^{-3}$ at 30°C and 22°C respectively. At the lowest irradiance, $10 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, the biomass ranged from $1.50\text{--}1.70 \text{ mg cm}^{-3}$ depending on the temperature.

Different tendencies were observed for MK10.10 (Figure 3b). On day 26, the highest d.w. was measured for cultures incubated at the higher temperature (30°C); at 30 and $80 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ it was $1.40 \pm 1.41 \text{ mg cm}^{-3}$ and $1.29 \pm 0.21 \text{ mg cm}^{-3}$ respectively. At 22°C , the MK10.10 biomass was $0.90 \pm 0.99 \text{ mg cm}^{-3}$, regardless of the irradiance used. The lowest d.w. were measured at the lowest irradiance; for the cultures incubated at 22°C and 30°C they were $0.60 \pm 0.57 \text{ mg cm}^{-3}$ and $0.50 \pm 0.42 \text{ mg cm}^{-3}$ respectively. Neither the effect of light nor the effect of temperature was statistically significant (at $p > 0.05$; ANOVA).

3.3. Microcystin production by MK10.10

HPLC analysis of *Microcystis* cell extracts showed that only the MK10.10 strain produced microcystins. Based on the retention time (10.5 min) and the UV spectrum, the toxin was identified as MC-LR. The gravimetric concentration of MC-LR was the highest in the culture with the lowest salinity. The maximum value of $3283.55 \mu\text{g mg}^{-1} \text{ d.w.}$ was recorded on day 14. Production of MC-LR by strain MK10.10 decreased

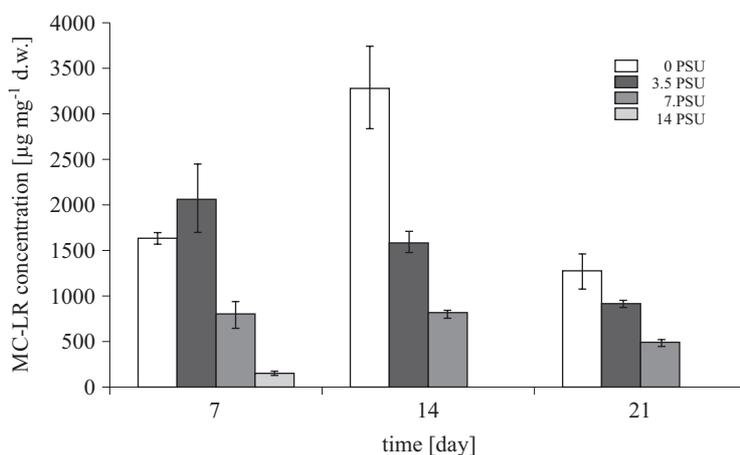


Figure 4. Effects of salinity on production of MC-LR by strain MK10.10. Vertical bars indicate standard errors

with increasing salinity and was the lowest in cultures grown at 14 PSU (Figure 4). At 14 PSU, the concentrations of MC-LR in MK10.10 cell extracts were lower than the detection limit, starting from day 14. Within the range of photon flux densities used in the experiment (10, 30 and 80 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$), irradiance had no significant effect on MC biosynthesis. The average gravimetric concentration of MC in MK10.10 cells grown at 22°C and 30°C was 1025.20 $\mu\text{g mg}^{-1} \pm 174.08 \mu\text{g mg}^{-1}$ d.w. and 574.75 $\mu\text{g mg}^{-1} \pm 102.67 \mu\text{g mg}^{-1}$ d.w. respectively. At 22°C, the concentration was 178.4% higher than the average concentration of the toxin in cells grown at 30°C. The effect of temperature on toxin production was statistically significant (at $p > 0.05$; ANOVA).

LC-MS/MS of the MK10.10 cell extract confirmed the presence of MC-LR with molecular ion $[\text{M}+\text{H}]^+$ at m/z (mass to charge ration) 995. Apart from this toxin, two other MC variants with molecular ions at m/z 981 and 1009 were detected. The m/z 981 ion corresponds to the demethylated MC-LR. However, in the fragmentation spectrum, the ions which in MC-LR contained Leu exhibited a m/z value 14 Da lower. This might indicate the presence of Val instead of Leu, e.g., 229 $[\text{Val-MeAsp}+\text{H}]^+$, 254 $[\text{Mdha-Ala-Val}+\text{H}]^+$, 383 $[\text{Glu-Mdha-Ala-Val}+\text{H}]^+$, 539 $[\text{Mdha-Ala-Val-MeAsp-Arg}+\text{H}]^+$, 556 $[\text{Mdha-Ala-Val-MeAsp-Arg-NH}_2 + 2\text{H}]^+$. The structure of the molecule was elucidated to be MC-VR. The fragmentation spectrum of the m/z 1009 ion gave ions at m/z 135 $[\text{Ph-CH}_2\text{CHOME}]$, 163 $[\text{C}_{11}\text{H}_{14}\text{O}+\text{H}]^+$, 213 $[\text{Glu-Mdha}+\text{H}]^+$, 282 $[\text{Mdha-Ala-Hil}+\text{H}]^+$, 303 $[\text{MeAsp-Arg}+\text{NH}_2+2\text{H}]^+$, 375 $[\text{C}_{11}\text{H}_{14}\text{O-Glu-Mdha}+\text{H}]^+$, 396 $[\text{Hil-MeAsp-Arg}+\text{H-NH}_3]^+$, 411 $[\text{Glu-Mdha-Ala-Hil}+\text{H}]^+$, 567 $[\text{Mdha-Ala-Hil-MeAsp-Arg}+\text{H}]^+$ and 599 $[\text{Arg-Adda-Glu}+\text{H}]^+$. The spectral data indicated the structure to be MC-HilR.

3.4. Genetic characteristics of *M. aeruginosa* isolates

The 16S rRNA and ITS PCR products of the two *Microcystis* isolates, MK10.10 and MAKR0205, from the Vistula Lagoon were sequenced. We analysed a fragment of the 16S rRNA gene (about 1200 bp) and the entire ITS (348 bp). The aligned PCR sequences from MK 10.10 and MAKR0205 were compared with the respective sequences from the five *Microcystis* morphospecies from GenBank. The 16S rRNA sequences of the three *Microcystis* strains – AM773534, FJ495667 and AB254437 – and the ITS sequences of *Microcystis* strains FJ495731 and FJ495802 were unambiguous.

The 16S rRNA regions of the toxic and non-toxic *Microcystis* isolate (MK10.10 and MAKR0205) were 99% similar and showed 97.9–99.8% similarity to the 16S rRNA sequences of the three *Microcystis* morphospecies from GenBank – AM773534, FJ495667 and AB254437.

For each of the two isolated strains, PCR amplification of the intergenic transcribed spacer between the 16S rRNA and 23S rRNA genes yielded one product upon electrophoresis in agarose gels which represented the true ITS region. The ITS sequences of MK10.10 and MAKR0205 were identical; they also showed 97.9–99.8% similarity to the ITS sequences of the two *Microcystis* strains from GenBank – FJ495731 and FJ495802. Using the primers specific to the analysed *mcy* genes (Table 1), respective PCR products were obtained only for the MK10.10 strain. The length of the products corresponded to their expected size.

4. Discussion

4.1. Taxonomic classification

One cyanobacterial genotype can be represented by more than one morphotype (Palińska et al. 1996, Bittencourt-Oliveira et al. 2001). *Microcystis* strains that are very close genetically may show high morphological variability (Otsuka et al. 2000, Bittencourt-Oliveira et al. 2001, Kurmayer et al. 2002). Under varying environmental conditions, both in nature and in cultures, some morphological features, such as colony formation, cell size and gas vesicle production, can be changed or lost (Otsuka et al. 2000). As a result, one strain can form colonies and cells typical of more than one species. Since classification of *Microcystis* based solely on morphology has turned out to be unreliable, genetic methods have to be applied in taxonomic studies. We used the 16S rRNA and 16S-23S ITS sequences to determine that the strains we isolated belonged to *Microcystis aeruginosa*. Generally, the sequence and length of the 16S-23S ITS in bacteria are more variable than the 16S rRNA regions and are used to discriminate between the genotypes. In cyanobacteria of the genus *Microcystis*, however, the 16S-23S ITS regions are still extremely similar and cannot be used to differentiate between toxic and non-toxic species. In the *Microcystis* isolates used in the current study (MAKR0205 and MK10.10) they were identical. Comparison with the same sequence of *Microcystis* strains from GenBank revealed a high level of genetic identity, strongly indicating that both strains belong to one and the same species. In the current study, the genes (*mcy*ABCE) that are necessary for microcystin biosynthesis were detected only in one strain (MK10.10) isolated from the Vistula Lagoon. The other isolate, MAKR0205, did not produce microcystins and apparently did not carry *mcy* genes. The results are in line with earlier findings on the patchy distribution of *mcy* genes among cyanobacteria. Recently, it has been suggested that lateral gene transfer and a series of gene losses might

explain the sporadic distribution of the ability to synthesise MCs in the *Microcystis* genus (Rantala et al. 2004). The proportions of MC-producing and non-producing genotypes have been determined in some water bodies. In the hypertrophic Lake Wannsee in Germany, the proportion of the *mcyB* genotype in the population of *Microcystis* fluctuated between 1 and 38% and did not change much between seasons (Kurmayer & Kutzenberger 2003). According to Yoshida et al. (2007), the proportion of the *mcyA* genotype in the total *M. aeruginosa* population in Lake Mikata, Japan, ranged from 0.5% to 35% and depended on nitrate concentration. The rise in nitrate level resulted in the increase in the *mcyA* genotype abundance. This finding indicates a higher risk of toxic *Microcystis* blooms in eutrophic waters such as the Vistula Lagoon.

4.2. Morphology

Kurmayer et al. (2002), Kurmayer & Kutzenberg (2003) and Via-Ordorika et al. (2004) demonstrated a positive correlation between *Microcystis* morphology and toxin production. The percentage of the *mcy* gene and microcystin was significantly higher in cyanobacteria forming bigger colonies. Kato et al. (1991) categorised *M. aeruginosa* strains into L (large)-type with a cell diameter of 4.58–5.05 μm and S (small)-type with a cell diameter of 3.25–4.19 μm . Later, the L-type cyanobacteria isolates from Lake Wannsee were identified by Rohrlack et al. (2001) as microcystin-producing, while the S-type did not produce the toxins. The results of our studies on *M. aeruginosa* isolates from the Vistula Lagoon confirmed the same relation between morphology and toxin production. Our morphological analysis shows that the microcystin-producing MK10.10 isolate belongs to the L-type and the non-toxic MAKR0205 belongs to the S-type. Additionally, even after four years of growing in culture, MK10.10 still tended to form colonies, unlike MAKR0205, which grows mostly in the form of single cells and for which aggregates larger than four cells have never been observed. The two isolates also differ with respect to the mucilaginous envelope; this was approximately 1 μm thinner in the colonies of the toxic MK10.10 isolate than in MAKR0205. Zhang et al. (2007) also examined colonial and disaggregated cyanobacteria belonging to *M. aeruginosa*. The colonies differed in ultrastructure and morphology and on the basis of these differences they were classified as various morphotypes. As in our study, the 16S-23S ITS sequences between the colonial and disaggregated strains were highly similar (83.4–100%) and were therefore classified as the same genotype.

4.3. Effect of light, temperature and salinity on *Microcystis aeruginosa* isolates

Although MAKR0205 and MK10.10 were isolated from the same phytoplankton sample and were classified as the same species, they reacted in a strain-specific manner to environmental conditions. However, although some tendencies in biomass changes were observed in the experiments with various salinity, light and temperature regimes, they were not statistically significant. The highest biomass of the non-toxic MAKR0205 was measured at $30 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$; but further increase to $80 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ resulted in lower dry weights. Temperature (22°C and 30°C) had no effect on MAKR0205 growth. In all our experiments, the non-microcystin producing isolate achieved a higher biomass than the toxic one. It might be supposed that toxic cyanobacteria need extra energy for the biosynthesis of microcystins. As a consequence, they grow more slowly and may be less competitive than non-toxic ones. The hypothesis of a higher energy requirement in the microcystin-producing isolate seemed to be supported by the reduction of the MK10.10 biomass at the lowest irradiance ($10 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$) compared to the higher irradiance and higher temperature (30°C). The average gravimetric concentration of MC-LR in MK10.10 exposed to 22°C was 178.4% higher than in the culture exposed to 30°C . The effect of light and temperature on growth and toxin production by *M. aeruginosa* was tested in a few studies. Reactions comparable to MK10.10 were reported by Watanabe & Oishi (1985), van der Westhuizen & Eloff (1985) and Imai et al. (2009). In their experiments, *M. aeruginosa* achieved a higher growth rate at the higher temperature used ($30\text{--}35^\circ\text{C}$). The temperature of 35°C was also optimal for the growth of *M. ichthyoblabe* (Sabour et al. 2009). The slightly higher toxicity observed in our studies at lower temperature was also documented by Watanabe & Oishi (1985) and van der Westhuizen & Eloff (1985).

Irradiance was reported to influence microcystin production by *M. aeruginosa* only under light-limited growth conditions. Usually, the microcystin content was greater when the irradiance was increased from approximately 7 to $40 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (van der Westhuizen & Eloff 1985, Watanabe & Oishi 1985, Wiedner et al. 2003). This tendency, however, was not observed in the current work. Probably, different *M. aeruginosa* isolates exhibit strain-specific reactions to environmental factors (Wiedner et al. 2003).

Being typical freshwater species, both *M. aeruginosa* isolates grew better in the BG-11 without added NaCl (0 PSU). However, the effect of higher salinities (3.5, 7 and 14 PSU) was not so clear-cut in the culture of toxic MK10.10. The decrease in biomass with increasing salinity was observed

on the last day of the experiment, but this tendency was not statistically significant. The most pronounced effects were observed in gravimetric toxin concentration per dry weight. The increasing salinity resulted in a lower MC-LR content in MK10.10. But even in this experiment, the highest MC-LR concentrations recorded at 0 PSU (1271.21–3283.55 $\mu\text{g mg}^{-1}$ d.w) were only four times higher than those measured at 7 PSU (479.11–802.80 $\mu\text{g mg}^{-1}$ d.w.). At 14 PSU, the MC-LR content in MK10.10 was sometimes below the limit of quantification by HPLC. According to Liu (2006), the tolerance level to salinity of *M. aeruginosa* is between 0 and 10 PSU, but only at 0 PSU was significant growth of the cyanobacterium observed during a 98 hour experiment. That author did not notice any significant correlation between salinity and toxin concentration, however.

The results of our experiments agree with the conclusions of other studies reporting that environmental conditions had only a minor direct effect on microcystin production (Orr & Jones 1998, Kurmayer & Christiansen 2009) and that the concentration of microcystins in cyanobacteria was modified by environmental conditions by a factor of no more than three to five (Sivonen & Jones 1999).

4.4. Microcystin production

In the natural environment, the abundance of *mcy* genotypes appeared not to vary much with changing conditions (Kurmayer & Kutzenberger 2003, Yoshida et al. 2007) and ranged from 0.5% to 35% of the population. A positive correlation was found between the concentration of nitrate and the contribution of toxic *M. aeruginosa* genotypes (Yoshida et al. 2007). In the case of *Microcystis*, the proportion of inactive *mcy* genotype was low (< 3%) and generally a good agreement between the presence of *mcy* and microcystin production was found (Kurmayer et al. 2002, Via-Ordorika et al. 2004). In this work, we also observed that only the strain carrying the *mcy* genes produced the toxins.

Cyanobacteria belonging to the genus *Microcystis* produce MC-LR, MC-RR and MC-YR as major microcystin variants. Their demethylated isoforms are detected less frequently (Via-Ordorika et al. 2004, Welker et al. 2004, Martins et al. 2009). One *Microcystis* strain can be responsible for the production of several microcystin isoforms; 7 isoforms have been reported in one strain by Martins et al. (2009). The presence of MC-HIIR with homoisoleucine (HII) and a molecular ion at m/z 1009, found in a cell extract of MK10.10, has already been reported in *Microcystis* spp. by Namikoshi et al. (1995). To our knowledge, the microcystin variant tentatively identified as MC-VR has never yet been detected in cyanobacteria. In *Microcystis aeruginosa* PCC7820, the production of MC-VF with Val at

position 2 (instead of Leu) and Phe at position 4 (instead of Arg) was demonstrated by Bateman et al. (1995). Other Val-containing microcystins, [D-Asp³]MC-VA, MC-VA, [D-Asp³]MC-LV and MC-LV, were identified in *Hapalosiphon hibernicus* BZ-3-1 (Fewer et al. 2007).

While *Microcystis* morphology can change as environmental conditions do so, the MC profile of individual cyanobacterial strains is a stable feature and is never lost under varying environmental conditions. This MC fingerprint is also an important element of studies into the diversity of cyanobacteria (Fastner et al. 2001, Welker et al. 2004, Martins et al. 2009). The profile of microcystin production can also be used to follow the cyanobacteria at the subpopulation level.

According to published data, the maximum gravimetric concentration of MCs in *M. aeruginosa* can reach $7.4 \mu\text{g mg}^{-1}$ d.w. (Orr & Jones 1998, Sivonen & Jones 1999). The MK10.10 is a highly toxic strain, with over $5 \mu\text{g mg}^{-1}$ d.w. at 0 PSU.

5. Conclusions

The two *Microcystis* isolates from the Vistula Lagoon share the same 16S-23S ITS sequence characteristic of the *M. aeruginosa* genotype but are classified as two different morphospecies and chemotypes; they also exhibited a slightly different reaction to light and temperature regimes. In fact, environmental conditions had only a minor effect on the growth of the isolates and on microcystin production by MK10.10. Our study confirmed that some morphological criteria can be very useful in preliminary assessing the potential toxicity of a *Microcystis* bloom.

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