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Interlaboratory comparison of Taq Nuclease Assays for the quantification of the toxic cyanobacteria *Microcystis* sp.

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Abstract

The application of quantitative real-time PCR has been proposed for the quantification of toxic genotypes of cyanobacteria. We have compared the Taq Nuclease Assay (TNA) in quantifying the toxic cyanobacteria *Microcystis* sp. via the intergenic spacer region of the phycocyanin operon (PC) and *mcy*B indicative of the production of the toxic heptapeptide microcystin between three research groups employing three instruments (ABI7300, GeneAmp5700, ABI7500). The estimates of *mcy*B genotypes were compared using (i) DNA of a *mcy*B containing strain and a non-*mcy*B containing strain supplied in different mixtures across a low range of variation (0–10% of *mcy*B) and across a high range of variation (20–100%), and (ii) DNA from field samples containing *Microcystis* sp. For all three instruments highly significant linear regression curves between the proportion of the *mcy*B containing strain and the percentage of *mcy*B genotypes both within the low range and within the high range of *mcy*B variation were obtained. The regression curves derived from the three instruments differed in slope and within the high range of *mcy*B variation *mcy*B proportions were either underestimated (0–50%) or overestimated (0–72%). For field samples cell numbers estimated via both TNAs as well as *mcy*B proportions showed significant linear relationships between the instruments. For all instruments a linear relationship between the cell numbers estimated as PC genotypes and the cell numbers estimated as *mcy*B genotypes was observed. The proportions of *mcy*B varied from 2 to 28% and did not differ between the instruments. It is concluded that the TNA is able to provide quantitative estimates on *mcy*B genotype numbers that are reproducible between research groups and is useful to follow variation in *mcy*B genotype proportion occurring within weeks to months.

Keywords: Genotype composition; Seasonality; Quantitative real-time PCR; Microcystin net production; Water monitoring

1. Introduction

The cyanobacterium *Microcystis* sp. is frequently found in freshwater all over the world. *Microcystis* sp. produces hepatotoxic microcystins that are known to inhibit eukaryotic protein phosphatases. Typically toxic strains and non-toxic strains co-occur but cannot be distinguished by morphological criteria in the microscope. The wax and wane of microcystin-producing vs. non-microcystin-producing strains has been suggested as a most important factor regulating microcystin net production in water (Sivonen and Jones, 1999). However, the quantitative assessment of strain composition in water samples has long time been impeded, mainly because of a lack of appropriate methods. Through the identification of the genes involved in microcystin synthesis (Dittmann et al., 1997), a new avenue in environmental research was opened and quantitative real-time PCR has been increasingly applied in order to quantify these genes directly in the environment (Foulds et al., 2002; Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003; Rinta-Kanto et al., 2005; Yoshida et al., 2006, 2007).

The Taq Nuclease Assay (TNA) was introduced to quantify *mcy* genotypes in water samples (Kurmayer and Kutzenberger, 2003). This approach was based on the quantification of the total population of *Microcystis* sp. by TNA amplifying the intergenic spacer region within the phycocyanin operon (PC) and a subpopulation by another TNA amplifying *mcy*B involved in microcystin synthesis. The proportion of *mcy*B

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genotypes is determined by dividing the number of cells of *mcy*B genotypes through the corresponding number of PC genotypes.

During the EU-project PEPCY (toxic and other bioactive peptides in cyanobacteria, QLRT-2001-02634, www.pepcy.de) a number of lakes in European countries were selected for quantifying microcystin genotypes. The analysis of the lake samples should be performed in several laboratories. Comparing and standardizing quantitative PCR results between laboratories prior to sample analysis was considered necessary because no experience on the reproducibility of the estimated proportion of specific genotypes via quantitative PCR is available. This issue is of particular relevance as quantitative PCR methodology uses linear-log calibration curves rendering the calculation of DNA concentrations sensitive to variations in the slope induced by minor variations of the C_t values. Consequently, the noise within C_t values induced by the semilogarithmic calibration algorithm alone can mask differences in genotype proportion in populations.

TNA results obtained in three laboratories (Helsinki, Mondsee, Vienna) were compared using DNA extracts of *Microcystis* strain HUB524 (with *mcy*B) and strain HUB53 (without *mcy*B) supplied in different mixtures from 0, 2, 4, 6, 8, 10% of strain HUB524 (low range of *mcy*B variation) and 20, 40, 60, 80, 100% of HUB524 (high range) at a constant cell density. Further, TNA results obtained from field samples were compared between the three research groups using DNA extracts obtained from water samples from a hypertrophic lake containing *Microcystis* sp. (Kurmayer and Kutzenberger, 2003).

2. Materials and methods

2.1. Sampling, cell harvesting and cell quantification

The unicellular strains HUB524 (with *mcy*B) and HUB53 (without *mcy*B) were isolated from the eutrophic lake Pehlitzsee (Brandenburg) in 1978 and kindly provided by M. Henning (Humboldt University Berlin, Germany). Both strains were grown in batch culture in Z medium (Zehnder and Gorham, 1960) and harvested during the late logarithmic growth phase. The DNA extracts of the field samples collected from Wannsee during 1999–2000 were those used previously by Kurmayer and Kutzenberger (2003) and were stored at −20 °C. In total 15 samples in monthly intervals were analysed.

Cells were harvested by filtering onto glass fiber filters (GF/C, Whatman, Kent, Great Britain) under vacuum pressure and stored frozen (−20 °C) until DNA extraction. For *Microcystis* strains aliquots were analysed for cell numbers using two independent methods: (i) electronic particle counting (Casy® 1, Schärfe system, Reutlingen, Germany) from formaldehyde fixed samples (2%, v/v), (ii) counting of cells from Lugol fixed samples by the inverted microscope technique as described previously (Kurmayer et al., 2003; Kurmayer and Kutzenberger, 2003). For strains HUB524 and HUB53 cell numbers determined by the particle counter correlated highly significantly with cell numbers determined in the microscope (Kurmayer and Kutzenberger, 2003).

Prior to the counting of field samples the colonies of *Microcystis* were identified from phytoplankton net samples according to the species key based on morphological criteria by Komarék and Anagnostidis (1999) as described (Kurmayer et al., 2002). Five morphospecies were identified: *M. aeruginosa*, *M. ichthyoblabe*, *M. flos-aquae*, *M. novacekii* and *M. wesenbergii*. Live counting of 400 randomly selected colonies in July 2000 showed a dominance of *M. aeruginosa*/*M. flos-aquae* (51%) and *M. ichthyoblabe* (41%).

For the counting of *Microcystis* cells in field samples the Lugol fixed samples were disintegrated by ultrasonication (40 impulses per second during 4 min in 10 ml sample) as described previously (Kurmayer et al., 2003). The samples were then diluted up to 100 fold (depending on the population density) and at least 400 single cells of *Microcystis* were counted at 400× magnification and the results were averaged from at least two transects per sedimentation chamber. Because it was impossible to discriminate between morphospecies during the counting of single cells, all morphospecies of *Microcystis* sp. were considered morphological variations of individuals of one population. This approach is in accordance with the unification 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 (Otsuka et al., 2001).

2.2. Preparation of DNA extracts

All research groups have used identical DNA extracts. DNA was extracted from cells collected on glass fiber filters using a standard phenol-chloroform DNA extraction procedure that has been described previously (Kurmayer et al., 2003). In a previous study the TNA results for PC and *mcy*B obtained from DNA extracted using the standard phenol-chloroform procedure have been compared with the TNA results using DNA extracted by a commercially available DNA purification kit (DNeasy®, Qiagen, Vienna). Although the DNeasy kit revealed quantitative data, the standard phenol-chloroform procedure has been found to be superior because it yields more accurate results (Schober and Kurmayer, 2006).

DNA extracts equivalent to 200 cells μl^{-1} either originating from strain HUB524 (with *mcy*B) or from strain HUB53 (without *mcy*B) were mixed at ratios with 0, 2, 4, 6, 8, 10% (low range of *mcy*B variation) and 20, 40, 60, 80, 100% (high range of *mcy*B variation) of HUB524. This concentration resulted in a total template of 1000 cells per reaction (5 μl of the template) and was shown to give most reliable results because it lies in the optimal range of the standard curves ($10–10^4$ cells, see Fig. 1 A in Kurmayer and Kutzenberger, 2003). For the strain mixture the ratios were adjusted and analysed by TNAs by each research group in triplicate. DNA extracts from field samples were also diluted down to a cell concentration of 200 cells μl^{-1} of DNA extract and analysed by each research group once. In order to reduce possible inhibitory influences from field samples down to a minimum, all extracts from field samples were diluted at least one hundred fold.

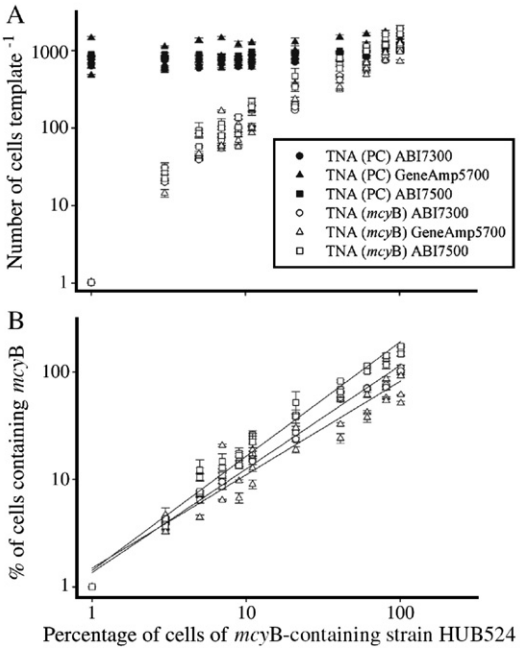

Fig. 1. The accuracy in determining genotype proportions using the TNA was tested by mixing two culture strains, one strain containing *mcy*B (HUB524) and one strain without *mcy*B (HUB53) in different ratios (0–100%) but a constant amount of DNA. (A) Mean ± 1SE cell numbers of PC genotypes (black symbols) and *mcy*B genotypes (white symbols) per template determined by three different instruments: ABI7300 (circles), GeneAmp5700 (squares), ABI7500 (triangles). (B) Mean ± 1SE proportions of *mcy*B genotypes.

2.3. Taq Nuclease Assay

For each gene standard curves based on pre-determined cell concentrations were established by relating the known DNA concentrations (in cell equivalents) to the threshold cycle of the diluted *Microcystis* sp. extract. The threshold cycle (C_t) is the PCR cycle number at which the fluorescence passes a set threshold level. The threshold value for the fluorescence of all samples was set manually at 0.1 according to the manufacturer's instructions. The research groups employed the following instruments: (i) ABI7300 Real Time PCR System (Helsinki), (ii) ABI GeneAmp5700 Sequence Detection System (Mondsee); (iii) ABI PRISM 7500 Sequence Detection System (Ingenetix, Vienna).

For all experiments TaqMan® Universal PCR Master Mix (ABI, #4304437) was used containing a twofold concentration of premixed PCR buffer, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP (instead of dTTP), AmpErase® UNG to prevent carry-over contamination and ROX (5-carboxy-X-rhodamine) as a passive reference dye. However, using ABI7500 an unexpected instability of the ROX signal was observed in field samples and a second independent reference signal (TAMRA, 6-carboxytetramethylrodamine) was used to correct for random fluctuations in the fluorescent signal. Primers and TaqMan probes were those used by Kurmayer and Kutzenberger (2003) and were synthesized by ABI (Vienna) and Sigma-Genosys (Helsinki). For the concentrations of the primers and probes see Kurmayer and Kutzenberger (2003). PCR was initiated for 2 min at 50 °C (AmpErase® UNG protection against carry-over contamination) followed by 10 min at 95 °C. Subsequently PCR was performed for 45 cycles consisting of a 95 °C denaturation step for 15 s and by a 60 °C annealing/extension step for 1 min.

In order to estimate the number of PC copies per cell the PCR product amplified during the TNA of PC-IGS (5′-GCTACTTC-GACCGCGCCGCCGCTGCTGTCGCCTAGTCCCTGGGGC-TAGTCTCAATTAAACCGTAGGA-3′, 67 bp) was also amplified from strain HUB524 by conventional PCR and purified using the QiaQuick purification kit (Qiagen, Vienna). The concentration of the purified oligonucleotide was determined using the Nanodrop ND-1000 spectral photometer (Peqlab Biotechnologie GmbH, Erlangen, Germany): 16.23–19.22 ng μl^{-1}. This concentration was diluted down to a working concentration of 1 fmol μl^{-1} (6.022×10^8 copies μl^{-1}) and dilutions ranged from 30.1 to 3.1×10^7 copies template^{-1}.

2.4. Statistical analyses

The linear calibration curves were fitted using the least square approximation and the associated statistical tests of Sigma Plot 2000 (V 6.10). To achieve normal distribution and homogeneity of variances cell numbers of genotypes and proportions of *mcy*B were log $(x+1)$ transformed.

The linear regressions between the proportion of strain HUB524 and the percentage of *mcy*B genotypes estimated in the strain mixture were compared in slope using a general factorial model of Analysis of Variance (ANOVA). The data were modelled as $y=\mu+\beta x+\epsilon$, where y is the measured proportion of *mcy*B, μ is the overall mean level, β is the effect of the instrument (ABI7300, GeneAmp5700, ABI7500), x is the effect of the cells of strain HUB524 in the strain mixture as a covariate, and ϵ is the random deviation, $N(0, \sigma 2)$ (Sokal and Rohlf, 1995). A SPSS statistical package (V 6.0 for Windows) was used for the covariance analysis.

The influence of the instruments and the low vs. the high range of *mcy*B variation on the estimation of *mcy*B proportions were statistically compared using a Two-Way ANOVA design. The data were modelled as $y=\mu+\alpha+\beta+\alpha\beta+\epsilon$ where y is the dependent variable estimated by TNA, μ is the overall mean level, α is the effect of the instrument (ABI7300, GeneAmp5700, ABI7500), β is the effect of the low range (0–10%) or high range (20–100%) of *mcy*B proportion of strain HUB524, $\alpha\beta$ is the interaction between both factors and ϵ is the unexplained part of the variance (Sokal and Rohlf, 1995). A statistical package from Statistica (V 7.1) was used for the Two-Way ANOVA.

3. Results

3.1. Standardization of TNA

Calibration curves obtained between C_t values (the PCR cycle number at which the fluorescence passes a set threshold level) and DNA (in cell number equivalents calculated from pre-determined cell concentrations) for PC and *mcy*B using DNA extracts obtained from strain HUB524 showed a good correspondence between the three instruments. The ABI7300 and ABI7500 instruments showed a higher sensitivity when compared with the GeneAmp5700 (Table 1). All calibration

Table 1
Linear calibration curves obtained with three instruments (ABI7300, GeneAmp5700, ABI7500) operated by three research groups (Helsinki, Mondsee, Vienna)

Instrument	Calibration curve	E (%)[a]	R^2	N	p
PC					
ABI7300	$y=36.21-3.50x$	93	0.99	5	<0.001
GeneAmp5700	$y=38.61-3.49x$	94	0.99	6	<0.001
ABI7500	$y=37.18-3.48x$	94	0.99	6	<0.001
mcyB					
ABI7300	$y=40.11-3.89x$	81	0.99	5	<0.001
GeneAmp5700	$y=46.14-4.07x$	76	0.99	5	<0.001
ABI7500	$y=40.81-3.93x$	80	0.99	6	<0.001
PC oligonucleotide					
GeneAmp5700	$y=42.84-3.34x$	99	0.99	7	<0.001

[a]The amplification efficiency (E) was calculated as follows: $E=(10^{-1/\text{slope}}-1)\times 100$.
y, the PCR cycle number (C_t) at the set fluorescence threshold level (0.1); x, the amount of starting DNA (given as $\log_{10}$ cell number equivalents) or the amount of phycocyanin (PC) copies per template (given as $\log_{10}$ copy number equivalents) for the oligonucleotide.

curves were similar in slope and showed the same amplification efficiency.

The regression curve obtained with the purified oligonucleotide for PC showed a higher amplification efficiency compared with the regression curves obtained from the DNA extracted from the cells (Table 1). The number of PC copies per cell was calculated by dividing the number of copies for a given C_t value through the number of cells resulting in the same C_t value taken from the linear calibration curve for PC (GeneAmp5700): 26.5±3.8 (SE), min=13, max=33, (n=7).

3.2. Quantification of mcyB in mixed populations of Microcystis strains

DNA extracts equivalent to 1000 cells per template either originating from strain HUB524 (with *mcyB*) or from strain HUB53 (without *mcyB*) were mixed at ratios of 0–100% and analysed by TNA as described above. For the total range of 0–100% of HUB524 the C_t values obtained for PC varied from 0.65 to 0.9 (ABI7300), 0.61 to 1.56 (GeneAmp5700), 0.46 to 0.75 (ABI7500) implying that the amount of DNA (in cell number equivalents) per template was relatively constant irrespective of the proportion of strain HUB524: 650 to 1100 cells (ABI7300), 800 to 1500 cells (GeneAmp5700), 780 to 1140 cells (ABI7500), (Fig. 1).

In contrast the cell numbers of *mcy*B genotypes decreased linearly with a decreasing proportion of HUB524 and *mcy*B genotypes were not detected by any of the three instruments at 0% HUB524. The proportion of *mcy*B genotypes was determined by dividing the number of cells of *mcy*B genotypes (as determined using the TNA for *mcy*B) through the corresponding number of PC genotypes. For all three instruments highly significant linear regression curves between the proportion of strain HUB524 and the percentage of *mcy*B genotypes were obtained (Table 2). However, the regression curves derived from the three instruments differed in slope, as the influence of the instrument was found highly significant (ANOVA, df=95, $p<0.001$). The slope of the regression line derived from ABI7300 was close to one resulting in relatively low underestimation or overestimation of *mcy*B proportions (9% underestimation–22% overestimation). In contrast the regression line derived from GeneAmp5700 underestimated *mcy*B proportions by 0–49% while the regression line derived from ABI7500 overestimated *mcy*B proportions by 0–73%.

Table 2
Regression curves between the proportion of *Microcystis* strain HUB524 (containing *mcy*B) and the percentage of *mcy*B genotypes in the strain mixture as determined via TNA

Instrument	Regression curve	R^2	N	p
ABI7300	$y=0.134+0.964x$	0.99	33	<0.001
GeneAmp5700	$y=0.174+0.869x$	0.92	33	<0.001
ABI7500	$y=0.152+1.064x$	0.98	33	<0.001

y, the $\log_{10}$ number of *mcy*B genotypes; x, the proportion of $\log_{10}$ cells of strain HUB524 (see also Fig. 1).

In order to find out whether the instruments differ in quantifying *mcy*B genotypes between the low range (0–10%) and the high range (20–100%) of *mcy*B variation, a Two-Way ANOVA testing the effects of both the instrument and the low vs. high range of *mcy*B proportion was performed. Overall the influence of the instrument on the average, minima and maxima of *mcy*B proportions was found marginally significant (Two-way ANOVA, df=93, $p=0.053$, Table 3). However, the interaction effect between both factors was never found significant implying that all three instruments produced accurate results both within the low range and the high range of *mcy*B variation ($p>0.5$).

Table 3
Mean±SE, minima and maxima of *mcy*B proportions estimated from mixed populations containing two *Microcystis* strains at fixed cell numbers (200 cells μl^{-1} of DNA extract), HUB524 (with *mcy*B) and HUB53 (without *mcy*B) in different proportions (0, 2, 4, 6, 8, 10% of strain HUB524=low range of *mcy*B variation, 20, 40, 60, 80, 100%=high range)

	*mcy*B %	Min % *mcy*B	Max % *mcy*B
ABI7300			
Low range(0–10%)	7.6 ± 1.2^{I}	$0–13.5^{I}$	$0–18.6^{I}$
High range (20–100%)	$65.4\pm6.9^{II,III}$	$21–96^{II,III}$	$23.4–121.4^{II,III}$
Total	$33.9\pm6^{a,b}$	$0–96^{a,b}$	$0–121.4^{a,b}$
GeneAmp5700			
Low range(0–10%)	7.4 ± 1.4^{I}	$0–18^{I}$	$0–25^{I}$
High range (20–100%)	49.1 ± 5.5^{II}	$16–86^{II}$	$20–100^{II}$
Total	26.4 ± 4.5^{a}	$0–86^{a}$	$0–100^{a}$
ABI7500			
Low range(0–10%)	10.5 ± 1.9^{I}	$0–22^{I}$	$0–33^{I}$
High range (20–100%)	102.4 ± 11.1^{III}	$31–161^{III}$	$40–208^{III}$
Total	52.3 ± 9.5^{b}	$0–161^{b}$	$0–209^{b}$
Two-Way ANOVA			
Instrument	$p=0.0532$	0.0459	0.044
Low vs. high range	$p<0.0001$	<0.0001	<0.0001
Interaction	$p=0.538$	0.6	0.529

[a,b] or [I, II, III] … homogeneous subsets not significantly different at $p<0.05$ (Fisher LSD post-hoc comparison).

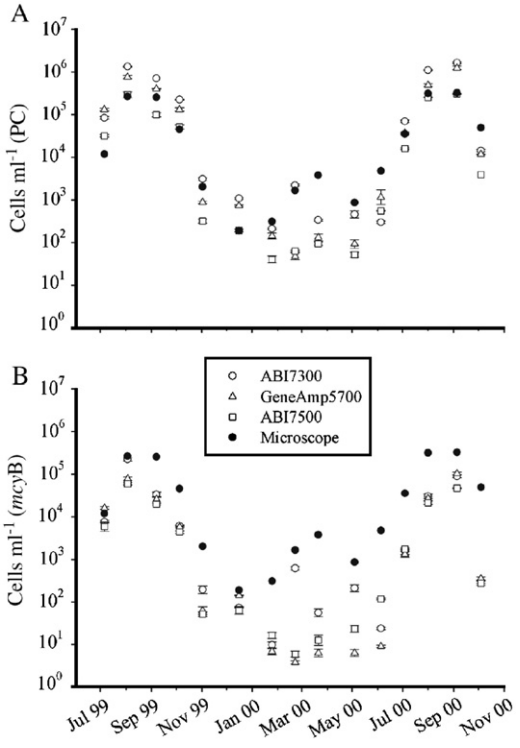

Fig. 2. *Microcystis* sp. cell numbers in hypertropic Lake Wannsee (Berlin. Germany) from July 1999 to October 2000 determined in the microscope and via (A) TNA (PC) and (B) TNA (*mcy*B) using three instruments (ABI7300, GeneAmp5700, ABI7500), mean (±1SE).

Post-hoc comparisons revealed a homogeneous subset of average, minima and maxima of *mcy*B proportions for the low range ($p>0.05$) but not for the high range of *mcy*B variation ($p<0.05$, Table 3, Fig. 1).

3.3. Application of TNA in field samples

Microcystis sp. was found on each sampling date with a minimum abundance in January (125 cells ml^{-1}) and a maximum abundance in August 2000 (6.1×10^5 cells ml^{-1}). Within cyanobacteria the genera *Planktothrix* (*P. agardhii*) and *Microcystis* sp. formed the major share of the biovolume, minor contributions were from *Aphanizomenon* spp. and several *Limnothrix* species. The diatoms (*Aulacoseira* spp., *Stephanodiscus* spp.) dominated the phytoplankton in early spring (March, April) and remained abundant during the whole season. Within cyanobacteria, *Microcystis* sp. dominated during August and September whereas *Planktothrix* was the dominant cyanobacterium in spring (together with *Limnothrix* species) and autumn. Since no other cyanobacteria that are morphologically similar to *Microcystis* sp. (e.g. *Anabaena* spp., *Woronichinia naegeliana*) co-occurred, the microscopical counting of *Microcystis* was considered unbiased.

Unexpectedly a number of DNA extracts obtained from field samples during 22 February–5 June 2000 showed unusual linear amplification curves during the amplification process of *mcy*B using ABI7500. Those unusual linear curves reached the fluorescence threshold set at 0.1 already after 6–8 cycles during amplification and failed to show the typical sigmoid character with a plateau phase. Consequently unreasonable low C_t values were calculated resulting in tremendous overestimation of cell numbers. Inspection of the ROX fluorescence signal revealed a constant decline of ROX fluorescence during the amplification process indicating that ROX – although designed as a passive reference dye – participated in the PCR reaction. In

Table 4
Regression curves between cell numbers determined in the microscope and cell numbers of PC and *mcy*B genotypes determined by three instruments: ABI7300 (Helsinki), GeneAmp5700 (Mondsee), ABI7500 (Vienna)

Instrument	Regression curve	R^2	N	p
PC				
ABI7300	$y=-0.682+1.201x$	0.82	15	<0.001
GeneAmp5700	$y=-1.349+1.292x$	0.81	15	<0.001
ABI7500	$y=-1.589+1.253x$	0.86	15	<0.001
mcyB				
ABI7300	$y=-1.23+1.052x$	0.74	15	<0.001
GeneAmp5700	$y=-2.473+1.264x$	0.71	15	<0.001
ABI7500	$y=-1.862+1.129x$	0.79	15	<0.001
PC vs. mcyB				
ABI7300	$y=-0.69+0.89x$	0.93	15	<0.001
GeneAmp5700	$y=-1.33+1.023x$	0.96	15	<0.001
ABI7500	$y=-0.536+0.93x$	0.97	15	<0.001

x, $\log_{10}$ cells ml^{-1} (microscope), y, $\log_{10}$ cells ml^{-1} (TNA). For the regression curves calculated between PC genotypes and *mcy*B genotypes y is the log cell number ml^{-1} calculated using *mcy*B (TNA) and x is the log cell number ml^{-1} calculated using PC (TNA).

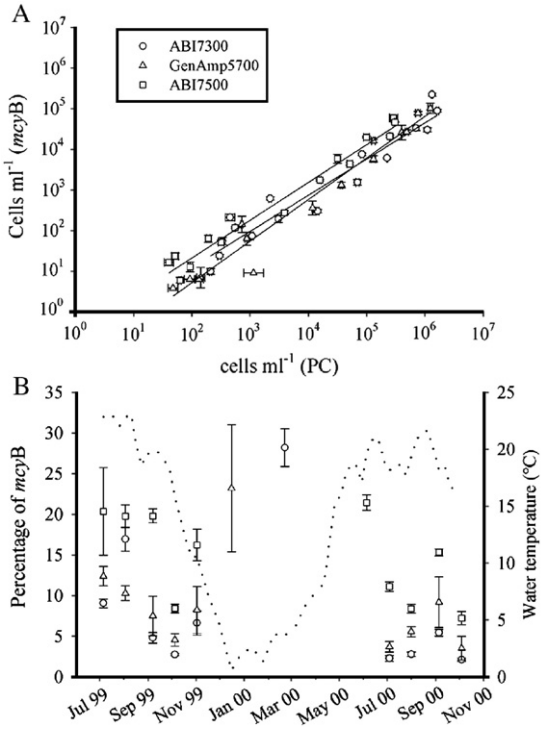

Fig. 3. (A) Relationship between the mean (±1SE) cell numbers determined via TNA (PC) and via TNA (*mcy*B) using three instruments (ABI7300, GenAmp5700, ABI7500). (B) Proportion of *mcy*B genotypes during the same period, mean (±1SE).

Table 5
Statistics on linear relationships on $\log_{10}$ cell numbers and *mcy*B proportions estimated via TNA (PC) and TNA (*mcy*B) between three instruments ABI7300 (Helsinki), GeneAmp5700 (Mondsee), ABI7500 (Vienna)

	GeneAmp5700 (x)	ABI7300 (x)	ABI7500
PC			
GeneAmp5700	–	$R^2=0.91$, $p<0.001$, $n=15$	$R^2=0.99$, $p<0.001$, $n=15$
ABI7300 (y)	$y=0.771+0.879x$	–	$R^2=0.94$, $p<0.001$, $n=15$
ABI7500 (y)	$y=-0.135+0.933x$	$y=-0.644+0.988x$	–
mcyB			
GeneAmp5700	–	$R^2=0.84$, $p<0.001$, $n=15$	$R^2=0.96$, $p<0.001$, $n=15$
ABI7300 (y)	$y=1.06+0.747x$	–	$R^2=0.82$, $p<0.001$, $n=15$
ABI7500 (y)	$y=0.512+0.831x$	$y=-0.137+0.94x$	–
mcyB proportion			
GeneAmp5700	–	$R^2=0.58$, $p=0.017$, $n=9$	$R^2=0.76$, $p=0.002$, $n=9$
ABI7300 (y)	$y=-2.59+1.17x$	–	$R^2=0.54$, $p=0.025$, $n=9$
ABI7500 (y)	$y=3.16+1.52x$	$y=9.22+0.83x$	–

The regression functions are given below the diagonal and the coefficients of determination are given above the diagonal. x, $y=\log_{10}$ cell number of cells of PC/*mcy*B genotypes or percent of *mcy*B genotypes of the dependent/independent variable; n, sample size; p, probability of erroneously rejecting the hypothesis of no linear relationship between the data sets compared between two instruments.

contrast the fluorescence signal of TAMRA remained stable and was used as an internal passive reference for the TNA using ABI7500 for all subsequent analyses in field samples.

In general PC genotypes and *mcy*B genotypes were detected on each sampling date throughout the whole study period (Fig. 2). For all three instruments cell numbers in field samples determined as either PC or *mcy*B genotypes showed a significant linear relationship with the cell numbers determined in the microscope (Table 4). For all three instruments the relationship between the cell numbers estimated via *mcy*B genotypes (dependent variable) and the cell numbers estimated via PC genotypes (independent variable) was found linear (Fig. 3A). The $\log_{10}$ cell numbers estimated via PC genotypes were by a factor of 0.3–1.7 (ABI7300), 0.7–2.1 (GeneAmp5700), 0.3–1.2 (ABI7500) higher when compared to the $\log_{10}$ cell numbers estimated via *mcy*B genotypes.

Due to the lower cell numbers of the total population during the period from 16 December 1999–5 June 2000 fluorescence signals were frequently exceeding the threshold of the calibration curves, i.e. $C_t>27.5$ (ABI7300), $C_t>30.5$ (GeneAmp5700), $C_t>29.5$ (ABI7500) equivalent to 10 cells per template. Consequently these values were omitted from calculating the proportion of *mcy*B genotypes. During the whole study period the proportions of *mcy*B genotypes varied from 2.1 to 28.2% (mean 8.1±2.7 (SE), $n=10$, ABI7300), 3.5 to 23.2% (8.8±1.9, $n=10$, GeneAmp5700), 7.2 to 21.4 (14.8±1.8, $n=10$, ABI7500), (Fig. 3B). The difference in *mcy*B proportions between instruments was not found significant (One-Way ANOVA, $p=0.06$, df=29). Cell numbers estimated via the TNA (PC) and via the TNA (*mcy*B) as well as *mcy*B proportions showed highly significant linear relationships between the instruments (Table 5).

4. Discussion

This study is the first that makes an effort in comparing the results obtained by TNA by different research groups and demonstrating the value of the TNA approach and its limitations. The variation in cell numbers in field samples was close to a factor of 10^4 (10^2 to 10^6 cells ml^{-1}) and quantitative PCR detection systems have been shown to give a good reproducibility when compared along a wide range of starting DNA concentration in the template (e.g. 10–10^5 cells per template, Becker et al., 2000; Zhang and Fang, 2006). In contrast the quantification of the proportion of subpopulations of toxic genotypes is more sophisticated, as the potential range of variations is only 0–100% implying that inaccuracies in cell number estimates are more visible. The only possibility to quantify the proportion of specific toxic genotypes is by relating cell numbers of the subpopulation to the cell numbers of the total population by two independent TNAs (Kurmayer and Kutzenberger, 2003). This approach minimizes the influence of a physiologically induced variation in the DNA content which can be more than tenfold (Kurmayer and Kutzenberger, 2003).

Notably, all three instruments showed the ability to discriminate between *mcy*B genotype proportions both in the low range of *mcy*B variation (0–10%) as well as in the high range (20–100%) of variation and highly significant linear regression curves between the proportion of the *mcy*B containing strain and the percentage of *mcy*B genotypes were obtained. On the other hand comparing the *mcy*B proportion between instruments documented that *mcy*B genotype proportions could either be overestimated (ABI7500) or underestimated (GenAmp5700) within the higher range of *mcy*B variation (20–100%). The maxima of the overestimates and underestimates recorded were 73% and 50%, each. Accordingly the ABI7500 instrument revealed also higher *mcy*B proportions in field samples when compared to the other instruments. Nevertheless, in this study the TNA results obtained by all three instruments led to the conclusion that the proportion of *mcy*B in the population of *Microcystis* in the study lake was low (1/5–1/10 of the population). It is concluded that the TNA is able to provide quantitative estimates on *mcy*B genotype numbers that are reproducible between research groups within the lower range of

variation of *mcy*B (0–10%) while proportions of *mcy*B genotypes vary up to a factor of two within the higher range of *mcy*B variation (20–100%). Despite these limitations in the quantitative estimates all instruments were able to follow the variation in *mcy*B genotype proportion both within the strain mixture as well as in the field occurring within weeks to months.

Over the whole study period the cell numbers of *mcy*B genotypes increased or decreased linearly with the cell numbers of PC genotypes implying that the average proportion of *mcy*B genotypes was constantly coupled to the increase or decrease in *Microcystis* cell numbers. On the other hand all three instruments revealed a short-term variation in *mcy*B genotype proportions within weeks to months. Recently Yoshida et al. (2006, 2007) reported 0.5–35% of seasonal variation of the *mcy*A genotype proportion in Lake Mikata, Japan. Biological agents influencing the *mcy* genotype proportion might explain this short-term variability in both lakes. The same authors reported cyanophages selectively infecting one toxic strain of *Microcystis aeruginosa* but other strains that were resistant (Yoshida et al., 2006, 2007). In the field viruses may result in the replacement of sensitive strains only while closely related resistant strains may gain a selective advantage (Fuhrman and Schwalbach, 2003). This mechanism will counteract a possible competitive exclusion occurring between closely related strains (Thingstad and Lignell, 1997) and would result in a constant co-adaptation process between the host population and the viruses.

It is concluded that the average proportion of *mcy*B genotypes in a specific lake estimated monthly during one year was found reproducible between research groups and the cell number of PC genotypes could be used to predict the number of *mcy*B genotypes in field samples. More studies on the seasonal proportion of *mcy*B genotypes in phytoplankton are needed to assist water monitoring purposes and early warning tasks. For example if *mcy*B genotypes are detected early during pre-bloom periods mitigation of microcystin net production could be attempted already in spring, e.g. by intensified mixing of the water column and reducing the growth of *Microcystis* sp. in favour of green algae and diatoms (Visser et al., 1996).

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