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Distribution of Microcystin-Producing and Non-Microcystin-Producing *Microcystis* sp. in European Freshwater Bodies: Detection of Microcystins and Microcystin Genes in Individual Colonies

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Summary

Microcystis is a well-known cyanobacterial genus frequently producing hepatotoxins named microcystins. Toxin production is encoded by microcystin genes (*mcy*). This study aims (i) to relate the *mcy* occurrence in individual colonies to the presence of microcystin, (ii) to assess whether morphological characteristics (morphospecies) are related to the occurrence of *mcy* genes, and (iii) to test whether there are geographical variations in morphospecies specificity and abundance of *mcy* genes. Individual colonies of nine different European countries were analysed by (1) morphological characteristics, (2) PCR to amplify a gene region within *mcyA* and *mcyB* indicative for microcystin biosynthesis, (3) matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) to detect microcystins. Almost one hundred percent of the colonies predicted to produce microcystins by PCR analysis were found to contain microcystins. A high similarity in microcystin variants in the different colonies selected from lakes across Europe was demonstrated. The different morphospecies varied in the frequency with which they contained *mcy* genes. Most colonies (>75%) of *M. aeruginosa* and *M. botrys* contained the *mcy* genes, whereas ≤20% of the colonies identified as *M. ichthyoblabe* and *M. viridis* gave a PCR product of the *mcy* genes. No colonies of *M. wesenbergii* gave a PCR product of either *mcy* gene. In addition, a positive relationship was found between the size of the colony and the frequency of those containing the *mcy* genes. It is concluded that the analysis of morphospecies is indicative for microcystin production, although the quantitative analysis of microcystin concentrations in water remains indispensable for hazard control.

Key words: cyanobacteria – hepatotoxicity – lake management – eutrophication – microcystin genotype distribution

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Introduction

Microcystis is a common planktonic freshwater cyanobacterium which frequently forms mass developments in surface waters used for recreation and as drinking water source. This genus produces toxins called microcystins which inhibit eukaryotic protein phosphatases type 1 and 2A, resulting in excessive phosphorylation of cytoskeletal filaments [2]. They have been implicated in deaths due to microcystin-induced liver failure of wild animals, livestock and fish [2], as well as in human illness [16] and even death due to exposure via haemodialysis [10]. In face of the widespread occurrence of microcystins the World Health Organisation has published a preliminary guideline value of 1 µg/l of microcystin-LR in drinking water [41]. Monitoring and potentially predicting microcystin concentrations in water has therefore become an important task not only for public health, but also for limnologists. Of particular interest is the question of regulation of microcystin concentrations in natural populations of microcystin-producing cyanobacteria such as *Microcystis*. These are composed of a variety of genotypes, with and without the genes for microcystin production.The microcystins are members of a peptide family which have the common structure cyclo(D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha), where X and Z are variable L-amino acids, Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methyl-aspartic acid and Mdha is N-methyldehydroalanine [33]. More than 70 structural variants of microcystins are known to date. Microcystins are synthesised by the thiotemplate mechanism like other non-ribosomal peptides (i.e. antibiotics such as gramicidin or tyrocidin) produced by bacteria and fungi [22]. The large enzyme complex encoded by the *mcy* gene cluster is composed of peptide synthetases, polyketide synthases and tailoring functions for microcystin biosynthesis. It has a modular structure, each module activating and incorporating specific constituents of the heptapeptide [3, 36]. The *mcyA*, *mcyB* and *mcyC* genes are responsible for the activation and incorporation of Mdha, D-Ala, L-X, D-MeAsp, L-Z of microcystins during biosynthesis [25, 36].

From the results of physiological work with laboratory cultures microcystin has been interpreted as being a cellular constituent, i.e. always present in microcystin-producing genotypes with cellular concentrations being modified by environmental factors usually two- to fourfold [1, 7, 20, 26]. In contrast a small number of *Microcystis* strains have been repeatedly shown to contain the biosynthesis genes but lack detectable microcystins [23, 11, 25, 37]. The reason why those strains do not synthesise microcystins is unclear. It has been hypothesized recently that the sporadic distribution of microcystin producers either between or within genera of cyanobacteria is the result of repeatedly loosing the ability to produce microcystin in the course of evolution [30]. The inactivation of microcystin synthesis found in an ever increasing number of strains might be considered as a first step in evolution towards loosing the ability to produce microcystin. A number of inactive microcystin genotypes have been found in the related genus *Planktothrix* spp. as well [18]. The frequency of occurrence of inactive microcystin genotypes in natural populations has only started to be explored very recently, and comparison of the presence of the *mcy* gene with microcystin occurrence in *P. rubescens* populations with a low (Lake Irrsee, Austria) or high density (Lake Mondsee, Austria) showed the proportion of inactive microcystin genotypes to be 5% and 21%, each [18]. Results for *Microcystis* sp. from the field comparing the presence of *mcy* genes with the occurrence of microcystin in individual colonies currently exist only from one lake, i.e. Wannsee (Berlin, Germany), where 28 of 29 (97%) of the colonies found to contain the *mcyB* gene also contained microcystins as shown by MALDI-TOF MS [19]. Wider investigations from a variety of field populations and types of water-bodies are needed to substantiate the relationship between the presence of microcystin genes and the occurrence of microcystin in *Microcystis* sp. This is important as basis for elucidating the mechanisms which determine microcystin concentrations in water-bodies.

Further, it is still unclear to which extend morphological characteristics are linked to the ability to produce microcystins, and previous studies came to differing conclusions: Watanabe and collaborators [38, 39] found a correlation of microcystin production with morphology, i.e., *M. viridis* and *M. wesenbergii* were generally considered as microcystin-producing and non-microcystin-producing, respectively. In contrast, Otsuka et al. [27] concluded that most distinguishable morphospecies included both microcystin-producing and non-microcystin-producing strains. These authors have also found transitions between morphological characteristics in culture [27]. However the frequent loss or change of morphological features during culture [35] has been interpreted as the result of unnatural culture conditions [29]. In field studies on lake Wannsee (Berlin) relationships between morphology and microcystin production have been found [5, 19]. However, more field data from a range of freshwater ecosystems are needed to assess whether or not this relationship can be generalised.

The study presented in the following analysed *Microcystis* colonies from a wide range of water-bodies across Europe (i) to clarify whether or not genotypes containing *mcy* genes always contain microcystin, and (ii) whether they tend to belong to certain morphospecies. These questions were addressed by an European workshop which isolated individual *Microcystis* colonies collected from natural populations from 13 water-bodies in 9 European countries, described and determined them morphologically and provided them directly for analysis of the presence of *mcy* genes by PCR as well as microcystins by MALDI-TOF MS.

Material and Methods

Sampling, isolation and determination of colonies

Sampling was carried out during summer 2001 in 9 countries and 13 water bodies by net hauls using a plankton net (40 µm mesh size). The sampled water bodies were in Austria

Table 1. Morphological characteristics of *Microcystis* colonies (morphospecies). Differential phenotype characters are marked by asterisks. Diameter of cells: (minimum) typical range (maximum).

	M. ichthyoblabe	*M. panniformis*	*M. flos-aquae*	*M. aeruginosa*	*M. botrys*	*M. viridis*	*M. wesenbergii*
diameter of cells	2–3.2 (3.8)*	(2.5) 3–4.6 (4.8)*	(3) 3.5–4.8 (5.6)	(3.5) 4–6.5*	4.9–7	(3) 4 – 7.9*	4–8.5 (10)*
shape of colonies	irregular, without holes, often flattened, forming up to large, irregular compact colonies, sometimes with small clusters of cells in their mass; disintegrating in mass of solitary cells*	± flattened up to monolayers, later spherical to irregular, with ± evenly aggregated cells, sometimes with small and indistinct holes; disintegrating in small groups*	irregular spheroidal, compact, not lobate, without holes, only with places with scarce cells*	irregular in outline, lobate, older with distinct holes*	± spherical subcolonies, often joined irregularly together, not distinctly lobate, without holes	composed from typical packet-like subcolonies, irregularly agglomerated and then usually elongated*	irregular in outline, typical spheroidal, lobate, elongated and with holes; sometimes composed of spherical subcolonies*
size of colonies	microscopic up to macroscopic; in massive development macroscopic and heavy "water blooms"	microscopic up to macroscopic, forming pulverous "water blooms"	microscopic, only in strong development (rare) forming fine, pulverous "water blooms"	initial stages microscopic, later macroscopic colonies, forming often heavy "water blooms"	microscopic, only agglomerations of colonies macroscopically visible; sometimes slight water-blooms	microscopic, only agglomerations of colonies macroscopically visible; usually participating on water-blooms of other species	microscopic up to macroscopic, sometimes forming slight (rarely strong) water-blooms
cell density	densely regularly (homogeneously) and evenly agglomerated	regularly densely and evenly agglomerated, sometimes in indistinct rows*	very densely and irregularly agglomerated cells*	± densely and irregularly agglomerated cells with irregularities at the margin*	very densely gathered cells; few solitary cells liberated into the enveloping mucilage*	almost cubically to irregularly arranged cells, not very densely, rarely densely*	sparsely to densely agglomerated cells, sometimes situated near the surface of subcolonies*
mucilaginous margin	indistinct, diffuse, slightly and irregularly overlapping cells	indistinct, diffuse, not overlapping clusters of cells; in old colonies irregular outline	indistinct, diffuse, not overlapping clusters of cells	diffuse, slightly overlapping cell agglomerations (usually about 5 μm wide)*	distinct, thick, wide, in form of radial, ± semi-globose, and sometimes with tubular structures (staining!)*	± narrow, hyaline, ± copying the cell clusters, with slightly refractive margin	distinct envelopes around irregular clusters of cells with delimited, refractive, smooth margin
young stages	small, spheroidal clusters of cells, rarely spherical, often with irregular outline	small clusters of cells, flat or circular in outline, soon developing in spheroidal and ± hollow agglomerations	irregular clusters of aggregated cells	small, ± spherical or irregular clusters of cells with various density, usually with distinct mucilaginous margin	small spheroidal groups of densely gathered cells, sometimes aggregated together	small, ± cubical groups of cells, with distinct mucilaginous envelopes, sometimes gathered together	small spherical colonies with few cells, enveloped by distinct, delimited mucilaginous envelopes

(Holzöstersee), Czech Republic (Brno Reservoir), Denmark (Lake Frederiksborg), Germany (Mueggelsee, Wannsee), Finland (Lake Tuusulanjärvi), Italy (Lake Arancio, Sicily), The Netherlands (t Joppe, Zegerplas), Portugal (City Park Pond) and United Kingdom (Balgavies, Dundee, Rescobie). The samples were taken a few days before the workshop from 2–7 July 2001 in Berlin and stored refrigerated. In the course of the workshop colony isolation and morphological description were standardized. For colony isolation, samples were diluted and individual colonies picked out by the means of tiny Pasteur pipettes or forceps under binocular microscopes. Colonies were washed 3 times in 50 µl of BG11 medium [34] at room temperature to eliminate other colonies or cyanobacteria, and absence of other cyanobacteria was checked by visual inspection using the microscope. Colony size (the largest diameter) was determined (with the exception of 54 colonies from Finland), and the cell size measured using an inverted microscope at a magnification of 400x. In total, 322 colonies were isolated. Morphological classification was done using the morphological criteria proposed by Komárek and Anagnostidis [13], Komárek and Komárková [14] (see Table 1). The characteristics for assigning colonies to the genus *Microcystis* were: Microscopic to macroscopic, floating, mucilaginous colonies with irregularly distributed cells (sometimes densely aggregated), a colourless mucilage, spherical cells, with obligatory presence of gas vesicles (aggregated to aerotopes) within whole cell volume (they are lacking only in dormant stages). Cell division in two or three perpendicular planes in subsequent generations. According to the current revision of all species of *Microcystis* Kützing ex Lemmerman 1907 (including *M. aeruginosa*, *M. ichthyoblabe*, *M. novackekii*, *M. viridis*, *M. wesenbergii*) under the Rules of the Bacteriological Code [28] in this study, all morphospecies are considered morphological variations of individuals of one population.

A number of colonies were documented photographically using the Image Analyzing System LUCIA M. Finally, the colonies were transferred in a reaction tube containing Millipore water or culture medium (final sample volume 4–30 µl). The presence of each colony in the tube was verified microscopically. The tubes were frozen (liquid nitrogen) and thawed several times at room temperature (without vortexing) in order to disintegrate the colonies, and stored at –20°C. Aliquots from each tube were then used for *mcy* amplification as well as MALDI-TOF MS.

Genetic characterisation

The presence of cyanobacterial DNA was checked by amplification of the phycocyanin *cpcBA* intergenic spacer region (PC-IGS, 685 bp) region using primers described by Neilan et al. [24]. Primer pairs allowing the amplification of part of the condensation domain (C) of *mcyA* (fragment size 291 bp, [8]) and the first adenylation (A1) domain of *mcyB* (fragment size 1312 bp, [19]), both indicative of the microcystin biosynthesis gene cluster, were used. DNA from *Microcystis* PCC7806 was added as positive control for *mcy* gene amplification during PCR analysis of colonies from field samples. Because it cannot be excluded that some colonies were lost during handling and in order to avoid false negatives as far as possible, all samples yielding no PCR product for PC-IGS, mcyB and mcyA were tested in triplicate. PCR for PC-IGS and *mcyB* amplification were carried out in parallel in the same thermal block of the cycler (PTC-200 apparatus, MJ Research, Watertown, Mass., USA) in a final volume of 50 µl with 1.4 µl of sample, 5 µl of PCR buffer 10× (containing 15 mM of $MgCl_2$, Qiagen, Hilden, Germany), 2 µl of $MgCl_2$ (25 mM, Qiagen), 1 µl of each primer (10 pmol), 1 µl of dNTP (10 mM), 38.4 µl distilled water and 0.2 µl Taq polymerase (5 U, Qiagen). The PCR thermal cycling protocol included an initial denaturation at 95 °C for 2min, followed by 33 cycles of (denaturation: 94 °C/30 sec; annealing: 51 °C/30 sec and elongation: 72 °C/1 min), and a final elongation step at 72 °C for 5 min. PCR products were analysed by electrophoresis in 1% (w/v) agarose gel (Seakem, BMA, USA) in 0.5 × TBE buffer at 120 V.

For *mcyA*, PCR was performed in a final volume of 20 µl with 1 µl of sample, 2 µl of PCR buffer 10× (containing 15 mM of $MgCl_2$), 1.2 µl of $MgCl_2$ (25mM, Qiagen), 0.5µl of each primer (10 pmol), 0.3 µl of dNTP (10 mM), 14.3 µl of distilled water and 0.2 µl of Taq polymerase (5U, Qiagen). The PCR thermal cycling was identical as for the other genes except the annealing step (59 °C/30 sec) and elongation step (72 °C/30 sec). PCR products were analysed by electrophoresis in 1.5% (w/v) agarose gel.

Microcystin analysis

1–4 µl of the cell suspension from each colony was prepared onto a stainless steel template and immediately 1 µl of matrix (10 mg/ml 2,5-dihydroxybenzoic acid in water/acetonitrile (1:1) with 0.03% trifluoroacetic acid) was added. The cell/matrix mixture was air dried at room temperature. Positive ion mass spectra were recorded from each colony using a MALDI-TOF mass spectrometer (Voyager DE-PRO, Applied Biosystems, USA) equipped with a reflectron, post-source decay (PSD) and collision-induced dissociation (CID) options. Mass spectra were obtained from 400 to 2000 Da comprising the mass range of

Table 2. Number of colonies analysed and 'drop-out rates', i.e. number (percentage) of negative samples during PCR (PC-IGS) and MALDI-TOF MS analysis of individual colonies with different colony size. Samples giving no PCR product were tested three times. Samples were considered negative for MALDI-TOF MS when no peaks of pigment degradation products or other substances except matrix peaks were detected.

Colony size (µm)	Number of colonies	Drop-out rates	
		no PC-IGS PCR signal	no MALDI signal
<200	18	3 (17%)	11 (61%)
201–400	71	7 (10%)	26 (37%)
401–600	47	1 (2%)	15 (32%)
601–800	37	1 (3%)	10 (27%)
801–1100	48	3 (6%)	14 (29%)
>1101	43	1 (2%)	2 (5%)
unknown size	58	1 (2%)	20 (35%)
Total	322	17 (5%)	98 (30%)

known microcystins. All analyses were carried out in the reflector and delayed extraction mode giving separation of isotopic peaks and a mass accuracy of at least 0.005%. Chlorophyll-a degradation products phaeophytine-a and phaeophorbide-a with mass values of *m/z* 871.57 and 593.27 Da were used for internal calibration. Mass signals indicative for known microcystins were in most cases further analysed by recording fragment ions using PSD. Structure assignment of microcystins was accomplished by comparing fragment spectra with those of known microcystins [4, 40].

Elimination of false-negative samples in PCR and MALDI-TOF MS

The PC-IGS region was amplified by PCR as reference and to confirm the presence of *Microcystis* in the aliquots. For MALDI-TOF the presence of chlorophyll *a* degradation products (phaeophytin and phaeophorbide) as well as other peptide peaks (in addition to the matrix peaks) in the mass spectra were used as a reference. PCR successfully amplified PC-IGS in 95% (305 colonies) of all colonies (Table 2). 5% (17 colonies) were found negative indicating the loss of colony during handling (i.e. "drop-out rate"). This was supported by MALDI-TOF analysis as no signals except of matrix peaks were detected in these samples. Furthermore, a relationship between colony detection and colony size was observed. Both for PCR of the PC-IGS region and for MALDI-TOF, the smallest colonies (<200 µm) showed the highest drop-out rate (proportion of negative samples, Table 2). The proportion of negative samples decreased with an increase in colony size, e.g. was lowest (<3%) for colonies larger than 400 µm with PCR analysis. For MALDI-TOF a minimum drop out rate (5%) for the largest colonies (>1100 m) was recorded. In general, the drop out rate was lower for PCR of the PC-IGS region (5%) than for MALDI-TOF (30%). For comparing the occurrence of *mcy* to the occurrence of microcystin 70% (224 colonies) of all 322 colonies, i.e. those testing positive not only for PCR but also for MALDI-TOF MS could be used.

Results and Discussion

Distribution of morphospecies

Seven morphospecies were identified: *M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. ichthyoblabe* (corresponding to the small cell size group identified in Japan, KATO et al. [12]), *M. panniformis*, *M. viridis* and *M. wesenbergii* (Fig. 1). These morphospecies differed in colony form, arrangement of cells, fragility and cell diameter. A small number (n = 20) of *Microcystis* colonies represented intermediate morphological characteristics and were classified as undetermined. The most frequently sampled morphospecies were *M. aeruginosa* and *M. ichthyoblabe* representing 149 and 60 colonies, respectively. Less frequently sampled morphospecies included *M. flos-aquae* (8), *M. wesenbergii* (21), *M. viridis* (17), *M. panniformis* (20) and *M. botrys* (27).

With the exception of the population in the Finnish lake, all *Microcystis* populations consisted of a mixture of *M. aeruginosa* and *M. ichthyoblabe* plus more rare morphospecies. The morphospecies *M. viridis* was mainly observed in Lake Tuusulanjärvi (Finland). *M. panniformis* was identified solely in freshwaters from Portugal and Sicily. This is consistent with the description of *M. panniformis* as a tropical morphospecies with similar populations being found in Southern Europe [15].

Comparison of mcy and microcystin occurrence in the colonies

Analysis of 224 colonies for both *mcyB* by PCR and microcystin by MALDI-TOF MS showed that a considerable proportion (48 of a total of 128) of microcystin containing colonies did not give a PCR product for *mcyB* (Fig. 2A). These 48 colonies were subsequently tested for *mcyA*. 42 of these colonies were found to contain *mcyA*, however, six colonies remained which did not give a PCR product for either the *mcyB* or the *mcyA* gene. Those six colonies included two determined as *M. aeruginosa* (Wannsee), two as *M. ichthyoblabe* (Brno reservoir), one determined as *M. panniformis* from Siciliy and one *M. botrys* from The Netherlands. Thus, for PCR analysis of *mcy*, these six colonies were "false negatives", and they amounted to 4.7% of all colonies found to contain microcystins by MALDI-TOF MS.

In order to check for sequence specific differences possibly resulting in the mismatch between microcystin and observed *mcyB* distribution the mcyBA1 primers used in this study and introduced by Kurmayer et al. [19] were blasted and found to match the corresponding gene region of all eight mcyBA1 sequences currently available in the DDBJ/ EMBL/GenBank database (October 2003). A factor accounting for the higher number of false negatives using amplification of *mcyB* when compared to *mcyA* is its larger amplicon size: generally amplification efficiency is higher for smaller PCR products, resulting in higher sensitivity when compared to larger amplification fragments, such as *mcyB*. Consequently, it is important that both primers (for the control gene and the *mcy* gene) exhibit the same amplification efficiency [17]. In this study, *mcyA* was closer in size to the PC-ITS gene fragment (685 bp) than *mcyB* (1312 bp). It is concluded that combining several gene probes to detect the *mcy* genes in colonies of *Microcystis* does provide reliable results on the distribution of microcystin-producing genotypes in the field.

Vice versa, a small percentage of colonies (3 colonies of 122, i.e. 2.5%) was found to contain *mcy* but did not show detectable microcystins. In general, sensitivity of MALDI-TOF was lower when compared to PCR (Table 2). This could be attributed to the sample preparation, for example unsuccessful splitting of individual colony samples for preparing the aliquots used for both methods. Many colonies remained intact in spite of the attempts to disintegrate the cell aggregates by repeated freezing and thawing. Because PCR analysis preceded MALDI-TOF it cannot be excluded that the removal of the sub-sample for PCR actually picked out the colony in total (particularly for small sized colonies). The total percentage of colonies found to contain *mcyB*/*mcyA* was 45% (from 305 non-empty colony samples). The total percentage of colonies with microcystin was 56% (from 224 non-empty colony samples). In summary, the correspondence of the PCR-amplification of either *mcy* gene and microcystin detection by MALDI-TOF was close.

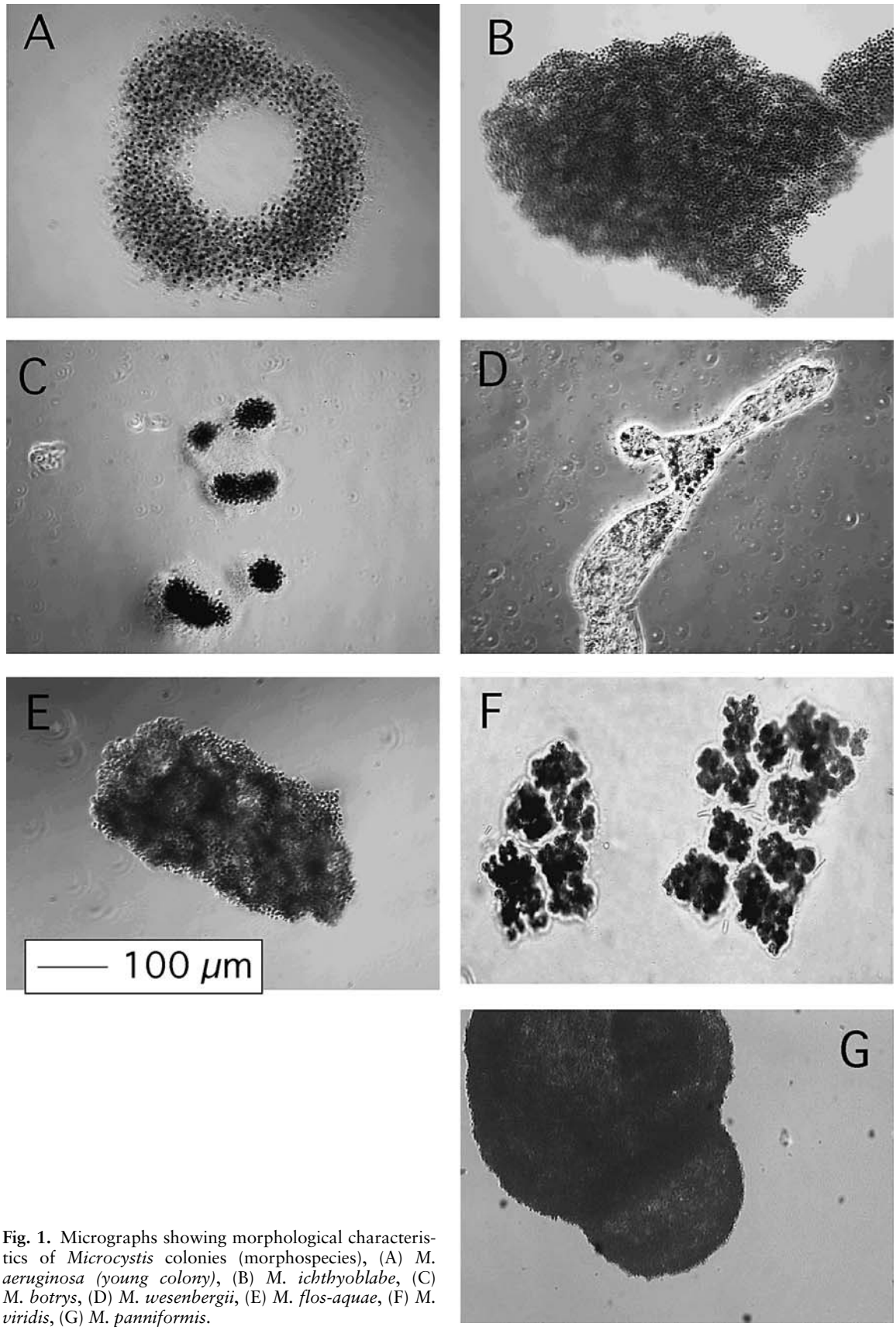

Fig. 1. Micrographs showing morphological characteristics of *Microcystis* colonies (morphospecies), (A) *M. aeruginosa (young colony)*, (B) *M. ichthyoblabe*, (C) *M. botrys*, (D) *M. wesenbergii*, (E) *M. flos-aquae*, (F) *M. viridis*, (G) *M. panniformis*.

Morphospecies and microcystin production

With the exception of *M. wesenbergii*, the *mcy* genes were amplified from all morphospecies (Fig. 2A). The highest proportion of colonies containing *mcy* and microcystin was found for *M. aeruginosa* (72%) and *M. botrys* (90%). Only about half of the colonies of *M. flos-aquae* and *M. panniformis* (50% and 53%) were identified as microcystin-producers. In contrast *M. ichthyoblabe* (20%) and *M. viridis* (17%) showed rather low proportions of microcystin producers. The low proportions of microcystin-producing genotypes shown by some morphospecies (particularly *M. wesenbergii, M. viridis and M. ichthyoblabe, see Fig. 2A*) correspond to the conclusions drawn by [38, 39]. In agreement with Harada et al. [6], the colonies assigned to *M. viridis* revealed a low proportion of microcystin producing genotypes. In contrast the proportion of microcystin producing colonies assigned to *M. aeruginosa* was high. In agreement with the earlier results from Lake Wannsee [5, 19] it is now proposed that in Europe a few morphospecies (*M. aeruginosa*, *M. botrys*) have a significantly higher proportion of microcystin producers (>70%) than others (*M. flos-aquae*, *M. ichthyoblabe*). In Europe, *M. wesenbergii* so far has never been found to produce microcystins.

Most field populations contained microcystin-producing as well as non-microcystin-producing colonies (Fig. 2B). Only in one lake, Lake Zegerplas (The Netherlands) all 19 colonies tested contained *mcy* and microcystins. The reverse was found for five water-bodies, from which the *Microcystis* colonies tested contained neither *mcy* nor microcystins, i.e. 5 colonies from Lakes Balgavies and 10 colonies from Lake Rescobie (Scotland), 9 colonies from Lake Frederiksborg (SlotssØ) in Denmark, 10 colonies from the City park pond from Porto (Portugal), and 5 colonies from Lake Morocco. Those water bodies are likely to exhibit low microcystin contents of the corresponding populations. In contrast, the samples originating from the Netherlands would indicate higher microcystin contents of the population. Consequently, linking microcystin production to morphological criteria might support hazard identification by local authorities in the future.

Taking all colonies together, a positive relationship between colony size and microcystin production (indicated either by the determination of *mcyB/mcyA* by PCR or the detection of microcystins by MALDI-TOF MS) and the colony size was observed (Table 3). The smallest colonies (<200 µm) showed the lowest proportion of *mcy* and microcystin producers (13% and 29% each). The frequency of *mcy* and microcystin production was positively related to colony size and the largest colony size class had a maximum proportion of the *mcy* (83%) and microcystin producers (90%). This finding confirms earlier results from Lake Wannsee [17] implying that the observation that the larger colonies of *Microcystis* sp. are the chief microcystin producers may be more generally valid. One explanation for this size dependency in frequency of *mcy* might be the difference in fragility of the colonies between morphospecies, i.e. *M. aeruginosa* has the highest percentage of *mcy* and also has been reported frequently to grow in firm and large colonies [9, 29, 32, 38].

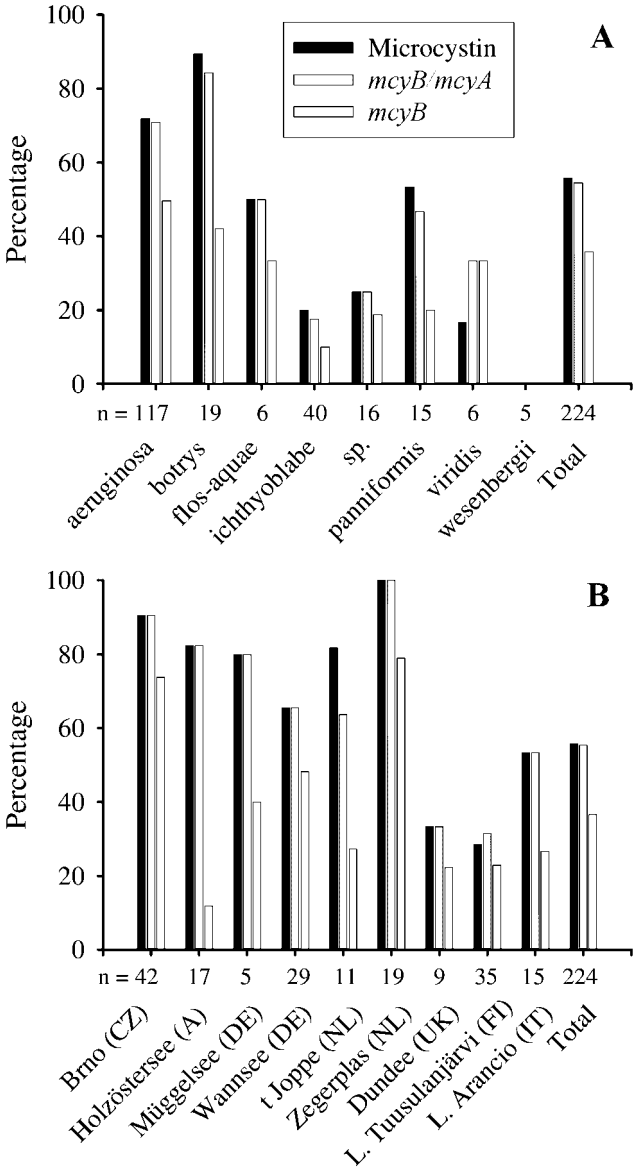

Fig. 2. Number of colonies tested (n) and their proportion containing microcystins (MALDI-TOF; solid shading) and microcystin *(mcy)* genes (PCR; grey columns show colonies giving a PCR-product for at least one of *mcyB* or *mcyA*; open columns show those testing positive for only *mcyB*): (A) for each morphospecies; (B) for each water-body.

Microcystin variants

MALDI-TOF analysis revealed the presence of 1–8 different microcystin variants (Table 4) and a high similarity in microcystin variants within and between lakes from Southern to Northern Europe. Microcystins frequently observed were microcystin-LR (MC-LR), MC-RR, MC-YR as well as their demethylated variants. The position of the demethylation could be determined only in some cases, while for most of the demethylated variants peak intensities of respective fragment ions were too

Table 3. Number of colonies analysed by PCR and MALDI-TOF MS in each size class (using only 'non-empty' colony samples) and number (percentage) of those containing the *mcyB/A* gene and microcystin (MC). N = number of colonies tested.

	PCR		MALDI-TOF MS	
Colony size (µm)	N	*mcy*	N	MC
<200	15	2 (13%)	7	2 (29%)
201–400	64	21 (33%)	45	15 (33%)
401–600	46	21 (46%)	32	21 (66%)
601–800	36	19 (53%)	27	18 (67%)
801–1100	45	24 (53%)	34	22 (65%)
>1101	42	35 (83%)	41	37 (90%)
Colonies of unknown size	57	16 (28%)	38	10 (26%)
Non-empty colonies	305	138 (45%)	224	125 (56%)
Empty colonies	17	–	98	–
Total	322	–	322	–

Table 4. Microcystins detected in *Microcystis* colonies by MALDI-TOF MS. Morphospecies: *M. aeruginosa* (M. aer.), *M. botrys* (M. bot.), *M. flos-aquae* (M. f.-aq.), *M. ichtyoblabe* (M. ich.), *M. panniformis* (M. pan.), *M. viridis* (M. vir.).

Site	Morpho-species	No of colonies	Microcystin variant							
			m/z 981 D-MC-LR	995 MC-LR	1024 D-MC-RR	1031 D-MC-YR	1038 MC-RR	1045 MC-YR	1049 [H4] MC-YR	1068 MC-WR
Dundee (SC)	*M. aer.*	2		x			x	x		
	M. ich.	1			x					
Brno reservoir (CZ)	*M. aer.*	34	x	x	x[1]		x	x		x
	M. ich.	5	x	x	x	x	x			
	M. sp.	1		x			x			
Lake Tuusulanjärvi (FL)	*M. aer.*	6	x[3, 4]		x[1,2]					
	M. sp.	3	x[3, 4]		x[1,2]					
	M. vir.	1		x						
Holzöstersee (A)	*M. aer.*	9	x	x						
	M. bot.	4	x	x			x			
	M. f.-aq.	1		x			x			
Zegerplas (NL)	*M. aer.*	12	x	x	x	x	x	x	x	x
	M. bot.	7		x	x	x		x		
t Joppe (NL)	*M. aer.*	5		x			x	x	x	
	M. bot.	4		x	x		x	x	x	
Mueggelsee (GER)	*M. aer.*	2	x		x	x				
	M. f.-aq.	1		x			x	x		
	M. ich.	1		x						
Wannsee (GER)	*M. aer.*	16	x	x	x	x	x	x	x	x
	M. bot.	1		x			x	x		
	M. ich.	1		x			x	x		
	M. f.-aq.	1		x			x			
Lake Arancio (I)	*M. pan.*	8	x	x	x			x		

[1] [Dha[7]]MC-RR, [2] [Asp[3]]MC-RR, [3] [Dha[7]]MC-LR, [4] [Asp[3]]MC-LR

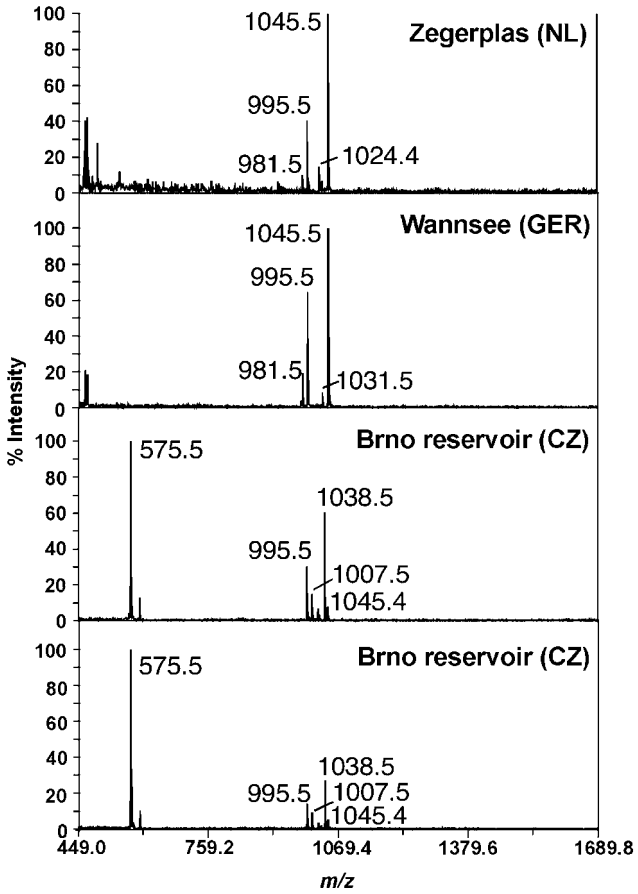

Fig. 3. Positive ion MALDI-TOF mass spectra of *Microcystis aeruginosa* for a single colony from Lake Zegerplas (NL), Lake Wannsee (GER) and two from Brno reservoir (CZ). Assignment of microcystins: 981 [M+H]+, demethylated microcystin-LR; 995 [M+H]+, microcystin-LR; 1024 [M+H]+, demethylated microcystin-RR; 1031 [M+H]+, demethylated microcystin-YR; 1038 [M+H]+, microcystin-RR; 1045 [M+H]+, microcystin-YR.

low for an unambiguous structure determination. Occasionally, [H_4] MC-YR and MC-WR were detected.

Colonies with similar microcystin profiles were detected among most of the European lakes investigated in this study (Table 4). Examples of positive ion mass spectra from *M. aeruginosa* colonies illustrate that in most colonies MC-LR, MC-RR and MC-YR showed the highest peak intensities suggesting highest concentrations of these variants (Fig. 3). HPLC analysis of total phytoplankton biomass confirmed the relative amount of major microcystins observed by the mass spectrometric analysis in the cases where sufficient *Microcystis* biomass was present (data not shown). In contrast, all colonies of *Microcystis aeruginosa* and other *Microcystis* sp. from Lake Tuusulanjärvi (FL) contained only demethylated microcystins as major variants.

While for the majority of the *Microcystis* populations world-wide MC-LR, MC-RR and MC-YR are reported as major variants, demethylated microcystins as major variants are rarely found in bloom samples and strains of this species. Interestingly, only Lake Tuusulanjärvi (Finland) differed from this general trend by the production of demethylated microcystins as major variants. Luukkainen et al. [21] detected demethylated variants of MC-LR and MC-RR as major microcystins in four strains and one bloom from Finish water bodies. It is possible that these differences in methylation are due to physiological effects, as the production of demethylated variants of MC-LR and MC-RR has been shown to depend on temperature in *Anabaena* [38].

Conclusions

The results show a very close relationship between the occurrence of *mcy* gene and the presence of microcystins in the cells for the genus *Microcystis* sp. The data also show a relationship between morphospecies and 'microcystin genotypes' as well as between morphospecies and microcystin production. Although one morphospecies (*M. wesenbergii*) was never found to contain microcystin, the distribution of microcystin production among morphospecies does not appear sufficiently clear cut to be used as taxonomic criterion. Although the microscopical analysis of morphospecies distribution in water is considered indicative for microcystin production, the quantitative analysis of microcystin concentrations remains the only reliable method for hazard control in water.

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