Development of sensitive and specific multiplex PCR method for the detection of microcystin producing cyanobacteria in spirulina food supplements

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Abstract Spirulina has emerged as the next-generation dietary supplement owing to its health benefits. Despite the advantages, there have been reports of contamination by cyanotoxins such as microcystins that can adversely affect human health. Hence, there is a need to develop a robust, efficient, and cost-effective method to detect microcystin-producing cyanobacteria in these food supplements. In this study, we have demonstrated a multiplex polymerase chain reaction (PCR) method for identification of microcystin-contamination in spirulina dietary supplements. This method involves simultaneous amplification of phycocyanin and microcystin B encoding genes (*pcb*, *mcyB*). The sensitivity of the multiplex PCR was assessed, and the limit of detecting *mcyB* along with *pcb* was found to be 250 fg/μL. The presence of microcystin was detected in five out of seven fish food supplements indicating poor culture conditions. Hence, rigorous quality control is required for monitoring the spirulina food supplements.

Keywords Spirulina food supplement · Multiplex PCR · Microcystin · *Arthrospira platensis* · Cyanotoxin

Introduction

Microalgae have a vast industrial potential and have emerged as the future of the food, fertilizer, drug, and cosmetic industries in the last decade (Park et al., 2018). *Arthrospira* sp. such as *Arthrospira maxima* and *Arthrospira platensis* are the microalgae most commonly used for the production of dietary food supplements as they are regarded as a rich source of high quality protein, vitamins (in particular vitamin B12 and provitamin β-carotene), phenolic acids, minerals, essential amino acids, pigments, and essential fatty acids (Joventino et al., 2012; Karkos et al., 2011; Kulshreshtha et al., 2010; Vichi et al., 2012). The algal food products derived from spirulina are advertised for weight loss, as abundant sources of energy, mood improvers, and also as detoxifier (Heussner et al., 2012). Researchers have studied their numerous pharmacological functions such as anti-nephrotoxic properties (Belay et al., 1993), anticancer properties (Mathew et al., 1995), blood glucose level control (Parikh et al., 2001), hypocholesterolemic activity (Samuels et al., 2002), activation of natural killer cell (Akao et al., 2009), and hypolipidemic activity (Mazokopakis et al., 2014). Food supplements containing blue-green algal supplements (BGAS) are sold to the customers as over the counter nutraceuticals in pharmacies, in supermarkets as well as through online stores all over the world which has made them more accessible (Vichi et al., 2012).

Arthrospira platensis and *A. maxima* are produced under controlled culture conditions as well as in open pond systems. However, certain other harmful cyanobacteria grow along with *Arthrospira* sps. and contaminating it. These cyanobacteria produce toxins (cyanotoxins) which are chemically diverse and are classified according to their mode of action as neurotoxins, hepatotoxins, and general

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cytotoxins. Some of the cyanotoxin producers are *Anabaena*, *Microcystis*, *Oscillatoria*, and *Nostoc* (Bittencourt-Oliveira, 2003; Bláha et al., 2009; Preece et al., 2015). They can produce microcystins (MCs), a group of monocyclic heptapeptides which have more than 60 structural forms (Soares et al., 2004). MCs mainly cause changes in the morphology and functioning of the hepatocytes thereby inhibiting the activity of protein phosphatase (Types 1 and 2A) both in vivo and in vitro. Controlling the growth of unwanted cyanobacterial species is attempted, but the contamination still occurs (Jiang et al., 2008). The presence of such toxins has been reported throughout the world, and it has been observed that hepatotoxic microcystins are found in 40–75% of cyanobacterial blooms (Corbel et al., 2014). Ingestion of contaminated food or water has led to increased exposure to cyanotoxins such as microcystins, and this has acquired increased attention around the world as a matter of public health concern (Gilroy et al., 2000). This calls for an urgent need to develop and validate accurate assays which can be used to detect even minute traces of the toxins. There have been several reported assays such as ELISA (Preece et al., 2015), cell-based bioassay (RCA) (Sorichetti et al., 2014), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Parker et al., 2015), reverse-phase high-performance liquid chromatography (Sangolkar et al., 2009), surface plasmon resonance biosensor (Yakes et al., 2015), ultra-high pressure liquid chromatography-tandem mass (Pekar et al., 2016), HRM multiplex qPCR (Manali et al., 2017), and MALDI-TOF (Valerio et al., 2010). However, these methods are either expensive, time-consuming, and require sophisticated instruments or highly trained personnel. Multiplex PCR based detection, on the other hand, is quick, sensitive, and economical. The primary goal of this work was to develop a specific and sensitive multiplex polymerase chain reaction (PCR) based assay for the rapid detection of microcystin in spirulina-based dietary supplements.

Materials and methods

Strains and medium used

Arthrospira platensis (CCC-477) was obtained from the Indian Agricultural Research Institute (IARI), New Delhi, India and grown in BG-11 medium at pH 8.5. *Microcystis aeruginosa* (NIES-843) was procured from the National Institute of Environmental Studies, Tsukuba, Japan and cultured in BG-11 medium at pH 7 (Rippka et al., 1979). Controlled culture room was used to maintain the growth conditions for the cyanobacteria (25 °C, 14/10 light and dark cycle). Regions of phycocyanin and microcystin B

genes were cloned and maintained in pGEM®-T Easy vector (Promega, Madison, Wisconsin, USA). *E. coli* strain Top10 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to maintain the pGEM®-T Easy vector with microcystin (*mcyB*) and phycocyanine (*pcb*) genes. All the chemicals used for the experiments were obtained from HiMedia Pvt. Ltd, Mumbai, India.

Food supplements

A total of seventeen spirulina food supplements were purchased online or from pharmacies in India. The spirulina food supplements were obtained in the form of capsules, flakes, mini pellets, powder, and tablets (Fig. 1). Among the seventeen products, ten human food supplements (F1 to F10) were obtained from Puducherry and Erode, India. Seven fish food supplements (F11 to F17) acquired were from Kolkata and Chennai, India.

Extraction of DNA from food supplements

The DNA was extracted from spirulina food supplements using the glass powder method (Ramakrishnan et al., 2017). 100 mg of food supplement was randomly collected from each package and homogenized using mortar with autoclaved glass powder until it turned into a fine powder. The isolation of DNA was done in triplicate for each sample to ensure that no disparity in the results occurred due to packaging errors or manufacturing defects. To check the quality and concentration of the isolated DNA, Spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used. The ratio of absorbance measured at 260/280 nm was used to assess the

Fig. 1 Various forms of Spirulina dietary supplements obtained from different parts of India (Clockwise: Tablets, pellets, flakes, tablets, powder and capsules)

purity of the extracted DNA which was then used for PCR and multiplex PCR experiments.

Cloning of *pcb* and *mcyB* in pGEM[®]-T easy vector

Primers specific to phycocyanin region (*pcb*) of *Spirulina platensis* were used (FP: 5'-GGAGATAAGTCCATGT TTGA-3', RP: 5' CTAGCTTAGGGCGTTGATCGC-3' (Yu et al., 2002) to amplify a region 1131 bp long. The PCR amplification was standardized for the following conditions: Initial denaturation step at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.2 min and a final extension step at 72 °C for 5 min. A specific region of microcystin gene (*mcyB*) was amplified using the FP: 5'-CCCCATCGGGGGTGAACATAGGG-3' and RP: 5'-CCCCCAGTTTGGGGAAGAAAAT-3' to give a product of length 212 bp using the conditions: Initial denaturation step 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 20 s and a final extension step at 72 °C for 5 min. The reaction without a template served as non-template control (NTC). PGEM[®]-T Easy Vector was used to clone the amplified regions of phycocyanin and microcystin genes, and these were then transformed into *E. coli* Top 10 competent cells. Colony PCR was carried out with the above-mentioned conditions to confirm the presence of *pcb* and *mcyB*. The plasmid isolated from the recombinant clones were used as a template for PCR and multiplex PCR reactions.

Sensitivity of polymerase chain reaction assay

The sensitivity of PCR was evaluated by performing analysis on serially diluted plasmids containing the target sequence (*pcb* and *mcyB*). The dilutions ranged for *pcb* from 300 ng/μL to 3 zg/μL, and for *mcyB* from 250 ng/μL to 2.5 zg/μL. Using PCR primers (Table 1) for *pcb* (FP: 5'-GCTTGCGTGACATGGAAATC-3', RP 5' TCTTCGAGA ACACTTGCGTC-3') and *mcyB* (FP-5' CAGGATTA

TGCTTATGTTTCTC-3', RP: 5'-CTCAAAAACGACCAA ACTC-3'), a reaction was set up with the following conditions: Initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, with an annealing temperature of 56 °C for *mcyB*, 60 °C for *pcb* for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The products were run on a 3.5%(w/v) agarose gel and visualized using gel documentation system (Gelstan; Mediacare, Chennai, India).

Specificity of the multiplex polymerase chain reaction assay

A multiplex PCR was set up to simultaneously detect two genes in a single reaction (Saker et al., 2007). Cross-reactivity between the two primer sets like the formation of primer dimers and hairpins in the multiplex reaction was checked using AutoDimer (Vallone and Butler, 2004). A standard concentration of *pcb* (3 ng) was spiked with varying concentration of *mcyB* template (2.5 ng/μL–2.5 zg/μL). The above-mentioned conditions were employed for a multiplex reaction set up with primers FP: 5'-CCCCATCGGGGGTAGAACATAGGG-3' and RP: 5'-CCCCCAGTTTGGGGAAGAAAAT-3' and an annealing temperature of 58 °C. The reaction without the template served as a non-template control (NTC). The products were run on a 3.5% (w/v) agarose gel and visualized using gel documentation system (Gelstan, Mediacare)

Evaluation of food supplements

The isolated DNA from spirulina dietary supplements was subjected to multiplex PCR to simultaneously detect the presence of phycocyanin gene specific to *Spirulina* sps., and microcystin B gene which encodes for microcystin biosynthesis. A reaction was set up for a total of 50 μL using the conditions mentioned above. The reaction without the template served as a non-template control (NTC). A standard *pcb* and *mcyB* template in a single reaction were used as the positive control. Sequencing of the PCR

Table 1 List of primers used in this study

Gene	Primer name	Sequence (5'–3')	Size (bp)	References
Phycocyanin	<i>pcb</i> Forward (L)	GGAGATAAGTCCATGTTTGA	1131	Yu et al. (2002)
	<i>pcb</i> Reverse (L)	CTAGCTTAGGGCGTTGATCGC		
Microcystin B	<i>mcyB</i> Forward(L)	CCCCATCGGGGGTAGAACATAGGG	212	Manali et al. (2017)
	<i>mcyB</i> Reverse (L)	CCCCCAGTTTGGGGAAGAAAA		
Microcystin B	<i>mcyB</i> Forward (S)	CAGGATTATGCTTATGTTTCTC	93	
	<i>mcyB</i> Reverse (S)	CTCAAAAACGACCAAATC		
Phycocyanin	<i>pcb</i> Forward (S)	GCTTGCGTGACATGGAAATC	76	
	<i>pcb</i> Reverse (S)	TCTTCGAGAACACTTGCGTC		

products was carried out using primers of *mcvB*. A BLAST search on GenBank (<https://blast.ncbi.nlm.nih.gov>) was done using the sequenced amplicons. All sequences were checked manually for the quality of the data.

Results and discussion

Sensitivity of polymerase chain reaction

To check for the sensitivity of PCR, serially diluted plasmids containing the target sequence (*pcb* and *mcvB*) was used. Using the primers designed to amplify a small region of the gene *mcvB* and *pcb*, it was observed that the *mcvB* gene with a product size of 93 bp was amplified and detected up to a concentration of 25 pg/ μ L (Fig. 2A) whereas the *pcb* was detected up to 30 ag/ μ L with a band of size 76 bp (Fig. 2B).

Specificity of the multiplex polymerase chain reaction

To simultaneously detect *pcb* and *mcvB* genes in a single reaction, a multiplex PCR was set up with varying concentrations *mcvB* spiked with a constant amount of *pcb* template. The goal of this assay was to check for the minimum concentration of the toxin-related gene that can be tested in the presence of the house-keeping gene. It was observed that up to 250 fg/ μ L of *mcvB* was amplified in the

presence of *pcb* beyond which the *mcvB* was not amplified (Fig. 3).

Analysis of food supplements

The food supplements obtained were subjected to a multiplex PCR reaction using the long primers for *mcvB* and short *pcb* primers. The spirulina food supplements available for human consumption (F1–F10), when subjected to a multiplex PCR, showed no microcystin contamination (Fig. 4). The positive control (a mixture *pcb* as well as *mcvB* templates) showed two bands at 212 bp and 76 bp. Five of the fish food supplements were contaminated with microcystin (Fig. 5) whereas two supplements which were devoid of any microcystin contamination showed no band for *mcvB*. The negative template was clear. A BLAST search revealed a 100% match to the microcystin gene (data not shown). This helped to confirm the presence of microcystin-producing cyanobacteria in the food supplements used in the study which were marketed for fish.

In this study, we have developed and evaluated a multiplex PCR assay for specific and simultaneous detection of microcystin toxin-producing algae in spirulina food supplements obtained from different parts of India. There have been several reports all over the world where multiplex polymerase chain reaction has been used as a method to detect microcystin-producing cyanobacteria (Barón-Sola et al., 2012; Douma et al., 2017; Saker et al., 2007). However, there have been no such reports across India. To the best of our knowledge, this is the first multiplex PCR assay developed for the detection of microcystin-producing cyanobacteria in blue-green algal dietary supplements in India. The primers designed for the detection of *mcvB* together with *pcb* amplify a single-copy gene making the assay even more specific.

A specificity test was carried out for the multiplex PCR assay. This was done to check the limit of simultaneous detection of the two genes considered for the study. It was seen that individually, *pcb* was detected up to a

Fig. 2 PCR amplification of *pcb* and *mcvB* genes with the diluted template DNA. (A) Tenfold dilutions of the PGEM[®]-T Easy Vector *mcvB* M- 100 bp Ladder, L1-25 ng/ μ L, L2-2.5 ng/ μ L, L3-250 pg/ μ L, L4-25 pg/ μ L, L5-2.5 pg/ μ L, L6-250 fg/ μ L, L7-NTC; (B) tenfold dilutions of the PGEM[®]-T Easy Vector *pcb* M-100 bp Ladder, L1-30 ng/ μ L, L2-3.0 ng/ μ L, L3-300 pg/ μ L, L4-30 pg/ μ L, L5-3.0 pg/ μ L, L6-300 fg/ μ L, L7-30 fg/ μ L, L8-3 fg/ μ L, L9-300 ag/ μ L, L10-30 ag/ μ L, L11-3 ag/ μ L, L12-300 zg/ μ L, L13-NTC

Fig. 3 Multiplex PCR analysis with constant PGEM[®]-T Easy Vector *pcb* template of 3 ng/ μ L (76 bp) and varying concentration of PGEM[®]-T Easy Vector *mcvB* template (212 bp) M-100 bp ladder, L1-2.5 ng/ μ L, L2-25 pg/ μ L, L3-250 fg/ μ L, L4-2.5 fg/ μ L, L5-25 ag/ μ L, L6-25 zg/ μ L, L7-2.5 ag/ μ L, L8-NTC

Fig. 4 Multiplex PCR with DNA extracted from Spirulina Food Supplements marketed for humans with *pcb* (76 bp) and *mcyB* (212 bp) M-100 bp Ladder, L1 to L10—FS1 to FS10 (Spirulina Food Supplements for Human), L11-Positive control (TA-*pcb* + TA-*mcyB*), L12-NTC

Fig. 5 Multiplex PCR with DNA extracted from Spirulina Food Supplements for fish with *pcb* (76 bp) and *mcyB* (212 bp). M-100 bp ladder, L1 to L5—FS11 to FS15 (Spirulina fish food supplements), L6-Positive control (TA-*pcb* + TA-*mcyB*), L7 and L8—FS16 and FS17 (Spirulina fish food supplements), L9-NTC

concentration of 30 ag/ μ L whereas *mcyB* was detected up to 25 pg/ μ L. The difference seen here might be a result of various aspects including the specificity and sensitivity of the primers. However, when a multiplex assay was performed, the detection of *mcyB* in a standard concentration of *pcb* was limited to 250 fg/ μ L. This has a significant implication: a minute amount, up to femtogram can be simultaneously detected in a fast and efficient manner. It was also observed that the human dietary supplement was not contaminated. However, the food supplement available for fish was found to have the microcystin-producing cyanobacteria. This might be attributed to the stringent precautions taken while culturing spirulina for human supplements than the fish food supplements. The quality control required for the production of algal dietary supplements should be rigorous and vigilant to avoid microcystin contamination (Grobbelaar, 2003). Multiplex PCR detected two diverse algal genes in a single reaction. This method could be further exploited for identifying different several different genes related to microcystin-producing cyanobacteria simultaneously.

In conclusion, we have validated multiplex polymerase chain reaction to be a rapid and economical method which can be used for the simultaneous detection of *pcb* (phycocyanin specific to photosynthetic pigment) and *mcyB* (microcystin B) in the spirulina-based food supplements marketed in India. This can be used as a prospective method to develop an on-field diagnostic kit for a rapid and

efficient detection microcystin in spirulina food supplement industries. Further, this method can also be used to detect various other cyanotoxins contaminating the spirulina food supplements.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

References

- Akao Y, Ebihara T, Masuda H, Saeki Y, Akazawa T, Hazeki K, Hazeki O, Matsumoto M, Seya T. Enhancement of antitumor natural killer cell activation by orally administered spirulina extract in mice. *Cancer Sci.* 100: 1494–1501 (2009)
- Barón-Sola A, Ouahid Y, del Campo FF. Detection of potentially producing cylindrospermopsin and microcystin strains in mixed populations of cyanobacteria by simultaneous amplification of cylindrospermopsin and microcystin gene regions. *Ecotoxicol. Environ. Saf.* 75(1): 102–108 (2012)
- Belay A, Ota Y, Miyakawa K, Shimamatsu H. Current knowledge on potential health benefits of spirulina. *J. Appl. Phycol.* 5: 235–241 (1993)
- Bittencourt-Oliveira M. Detection of potential microcystin-producing cyanobacteria in brazilian reservoirs with a *mcyb* molecular marker. *Harmful Algae* 2(1): 51–60 (2003)
- Bláha L, Babica P, Maršálek B. Toxins produced in cyanobacterial water blooms—toxicity and risks. *Interdiscip. Toxicol.* 2: 36–41 (2009)
- Corbel S, Mougin C, Bouaicha N. Chemosphere cyanobacterial toxins: modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops. *Chemosphere* 96: 1–15 (2014)
- Douma M, Ouahid Y, Loudiki M, del Campo FF, Oudra B. The first detection of potentially toxic microcystis strains in two middle Atlas mountains natural lakes (Morocco). *Environ. Monit. Assess.* 189(1): 39 (2017)
- Gilroy DJ, Kauffman KW, Hall RA, Huang X, Chu FS. Assessing potential health risks from microcystin toxins in blue-green algae dietary supplements. *Environ. Health Perspect.* 108: 435–439 (2000)
- Grobbelaar JU. Quality control and assurance: crucial for the sustainability of the applied phycology industry. *J. Appl. Phycol.* 15: 209–215 (2003)
- Heussner AH, Mazija L, Fastner J, Dietrich DR. Toxin content and cytotoxicity of algal dietary supplements. *Toxicol. Appl. Pharmacol.* 265: 263–271 (2012)
- Jiang Y, Xie P, Chen J, Liang G. Detection of the hepatotoxic microcystins in 36 kinds of cyanobacteria spirulina food products in china. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 25: 885–894 (2008)
- Joventino IP, Alves HG, Neves LC, Pinheiro-Joventino F, Leal LK, Neves SA, Ferreira FV, Brito GA, Viana GB. The microalga *Spirulina platensis* presents anti-inflammatory action as well as hypoglycemic and hypolipidemic properties in diabetic rats. *J. Complement. Integr. Med.* 9: 1 (2012)

- Karkos PD, Leong SC, Karkos CD, Sivaji N, Assimakopoulos DA. Spirulina in clinical practice: evidence-based human applications. *Evid. Based Complement. Alternat. Med.* 2011; 4 (2011)
- Kulshreshtha A, Zacharia AJ, Jarouliya U, Bhadauriya P, Prasad GB, Bisen PS. Spirulina in health care management. *Curr. Pharm. Biotechnol.* 9: 400–405 (2010)
- Manali KM, Arunraj R, Kumar T, Ramya M. Detection of microcystin producing cyanobacteria in spirulina dietary supplements using multiplex HRM quantitative PCR. *J. Appl. Phycol.* 29: 1279–1286 (2017)
- Mathew B, Sankaranarayanan R, Nair PP, Varghese C, Somanathan T, Amma BP, Amma NS, Nair MK. Evaluation of chemoprevention of oral cancer with *Spirulina fusiformis*. *Nutr. Cancer.* 24: 197–202 (1995)
- Mazokopakis EE, Papadomanolaki MG, Foustieris AA, Kotsiris DA, Lampadakis IM, Ganotakis ES. The hepatoprotective and hypolipidemic effects of spirulina (*Arthrospira platensis*) supplementation in a Cretan population with non-alcoholic fatty liver disease: a prospective pilot study. *Ann. Gastroenterol.* 27: 387–394 (2014)
- Parikh P, Mani U, Iyer U. Role of spirulina in the control of glycemia and lipidemia in type 2 diabetes mellitus. *J. Med. Food.* 4: 193–199 (2001)
- Park JH, Lee SI, Kim IH. Effect of dietary *Spirulina* (*Arthrospira platensis*) on the growth performance, antioxidant enzyme activity, nutrient digestibility, cecal microflora, excreta noxious gas emission, and breast meat quality of broiler chickens. *Poult. Sci.* 97: 2451–2459 (2018)
- Parker CH, Stutts WL, Degrasse SL. Development and validation of a liquid chromatography-tandem mass spectrometry method for the quantitation of microcystins in blue-green algal dietary supplements. *J. Agric. Food Chem.* 63: 10303–10312 (2015)
- Pekar H, Westerberg E, Bruno O, Lääne A, Persson KM, Sundström FL, Thim A. Fast, rugged and sensitive ultra-high pressure liquid chromatography tandem mass spectrometry method for analysis of cyanotoxins in raw water and drinking water—first findings of anatoxins, cylindrospermopsins and microcystin variants in Swedish source waters and infiltration pond. *J. Chromatogr. A.* 1429: 265–276 (2016)
- Preece EP, Moore BC, Swanson ME, Hardy FJ. Identifying best methods for routine ELISA detection of microcystin in seafood. *Environ. Monit. Assess.* 187: 12 (2015)
- Ramakrishnan GS, Aliya FA, Ramya M. A rapid and efficient DNA extraction method suitable for marine macroalgae. *3 Biotech.* 7(6): 364 (2017)
- Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* 111: 1–61 (1979)
- Saker ML, Welker M, Vasconcelos VM. Multiplex PCR for the detection of toxigenic cyanobacteria in dietary supplements produced for human consumption. *Appl. Microbiol. Biotechnol.* 73: 1136–1142 (2007)
- Samuels R, Mani UV, Iyer UM, Nayak US. Hypocholesterolemic effect of spirulina in patients with hyperlipidemic nephrotic syndrome. *J. Med. Food.* 5: 91–96 (2002)
- Sangolkar LN, Maske SS, Muthal PL, Kashyap SM, Chakrabarti T. Isolation and characterization of microcystin producing microcystis from a central Indian water bloom. *Harmful Algae* 8: 674–684 (2009)
- Soares RM, Magalhaes VF, Azevedo S. Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in *Tilapia rendalli* (Cichlidae) under laboratory conditions. *Aquat. Toxicol.* 70(1): 1–10 (2004)
- Sorichetti RJ, McLaughlin JT, Creed IF, Trick CG. Suitability of a cytotoxicity assay for detection of potentially harmful compounds produced by freshwater bloom-forming algae. *Harmful Algae* 31: 177–187 (2014)
- Valerio E, Chambel L, Paulino S, Faria N, Pereira P, Tenreiro R. Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples. *Environ. Toxicol.* 25(3): 251–260 (2010)
- Vallone PM, Butler JM. Autodimer: a screening tool for primer-dimer and hairpin structures. *BioTechniques.* 37: 226–231 (2004)
- Vichi S, Lavorini P, Funari E, Scardala S, Testai E. Contamination by microcystis and microcystins of blue-green algae food supplements (BGAS) on the Italian market and possible risk for the exposed population. *Food Chem. Toxicol.* 50: 4493–4499 (2012)
- Yakes BJ, Handy SM, Kanyuck KM, DeGrasse SL. Improved screening of microcystin genes and toxins in blue-green algal dietary supplements with PCR and a surface plasmon resonance biosensor. *Harmful Algae* 47: 9–16 (2015)
- Yu P, Li JR, Cen PL. Cloning and sequencing of the phycocyanin gene from *Spirulina maxima* and its evolutionary analysis. *J. Appl. Phycol.* 14: 307–310 (2002)