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Rapid quantification of *mcyB* copy numbers on dry chemistry PCR chips and predictability of microcystin concentrations in freshwater environments

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ABSTRACT

Microcystin-producing cyanobacteria cause serious water quality problems worldwide, which has led to growing pressure for more intensive monitoring. Molecular biology methods that are based on identification and enumeration of biosynthetic genes, such as quantitative PCR, show promise in this respect. To be practical in a wide range of settings, these methods need to be usable also by laboratory personnel who do not have previous experience in PCR setup. Here we present a real-time quantitative *mcyB* dry chemistry PCR assay capable of identifying the three globally most common microcystin-producing cyanobacterial genera, *Anabaena*, *Microcystis* and *Planktothrix*. It minimizes the amount of liquid handling and avoids direct contact with the PCR reagents at the time of analysis. Large quantities of virtually identical chips can be manufactured, improving the comparability of results. Using the dry chemistry PCR chips, freshwater environmental samples from Finnish and Estonian lakes, rivers and reservoirs were analyzed for *mcyB*. The chip format was found to be highly suitable for water sample analysis due to its ease-of-use, good sensitivity and amplification efficiency. Significant positive correlation (Spearman's rank correlation, $\rho > 0.66$, $P < 0.001$) was observed between combined *mcyB* copy numbers from *Microcystis*, *Anabaena*, *Planktothrix* and total microcystin concentrations, regardless of the method used to measure the toxins (ELISA or LC–MS). Positive correlations were observed also for single lakes.

1. Introduction

Mass occurrences of toxic cyanobacteria are increasingly recognized as a serious risk to public health, and they are expected to become more frequent and severe with increasing nutrient loads and the changing climate (Paerl and Paul, 2012). Among the most common cyanobacterial toxins are microcystins, a group of acutely hepatotoxic cyclic peptides, which are encountered in freshwater environments worldwide. Microcystin exposure in humans can occur via many routes (Falconer et al., 1999), but the most common are drinking water and recreational use of waterbodies which suffer from toxic blooms. The World Health Organization has set a provisional guideline value for the maximum amount of microcystin-LR allowed in drinking water, 1 $\mu g\ L^{-1}$ (WHO, 2011). However, since microcystins have been linked to increased incidence of hepatocellular carcinoma (Yu, 1995; Svircev et al., 2009), and MC-LR has recently been classified as possibly carcinogenic to humans (IARC, 2010), the range of potentially harmful toxin amounts is not limited to moderate or high concentrations only.

Over 90 microcystins have been identified (Welker and von Döhren, 2006) and new structural variants are constantly being discovered. These variants differ in their toxicity, and the differences can be over 10-fold for variants commonly found in the environment, such as MC-LR (50 $\mu g\ kg^{-1}$, Krishnamurthy et al., 1986) and MC-RR (600 $\mu g\ kg^{-1}$, Watanabe et al., 1988). Microcystins are produced by strains of several bloom-forming cyanobacterial genera, globally most common of which are *Microcystis*, *Anabaena* and *Planktothrix* (Sivonen and Jones, 1999). With the growing awareness of the toxins and their producers, the demand for risk assessment and monitoring methods is increasing. Since the discovery of the genetic background of microcystin biosynthesis (Nishizawa et al., 1999; Tillett et al., 2000; Christiansen et al., 2003;

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Rouhiainen et al., 2004), molecular approaches have shown promise in this respect, and a number of qualitative and quantitative methods based on the detection of microcystin synthetase genes have been described (see the review by Sivonen, 2008).

When genetic detection methods are to be used in localized and on-site monitoring applications, fast and convenient reaction setup is essential. All PCR reagents, including the DNA polymerase, can be successfully dried to produce ready-to-use reaction vessels (von Lode et al., 2007). The technology has previously been applied to diagnostics of human diseases (Kiviniemi et al., 2009; Hirvonen et al., 2012), but not to environmental monitoring. Since some studies have indicated positive correlations between microcystin concentrations and the amount of *mcy* positive genotypes in the environment (Vaitomaa et al., 2003; Fortin et al., 2010; Ostermaier and Kurmayer, 2010), the DNA-based monitoring of toxin producers would benefit from an approach similar to medical diagnostics.

We have previously developed a quantitative PCR assay for the *mcyB* gene, combined with an effective and simple heat-treatment based sample preparation method (Hautala et al., 2013). The aims of this study were to assess the suitability and performance of an easy-to-use dry chemistry PCR method in water sample analysis, and to determine the predictability of microcystin concentrations in the environment based on genus-specific detection and quantification of *mcyB*. LC–MS and ELISA microcystin measurements were compared to *Anabaena*, *Microcystis* and *Planktothrix* specific gene copy numbers in water samples collected from Southern and Southwestern Finland as well as Northern Estonia.

2. Materials and methods

2.1. Environmental samples and sample preparation

In total, 98 water samples were collected from on Åland Islands, mainland Southwestern Finland, Southern Finland and northern Estonia during June–November 2009. Sampling locations consisted of 34 freshwater lakes, two rivers and three drinking water reservoirs of varying size and trophic status. Three of the Finnish locations were followed for longer periods over time, namely Hauninen Reservoir ($n = 16$) and Lake Littoistenjärvi ($n = 9$) in the Turku region and Lake Tuusulanjärvi ($n = 9$) in Tuusula. All lakes and rivers were sampled near the shoreline, from the depth of 10–20 cm, avoiding possible intense surface scums. All samples were collected manually into 50 ml plastic tubes. The drinking water reservoirs were sampled similarly at water intake pipes, and represent depths of 0–2 m. Samples were filtered immediately or refrigerated and processed within 4 h of sampling. For qPCR analysis, 10 ml aliquots of the water samples were filtered through binder-free fiberglass filters (25 mm, GF/C, Whatman, UK) using 20 ml disposable plastic syringes and sterilizable 25 mm polycarbonate filter holders (Sartorius Stedim Biotech, Göttingen, Germany). Samples with a high density of cells or other solid material were filtered in smaller aliquots, and the volume passed through the filter was recorded. The filters were rinsed and excess water removed by first filtering 20 ml of sterile deionized water and then pressing a full syringe of air through the filter. Filters were removed and placed in 2 ml plastic tubes, and either processed further immediately, or stored and transported frozen (−20 °C). After filtration, 1×10^5 cells of *Bacillus subtilis* ATCC 6633 (Difco Laboratories, Detroit, MI, USA), used as a qualitative internal amplification control (IAC) for heat-treatment and PCR chips (see Section 2.4), were added onto the membrane, and 1.5 ml of sterile deionized water was added to the tube. Tubes were heated at 80 °C for 10 min, after which all liquid was transferred to clean, sterile tubes and stored frozen (−20 °C) until analysis. For chromatographic and ELISA, 20 ml of water sample was filtered as described above, without rinsing. Excess water was removed by pressing a full syringe of air through the filter, which were then stored as above.

2.2. Microcystin analysis

Extraction of microcystins from the filters and LC–MS analysis was carried out as described previously (Spoof et al., 2003; Spoof and Meriluoto, 2005; Hautala et al., 2013). The experiments were performed on an Agilent 1200 Rapid Resolution (RR) LC coupled to a Bruker Daltonics HCT Ultra ion trap mass spectrometer (Bremen, Germany) with an electrospray ion (ESI) source. Using the same extracts, the amount of intracellular microcystins was also analyzed with a Quantiplate Microcystin ELISA kit (Envirologix, Portland, ME, USA) according to the protocol provided by the manufacturer.

2.3. PCR chip preparation

Primer, probe, and quencher probe sequences are listed in Table 1. All detection probes were labeled with intrinsically fluorescent lanthanide chelates as described previously (Nurmi et al., 2002). The mcyB-aP, mcyB-mP and mcyB-pP probes were labeled at the 5′ end aminolinker with a Tb(III) chelate ({2,2′,2″,2‴-{{6,6′-{4″-[2-(4-Isothiocyanatophenyl)ethyl]pyratzole-1″,3″-diyl}bis(pyridine)-2,2′-diyl}bis(methylenenitrilo)}tetrakis(acetato)}terbium(III)) and the IAC probe BsP with a corresponding Eu(III) chelate. The chelates were synthesized at the University of Turku, Turku, Finland. Four

Table 1
Primers and probes used in this study.

Oligonucleotide name	Sequence, 5′–3′	Oligonucleotide type	Target	Reference
mcyBHF03A	GCTTTAATCCACAAGAAGCTTTATTAGC	Forward primer	All *mcyB*, prim. *Anabaena*	Hautala et al. (2013)
mcyBHF03M	AGATTTTAATCCACAAGAAGCTTTATTAGC	Forward primer	All *mcyB*, prim. *Microcystis*	Hautala et al. (2013)
mcyBHF03P	GGTTTAATCAACAAGAGGCTTTATTAGC	Forward primer	All *mcyB*, prim. *Planktothrix*	Hautala et al. (2013)
mcyBHR04	CTGTTGCCTCCTAGTTCAAAAAATGACT	Reverse primer	All *mcyB*	Hautala et al. (2013)
mcyB-aP	amC6-ACTGAATTATTGGAGGTAGAGGTGAGTGATAC-Phos	Detection probe	*Anabaena*	Hautala et al. (2013)
mcyB-aQ	CCTCTACCTCCAATAATTCA-BHQ1®	Quencher probe	*Anabaena*	Hautala et al. (2013)
mcyB-mP	amC6-GGGTGAGTTATTAGAAGCAGAAGTTAGTAACAG-Phos	Detection probe	*Microcystis*	Hautala et al. (2013)
mcyB-mQ	TTCTGCTTCTAATAACTCACC-BHQ1®	Quencher probe	*Microcystis*	Hautala et al. (2013)
mcyB-pP	amC6-GGGGTGAATTATTAGAAATAGAAGTAAGTGACAA-Phos	Detection probe	*Planktothrix*	Hautala et al. (2013)
mcyB-pQ	TTACTTCTATTTCTAATAATTCACC-BHQ1®	Quencher probe	*Planktothrix*	Hautala et al. (2013)
Bs5′	GCGGAGCAAGCTTCGTACCTTCT	Forward primer	*B. subtilis*	Korpimäki et al. (2007)
Bs3′	CTAACGCCCAGAACACCGATTGAGT	Reverse primer	*B. subtilis*	Korpimäki et al. (2007)
BsP	amC6-CCATACCAGGACGGCAGTTCTCAGC-Phos	Detection probe	*B. subtilis*	Korpimäki et al. (2007)
BsQ	CTGCCGTCCTGGTATGG-BHQ2®	Quencher probe	*B. subtilis*	Korpimäki et al. (2007)

types of chips (empty chips provided by Abacus Diagnostica, Turku, Finland) were prepared: three different *mcyB* detection chips, specific for *Anabaena*, *Microcystis* or *Planktothrix*, and IAC chips. All four chip types contained the same basic reagents: 14 nmol of each dNTP and 2 U DyNAzyme II polymerase (Finnzymes, Espoo, Finland), and a buffer including 35 μg BSA (Sigma, St. Louis, MO), 192.5 nmol $MgCl_2$, 350 nmol Tris, 1.75 mmol KCl, pH 8.3. All reagents were of molecular biology grade. Additionally, all three *mcyB* chip types contained a mix of the forward primers mcyBHF03A, mcyBHF03M and mcyBHF03P, 5.85 pmol each, and the reverse primer mcyBHR04, 17.5 pmol. Each mcyB chip type also contained its respective probe–quencher pair: *Anabaena*, mcyB-aP–mcyB-aQ (525 fmol and 6.3 pmol, respectively), *Microcystis*, mcyB-mP–mcyB-mQ (525 fmol and 5.25 pmol, respectively) and *Planktothrix*, mcyB-pP–mcyB-pQ (525 fmol and 5.25 pmol, respectively). Correspondingly, the IAC chips contained 17.5 pmol both the Bs5′ and Bs3′ primers, as well as 525 fmol BsP-probe and 5.25 pmol BsQ-quencher. The reagents were dispensed as two separate droplets, the chips dried under a vacuum and then sealed with aluminum foil as described previously by von Lode et al. (2007). The chips were stored in sealed aluminum foil bags in dry conditions at room temperature until use, or a maximum of 4.5 months.

2.4. Production and enumeration of amplification control

The amplification control, *Bacillus subtilis* strain ATCC 6633, was grown overnight at 37 °C, in a shaking incubator (300 rpm), in LB medium. The bacterium was chosen as a control based on its behavior in the sample heat-treatment stage, which was similar to that of the target cyanobacteria, studied previously (Hautala et al., 2013). Cells were harvested by centrifugation (5 min, 3220 g, Eppendorf 5810R, Eppendorf, Ulm, Germany), washed 3 times with 5 ml of sterile deionized water and stored in small aliquots at −20 °C until use. Cells were enumerated using quantitative real-time PCR with purified amplicons as standards. The qPCRs contained 1× DyNAzyme II HotStart buffer (Finnzymes), 4 pmol dNTPs, 6 pmol Bs5′ and Bs3′ primers, 0.3 pmol BsP, 3 pmol BsQ, 0.2 U DyNAzyme II HotStart enzyme (Finnzymes). Templates were added in a volume of 4 μl, and reactions were filled to 20 μl with sterile deionized water. Reactions were run on ThermoFast 96 Robotic PCR Plates (Abgene, Surrey, UK) with Applied Biosystems Optical Caps (Foster City, CA, USA). The qPCRs were run as follows: 95 °C 5 min, 8 cycles of 95 °C 30 s and 62 °C 1 min, 16 cycles of 35 °C 15 s (during which the fluorescence was measured), 95 °C 30 s, 62 °C 1 min, 95 °C 30 s, 62 °C 1 min and a final measurement at 35 °C 15 s. Measurements were carried out with a Victor^2 1420 Multilabel Counter (PerkinElmer Life Sciences Wallac, Turku, Finland) using the manufacturer's standard TRF-Eu protocol. The number of subtilisin gene copies per cell (Bach et al., 2002), was taken into account when determining cell concentrations. The recovery of the amplification control in the absence of environmental samples was assessed by filtering and heat-treating four replicates of *B. subtilis* suspension corresponding 1×10^5 cells per PCR and by testing the outcome with qPCR as described above. The suitability of *B. subtilis* cells as internal control was investigated similarly by screening randomly selected waters samples ($n = 30$) for amplification of the target subtilisin gene. The sample preparation was done as described above, but without the addition of IAC.

2.5. Analysis of environmental samples on PCR chips and interpretation of results

The GenomEra automated nucleic acid analyzer platform (Hagren et al., 2008) was used to run the chip based PCR assays. The analyzer is designed specifically for the PCR chips used. The metal foil laminated, flat reaction vessels are moved between pre-heated thermal blocks, which allows for a rapid rate of temperature change and fast thermal cycling. The standards and environmental samples were added onto the dried reagents in a 35 μl volume, and the chips were irreversibly heat-sealed at the beginning of the program to avoid any cross-contamination from amplification products. The program consisted of a 2 min initial denaturation at 100 °C, 10 cycles of 62 °C 16 s and 100 °C 4.5 s and 18 cycles of 40 °C 20 s (measurement), 62 °C 25 s, 100 °C 4.5 s, 62 °C 16 s and 100 °C 4.5 s. The assay performance was assessed by analyzing a series of 1, 5, 10, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 copies of each *mcyB* standard and a series of 10, 1×10^2, 1×10^3, 1×10^4 and 1×10^5 *B. subtilis* cells, all in triplicate. One set of standards in three to four replicates was run for each chip batch produced. Each environmental sample was analyzed using all four chip types (*mcyB Anabaena*, *mcyB Microcystis*, *mcyB Planktothrix* and IAC) in a single qPCR run. The recoveries for amplification controls were calculated for each sample.

2.6. Statistical analysis of results

Due to the non-normal distribution of the measurement results, Spearman rank correlation was used to study the relationship between total *mcyB* copies and microcystin concentrations in the samples. Copy number differences between sample groups allocated according to total microcystin concentration were analyzed with pair-wise Mann–Whitney *U* tests. Correspondence analysis was used to study whether certain variants of microcystin co-occurred with *Microcystis*, *Anabaena* or *Planktothrix*. All analyses were conducted using R 2.15.1 (R Core Team, 2012) with the ca package (Greenacre and Nenadic, 2013) for correspondence analysis installed.

3. Results

3.1. mcyB *PCR chips*

The performance of *mcyB* PCR chips was further improved compared to the 96-well plate format assay that we previously described (Hautala et al., 2013). The PCR chips were more sensitive, with a detection limit of 5 copies of *mcyB* per reaction compared to 10 copies per reaction for all target cyanobacterial genera as determined by standard qPCR (Fig. 1), corresponding to 21 copies of the target gene per ml of environmental sample. The quantification range of PCR chips was 5×10^0 to 1×10^7 copies of *mcyB* per reaction, and PCR efficiencies varied between 99 and 103% in PCR chips compared to 91–95% in the 96-well format. Higher signal to background ratios were also observed in PCR chips, on average 2–2.5 times as high as on a plate. Total assay duration from untreated water sample to *mcyB* quantification results was approximately 1 h 30 min, including the PCR cycling and measurements (55 min).

3.2. Amplification control

The mean recovery of IAC, *Bacillus subtilis*, in the absence of environmental samples was 84% (sd 18%). Considerably larger variation in the recovery percentages (63%, sd 48%) was observed when analyzing environmental samples. However, complete inhibition of amplification was not observed, and thus inconclusive results were not received. The assay sensitivity was 10 cells per reaction, and the log-linear range 1×10^1 to 1×10^5 cells per reaction ($y = -3.69x + 39.33$, $R^2 = 0.99$). No amplification of target *B. subtilis* gene was observed from environmental samples ($n = 30$) without the addition of cells at the sample preparation stage.

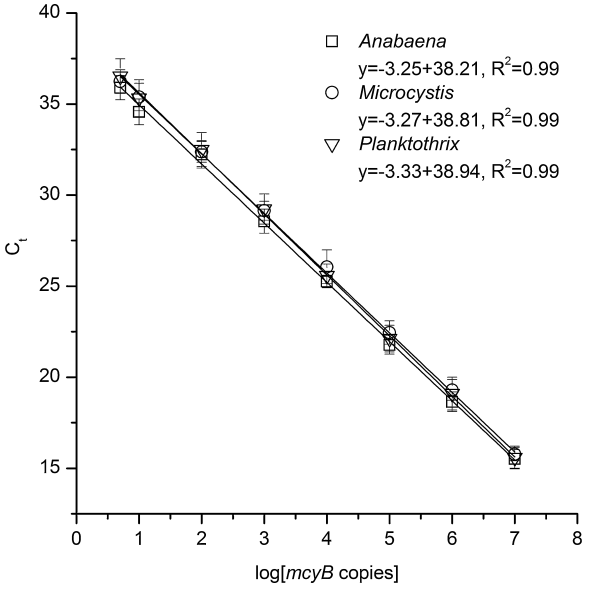

Fig. 1. Standard curves for the *mcyB* chip PCR. Error bars indicate the standard deviations between three to four replicates run on three separate PCR instruments.

3.3. Microcystin analysis of the environmental samples

Detectable microcystins, determined with ELISA and LC–MS, were present in 90% and 71% of the samples, respectively ($n = 98$). Concentrations ranged from 0 (not detected) to 30.4 (mean 2.3) and 0 to 40.9 (mean 2.0) $\mu g\,L^{-1}$ with ELISA and LC–MS, respectively. Based on LC–MS analysis, dmMC-RR was the most commonly occurring microcystin variant, present in 66% of all samples examined and 93% of microcystin-containing samples (Table 2). Other common variants were MC-RR, MC-LR, dmMC-LR and MC-YR. Desmethyl microcystin-YR was observed in samples collected from Hauninen Reservoir. Didesmethyl variants were present in three samples, and one sample from Åland contained MC-LF, MC-LW and MC-LY.

Correspondence analysis modeling showed that the presence of didesmethyl variants of MC-LR and MC-RR was strongly correlated with *mcyB*-positive *Anabaena* (Fig. 2). However, the low number of examples in this case does not exclude possible associations with other genera. MC-RR, MC-LR and MC-YR were more likely to co-occur with *Anabaena* and *Microcystis* than *Planktothrix*. On the other hand, dmMC-LR and dmMC-YR were more likely to be present in conjunction with *Microcystis* and *Planktothrix*. Desmethyl MC-RR was almost equally present with all studied genera.

Table 2
The prevalence of microcystin variants in water samples as determined by LC–MS.

Microcystin variant	Variant present in the sample, no. of samples (percentage of all)	Variant dominant in the sample, no. of samples (percentage of all)
dmMC-RR	65 (66%)	40 (41%)
MC-RR	43 (44%)	23 (24%)
dmMC-LR	36 (37%)	–
MC-LR	33 (34%)	2 (2%)
MC-YR	17 (17%)	1 (1%)
dmMC-YR	14 (14%)	–
didmMC-RR	3 (3%)	–
didmMC-LR	2 (2%)	–
MC-LF	1 (1%)	–
MC-LW	1 (1%)	–
MC-LY	1 (1%)	–

Note: More than one microcystin variant was present in almost all samples.

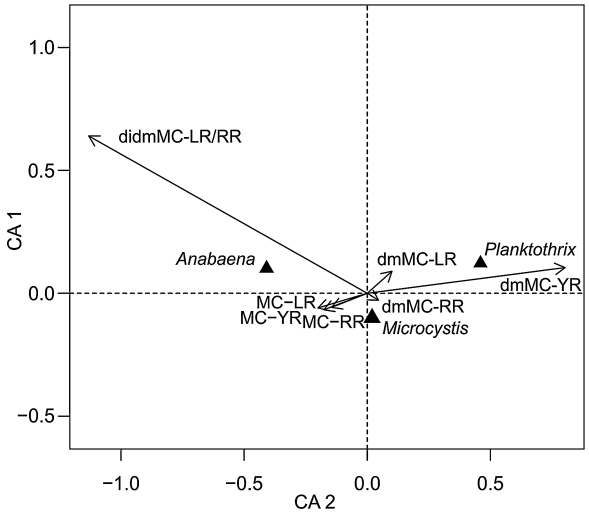

Fig. 2. Correspondence analysis plot of the co-occurrence of specified microcystin variants and genus-specific *mcyB* genotypes in the environmental samples ($n = 98$).

3.4. PCR chip analysis of environmental samples

According to PCR chip analysis, only 7% of all samples ($n = 98$) did not contain any of the targeted microcystin-producing cyanobacteria. *Microcystis* was the most common of the three genera, present as the only potential microcystin producer in 30% of the samples. *Planktothrix* or *Anabaena* was present as the only potential microcystin producer in 3% and 8% of the samples, respectively. In the cases where more than one genus was present (52% of all samples), *Microcystis* together with *Planktothrix* or *Anabaena* were equally common combinations (26% and 25%, respectively), while the combination *Anabaena–Planktothrix* was considerably more rare (1%). All three target genera were present simultaneously in one sample only (1%).

Total *mcyB* copy numbers showed significant positive correlation (Spearman rank correlation, $P < 0.001$) to observed microcystin concentrations in the samples, regardless whether ELISA or LC–MS was used for microcystin analysis (Table 3). Combining *mcyB* copy numbers from several genera resulted in a stronger correlation than when each genus was studied separately. Similar results were obtained also when studying three separate lakes which had been sampled nine or more times. Significant positive correlation between total *mcyB* copy numbers and microcystin concentrations was observed at Lake Littoistenjärvi ($\rho = 0.70$, $P = 0.043$; $\rho = 0.73$, $P = 0.031$, ELISA and LC–MS, respectively) and Hauninen Reservoir ($\rho = 0.51$, $P = 0.037$; $\rho = 0.48$, $P = 0.051$, ELISA and LC–MS, respectively). At Lake Tuusulanjärvi, *mcyB* copy numbers were positively correlated with toxin concentrations. The correlation was statistically significant when toxin concentrations were measured with ELISA ($\rho = 0.74$, $P = 0.023$), but not when LC–MS was used ($\rho = 0.30$, $P = 0.43$).

Table 3
Total microcystin and *mcyB* copy number correlation.

Microcystin analysis method	Source of *mcyB* copies	Spearman's ρ	P
ELISA	All target genera	0.68	<0.001
	Microcystis	0.50	<0.001
	Planktothrix	0.38	<0.001
	Anabaena	0.17	0.087
LC–MS	All target genera	0.67	<0.001
	Microcystis	0.47	<0.001
	Planktothrix	0.35	<0.001
	Anabaena	0.18	0.079

The samples were divided into three categories according to measured total microcystin content: the first group included samples with microcystin concentration less than 0.1 μg L^{-1}, including those with no detectable toxin, the second up to, but not including concentrations of 1 μg L^{-1} and the third concentrations of 1 μg L^{-1} and above. Significant (Mann–Whitney *U* test, $P < 0.001$) differences between the total *mcyB* copy numbers in these categories were found, regardless of the microcystin analysis method used (Fig. 3).

4. Discussion

One of the aims of this study was to assess the suitability of dry chemistry PCR chips to environmental water sample analysis. Since there are no differences in morphology between toxic and non-toxic strains, and mixed blooms are common (Vezie et al., 1998), identification of potentially toxic cyanobacteria by microscopy alone is not reliable as the only detection method. In principle, genetic testing based on conventional PCR is relatively simple. However, the current instrumentation and PCR methods require training and expertise in the field of molecular biology, and amplicon carry-over contamination is an ever-present risk. These limitations can be circumvented by applying a more automated approach, making molecular monitoring available for larger user groups. Dry chemistry PCR chips have several benefits over the more conventional 96-well plate format. The handling of PCR reagents at the time of analysis can be completely avoided, reducing the contamination risk, and large batches of chips can be manufactured which further decreases the chance of operator error as well as ensures reproducibility and comparability of results. Here, in the case of *mcyB* quantification, and compared to a traditional qPCR setup, a more sensitive assay was achieved through increased signal-to-background ratios and more efficient amplification, due to the choice of reaction vessel and instrumentation. Since all PCR reagents have been pre-dried on the chip, sample volume could also be significantly increased, making the detection of low target copy numbers more reliable. On the other hand, at the same time the concentration of possible PCR inhibitors and the probability of false negative results increase. This drawback can be overcome by including an amplification control, as was done in this study. To the best of our knowledge, amplification controls added at the sample preparation stage have not been previously used to aid the unambiguous detection of potentially toxic cyanobacteria, although water samples may contain inhibiting agents that can lead to false negative results (Wilson, 1997). Although we indeed observed larger variation in amplification control recovery in the presence of environmental samples when compared to the control reactions with just the IAC, which suggests a degree of inhibition in the some of the samples studied, with this information problematic samples can be pinpointed and examined further accordingly.

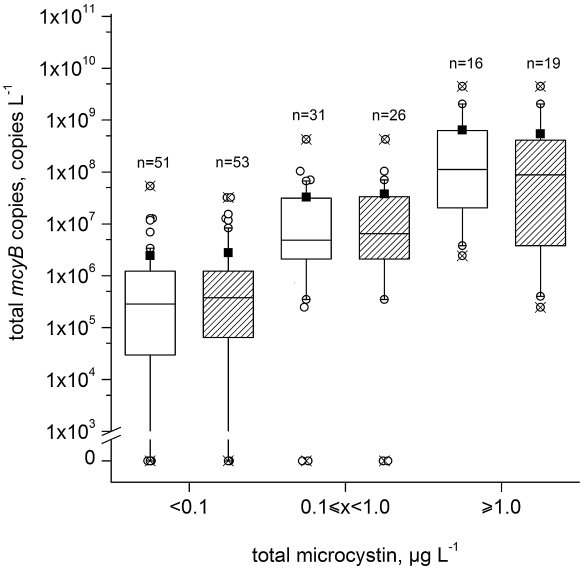

Fig. 3. The distribution of total *mcyB* copy numbers according to sample microcystin content measured by either LC–MS (white) or ELISA (pattern). The boxes indicate the range from 25th to 75th percentiles, medians are indicated with a line crossing the box. The whiskers range from the 10th to the 90th percentile. Minimum and maximum values (×), value means (■) and outliers (○) are indicated.

The prevalence of hepatotoxic cyanobacteria in Finnish lakes was estimated to be 30% in mid-1980s (Sivonen et al., 1990). However, more recently this figure has been set much higher, up to 70%–90% (Rantala et al., 2006), and a similar prevalence was observed also in the current study. The difference to early results can be explained by the previous use of less sensitive screening methods, such as the mouse bioassay. Elsewhere in Europe the occurrence rates have also been similar (Henriksen and Moestrup, 1997; Fastner et al., 1999b; Utkilen et al., 2001). The most common microcystin variants in the samples analyzed in this study, MC-RR, -LR, -YR and desmethyl variants of the first two, are also commonly encountered throughout the continent. Different genera of microcystin-producing cyanobacteria often exhibit typical toxin profiles. *Microcystis* spp. dominated samples have been associated with the occurrence of MC-RR, -LR and -YR, and likewise field samples of *Planktothrix agardhii* have contained mainly desmethyl variants of MC-LR and -RR (Fastner et al., 1999a). Our observations on the presence of genus-specific *mcyB* sequences and different microcystin variants corroborate these findings. Desmethyl variants of MC-LR and -RR were commonly found in samples positive for *Planktothrix mcyB*, which has been shown to be typical for Finnish strains (Luukkainen et al., 1993). Didesmethyl microcystins were exclusively present with *Anabaena*. These variants have been found before in *Anabaena* strains isolated from lake Vesijärvi, Lahti, Finland (Sivonen et al., 1992), and *mcyA* deletion mutants of *Anabaena* are known to incorporate predominantly dehydroalanine (Dha), instead of methyldehydroalanine (Mdha) during microcystin synthesis (Fewer et al., 2008).

Over 90% of the samples analyzed here contained *mcyB* copies from at least one of the target genera, and half of these were positive for more than one genus. *Microcystis* was found to be the most commonly encountered microcystin-producing genus, followed by *Anabaena* and *Planktothrix*. These results are similar to previous observations (Rantala et al., 2006), and show that potential microcystin producers are very common in Finnish and Estonian freshwaters. The amount of positive samples is much larger than would be estimated on the prevalence of cyanobacterial blooms alone (50%, Lindholm et al., 2003), which supports the fact that monitoring of toxic cyanobacteria cannot be left to bloom observations alone. In some cases, *mcyB* copies were observed in samples that contained none, or very little detectable microcystin. Genotypes with partially inactive microcystin biosynthesis genes have been described (Kurmayer et al., 2004; Tanabe et al., 2007; Ostermaier and Kurmayer, 2009), and the presence of such cannot be excluded as a possible reason for the absence of measurable toxin in the presence of apparently *mcy* positive cyanobacteria. Variation in the activity of the microcystin biosynthesis pathway would provide an alternative explanation for this phenomenon. Environmental factors, such as the availability of light (Kaebernick et al., 2000; Tonk et al., 2005; Straub et al., 2011) or nutrients (Vezie et al., 2002; Downing et al., 2005) have been shown to have an effect on *mcy* gene expression and toxin production. Microcystins could therefore have been produced, but at such low levels, that the analytical methods used were unable to detect them.

The current World Health Organization recommendation for maximum allowed MC-LR concentration in drinking water is 1 $\mu g\,L^{-1}$. In this study we have shown that raw water with microcystin concentrations exceeding this value most often contain significantly higher amounts of potentially microcystin-producing cyanobacteria compared to water samples with less microcystin. This is an important observation if a cut-off limit for potentially microcystin-producing cyanobacteria corresponding this recommendation is to be determined. Moreover, a similar distinction can be seen in samples with some toxin (0.1–1 $\mu g\,L^{-1}$) and those with very small concentrations or none at all ($<0.1\ \mu g\,L^{-1}$), equally important since long-term exposure to the toxins may also be harmful. The significant positive correlation between microcystin synthetase gene copies and microcystin concentrations observed in the waterbodies included in this study suggests that it is possible to estimate microcystin concentrations in the environment based on the amount of biosynthetic genes. These results are in line with previous studies, which have demonstrated similar correlations between either *Microcystis* or *Planktothrix* populations and toxin amounts in European and North American lakes (Rinta-Kanto et al., 2009; Fortin et al., 2010; Ostermaier and Kurmayer, 2010; Martins et al., 2011) although microcystin concentrations are still rarely compared to the combined gene copy numbers of three major microcystin-producing genera. According to this study, determining copy numbers from several genera is clearly beneficial when the aim is to estimate toxin concentrations as accurately as possible. Each added genus strengthened the observed correlation, and since *mcyB* gene copies originating from more than one genus were observed in almost half of the samples, it is well justified to take into account the combined impact. Genus-specific detection confers additional advantages compared to universal detection of all hepatotoxin producers, such as more detailed information on cyanobacterial population dynamics. Plans on measures aimed at preventing and mitigating harmful cyanobacterial occurrences may also benefit from more detailed information on the cyanobacterial population – different strategies would be required by e.g. non-nitrogen-fixing cyanobacteria compared to nitrogen-fixing genera. The same correlation of measurable microcystin to genetic determinants was generally retained when moving from a larger set of lakes to single sampling locations, which showed that independent of the scale of the monitoring, quantitative PCR has potential both as a predictive and a follow-up tool.

In conclusion, dry chemistry PCR chips were shown to be well suited for water sample analysis, and the introduction of an amplification control confirmed that false negative results were not obtained. The assay format makes it possible to apply the method to monitoring programs of microcystin-producing cyanobacteria on a wider scale, since no specialized expertise in PCR techniques is required from the operator and assays can be conducted outside molecular biology laboratories. A strong positive correlation between the combined *mcyB* copy numbers from three commonly microcystin-producing genera and microcystin concentrations was observed, which suggests that measuring genetic determinants in water can be a very useful tool when predicting microcystin concentrations in the environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2014.08.007.

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