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Application of Real-Time PCR To Estimate Toxin Production by the Cyanobacterium *Planktothrix* sp.▽†

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Quantitative real-time PCR methods are increasingly being applied for the enumeration of toxic cyanobacteria in the environment. However, to justify the use of real-time PCR quantification as a monitoring tool, significant correlations between genotype abundance and actual toxin concentrations are required. In the present study, we aimed to explain the concentrations of three structural variants of the hepatotoxin microcystin (MC) produced by the filamentous cyanobacterium *Planktothrix* sp., [Asp, butyric acid (Dhb)]-microcystin-RR (where RR means two arginines), [Asp, methyl-dehydro-alanine (Mdha)]-microcystin-RR, and [Asp, Dhb]-microcystin–homotyrosine-arginine (HtyR), by the abundance of the microcystin genotypes encoding their synthesis. Three genotypes of microcystin-producing cyanobacteria (denoted the Dhb, Mdha, and Hty genotypes) in 12 lakes of the Alps in Austria, Germany, and Switzerland from 2005 to 2007 were quantified by means of real-time PCR. Their absolute and relative abundances were related to the concentration of the microcystin structural variants in aliquots determined by high-performance liquid chromatography (HPLC). The total microcystin concentrations varied from 0 to 6.2 μg liter^{-1} (mean ± standard error [SE] of 0.6 ± 0.1 μg liter^{-1}) among the samples, in turn resulting in an average microcystin content in *Planktothrix* of 3.1 ± 0.7 μg mm^{-3} biovolume. Over a wide range of the population density (0.001 to 3.6 mm^3 liter^{-1} *Planktothrix* biovolume), the Dhb genotype and [Asp, Dhb]-MC-RR were most abundant, while the Hty genotype and MC-HtyR were found to be in the lowest proportion only. In general, there was a significant linear relationship between the abundance/proportion of specific microcystin genotypes and the concentration/proportion of the respective microcystin structural variants on a logarithmic scale. We conclude that estimating the abundance of specific microcystin genotypes by quantitative real-time PCR is useful for predicting the concentration of microcystin variants in water.

During the last decade, genetic methods have significantly increased our understanding of the distribution of genes that are involved in the production of toxins within cyanobacteria that occur in fresh and brackish water (45). Although genetic methods can indicate only the potential risk of toxin synthesis and do not provide information about the actual toxin concentrations, quantitative real-time PCR has been increasingly applied for monitoring the toxin-producing genotypes of cyanobacteria in water (26, 33, 44). The development of real-time PCR methods was driven primarily by its potential (i) as an early-warning tool as well as to monitor toxin-producing cyanobacteria and (ii) to identify those factors that lead to a dominance/repression of toxin-producing genotypes versus nontoxic genotypes. For the first aim, it is essential that the abundance of toxin-producing cyanobacteria can be related to the concentration of the respective toxic substance in water. A few studies showed that the concentration of certain toxic genotypes was linearly related to the respective toxin concentrations, e.g., for the most common group of hepatotoxins, the microcystins (MCs) (7, 12, 14), and for the related nodularin (19). Both microcystins and nodularins are known to be potent inhibitors of eukaryotic protein phosphatases 1 and 2A, resulting in a health hazard to humans and the environment (9). In contrast, no correlation was found (37, 50), or even the opposite was reported, by other studies, i.e., that the measurement of microcystin-producing genotypes is not a satisfactory method for use in monitoring programs in order to predict the toxic risk associated with cyanobacterial proliferation (3). For microcystins, these contrasting results may be due to several reasons: (i) several genera producing microcystins frequently coexist in water bodies, and therefore, not all microcystin producers may have been identified; (ii) the semilogarithmic calibration curves limit the accuracy in estimations of genotype numbers and proportions (for example, the only laboratory comparison carried out so far revealed that among the three laboratories tested, the proportions of toxic genotypes were overestimated or underestimated by 0 to 72% and 0 to 50%, respectively [42]); and (iii) inactive mutants that contain the respective genes, however, which have been inactivated in toxin production through the insertion of transposable elements, may co-occur and decrease toxin production in a given population (6). Nevertheless, the real-time PCR technique is the only quantitative technique available for estimating the proportion of potential toxin-producing genotypes in water. The development of automated and field-applicable real-time PCR methods (e.g., see reference 35), in particular, may contribute to a more widespread integration of real-time PCR into routine monitoring programs in the future.

In the present study, we attempted to quantify microcystin-producing genotypes in total as well as quantify the specific

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genotypes that were shown to encode different microcystin structural variants characterized for strains isolated from lakes in the Alps (23): (i) the methyl-dehydro-alanine residue (Mdha) genotype, which was found to synthesize structural variants containing only Mdha in position 7; (ii) the butyric acid (Dhb) genotype, which was found to contain Dhb instead of Mdha in the same position; and (iii) the homotyrosine (Hty) genotype, which was found to contain Hty and Leu in position 2 but never Arg. The Hty variant has always been found to co-occur with Dhb in position 7 of the molecule (24). Consequently, the Hty genotype forms a subgroup of the microcystin-producing population composed of the Mdha and Dhb genotypes. The following hypotheses were tested: (i) as only one microcystin-producing organism (*Planktothrix* sp.) is of quantitative importance in those lakes (32), the total microcystin concentration should be predictable from the sum of Mdha and Dhb genotypes; (ii) given that all *Planktothrix* genotypes are amenable to cultivation, all the structural microcystin variants found in the field samples should have been described for the strains isolated previously (23); and (iii) as, on average, the proportion of the inactive microcystin genotypes was found to be low and rather stable (<6.5% [32]), their occurrence should not reduce the ability to predict microcystin concentrations from genotype abundance. For this purpose, the phytoplankton in 12 lakes of the Alps in Austria, Germany, and Switzerland was monitored both with an inverted microscope as well as by means of real-time PCR over the course of 2 years (2005 to 2007). In parallel, microcystin concentrations in aliquots were determined by means of high-performance liquid chromatography (HPLC). We show that the abundance of specific microcystin genotypes can be related to the corresponding microcystin concentrations in water on a logarithmic scale over a range of trophic conditions. The proportion of certain genotypes encoding the synthesis of a specific microcystin variant significantly correlates with the concentration of the respective microcystin variant. We argue that these genotype-toxin concentration relationships are of great importance for the justification of real-time PCR use in monitoring programs.

MATERIALS AND METHODS

Sampling procedure. Phytoplankton sampling was performed in 12 lakes of the Alps (Afritzersee, Attersee, Fuschlsee, Irrsee, Mondsee, Offensee, Schwarzensee, Wolfgangsee, and Wörthersee in Austria; Ammersee in Germany; and Hallwilersee and Zürichsee in Switzerland) differing in trophic states from oligotrophic to mesoeutrophic (for a detailed morphometric and trophic description of the lakes, see Table 1 in reference 32). Water samples were taken between spring 2005 and autumn 2007 by pooling 1 liter of water every 2 m from the surface to a depth of 20 m (depth-integrated samples). Since some lakes had a rather low population density (0 to 0.0026, 0 to 0.0058, and 0.0011 to 0.0136 mm^3 $liter^{-1}$ [minimum to maximum {min-max}] for Attersee, Wolfgangsee, and Schwarzensee, respectively), three vertical plankton net hauls (30-μm mesh size) from a depth of 20 m to the surface were taken in parallel (net samples). As estimated from the diameter (0.5 m) and length (1.5 m) of the conical net, taking one net haul from a depth of 20 m up to the surface results in the filtering of 1,300 liters. In total, 80 depth-integrated and 79 net samples were analyzed. Aliquots of each sample (2 to 4 liters of integrated samples and 20 to 100 ml of net samples) were filtered onto glass fiber filters (BMC; Ederol, Vienna, Austria) under vacuum pressure and stored frozen (−20°C) for subsequent DNA and microcystin analyses. For microscopic counting, aliquots (100 ml) of the samples were preserved with Lugol solution and formaldehyde (2% final concentration). To characterize the trophic state, the total phosphorus concentration and the chlorophyll *a* concentration were determined according to standard methods (48).

Culturing of strains. *Planktothrix rubescens* strains PCC 7821 (a microcystin-producing genotype with dehydrobutyrine in position 7 from Lake Gjersjøen, Norway), number 21/1 (a microcystin-producing genotype synthesizing Hty/Leu in position 2, from Lake Figur, Austria), and No. 40 (a microcystin-producing genotype synthesizing Mdha in position 7, from Lake Mondsee, Austria) were all grown under continuous light (5 to 15 μmol m^{-2} s^{-1}) (Osram type L30W/77; Fluora) at 20°C in BG11 medium (38). Cells from cultures in the exponential growth phase were harvested as described above. Aliquots of cultures (100 ml) were preserved by the addition of formaldehyde, and the cells were enumerated by use of epifluorescence microscopy. Cell numbers (converted to biovolume equivalents) were related to the threshold cycle (C_T) value measured by real-time PCR from a series of DNA dilutions of the respective strains.

Microscopic counting. Lugol-fixed, integrated samples were counted in sedimentation chambers by use of an inverted microscope. At least three transects per chamber were screened to enumerate *Planktothrix* at a ×100 magnification and the other phytoplankton at a ×400 magnification. Filaments were assigned to the genus *Planktothrix* according to morphological criteria (18). *Planktothrix* filaments in the net samples were enumerated by use of epifluorescence microscopy (Zeiss Axioscop 40). The biovolume was then calculated by approximating the geometrical shape (48). Other phytoplankton species were enumerated from integrated samples only. Only phytoplankton taxa that were visible at a 400-fold magnification were considered, while small cyanobacteria such as *Synechococcus* sp. could not be detected quantitatively.

Genetic analysis. The DNA that was collected on filters was isolated quantitatively by use of a standard chloroform-phenol protocol described previously (21). In order to quantify the three genotypes differing in microcystin syntheses as well as the total population of *Planktothrix*, a TaqMan assay was used as described previously (32). The TaqMan assay for the detection of each microcystin genotype was designed for genotypes that were characterized for their microcystin production (23): (i) the Mdha genotype (long *mcyAA1* variant [GenBank accession no. AJ749248.1 to AJ749259.1 and AJ441056.1]), (ii) the Dhb genotype (short *mcyAA1* variant [accession no. AJ749260.1 to AJ749266.1 and AJ749254.1 to AJ749256.1]), and (iii) the Hty genotype (accession no. AJ890275.1 to AJ890279.1, AJ749273.1, AJ749275.1, AJ749278.1, and AJ863131.1 to AJ863134.1). Those DNA sequences were aligned (ClustalW 1.8), and the target regions with complete identity within a genotype and the maximum difference from the other genotypes were identified. For each of these gene regions, primers and TaqMan probes were designed by use of Primer Express software (version 2.0; ABI). Prior to synthesis, all oligonucleotides were tested for stem-loop formation, the formation of primer dimers, and heat stability and were modified, if necessary, by TIB Molbiol (Berlin, Germany). This resulted in the design of the following forward primers, TaqMan probes, and reverse primers, respectively: (i) mdha+ (5′-AAGTCAA TCTCTTACATCCTTGT-3′), Mdha (5′-ACCGGCTAATCTAGCTAAAATT ATCTGC-3′), and mdha− (5′-CAATGAGATCCCAATCACTAT-3′) (amplicon length of 77 bp) for Mdha; (ii) dhb+ (5′-CCTCAACATCAAGCGAGTA TTAT-3′), Dhb (5′-TACAGAATGGGAAAAAATTACTCAAGAGAA-3′), and dhb− (5′-CCACTTTCCGGGTTTG-3′) (83 bp) for Dhb; and (iii) HtyS (5′-AAACCGATGATCCCGTCATTTCT-3′), Hty (5′-CCCATTGCCAATAA CCAAATATACATTCTTGATCC-3′), and HtyA (5′-CACAATGCCGACAGG AACG-3′) (98 bp) for Hty. Concentrations of the forward primer, reverse primer, and TaqMan probe were optimized according to the manufacturer's instructions (ABI TaqMan universal PCR master mix) and adjusted to 200, 200, and 100 fmol μl^{-1} for the Mdha and Hty genotypes and 200, 200, and 250 fmol μl^{-1} for the Dhb genotype, respectively. All samples were measured in triplicate with an Eppendorf Master Cycler Ep Realplex system. The reaction mixtures were 25 μl in total volume, consisting of 12.5 μl master mix (TaqMan Universal PCR master mix; ABI, Austria), 5 μl of template DNA, and the optimized concentrations of the primers and probe. Following the initial denaturation step of 10 min at 95°C, 50 cycles of a two-step PCR were run, with alternating steps of 15 s of denaturation at 95°C, and a 1-min annealing and elongation step at 60°C for Hty and at 55°C for Dhb and Mdha. The calibration curves were established as described previously (32) and are listed in Table S1 in the supplemental material. The specificity of the TaqMan assays was tested by the addition of DNAs of *Microcystis* strains HUB53 and HUB524, which were added at the same concentration as the target DNA (equivalent to 460 cells per template) and also at a 100-fold-lower concentration (32). In no case did the C_T values of the calibration curve show a significant deviation in the presence of the DNA background (see Table S2 in the supplemental material). In addition, the specificity of the primer sets was tested by endpoint PCR by using the DNA of 18 *Planktothrix* strains differing in the production of specific microcystin variants and the DNA of different species of cyanobacteria (*Aphanizomenon gracile*, *Aphanizomenon flexuosum*, *Microcystis aeruginosa* HUB524, *Microcystis flos-aquae*, *Nostoc* sp. strain PCC 7120, and *Synechococcus* sp. strain MW-10). All

primer pairs showed the specific PCR products only. For each TaqMan assay, the lower limit of detection, corresponding to 1 copy template^{-1}, was determined by measurements of a dilution series of purified PCR products from the strains, as described previously (32).

Microcystin analysis. Microcystins were extracted from filters by using 75% (wt/vol) aqueous methanol (10). Extracts were analyzed by HPLC with diode array detection (DAD) for their microcystin compositions by using a linear gradient of acetonitrile (0.05% trifluoroacetic acid) against water (27). Microcystin variants were identified by their respective retention times and molecular masses as described previously (22, 23). Microcystin variants were quantified as equivalents of [D-Asp, Mdha]-microcystin-LR (Cyanobiotech GmbH, Berlin, Germany). All aqueous methanolic extracts that were found to be negative for microcystin, as revealed by HPLC, were subsequently tested by an indirect competitive enzyme-linked immunosorbent assay (ELISA; Abraxis, United Kingdom) that was targeted at the ADDA group of the molecule (11) detecting microcystin variants independent of structural variation. The ELISA has good cross-reactivity with a range of microcystin structural variants, such as microcystin-LR, -RR, -YR, -LW, -LF, D-Asp-LR, D-Asp-RR, and even nodularin. The sensitivity of the assay ranged from 0.15 to 5 ng ml^{-1}. To prevent the inhibition of the assay by methanol (30), the extracts were dried and resuspended in Millipore water. The ELISA was performed in triplicate for each sample according to the manufacturer's instructions.

RESULTS

Phytoplankton composition. According to the total phosphorus and chlorophyll *a* concentrations, the 12 lakes were classified as oligotrophic, oligomesotrophic, mesotrophic, and mesoeutrophic (see Table 1 in reference 32). In the vast majority of the samples, *Planktothrix* filaments were observed. There was no *Planktothrix* detected in a sedimentation volume of 25 ml for only 10 integrated samples, which were obtained from the lakes showing the lowest population densities (Attersee [four samples], Irrsee [three samples], Offensee [one sample], and Wolfgangsee [two samples]). During the study period, the 12 lakes varied in phytoplankton compositions and in the proportions of *Planktothrix* biovolume, ranging from 0.2% (Attersee) to 95.6% (Hallwilersee) (Fig. 1). Other cyanobacteria (*Anabaena*, *Lyngbya*, and *Aphanizomenon* spp.) made up no more than 3.6% of the total amount of phytoplankton. The second most common phytoplankton group was diatoms (*Aulacoseira*, *Fragillaria*, *Tabellaria*, and *Stephanodiscus* spp.), ranging from 1% (Hallwilersee) to 71.9% in Wolfgangsee. Cryptomonads (*Cryptomonas* sp.) and dinoflagellates (*Ceratium* sp.) contributed 0.8% (Hallwilersee) to 22.5% (Schwarzensee) and 1.3% (Afritzersee) to 11.8% (Zürichsee) to the total phytoplankton counts, respectively. On average, chrysomonads (*Dinobryon* sp.) and green algae (*Ankistrodesmus* and *Elakatothrix* spp.) never constituted more than 10% of the total phytoplankton community.

Abundance of microcystin-producing genotypes. For a total of 159 samples, a linear correlation between the total *Planktothrix* biovolume estimated by a 16S rRNA gene assay and the biovolume calculated from counting with a microscope was found: $y = -0.47 + 0.93x$ ($R^2 = 0.82$; $n = 145$; $P < 0.001$) (where x is the $\log_{10}$ biovolume [mm^3 liter^{-1}] calculated by counting by microscopy and y is the $\log_{10}$ biovolume estimated by 16S rRNA gene assay) (see Fig. S1 in the supplemental material). Compared to microscopy, real-time PCR showed a higher sensitivity: no *Planktothrix* could be detected for only three integrated samples (Attersee, Fuschlsee, and Wolfgangsee) and three net samples (one sample from Wolfgangsee and two from Attersee).

In order to find out whether the microcystin-producing genotypes of *Planktothrix* sp. contributed equally in all populations, the proportions of the sum of the Mdha genotype and the Dhb genotype were compared between lakes. On average, the Mdha and Dhb genotypes contributed 42.4% ± 3% (min-max, 16.3 to 63.2%) to the total population. In general, the proportion of the Dhb genotype was the highest (mean, 28.7% ± 2%; min-max, 6.1 to 49.8%), and on average, the populations showed a significantly lower proportion of the Mdha genotype ($P < 0.001$ by Kruskal Wallis one-way analysis of variance [ANOVA]; mean, 13.7% ± 1%; min-max, 0.3 to

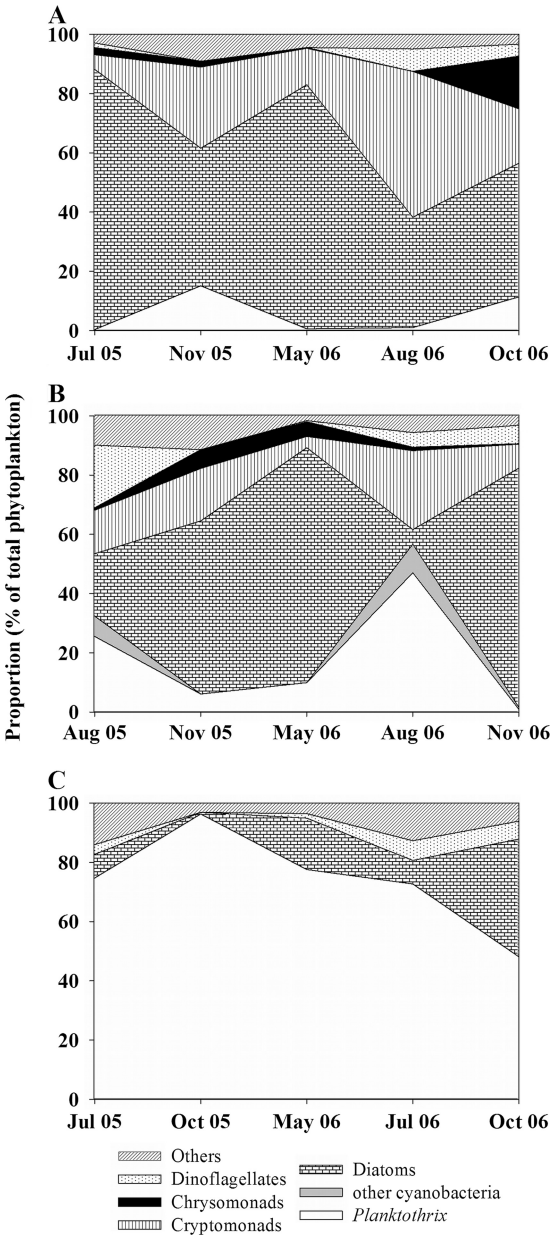

FIG. 1. Phytoplankton compositions in the study lakes classified into three groups depending on the average proportions of *Planktothrix* in the total phytoplankton biovolume ($n = 5$): <10% of the average proportion of *Planktothrix* in total phytoplankton, e.g., Schwarzensee (A); 10 to 50%, e.g., Mondsee (B), and 50 to 90%, e.g., Wörthersee (C). Phytoplankton groups that contributed ≤5% to the total biovolume are shown as "others."

TABLE 1. Average proportions of microcystin genotypes synthesizing different structural variants determined by real-time PCR and the corresponding microcystin variants detected by HPLC analysis over the study period

Lake	Trophy[a]	No. of samples used for real-time PCR[b]	Mean % biovolume of genotype ± SE			No. of samples used for HPLC[b]	Mean % proportion of microcystin variant ± SE					Mean total MC (μg liter^{-1}) ± SE[c]
			Mdha	Dhb	Hty		[Asp, Mdha]-microcystin-RR	[Asp, Dhb]-microcystin-RR	[Asp]-microcystin-HtyR	[Asp]-microcystin-LR	Unknown MCs	
Attersee	O	6	32.3 ± 10	25.2 ± 11.4	12.3 ± 11.9	1	0	0	0	100	0	0[d]
Wolfgangsee	O	9	6.0 ± 2.9	22.7 ± 1.4	7.8 ± 3.2	0	0	0	0	0	0	0[d]
Schwarzensee	O	13	0.3 ± 0.2	28.1 ± 4.6	4.8 ± 2.0	7	0	100	0	0	0	0[d]
Offensee	O	13	2 ± 1.8	44.4 ± 11.7	35.3 ± 4.9	8	0	0	42.2 ± 2.2	57.8 ± 2.2	0	0.05 ± 0.02
Ammersee	O-M	12	29.3 ± 6.8	17.7 ± 3.5	3 ± 0.4	12	67.3 ± 5.4	25.3 ± 5.3	0.8 ± 0.5	6.6 ± 2.9	0	0.11 ± 0.04
Fuschlsee	O-M	12	8.5 ± 1.8	23.1 ± 11.5	16.7 ± 13.6	12	41.0 ± 1.9	43.8 ± 3.4	1.2 ± 0.6	10.8 ± 3.1	3.3 ± 2.2	0.11 ± 0.04
Irrsee	O-M	13	22.2 ± 6.4	19.7 ± 5.2	7.6 ± 6.0	6	15.2 ± 15.2	48.6 ± 21.8	10.2 ± 10.2	26.0 ± 15.9	0	0.04 ± 0.03
Mondsee	O-M	12	10.2 ± 2.6	6.1 ± 1.9	2.1 ± 0.9	8	56.2 ± 2.1	38.8 ± 2.8	0.7 ± 0.7	4.3 ± 2.2	0	0.05 ± 0.02
Wörthersee	M	12	15.1 ± 3.1	32 ± 8.9	5.4 ± 1.3	12	27.9 ± 1.7	48.9 ± 1.9	6.4 ± 0.6	14.1 ± 1	2.7 ± 1.4	1.09 ± 0.29
Afritzersee	M	12	17.9 ± 4.5	34.4 ± 7.6	6.3 ± 0.9	12	39.9 ± 2.7	38.1 ± 2.5	7.3 ± 1.4	12.6 ± 0.7	2 ± 1	0.85 ± 0.24
Zürichsee	M	15	15.5 ± 1.1	36.7 ± 3.9	9.1 ± 1.1	15	33.7 ± 2.1	52.8 ± 2.3	3.3 ± 0.8	7.3 ± 1.7	2.9 ± 1.3	2.08 ± 0.73
Hallwilersee	M-E	11	13.4 ± 4.7	49.8 ± 8.3	16 ± 2.6	11	20.6 ± 5.8	57 ± 5.8	7.9 ± 1.0	14.4 ± 1.2	0.2 ± 0.1	3.82 ± 0.80
Total		140	13.7 ± 1.4	28.7 ± 2.3	10.6 ± 1.6	104	32.6 ± 2.3	44.2 ± 2.6	7.0 ± 1.2	14.9 ± 1.9	1.4 ± 0.4	0.60 ± 0.14

[a] O, oligotrophic; O-M, oligomesotrophic; M, mesotrophic; M-E, mesoeutrophic.
[b] Depth-integrated and plankton net samples were combined.
[c] Total microcystin concentrations (μg liter^{-1}) from integrated samples.
[d] Microcystin positive as revealed by ELISA.

32.3%). Extremely low proportions of the Mdha genotype occurred in oligotrophic lakes, i.e., Offensee, Wolfgangsee, and Schwarzensee (Table 1). Consequently, the proportions of the Mdha genotype were significantly positively related with either total phosphorus concentrations or chlorophyll *a* concentrations ($P < 0.05$). However, the correlation coefficients were always low ($R^2 \leq 0.20$). The Hty genotype generally constituted the lowest proportion in all the populations (10.6% ± 2%). Only for Offensee, Fuschlsee, and Hallwilersee was a higher proportion of the Hty genotype recorded. The proportion of the Hty genotype did not depend on the total phosphorus or chlorophyll *a* concentrations.

Altogether, the abundance of the three microcystin genotypes was significantly positively related to the total *Planktothrix* biovolume revealed by 16S rRNA gene analysis. For the sum of the Mdha and Dhb genotypes, the linear relationship was $y = -0.52 + 0.99x$ ($R^2 = 0.94$; $n = 138$; $P < 0.001$), where

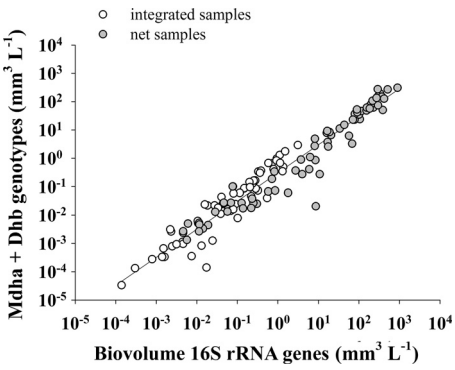

FIG. 2. Relationship between the total *Planktothrix* biovolume (mm^3 liter^{-1}) determined by real-time PCR via 16S rRNA genes and the microcystin genotypes consisting of the Mdha and Dhb genotypes (synthesizing either Mdha or Dhb in position 7 of the microcystin molecule). For details on the regression curves, see the text.

x is the $\log_{10}$ biovolume estimated by 16S rRNA gene assay and y is the sum of the $\log_{10}$ biovolume of the Mdha and the Dhb genotypes (Fig. 2). The regression curves for each of the three genotypes considered separately were $y = -0.97 + 0.94x$ ($R^2 = 0.92$; $n = 111$; $P < 0.001$) for Mdha, $y = -0.67 + 0.97x$ ($R^2 = 0.93$; $n = 126$; $P < 0.001$) for Dhb, and $y = -1.22 + 0.94x$ ($R^2 = 0.88$; $n = 113$; $P < 0.001$) for Hty, where x is the $\log_{10}$ biovolume estimated by 16S rRNA gene assay and y is the $\log_{10}$ biovolume of the respective microcystin genotype (see Fig. S2 in the supplemental material). Consequently, the population density was a significant factor related to the abundance of all microcystin genotypes.

Microcystin concentrations. Microcystin concentrations varied from 0 to 6.2 μg liter^{-1} (mean, 0.6 ± 0.1 μg liter^{-1}; $n = 79$) among the integrated samples and from 0 to 4,994 μg liter^{-1} (mean, 420 ± 90 μg liter^{-1}; $n = 80$) among the net samples. No microcystins were detected for 34% of all the integrated samples and 35% of all the net samples by means of HPLC (see Table S2 in the supplemental material). Subsequent ELISAs revealed the presence of microcystins in all of those samples. The microcystin concentrations determined by ELISA varied from 0.001 to 10.5 μg liter^{-1} (mean, 0.412 ± 0.4 μg liter^{-1}; $n = 27$) for the integrated samples and from 0.084 to 13.3 μg liter^{-1} (mean, 2.1 ± 0.6 μg liter^{-1}; $n = 28$) for the net samples. The average microcystin content in *Planktothrix* was 3.1 ± 0.7 μg mm^{-3} biovolume (0.2% ± 0.05% of the cellular biovolume). There was no statistically significant difference in the average microcystin contents between integrated and net samples ($P = 0.96$ by t test).

Microcystins consisted of [Asp, Dhb]-microcystin-RR (44.2% ± 3%), [Asp, Mdha]-microcystin-RR (32.6% ± 2%), [Asp]-microcystin–homotyrosine-arginine (HtyR) (7% ± 1%), and [Asp]-microcystin-LR (14.9% ± 2%). Unknown microcystins generally contributed less than 1.4%. Corresponding to the low proportion of the Mdha genotype in oligotrophic lakes, the proportion of [Asp, Mdha]-microcystin-RR was significantly posi-

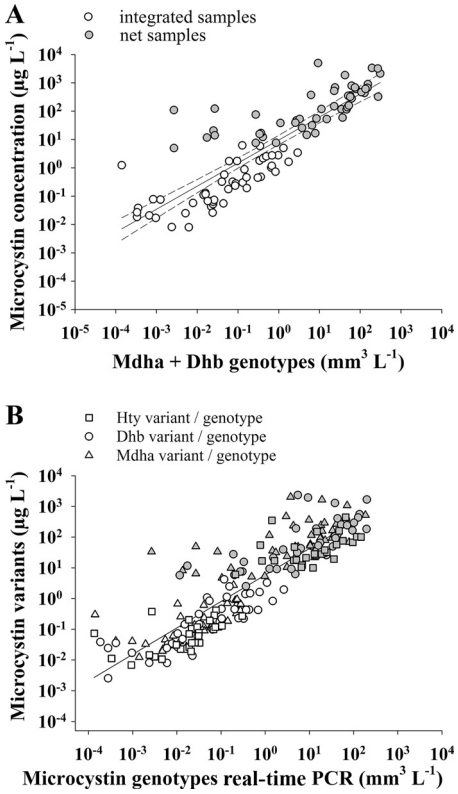

FIG. 3. (A) Relationship between the total of the microcystin genotypes (Mdha and Dhb) determined by real-time PCR (biovolume in mm^3 $liter^{-1}$) and the total microcystin concentration estimated by HPLC (μg $liter^{-1}$) from integrated (white symbols) and net (gray symbols) samples. Broken lines indicate 95% confidence limits. (B) Relationship between each of the microcystin genotypes and the corresponding microcystin variants for the same data set. For details on the regression curves, see the text.

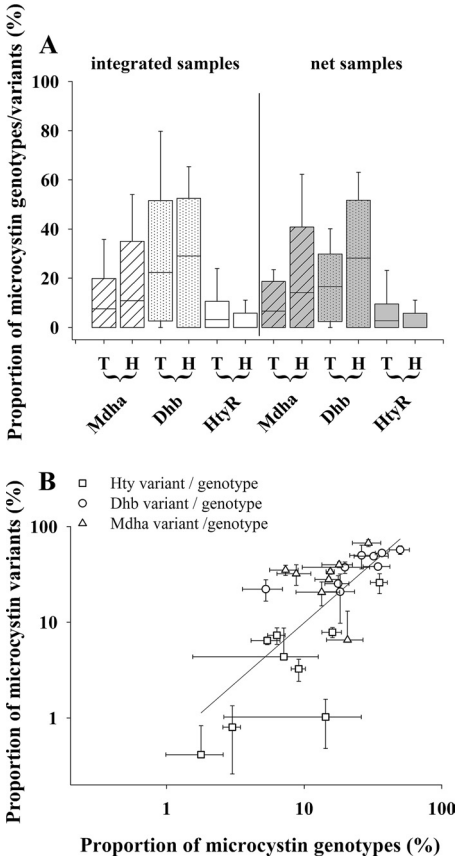

FIG. 4. (A) Proportions of genotypes encoding the synthesis of a specific microcystin variant (Mdha, Dhb, and Hty) revealed by real-time PCR (T) and of the respective microcystin structural variants revealed by HPLC (H). Box plots show the median and 25% to 75% percentiles. (B) Relationship between the average proportion (±SE) of microcystin variants determined by HPLC and the average proportion (±SE) of microcystin genotypes estimated by real-time PCR for each lake. For details on the regression curve, see the text.

tively related to the trophic state, as indicated by the total phosphorus or chlorophyll *a* concentrations ($P < 0.05$; $R^2 \leq 0.27$). The proportions of the other MC variants did not show any relation to the trophic state.

Relationship between microcystin genotype abundance and microcystin concentration. There was a linear correlation between the abundance of each of the three genotypes and the concentrations of the respective microcystin variants ($y = 0.97 + 0.85x$ [$R^2 = 0.72$; $n = 79$; $P < 0.001$] for Mdha, $y = 0.75 + 0.89x$ [$R^2 = 0.8$; $n = 80$; $P < 0.001$] for Dhb, and $y = 0.5 + 0.85x$ [$R^2 = 0.84$; $n = 54$; $P < 0.001$] for HtyR, where x is the $\log_{10}$ of the respective microcystin genotype measured by real-time PCR [mm^3 $liter^{-1}$] and y is the $\log_{10}$ of the corresponding microcystin variant measured by HPLC [μg $liter^{-1}$]). The sum of Mdha and Dhb genotypes was significantly related to the total concentration of microcystin following the regression $y = 0.98 + 0.81x$ ($R^2 = 0.73$; $n = 100$; $P < 0.001$) (Fig. 3A). Furthermore, the abundance of each of the microcystin genotypes was significantly related to the microcystin concentration assigned to the corresponding microcystin variant, $y = 0.77 + 0.86x$ ($R^2 = 0.77$; $n = 213$; $P < 0.001$), where x is the $\log_{10}$ biovolume of a specific genotype and y is the $\log_{10}$ of the concentration of the corresponding microcystin variant (μg $liter^{-1}$) (Fig. 3B). Corresponding to the highest Dhb genotype proportion (36.8% ± 4% among the integrated samples and 20.9% ± 2% among the net samples), the Dhb microcystin constituted 46.2% ± 4% of the integrated samples and 43.9% ± 4% of the net samples (Fig. 4A). Correspondingly, the lower Mdha genotype proportion (14.2% ± 2% of the integrated samples and 13.2% ± 2% of the net samples) resulted in a lower proportion of the Mdha microcystin (30.8% ± 3% of the integrated samples and 35.5% ± 4% of the net samples). In contrast, the lowest Hty genotype proportion (10.4% ± 2% of the integrated samples and 10.7% ± 3% of the net samples) resulted in a low proportion of the Hty microcystin both among the integrated samples (8% ± 2%) and among the net samples (6.3% ± 2%). On average, the proportion of microcystin genotypes in the lakes was related to the concentrations of the corresponding microcystin variant following the regression $y = -0.26 + 1.26x$ ($R^2 = 0.54$; $n = 26$), where x is the $\log_{10}$ of the average proportion of the genotypes and y is the $\log_{10}$ of the average proportion of the corresponding microcystin variants of the lakes (Fig. 4B).

DISCUSSION

Correlation between microcystin concentrations and the abundance of microcystin genotypes. In the present study,

the concentrations of microcystins in total as well as each of the three structural variants could be predicted from the corresponding genotype concentrations. We did not find that a specific genotype concentration was significantly better at predicting the corresponding microcystin concentration than the total population, as determined via 16S rRNA gene assay or via microscopic counting. For all predicting variables, the slope of the regression curve was close to 1, and the coefficient of determination was close to 0.9. This is probably because the populations that were investigated were absolutely dominated by the microcystin-producing genotype (22). Accordingly, Briand et al. (4) previously reported a highly significant correlation between the individual growth rate and the microcystin production rate of *Planktothrix* in Lac du Bourget, France. In that study, the high correlation coefficients observed were favored by the occurrence of only one microcystin-producing organism. Since the real-time PCR assays that were applied were *Planktothrix* specific, the presence of other microcystin-producing taxa would have deteriorated the quality of the regression curves. If other taxa containing the *mcy* gene cluster would be found to be co-occurring, more general probes and primers, for example, targeted at the *mcyE* gene (16, 34), would need to be applied. In general, the polyketide part of the *mcy* synthetase gene cluster shows less genetic variation between genera and is most suitable for the design of primers binding to the *mcyE* gene of all taxa. The *mcyE* abundance would then need to be referred to by applying another real-time PCR assay targeted at cyanobacterial 16S rRNA genes (36). The sequencing of the *mcyE* products would then lead to the identification of the corresponding microcystin-producing organisms.

In addition, from the results of the present study, it must be concluded that all microcystin variants produced by *Planktothrix* spp. that were observed under field conditions were also observed in culture, and the proportion of the unexplained microcystin variants occurring under natural conditions was low ($\leq$5.1%) (see Table S1 in the supplemental material). Recently, by comparing peptide compositions between the single isolates and field samples, the isolation of all major chemotypes occurring in Lake Steinsfjorden in Norway was reported (39). This high correlation in peptide occurrence between the laboratory and field conditions is remarkable, as the cultivation of bacteria in general is considered biased, i.e., favoring those species and/or genotypes that are able to cope with the high concentrations of nutrients that are provided (1). It was speculated that the positive phototaxis of *Planktothrix* filaments on agar, which allows the cutting out of single filaments by means of a microspade (38), leads to a much higher rate of success in the isolation of individual strains compared to immotile cyanobacteria. It is speculated that due to its accessibility to cultivation, the genus *Planktothrix* is highly suitable for the analysis of the genetic regulation of toxin production both in the laboratory and in the field.

Low proportion of inactive microcystin genotypes. In a previous study, we reported the proportion of inactive microcystin genotypes carrying either deletions or insertions within the *mcy* gene cluster in samples from the same lakes (32). On average, a rather low proportion of the mutant genotypes carrying either the deletion (3.7%) or several insertions (2.8%) was found. In this study, the high correlation coefficient between the individual microcystin genotypes and the concentration of the corresponding microcystins (R^2 = 0.72 to 0.84) implies that the proportion of inactive microcystin genotypes did not vary significantly and that the occurrence of selective sweeps leading to a dominance of inactive genotypes did not occur. Recently, Manganelli et al. (28) suggested an increase in *Planktothrix* genotypes lacking the *mcy* gene cluster under conditions that favor blooms in an Italian lake. The highest densities recorded were 44.4×10^6 cells liter^{-1}, corresponding to 1.47 mm^3 liter^{-1} (assuming a cellular *Planktothrix* biovolume of 33.4 μm^3). In this study, these higher *Planktothrix* cell densities were observed frequently in depth-integrated samples of Lake Wörthersee, Lake Zürichsee, and Lake Hallwilersee. For a much more densely growing green-pigmented *Planktothrix* population flourishing in Lake Base Nautique de Viry (Paris, France), an increase in the non-*mcy*-containing genotype from a blooming period (50 mm^3 liter^{-1}; 36 to 60% of the *mcyA* genotype) to a period with the lowest cell numbers (0.7 to 8 mm^3 liter^{-1}; 56 to 73%) was reported (3). From this data set, the change in the proportion of the *mcy*-containing genotype seems rather minor compared with the large variation in population cell density.

At present, it is difficult to explain which factors keep the proportion of the inactive microcystin genotypes low. It is frequently argued that the production of secondary metabolites, including enzymes and toxins, can be considered a common good that is beneficial to the whole population of a particular prokaryotic organism, e.g., *Myxococcus xanthus* or *Bacillus subtilis* (the multicellular organism hypothesis [43]). In these populations, the occurrence of mutants that are deficient in secondary metabolite production but that show faster growth rates that lead to an ultimate outgrowth of the wild type has been frequently reported (47). Recently, it was shown that so-called nontoxic cheaters occurring in populations of *Pseudomonas fluorescens* show a negative frequency-dependent fitness, implying that the nontoxic mutants indeed rely on the exotoxins produced by wild-type bacteria (15). For *Planktothrix*, it could be argued that predators such as aquatic crustaceans keep the proportion of inactive microcystin genotypes low. Unfortunately for this particular hypothesis, the vast majority of *Planktothrix* filaments exceed the ingestible size range for pelagic herbivorous crustaceans, and the ingestion rates that have been determined for *Planktothrix* filaments are close to zero compared with those of other food items (25). Another selective factor promoting microcystin production in *Planktothrix* sp. might be chronic infection by parasites, such as by the chytrid fungus *Rhizophidium megarrhizum* Sparrow (5). According to that study, infected filaments can be identified by one or several zoospores settling at the apex of the filament. In our study, a low proportion of infected filaments was observed (only two filaments were found to carry zoospores during the enumeration of *Planktothrix* in the 159 samples). It remains to be seen whether it is indeed the inactive microcystin genotype that was infected by the parasite. In the future, single filaments should be isolated and probed for the presence of *mcy* gene cluster mutations and the co-occurrence of potential parasites by means of single-filament PCR (22).

Water quality monitoring. The results of the present study are of relevance to justify the application of toxic genotype monitoring by means of real-time PCR in order to predict

microcystin concentrations in water. Following the overall regression curve (depicted in Fig. 3A), a biovolume of 10 mm^3 $liter^{-1}$ of microcystin genotypes corresponds to a microcystin concentration of 62.3 ± 1 μg (confidence interval, 45.8 to 84.8 μg) microcystin-LR equivalents. Since microcystin-producing strains of *Microcystis* and *Planktothrix* do not differ principally in their microcystin contents (20), such a regression curve may also be applicable to water samples that are dominated by *Microcystis*. While for both taxa, comparable ranges of cellular microcystin contents have been reported (e.g., see data in reference 49 versus data in reference 46), we hereby emphasize, however, that the applicability of the regression curve (Fig. 3A) to freshwater that is dominated by *Microcystis* still needs to be confirmed.

Currently, drinking water/recreational water guideline values are calculated based on microcystin-LR equivalents. It has been repeatedly shown that microcystin structural variants can differ profoundly in their toxicodynamic and toxicokinetic properties (2, 13), and therefore, it was suggested that risk assessment schemes that are based solely on microcystin-LR are insufficient (8). According to a report by Blom et al. (2), the inhibition of protein phosphatases 1 and 2A was much weaker for [Asp, Dhb]-microcystin-RR than for the other structural variants, while the toxicity was one of the highest. Consequently, it might be useful to differentiate the genotypes encoding those microcystin variants, such as [Asp, Dhb]-microcystin-RR, which differ in toxicity compared with microcystin-LR.

Frequently, the length of the shorelines that need to be observed constitutes a major factor that increases the monitoring costs and response time due to sample transportation (45). Since portable real-time PCR instruments have been shown to be field applicable (35), it is conceivable that during future monitoring, those measurements will be confirmed directly on site subsequent to the filtration of water samples and the rapid extraction of the DNA from filters according to previously established protocols (41). The results would then be directly forwarded to the respective authorities and distributed to the public via websites, newspapers, and radio (9). This approach would reduce not only the effort of sample transportation but also the sample analysis time until an early warning is possible. Various real-time PCR assays quantifying the microcystin-producing genotypes of the other microcystin-producing genera *Microcystis* and *Anabaena* and the nodularin-producing genotypes of the genus *Nodularia* have been developed (see reference 33 for a review). Analogously, in parallel to the description of the gene cluster involved in cylindrospermopsin synthesis (31, 40), real-time PCR assays for the identification of the cylindrospermopsin-producing genera *Cylindrospermopsis* and *Aphanizomenon* have been applied (35). The gene clusters encoding the synthesis of other toxic metabolites have been elucidated more recently, i.e., saxitoxin (17) and anatoxin-a (29). This sequence information forms the basis for the establishment of a real-time-based monitoring approach that targets all the toxic genotypes of cyanobacteria in surface water.

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