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MINI-REVIEW

Use of qPCR for the study of hepatotoxic cyanobacteria population dynamics

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Abstract Toxic cyanobacteria blooms are increasingly frequent and object of greater concern due to its ecological and health impacts. One important lack in the toxic cyanobacteria research field is to understand which parameters influence most and how they operate to regulate the overall levels of cyanotoxins in a body of water. MC concentration is believed to be influenced by changes in several seasonal environmental factors that influence the succession of toxic cyanobacteria. In the last years, qPCR (quantitative polymerase chain reaction) has been applied to determine the seasonal and temporal shifts in the proportions of MC-producing and non-MC-producing subpopulations by quantifying both *mcy* genotypes and total population numbers. We discuss the most prominent and recent studies using qPCR to address hepatotoxic cyanobacteria population dynamics and evaluate how they helped understanding the factors promoting the growth of toxic strains in situ and the succession of hepatotoxin-producing genera in natural populations.

Keywords qPCR · Cyanobacteria · Cyanotoxins · Monitoring · Dynamics

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Introduction

Cyanobacteria are ubiquitous organisms known for the ability to produce a broad range of secondary metabolites, some of which toxic. Blooms of toxic cyanobacteria are becoming increasingly important in fresh, marine and brackish waters worldwide due to the ecological impacts and the human and animal health risks that toxins represent. For one side, cyanobacteria blooms cause oxygen depletion, alter food webs and threaten freshwater bodies worldwide used for drinking, agriculture, fishing and recreation (Paerl and Huisman 2009). On the other hand, toxin production by cyanobacteria endangers both animal and human health, with several cattle and human poisoning incidents reported (Chorus and Bartram 1999; Sivonen and Jones 1999; Pearson et al. 2010).

The increased knowledge gathered in the last years on the genetic bases underlying major cyanotoxins production can lead to the development of new drinking and recreational waters monitoring methods. The gene clusters responsible for the synthesis of the hepatotoxins, microcystins (MC), *mcyS*, nodularin (NOD), *ndaS* and cylindrospermopsin (CYN), *cyr*, have been described (Tillett et al. 2000; Christiansen et al. 2003; Moffitt and Neilan 2004; Rouhiainen et al. 2004; Mihali et al. 2008). Furthermore, the genes putatively responsible for the biosynthesis of saxitoxin (STX), anatoxin-a and homoanatoxin-a have recently been identified (Kellmann et al. 2008; Méjean et al. 2009). Genetic-based methods enhanced the understanding of the natural distribution of genes that are involved in cyanotoxins production, despite only indicating potential toxin production (Sivonen and Börner 2008). Quantitative real-time PCR (qPCR) has been increasingly applied to monitor potentially toxic cyanobacteria population shifts in diverse aquatic ecosystems worldwide

(Pearson and Neilan 2008; Kurmayer and Christiansen 2010; Ostermaier and Kurmayer 2010).

This review will mainly address the use of qPCR to study hepatotoxin-producing cyanobacteria population dynamics and toxin production regulation, mainly focusing on MC, CYN and NOD.

qPCR: the fundamentals

The polymerase chain reaction (PCR) is one of the most potent technologies in molecular biology. Using PCR, a specific sequence within a DNA template can be amplified up to a million fold. The initial target DNA fragment is exponentially increased by multiple amplification cycles, starting with an initial DNA denaturation, followed by annealing of oligonucleotide primers targeting specific sequences and ending with extension of a complementary strand from each annealed primer by a thermo stable DNA polymerase.

QPCR is a potent advancement of the basic PCR technique, although sharing the same basic principles. In qPCR, instead of the endpoint analysis given by conventional PCR in which PCR product molecules (amplicons) are only analysed after conclusion of the final PCR cycle, the amount of DNA is measured after each cycle by the use of fluorescent markers that are incorporated into the PCR product. The increase in fluorescent signal is directly proportional to the number of amplicons generated in the exponential phase of the reaction (Heid et al. 1996). Several different fluorescence reporter systems are available for qPCR. The qPCR assays using intercalating dyes, like SYBR green (Wittwer et al. 1997) and the probe based assays, e.g., the TaqMan hybridization probe system (Holland et al. 1991; Livak et al. 1995), are the two most frequently used, while the recently developed primer-based fluorescence detection technologies (Zhang et al. 2003) have been less used.

The SYBR green intercalating dye binds to all double-stranded (ds) DNA via intercalation between adjacent base pairs. This assay, although simple can lack specificity since the dye binds to all dsDNA and not only to the target DNA. Nevertheless, the specificity of each reaction can be assessed using post-PCR melting (dissociation) curve analysis. In this process, the dsDNA is heated over a temperature gradient and fluorescence is measured at each distinct temperature point. As the dsDNA is heated, it denatures, resulting in a corresponding decline in fluorescence due to SYBR green dissociation (Giglio et al. 2003; Gonzalez-Escalona et al. 2006). The temperature at which 50% of the double-stranded template is denatured can be used to confirm the presence of the expected PCR product and the absence of other non-specific templates or primer dimers.

The TaqMan probe method utilises a probe labelled with a fluorescent dye that hybridizes to a further conserved region within the target amplicon sequence (Livak et al. 1995). This method provides highly sensitive and specific quantifications, however, dual labelling and complex design specifications make it more expensive and difficult to use than other systems.

In both assays, quantification of gene copy numbers by qPCR is determined during the exponential phase of the amplification, when the amount of amplified target is proportional to the starting template. This assures a more accurate determination of the target DNA initial amount (Smith and Osborn 2009). Quantification of unknown initial target sequences amount (gene copy numbers) is extrapolated from the Ct (threshold cycle) values. The Ct is the cycle number at which the fluorescent signal shows a statistically significant increase over the calculated background level (Heid et al. 1996). The higher the initial target template concentration, the lower will be the Ct.

Use of qPCR in cyanobacterial ecology

The qPCR-based methods started to be commonly used in microbial ecology since 2000 (Becker et al. 2000; Suzuki et al. 2000; Takai and Horikoshi 2000; Smith and Osborn 2009). Molecular biology application in this research field translates mainly in the use of molecular genetic markers to address such ecological aspects as structure and composition of microbial communities and its correlation with the biological function of individual species or functional systems (Smith and Osborn 2009). A broad range of genetic markers has been used in studies of cyanobacterial ecology (Garcia-Pichel 2008). These genetic markers include genes that are exclusively found in cyanobacteria, (e.g. genes coding for the phycocyanin (PC) operon) or universal genes that are specifically targeted by cyanobacteria-specific primers (e.g. 16S rRNA and RNA polymerase genes) (Garcia-Pichel 2008). Currently, qPCR is broadly used to quantify specific genes copy numbers and thus quantify structural and functional properties of cyanobacterial communities in both field and laboratory conditions (Garcia-Pichel 2008). qPCR assays targeting conserved regions of the 16S rRNA gene have been developed to determine total cyanobacteria numbers, while assays targeting highly variable regions of this gene have been used to quantify taxa-specific sequences, from phylum to species levels (Rinta-Kanto et al. 2005; Ha et al. 2009; Rinta-Kanto et al. 2009; Smith and Osborn 2009). However, the difficulties in developing qPCR protocols based on the 16S rRNA gene for cyanobacteria and *Microcystis* quantification are well known. These complications have mainly been attributed to the variable copy

number of 16S rDNA operons and their sequence heterogeneity in cyanobacterial cells in natural populations (Crosby and Criddle 2003; Rinta-Kanto et al. 2009). This may result in flawed estimates of cell density, originating surreal results like distinct populations, e.g., *Microcystis*, representing more than the overall cyanobacteria abundance (Rinta-Kanto et al. 2005, 2009). Alternatively, PC (phycocyanin) genes have been also used as markers for *Microcystis aeruginosa* and *Planktothrix* total numbers estimation (Kurmayer and Kutzenberger 2003; Yoshida et al. 2007; Briand et al. 2008, 2009). Fairly good correlations have been obtained between qPCR estimates and the several other counting methods, whether they are light microscopy, fluorescence microscopy or electronic particle counting (Kurmayer and Kutzenberger 2003; Briand et al. 2008, 2009; Ha et al. 2009). Nevertheless, some discrepancies have been observed, as for the *C. raciborskii* cell concentration and the *C. raciborskii* identifier gene, *rpoC1* (Rasmussen et al. 2008; Orr et al. 2010). Despite one cannot discard the lack of accuracy of the more traditional cell counting methods, one of the main problems typically associated with qPCR methods is in fact the difficulty to convert gene quantities to quantities of cells that carry these genes. Nevertheless and perhaps more important than determining overall cyanobacterial numbers, qPCR has also been used to quantify genes that encode enzymes in key metabolic or catabolic pathways and thus allowing the evaluation of its potential function in a given environment (Smith and Osborn 2009). Namely, quantification of genes involved in hepatotoxins synthesis has been used to determine the relative abundance of potential to non-potential toxin-producing genotypes in water (Koskenniemi et al. 2007; Briand et al. 2008, 2009; Rinta-Kanto et al. 2009; Orr et al. 2010). These data have been fundamental to better evaluate and understand the dynamics of these subpopulations during bloom formation and so to better predict the occurrence of toxic blooms.

Assessment of spatial and temporal dynamics of MC-producing cyanobacteria communities by qPCR

Among the hepatotoxins-producing cyanobacteria, MC producers have been the most studied using qPCR, mainly due to their worldwide distribution menacing water quality in several countries (Sivonen and Jones 1999). Furthermore, the genetic bases for MC production are well known for quite some time (Tillett et al. 2000). MC production has been documented for several genera of cyanobacteria, including *Microcystis*, *Anabaena*, *Oscillatoria*, *Planktothrix*, *Chroococcus*, *Nostoc, Arthrospira*, *Phormidium*, *Plectonema*, *Pseudanabaena*, *Synechococcus* and *Synechocystis* (Kurmayer and Christiansen 2010; Pearson et al. 2010). *Microcystis* is probably the MC producer with a wider distribution on earth, with MC-producing strains being found in all continents (Sivonen and Börner 2008).

MCs concentration in water during bloom development is known to vary considerably. This happens, at least partially, due to the different proportions of MC-producing and non-MC-producing subpopulations present at a time (Kurmayer et al. 2004). In the last years, qPCR has been several times applied to determine the spatial and temporal variations in proportions of MC-producing cyanobacterial subpopulations in natural conditions (Hotto et al. 2008; Briand et al. 2009; Ha et al. 2009; Rinta-Kanto et al. 2009; Sabart et al. 2010). Several genes were used as markers to quantify the number of toxic (*mcy*) genotypes and total cyanobacteria population numbers (Table 1).

From the several works most recently published, emerges the extraordinarily spatial–temporal variability in the proportions of toxic genotypes, found in water systems worldwide. For instance, Kurmayer and Kutzenberger (Kurmayer and Kutzenberger 2003) reported that the percentage of toxic (*mcyB*) genotypes varied from 1.7 to 71.0% of the total *Microcystis* population during its seasonal cycle (from June 1999 to October 2000) in Lake Wannsee (Berlin, Germany). A seasonal stability between years was found with the lowest abundances occurring in winter and the highest densities in summer. An even higher variability was found for the western basin of Lake Erie, USA (Rinta-Kanto et al. 2005), with the proportion of toxic (*mcyD*) genotypes ranging from just 0.3% to comprising 100% the *Microcystis* population. Also in Lake Erie, a large-scale survey performed in the summer months of 2003, 2004 and 2005, showed that the relative abundances of potentially MC-producing genotypes (*mcyD*) vary between 0.0 and 48.0% of the overall *Microcystis* population. The area where *Microcystis* blooms are more frequent presented the highest percentage of toxic genotypes reaching 60.0% of the total population. Although no clear pattern between years seasonal succession could be observed, the mean variability in toxic genotype proportions was greater between sites than the seasonal variability. Proportions of *mcyA* subpopulation shifted between 0.5 and 35.0% from July 2004 to October 2005 in Lake Mikata, Japan (Yoshida et al. 2007), and between 0.7 and 41.0% from June to November 2007 in the Hirosawa-no-ike fish pond, Kyoto, Japan (Ha et al. 2009). More recently, Baxa and co-workers (Baxa et al. 2010) showed that in the estuarine environment of the San Francisco estuary, USA, the proportions of potentially toxic (*mcyD*) *Microcystis* subpopulations varied significantly between sites and bloom development stages, in both water column and ambient surface waters, constituting between 0.01 and 27.00% of the overall *Microcystis* natural population. In 4 different lake systems in the Northeast US, for 2 years, toxic (*mcyD*) *Microcystis* cells comprised between 0.01% and 100% of the

Table 1 Chronological presentation of important studies using qPCR for genotypical characterisation of potential MC-producing cyanobacterial natural populations

Target gene	Primers and probes[a]	% Toxic genotypes	qPCR method	Study site	Study period	Reference
mcyE	mcyE-F2a: GAA ATT TGT GTA GAA GGT GC MicmcyE-R8: CAA TGG GAG CAT AAC GAG AnamcyE-12R: CAA TCT CGG TAT AGC GGC	n.d.	SYBR Green I	Lake Tuusulanjärvi and Lake Hiidenvesi, Finland	June–Oct 1999	Vaitomaa et al. (2003)
mcyB	30F: CCTACCGAGCGCTTGGG 108R: GAAAATCCCCTAAAGATT TNA probe: CACCAAAGAAACACCCGAATCTGAGAGG	1.7–71	TNA (Taq nuclease assay)	Lake Wannsee (Berlin, Germany)	June 1999–Oct 2000	Kurmayer and Kutzenberger (2003)
Phycocyanin	188F: GCTACTTCGACCGCGCC 254R: TCCTACGGTTTAATTGAGACTAGCC TNA probe: CCGCTGCTGTCGCCTAGTCCCTG					
Cyanobacterial 16S rRNA	CYAN108F: ACGGGTGAGTAACRCGTRA CYAN377R: CCATGGCGGAAAATTCCCC CYAN328R TNA probe: FAMa-CTCAGTCCCAGTGTGGCTGNTC-BHQ	0 to >100	TNA	Western Basin of Lake Erie, North America	Aug 2003 and Aug 2004	Rinta-Kanto et al. (2005)
Microcystis 16S rRNA	MICR184F: GCCGCRAGGTGAAAMCTAA MICR431R: AATCCAAARACCTTCCTCCC MICR228F TNA probe: FAMa-AAGAGCTTGCGTCTGATTAGCTAGT-BHQ					
mcyD	mcyD F2: GGTTCGCCTGGTCAAAGTAA mcyD R2: CCTCGCTAAAGAAGGGTTGA mcyD TNA probe: FAMa-ATGCTCTAATGCAGCAACGGCAAA-BHQ					
mcyA	MSF F: ATCCAGCAGTTGAGCAAGC MSR R: TGCAGATAACTCCGCAGTTG MSR-2R: GCCGATGTTTGGCTGTAAAT	n.d.	SYBR Green I	Lake Kitaura and Lake Kasumigaura, Ibaraki, Japan	Sept 2005	Furukawa et al. (2006)
mcyA (*Microcystis aeruginosa*)	M1r-F: AGC GGT AGT CAT TGC ATC GG M1r-R: GCC CTT TTT CTG AAG TCG CC	0.5–35.0	SYBR Green I	Lake Mikata, Japan	July 2004–Oct 2005	Yoshida et al. (2007)
PC-IGS (*Microcystis aeruginosa*)	188F: GCT ACT TCG ACC GCG CC 254R: TCC TAC GGT TTA ATT GAG ACT AGC C					
mcyA (*Microcystis aeruginosa*)	As previously described (Yoshida et al. 2007)	0.5–47.1	SYBR Green I	Lake Mikata, Japan	April–Dec 2006	Yoshida et al. (2008)
PC-IGS (*Microcystis aeruginosa*)						
Cyanophage g91	SheathRTF: ACA TCA GCG TTC GTT TCG G SheathRTR: CAA TCT GGT TAG GTA GGT CG					

Table 1 continued

Target gene	Primers and probes[a]	% Toxic genotypes	qPCR method	Study site	Study period	Reference
Microcystis 16S rRNA *Microcystis* mcyD pGEM plasmid	As previously described (Rinta-Kanto et al. 2005) pGEM F: CCCAGTCACGACGTTGTAAAACG pGEM R: TGTGTGGAATTGTGAGCGGA TNAprobe: NED-CACTATAGAATACTCAAGCTTGCATGCCTGCA-MGBNFQ	0.0–37.0	TNA	Oneida Lake, North America	June–Oct 2002 and 2003	Hotto et al. (2008)
mcyA (*Planktothrix*)	MAPF: CTAATGGCCGATTGGAAGAA MAPR: CAGACTATCCCGTTCCGTTG MAP TNA probe: CTCTGCGGTTACAGCTAACGGGTGG-BHQ	31.0–83.0	TNA	Base Nautique de Viry, Paris, France	Mar 2004–Jan 2006	Briand et al. (2008, 2009)
Phycocyanin (*Planktothrix*)	FPC1: AACCCATAGGGAGATAACTC RPC1: GCTTTGGCTTGACGGAAACG PPC TNA probe: CYA-ATTCTCAAGGCCGTTTCCTGAGCAG-BHQ					
mcyA Microcystis	MSF: ATCCAGCAGTTGAGCAAGC MSR: TGCAGATAACTCCGCAGTTG	0.7–41.0	SYBR Green I	Hirosawa-no-ike fish pond, Kyoto, Japan	June–Nov 2007	Ha et al. (2009)
16S rRNA *Microcystis*	209F: ATGTGCCGCGAGGTGAAACCTAAT 409R: TTACAATCCAAAGACCTTCCTCCC					
Cyanobacterial 16S rRNA *Microcystis* 16S rRNA *mcyD*	As previously described (Rinta-Kanto et al. 2005)	0.0–60.0	TNA	Lake Erie, North America	Summer seasons 2003–2005	Rinta-Kanto et al. (2009)
mcyB region (*Microcystis*)	30F: CCTACCGAGCGCTTGGG 108R: GAAAATCCCCTAAAGATTCCTGAGT TNA probe: FAM-CACCAAAGAAACACCCGAATCTGAGAGG-TAMRA	6.0–93.0	TNA	Grangent reservoir, France	Every 2 weeks from 18 April to 1 Aug 2007; 6 stations	Briand et al. (2008)
PC gene region (*Microcystis*)	188F: GCTACTTCGACCGCGCC 254R: TCCTACGGTTTAATTGAGACTAGCC TNA probe: CYA-CCGCTGCTGTCGCCTAGTCCCTG-BHQ					
mcyA	mcyA-Cd 1R: AAAAGTGTTTTATTAGCGGCTCAT mcyA-Cd 1F: AAAATTAAAAGCCGTATCAAA	n.d.	SYBR Green I	Meilang Bay, Lake Taihu, China	April 2006–March 2007	Ye et al. (2009)
mcyE/ndaF	HEP-F: TTTGGGGTTAACTTTTTTGGGCATAGTC HEP-R: AATTCTTGAGGCTGTAAATCGGGTTT	n.d.	SYBR Green I	Nile River Delta, Egypt		Amer et al. (2009)
Microcystis 16S rRNA *Microcystis* mcyD pGEM plasmid	As previously described (Hotto et al. 2008; Rinta-Kanto et al. 2005)	0.01–100.00	TNA	Lake Champlain, Lake Agawam, Mill Pond, Lake Ronkonkoma, USA	May–Nov 2005 and 2006	Davis et al. (2009)

Table 1 continued

Target gene	Primers and probes[a]	% Toxic genotypes	qPCR method	Study site	Study period	Reference
Cyanobacterial 16S rRNA	CYA16S F: TCG CCC ATT GCG GAA A CYA16S R: AGA CAC GGC CCA GAC TCC TA CYA16S TNA probe: TTC CCC ACT GCT GCC	0.4–20.2	TNA	San Francisco estuary, USA	July–Sept 2007	Baxa et al. (2010)
Microcystis 16S rRNA	MIC16S F: AAA GCG TGC TAC TGG GCT GTA MIC16S R: CCC TTT CGC TCC CCT AGC T MIC16S TNA probe: CTG ACA CTC AGG GAC G					
Microcystis mcyD	As previously described (Rinta-Kanto et al. 2005)					
mcyB region (*Microcystis*)	As previously described (Briand et al. 2008)	0.5–66.6	TNA	5 freshwater lakes in Uganda	May 2007–April 2008	Okello et al. (2010)
PC gene region (*Microcystis*)						

n.d. not determined

TNA Taq nuclease assay

[a] 5′-3′

total *Microcystis* population (Davis et al. 2009), a larger range than those found in prior studies (Kurmayer and Kutzenberger 2003; Rinta-Kanto et al. 2005; Hotto et al. 2008; Rinta-Kanto et al. 2009). In this study, it was further found that the higher ratios were observed in the only stratified lake studied, Lake Ronkonkoma, where toxic *Microcystis* comprised between 12.0 and 100.0% of total *Microcystis* cells. In five freshwater lakes in Uganda, *Microcystis* cells containing *mcyB* comprised, on average, around 20.0% of the total *Microcystis* population (Okello et al. 2010). The between sites variation ranged from 0.5 to 66.6% of the overall *Microcystis* population, being more significant than the seasonal variation within each of the lakes. This induces a stable variance in MC production by *Microcystis* between the spatially isolated populations. These results are especially relevant regarding biogeography and how it can influence our understanding on toxin production in cyanobacteria (Okello et al. 2010). In Grangent reservoir, Loire river, central France, the proportions of toxic (*mcyB*) genotypes changed significantly over time and between sites, ranging from 6.0 to 93.0% of the total *M. aeruginosa* population (Briand et al. 2009). The highest proportions were observed at the beginning and after the decline of the bloom, while the lowest proportions were recorded at the height and at the end of the bloom. The marked spatial variations identified during bloom development were attributed to greater cyanobacterial growth in areas displaying better environmental growth conditions or to population displacement caused by currents or winds.

Although less studied, toxic *Planktothrix* population dynamics has also been characterised by qPCR. The subpopulation dynamics of potentially MC-producing and non-MC-producing strains of *P. agardhii* were evaluated twice monthly at one sampling station, from 23 March 2004 to 21 March 2006, in an artificial, shallow, eutrophic French lake (Briand et al. 2008, 2009). The proportion of *mcyA* containing *P. agardhii* cells varied considerably from 31.0% to 83.0% of the total population. This variation in *mcyA* genotype proportion was mainly attributed to changes in the total *P. agardhii* cell densities, although both parameters correlated negatively. Furthermore, the observed changes in MC levels could not be explained only by toxic genotypes proportion variation. Between spring 2005 and autumn 2007, in 12 lakes of the Alps, proportions of potential MC-producing *Planktothrix* sp. genotypes ranged between 18.4 and 81.7%, with an average of 52.9% (Ostermaier and Kurmayer 2010). A significant correlation was found between each of the toxic genotypes quantified and the correspondent MC structural variant level. These proportions seem to be, in average, higher than the ones observed for MC-producing strains in *Microcystis* natural populations.

The development of new qPCR assays using new genetic markers for different toxin-producing cyanobacteria will certainly help elucidating how the internal population dynamics is ruled and which effects it produces in the global ecosystem dynamics. On the other hand, it is important to know how the environment affects these spatial and temporal population shifts.

Influence of environmental parameters on the internal dynamics of MC-producing cyanobacteria communities

The influence of environmental parameters in the internal dynamics of cyanobacterial communities is still poorly understood. The qPCR technique is a helpful tool to better understand these relationships. By establishing correlations between environmental parameters and numbers/proportions of toxic genotypes, it is possible to determine which conditions affect most the internal organisation of potential MC-producing cyanobacteria communities.

A positive correlation between toxic genotypes (*mcyA*) and nitrate (NO_3-N) concentrations was observed by Yoshida and co-workers in lake Mikata, Japan (Yoshida et al. 2007), while temperature and orthophosphate (PO_4-P) concentration seem not to influence *mcyA* abundance (Yoshida et al. 2007). In the same lake, an evident increase in cyanophages numbers following the *M. aeruginosa* numbers decline was observed, and a direct influence of the cyanophage assemblage in shifts of MC-producing and non-MC-producing subpopulations was suggested (Yoshida et al. 2008). NH_4 and NO_3 were also shown to increase toxic (*mcyA*) *Microcystis* strains abundance and MC concentrations in the Hirosawa-no-ike hypereutrofic pond (Ha et al. 2009), without affecting the total *Microcystis* abundance. Total phosphorus was shown to positively correlate with both toxic (*mcyD*) and total *Microcystis* abundance in Lake Erie, USA (Rinta-Kanto et al. 2009). The abundance of *mcyD* genotypes in this lake, on the other hand, correlated negatively with NO_3, total nitrogen, nitrogen to phosphorus ratio and pH (Rinta-Kanto et al. 2009). Phosphorus was also shown to correlate positively with the relative abundance of potentially hepatotoxic (*mcyE*/*ndaF*) cyanobacterial community of river Nile, Egypt, at a site showing strong phosphorus limitation (Amer et al. 2009). The effects of increased temperature levels (+4°C), nitrogen and phosphorus on the growth rates of toxic and non-toxic strains of *Microcystis* were further analysed In a multi-site survey including four lakes across the Northeast USA (Davis et al. 2009). Simultaneous increases in temperature and phosphorus concentrations produced the highest growth rates of toxic *Microcystis* cells. This may lead in the future to the formation of blooms with higher MC content due to the increasing eutrophication and climatic warming that may cumulatively promote the growth of MC-producing, rather than non-MC-producing *Microcystis* populations (Davis et al. 2009).

As seen, the results reported so far about the influence of environmental factors on numbers/proportions of toxic genotypes give contradictory information. This may reflect that although some parameters like phosphorus and nitrogen contents are significant factors governing *Microcystis* abundances and MC production, the weight of each of these factors may vary significantly in different environments (Rinta-Kanto et al. 2009). Moreover, the intra-cellular mechanisms controlling MC production may contribute to the different way how these parameters affect the relative proportion of potentially toxic genotypes in the overall *Microcystis* population (Rinta-Kanto et al. 2009). Other aspects like nutrient availability, water temperature, eutrophication, light availability and spring–summer period length have been shown to specifically affect the growth and to alter interactions between MC-producing and non-MC-producing *Microcystis* strains or affect the ability of potentially MC-producing genotypes to in fact produce MC (Kardinaal et al. 2007; Davis et al. 2009; Baxa et al. 2010). On the other hand, a combination of factors including mixing processes produced by stream flow, wind and tides has been previously shown to affect phytoplankton bloom spreading and enhance aggregation of *Microcystis* biomass in shallow or low flow water reaches (Huisman and Hulot 2005; Lehman et al. 2008). Considering this, it becomes imperative to enlighten the physiological basis governing toxin production in the different natural scenarios. Specifically, more research on the processes coupling gene expression to toxin production is lacking.

Assessment of hepatotoxin biosynthetic genes transcriptional response by RT–qPCR

Besides being influenced by the internal dynamics of toxic populations, MC concentration in a water body also depends on toxin synthesis rate variability inside a given subpopulation and/or on different biosynthetic genes expression levels. To better understand the regulatory factors governing toxin biosynthesis, the upstream-signalling pathways that target toxin synthesis and the molecular mechanism(s) by which toxin production is controlled, it is essential to analyse the transcriptional pattern of the genes involved in hepatotoxin synthesis (Dittmann and Wiegand 2006). Reverse transcriptase PCR (RT–PCR) is used to amplify RNA by converting it to cDNA using a reverse transcriptase before PCR amplification. By combining qPCR with this initial reverse transcriptase reaction, it is possible to quantify RNA transcripts (rRNA or mRNA) and study gene expression profiles both in natural and laboratory conditions. So,

RT–qPCR (reverse transcriptase quantitative PCR) enables to quantitatively estimate the activity of specific taxa or functional guilds within cyanobacterial communities (Garcia-Pichel 2008; Smith and Osborn 2009).

The influence of several environmental parameters like temperature, irradiance, macronutrients (nitrate, ammonium, phosphate), trace elements (iron and others), salinity, CO_2 and pH, on MC synthesis ability, has been previously addressed with conflicting results being often obtained (Utkilen and Gjolme 1995; Rapala et al. 1997; Sivonen and Jones 1999; Long et al. 2001; Dai et al. 2008). Physical mixing processes are known to affect population dynamics of phytoplankton, being vertical mixing a major determinant of harmful cyanobacteria surface blooms development (Huisman and Hulot 2005). Transcriptional regulation studies using RT–qPCR (reverse transcriptase quantitative PCR) revealed that the promoter region of the *mcyS* gene cluster of *Microcystis* is bi-directional, with two different transcription initiation sites under high and low light intensity conditions. Increased transcription under high light and red light was observed, while under low light and dark conditions transcription decreased (Nishizawa et al. 1999; Kaebernick et al. 2000). Blue light and artificial stress factors also reduced the *mcy* transcription, and an inter-relationship between irradiance and temperature on the *mcy* transcript level was observed (Kim et al. 2005). The *mcyS* promoter region contains binding regions for transcription factors, such as a ferric uptake regulator (Fur) that usually represses gene expression in the presence of Fe^{2+}, and an in vivo inter-relationship between the extracellular iron availability and *mcy* transcription rate has been reported (Sevilla et al. 2008). Transcriptional results confirm that synthesis of MC is related to cell division and growth, although the amount of produced MC, is regulated in response to environmental factors such as light (Kaebernick et al. 2000; Kim et al. 2005; Tonk et al. 2005) and trace elements (iron) (Sevilla et al. 2008). On the contrary, availability of macronutrients (nitrogen), although being often showed to affect MC synthesis (Lee et al. 2000), does not seem to influence *mcyD* expression (Sevilla et al. 2010). Under phosphate starvation, the nine genes of the *ndaS* cluster are constitutively overexpressed, up to a 2.73-fold, in *N. spumigena* strain AV1, probably due to transcription from the single bidirectional promoter (Jonasson et al. 2008). Excess NOD synthesised due to continuous *ndaS* cluster expression could be downregulated either by intracellular degradation, cellular excretion or any unknown post transcriptional regulatory mechanism affecting NOD biosynthesis and/or maturation or enzyme activity levels (Jonasson et al. 2008). Ammonium supplementation repressed *ndaS* cluster gene expression, but once again without changing NOD levels suggesting the existence of some regulatory mechanism controlling intracellular NOD contents (Jonasson et al. 2008). RT–qPCR has been also used to detect and quantify specific mRNA transcripts in complex environmental samples. Detection and quantification of specific environmental cyanobacterial gene transcripts have been successfully achieved in a range of aquatic ecosystems (Boström et al. 2007; Jonasson et al. 2008; Steunou et al. 2008; Sipari et al. 2010), with some results confirming the laboratory data (Jonasson et al. 2008). It seems to be a wide array of factors and processes governing MC production by cyanobacteria, suggesting the possible role of MC in a variety of functions like cell-to-cell communication, defence against grazing or nitrogen nutrients (Briand et al. 2008, 2009). An interesting work brought up the question of toxic and non-toxic cyanobacteria cells displaying different fitness under different environmental conditions, suggesting that the benefits of producing MC are particularly important under growth-limiting conditions and that this benefit is outweighed by MC production costs when environmental conditions favour cell growth (Briand et al. 2009).

Quantification of potential CYN and NOD-producing cyanobacteria by qPCR-based approaches

Cylindrospermopsin (CYN) is considered the second most widely occurring cyanobacteria produced hepatotoxin. CYN is produced by several cyanobacteria species, including *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Umezakia natans*, *Rhaphidiopsis curvata*, *Anabaena bergii*, *Anabaena lapponica* and *Lyngbya wollei* (Harada et al. 1994; Banker et al. 1997; Li et al. 2001a, b; Schembri et al. 2001; Preußel et al. 2006; Spoof et al. 2006; Seifert et al. 2007; Pearson et al. 2010). Nodularin (NOD) is typically produced by *Nodularia spumigena*, a worldwide distributed hepatotoxic cyanobacterium. *N. Spumigena* is known to originate toxic masses, especially in the Baltic Sea and in some Australian lakes and estuaries (Sivonen et al. 1989; Heresztyn and Nicholson 1997; Pearson et al. 2010).

Despite less frequently, qPCR has also been applied for the study of NOD and CYN-producing cyanobacterial communities (Table 2). A specific and sensitive qPCR method was developed by Koskenniemi and co-workers (Koskenniemi et al. 2007), targeting subunit F of the nodularin synthetase gene (*ndaF*) to estimate abundances of potentially NOD-producing *Nodularia* cells in the Baltic Sea. This method proved to be useful for *Nodularia* blooms monitoring since the earliest stages, enabling detection of as few as 30 *ndaF* gene copies/mL. The *ndaF* copy numbers correlated significantly with NOD concentrations, further revealing a relatively constant production of NOD by *Nodularia* in the Baltic Sea. A TNA duplex qPCR protocol was developed to detect and quantify *Cylindrospermopsis*

Table 2 Studies using qPCR for detection and quantification of potential NOD and CYN-producing cyanobacteria

Target gene	Primers and probes	qPCR method	Study site	Study length	Reference
NOD					
ndaF Nodularia spumigena	ndaF8452: GTG ATT GAA TTT CTT GGT CG ndaF8640: GGA AAT TTC TAT GTC TGA CTC AG	SYBR Green I	Baltic Sea	July 2004	Koskenniemi et al. (2007)
CYN					
rpoC1 C. raciborskii	cyl2: GGCATTCCTAGTTATATTGCCATACTA cyl4: GCCCGTTTTTGTCCCTTTGCTGC RPOC1 TNA probe: ROX-TCCTGGTAATGCT GACACACTCG-BHQ2	Duplex TNA assay	South Australia, and New South Wales, Australia	March 2004–June 2005	Rasmussen et al. (2008)
pks	k18: CCTCGCACATAGCCATTTGC m4: GAAGCTCTGGAATCCGGTAA PKS TNA probe: FAM-CGGCAGCAACA CTCACATCAGT-BHQ1				
cyrC *rpoC1 C. raciborskii* ribotype specific primers	As previously described (Rasmussen et al. 2008) AusNZ-PR-F (GAACAGAAAGCAAACAGAATTGATG) AusNZ-PR-R (CAGAAATAGAAGCTGACATTCAAGG) TNA probe (TGCCCACTCTGTCATTTATGTCGG)	Triplex TNA assay	Southeast Queensland, Australia	Feb/March 2007	Orr et al. (2010)
Cyanobacterial 16S rRNA	CYAN108F: ACGGGTGAGTAACRCGTRA CYAN377R: CCATGGCGGAAAATTCCCC CYAN328R TNA probe: FAMa- CTCAGTCCC AGTGTGGCTGNTC-BHQ-	Singleplex TaqMan nuclease assay			

raciborskii and potential CYN-producing cyanobacteria (Rasmussen et al. 2008). This qPCR protocol proved to be a sensitive and rapid method to detect potential cylindrospermopsin-producing cyanobacteria in Australian lakes, reservoirs and rivers, constituting a powerful tool to more efficiently monitor this rising threat to water quality. This protocol was recently improved and used to assess spatial and temporal variability of *C. raciborskii* and potential CYN-producing cyanobacteria in three drinking water reservoirs in Queensland, Australia (Orr et al. 2010). The intra-cellular CYN cell quotas strongly correlated with the *cyrC* gene cell quotas, indicating that qPCR analysis of the *cyrC* gene could be used to indicate the presence of intracellular CYN. Contrarily to most of the qPCR methods applied for MC producers quantification, this method proved not to be adequate to quantify either 16S rRNA or *rpoC1* gene cell quotas as an alternative way to estimate *C. raciborskii* abundance (Orr et al. 2010).

Potential use of qPCR methods for water quality monitoring programs

The predicted increase in the incidence of toxic cyanobacteria blooms in aquatic ecosystems worldwide (Davis et al. 2009) will certainly bring additional health problems to human beings and animals. New and more efficient monitoring systems enabling rapid, reliable and accurate identification of toxins, and toxin producers in the environment are needed to protect water users (Sivonen 2008). Several studies support the application of toxic genotype monitoring by qPCR to predict MC concentrations in water. Densities of potentially toxic *Microcystis* cells significantly correlated with MC concentrations (Davis et al. 2009; Ha et al. 2009; Rinta-Kanto et al. 2009) being suggested that it could be a better predictor of MC concentrations in aquatic ecosystems than the currently recommended parameters, total cyanobacteria cell counts, total *Microcystis*, or chlorophyll a (Davis et al. 2009). Furthermore, the proportion of *mcyA* containing *P. agardhii* cells was shown to significantly correlate with MC values (Briand et al. 2008, 2009) and that different MC variant concentrations could be predicted from the corresponding genotype concentrations (Ostermaier and Kurmayer 2010).

The widely occurrence of *C. raciborskii* and its invasive nature makes CYN production a global water management problem (Neilan et al. 2003; Pearson et al. 2010). It was suggested that qPCR analysis of the *cyrC* gene can be used to detect and quantify intra-cellular CYNs in bloom material (Rasmussen et al. 2008; Orr et al. 2010).

Nevertheless, some drawbacks emerge for the use of qPCR in efficient water quality monitoring programmes. Discrepancies between toxic genotype numbers and MC contents have been reported (Vaitomaa et al. 2003; Yoshida et al. 2007; Rinta-Kanto et al. 2009; Ye et al. 2009). Several explanations were suggested, namely different intra-cellular MC concentrations between different strains in different time periods, and the accumulation of DNA originating from lysed cells was suggested as possible causes (Vaitomaa et al. 2003). The possibility that non-toxic strains may, however, contain fragments of MC synthetase genes or mutations within these genes, still enabling PCR amplification (Neilan et al. 1999; Tillett et al. 2001) has also been raised as a possible explanation for such discrepancies (Vaitomaa et al. 2003). The presence of other MC-producing cyanobacteria or of different MC congeners can potentially enhance differences in total MC concentrations, thus leading to the lack of correspondence with qPCR toxic genotypes estimates (Baxa et al. 2010). Furthermore, some authors suggested that MC is mainly produced by few highly productive species representing only a small proportion of the overall potential MC-producing population (Ye et al. 2009). The presence of toxic genotypes during non-bloom season, despite absence of MC, suggests that toxic genotypes may survive the winter but that under low growth or low metabolism there is no MC production (Ye et al. 2009). These aspects raise the problem of potential MC threat even during non-bloom seasons making important to adapt monitoring programmes so to include non-bloom seasons. Thus, the use of qPCR as a early-warning monitoring tool gains extra relevance by enabling detection of just a small number of toxic genotypes long before a bloom becomes apparent. Moreover, qPCR-based methodologies have been shown useful as follow-up tool to monitor the restoration strategies impact on cyanobacterial communities by quantitatively enable evaluation of the shifts in toxic strains number (Vaitomaa et al. 2003).

Conclusions

The data available so far indicate the urgent need in developing new and better qPCR assays to assure more accurate quantifications of the different toxin-producing cyanobacteria and biosynthetic gene transcripts under different environmental conditions. Nevertheless, despite the known caveats of the method, qPCR is undoubtedly an efficient tool to evaluate the temporal and spatial shifts of potential MC-producing cyanobacteria in natural conditions. When combined with other methods, like DGGE, it gives us a comprehensive picture of the internal dynamics of specific cyanobacterial populations, in any given water body, both in terms of quantities as of genotypes diversity. Furthermore, it enables the establishment of correlations with the biotic and abiotic parameters found. It is important

to notice, however, that several methodological issues can limit or enhance the value of this technique or even distort the observed reality. The design of a proper sampling plan is essential to assure that the results obtained are representative. For instance, the advantages of multi-point sampling for estimating and better understanding cyanobacteria population dynamics in freshwater ecosystems are well recognised (Briand et al. 2009). Furthermore, the adoption of the most efficient and reproducible nucleic acids extraction method is of major importance to avoid creating artificial bias. Biases related to genomic DNA extraction and PCR amplification are well documented (Polz and Cavanaugh 1998; Frostegard et al. 1999; Miller et al. 1999; Martin-Laurent et al. 2001; Crosby and Criddle 2003). The standard phenol–chloroform DNA extraction procedure (Kurmayer et al. 2003) was proved to be reliable and accurate for qPCR quantifications. To use qPCR methodologies as routine water quality monitoring tools, it is mandatory to standardize all the steps from sample collection to qPCR data analysis. On the other hand, further research on the expression of cyanotoxins-related genes and its regulation, assessed by qPCR, will certainly help elucidating the physiological processes defining overall toxin production in natural populations and so better understand its role in the environment. Furthermore, this knowledge will help designing more efficient monitoring programmes and adopting the most adequate restoration strategies in water systems worldwide.

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