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Detection of microcystin synthetase genes in health food supplements containing the freshwater cyanobacterium *Aphanizomenon flos-aquae*

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Abstract

In this study we investigated the presence of toxin-producing cyanobacterial contaminants in food supplements manufactured from blooms of the non-toxic freshwater cyanobacterium *Aphanizomenon flos-aquae*. Previous reports investigating the contamination of health food supplements with toxin-producing cyanobacteria have used chemical and or biochemical methods such as HPLC, ELISA and protein phosphatase assays. Whilst these studies have drawn attention to the presence of hepatotoxic microcystins in some commercially available food supplements, the methods used do not provide any information on the source of the contaminant. Such information would be useful for the quality control of food supplements produced for human consumption. In this study we applied a molecular technique, involving the amplification of the 16s rRNA gene, the phycocyanin operon, and two genes of the microcystin synthetase gene cluster to show that all 12 food supplement samples, sourced from various internet distributors and containing non-toxic *A. flos-aquae*, also contained toxigenic cyanobacteria. Sequencing of the microcystin synthetase genes detected in all of the food supplements showed that *M. aeruginosa* was the organism responsible for the production of microcystins in the samples. The presence of microcystins in the food supplements was confirmed by ELISA, with concentrations within the range of 0.1–4.72 $\mu g\ g^{-1}$ (microcystin-LR equivalents). Given that the molecular methods applied here are highly sensitive, and show good agreement with the results obtained from ELISA, we believe that they could potentially be used as a quality control technique for food products that contain cyanobacteria.
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Keywords: Aphanizomenon; Microcystin; *Microcystis*; Health food supplements

1. Introduction

The presence of cyanobacteria in freshwaters is of increasing concern worldwide due to the ability of many species to produce toxins that are harmful to humans and other animals. Of over 1500 known species of cyanobacteria, less than 30 have been found to produce toxic compounds. Among the potentially toxic species, several chemically and toxicologically diverse compounds have been isolated, purified and characterized (Sivonen and Jones, 1999).

Microcystis aeruginosa is one of the most common freshwater bloom-forming species of cyanobacteria. This

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organism produces a group of compounds known as cyclic peptides (also known as microcystins), which have molecular weights ranging from 800 to 1100. Other genera of cyanobacteria including *Anabaena* and *Planktothrix* are also known to produce these compounds. To date, more than 60 microcystin variants have been chemically identified, with the major differences between variants related to either the substitution, epimerization or the demethylation of amino acids (Burns et al., 2004). These compounds have LD_{50} values (mouse i.p.) in the range of 50–500 mg kg^{-1}. *M. aeruginosa* has been implicated in the death of wild and domestic animals (Ressom et al., 1994) and the death of humans (Jochimsen et al., 1998; Kuiper-Goodman et al., 1999). Furthermore, these toxins have been shown to be tumor promoters in mice and have been linked to liver cancer in humans (Nishiwaki-Matsushima et al., 1992). In recognition of their toxicity, the World Health Organization has implemented a maximum allowable concentration of microcystin in drinking water of 1 μg L^{-1} (WHO, 1998).

Although exposure to microcystins through drinking water and recreational activities is widely recognized, there have been few studies investigating the potential for exposure to these toxins through the consumption of contaminated food. One possible route involves the consumption of food supplements containing cyanobacteria. In the past two decades, there has been increasing interest in the potential health benefits that can be gained by the consumption of *Aphanizomenon flos-aquae* (Ralfs ex Born. & Flah). Although some strains of this species have been reported to produce PSP (Ferreira et al., 2001; Pereira et al., 2000) toxins and anatoxin-a (Rapala et al., 1993), more recent studies have shown that in many cases, toxin-producing *Aphanizomenon* species are misidentified as toxic strains of *A. flos-aquae* when they in fact belong to another closely related species, *A. issatschenkoi* (Li et al., 2003). Among the reported health benefits of consuming *A. flos-aquae* are; weight loss, increased energy, mental clarity, 'HIV-improvement', elevated mood and general 'detoxification' (http://www.bluegreenfoods.com/). Whilst many of these claims are unfounded based on scientific evidence, studies have shown that *A. flos-aquae* produces compounds with anti-bacterial (Østensvik et al., 1998) and anti-mutagenic properties (Lahitova et al., 1994) and is a rich source of polyunsaturated fatty acids (Kushak et al., 2000). Most of the *A. flos-aquae* that is available commercially is harvested from Lake Klamath, Oregon, USA, where this cyanobacterium grows to high cell densities under natural conditions. In contrast to other cyanobacteria that are used for human consumption such as *Spirulina* (which is usually grown in artificial ponds), *A. flos-aquae* is collected directly from the natural environment. The potential therefore exists for the inclusion of other organisms, including toxin-producing cyanobacteria, in the health food supplements. The process of harvesting and incorporation of *A. flos-aquae* into tablets and capsules for distribution to consumers has been described in detail by Carmichael et al. (2000).

In 1996, a bloom of *M. aeruginosa* in Lake Klamath alerted health care workers to the contamination of *A. flos-aquae*-containing with a toxin-producing cyanobacterium. A subsequent survey of commercially available food supplements containing *A. flos-aquae* showed that of 87 samples collected from various sources, 85 contained microcystins at concentrations up to 16.4 μg g^{-1} (Gilroy et al., 2000). Sixty-three of the 87 samples (72%) contained concentrations greater than 1 μg g^{-1}. As a result of that study, and based on the results of a risk assessment analysis, the Oregon State Health Department has enforced a maximum allowable concentration of microcystin-LR in health food supplements of 1 μg g^{-1}. Another study, conducted by Health Canada, further highlighted the prevalence of microcystins in *A. flos-aquae*-containing products distributed throughout Canada and North America (Lawrence et al., 2001).

In these studies, toxins were characterized using ELISA and HPLC techniques. Whilst these analyses are useful for identifying potentially dangerous concentrations of cyanobacterial toxins such as microcystins, they provide no information regarding the source of the contaminant. In this study we applied molecular techniques in an attempt to identify the contaminating organisms present in health food supplements. DNA was extracted from the health food supplements, and polymerase chain reaction (PCR) was then used to amplify two micrcystin synthetase genes. One of the primer sets used in this study was designed to target the aminotransferase gene of microcystin and nodularin synthetases from all hepatotoxic cyanobacteria (Jungblut and Neilan, unpublished). The second set was based on a sequence of the *mcyA* module of microcystin synthetase from *M. aeruginosa* PCC7806. Sequences obtained from the purified PCR reaction products were then used to identify the contaminating organism, by comparison with other published sequences, using the BLAST search on GenBank. The concentration of microcystins in the food supplements was then measured using ELISA. The molecular techniques described here were found to be useful for the identification of contaminating organisms present in the food supplements. Similar techniques have been used for the identification of toxin-producing cyanobacteria strains in mixed-species phytoplankton samples (Pan et al., 2002; Kurmayer et al., 2003; Burns et al., 2004), and have been used to provide an 'early warning system' for cyanobacterial bloom populations in drinking water supplies (Baker et al., 2001). This study is the first report of their application to food products used for human consumption.

2. Methods

2.1. Source of sample

Samples of *A. flos-aquae*-containing food supplements were obtained from several sources. Samples 1–7 (Table 1)

Table 1
Concentrations of microcystins detected in food supplements sold for human dietary use

No.	Sample	ELISA (present study) μg g^{-1}	Microcystins HPLCa, μg g^{-1}	ELISA, μg g^{-1a}
1	OR-01a	1.03	LR+LA (1.4)	2.1–2.2
2	OR-04a	1.03	LR+LA (1.9)	2.9
3	OR-06a	0.10	LR+LA (5.6)	0.4
4	OR-14a	0.76	LR+LA (1.4)	2.2
5	OR-22a	4.73	LR+LA (5.7)	5.8–6.2
6	OR-28a	0.46	LR+LA (1.8)	2.3
7	WR-21a	3.49	LR+LA (2.8–4.2)	6.1
8	KB-01	0.02	–	–
9	DT-01	0.22	–	–
10	SN-01	0.10	–	–
11	SN-02	0.38	–	–
12	KS-01	0.14	–	–

[a] Data from Lawrence et al. (2001).

were originally included in a survey of microcystin contamination of health food products throughout the USA and Canada, carried out by the Food Research Division of Health Canada (Lawrence et al., 2001). Since their collection, samples were maintained at low temperatures (−20 °C). Samples 8–12 were obtained from internet distributors in the USA and Europe during May 2004. All *A. flos-aquae*-containing health food supplements that are available commercially are harvested, processed and distributed by a small number of companies that operate at Upper Klamath Lake, Klamath Falls, Oregon, USA. This lake is characterized by seasonal blooms of *A. flos-aquae*, which dominate the phytoplankton from June to October. Other cyanobacteria including *Anabaena flos-aquae*, *Coelosphaerium*, *Microcystis aeruginosa* as well as species belonging to the Order Oscillatoriales are also known to occur (Carmichael et al., 2000).

2.2. DNA extraction

The samples of *A. flos-aquae*, obtained from various sources in the form of powders, capsules and tablets, were homogenized with a mortar and pestle. Approximately, 10 mg of each sample was used for extraction of DNA. Samples were combined with 500 μl of XS buffer (1% potassium–methylxanthogenate; 800 mM ammonium acetate; 20 mM EDTA; 1% SDS; 100 mM Tris–HCl, pH 7.4) and incubated at 65 °C for 2 h (vortexed after 1 h). The solutions were then placed on ice for 10 min, and centrifuged at 12,000*g* for 10 min. The supernatant was collected and the DNA precipitated by adding 1 volume of isopropanol and 1/10 volume of 4 M KOAc for 15 min at 4 °C followed by a centrifugation step at 12,000*g* for 20 min. DNA was washed with 70% ethanol, centrifuged at 12,000*g* for 15 min, dried, and resuspended in 50 μl of milli-Q water.

2.3. Polymerase chain reaction (PCR)

16S rDNA amplification was performed in 20 μl reactions using primers 27F and 809R (Table 2), with an initial denaturation step at 92 °C for 2 min followed by 35 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 1 min and a final extension step at 72 °C for 5 min (Jungblut et al., 2005).

The phycocyanin gene was amplified using PCβF and PCαR primers (Table 2) with 35 cycles of 92 °C for 20 s, 52 °C for 30 s and 72 °C for 1 min (Neilan et al., 1995).

Hepatotoxin (Hep)-PCR reactions were performed using primers HepF and HepR (Table 2). The initial denaturation step at 92 °C for 2 min was followed by 35 cycles of denaturation at 92 °C for 20 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min (Jungblut and Neilan, unpublished). All Hep-PCR reactions were performed using 0.2 units of *Taq* polymerase (Fischer Biotech, Perth, Australia) in a 20 μl reaction volume containing 2.5 mM $MgCl_2$, 1x *Taq*-

Table 2
Oligonucleotide primers used in this study for the amplification of cyanobacterial 16S rDNA (27F, 809R), phycocyanin operon (PCβF/PCαR) and cyanobacterial hepatotoxin synthetase genes (QmetF/R, HepF/R)

Designation	Target gene	Sequence	Annealing temp. (°C)	Reference
27F, 809R	16S rDNA	5′-AGAGTTTGATCCTGGCTCAG-3′, 5′-GCTTCGGCACGGCTCGGGTCGATA-3′	52	Salmon and Neilan (unpublished)
PCβF, PCαR	Phycocyanin	5′-GGCTGCTTGTTTACGCGACA-3′, 5′-CCAGTACCACCAGCAACTAA-3′	50	Neilan et al. (1995)
QmetF, QmetR	*mcy*A	5′-TTATTCCAAGTTGCTCCCCA-3′, 5′-GGAAATACTGCACAACCGAG-3′	55	Gehringer and Neilan (unpublished)
HepF, HepR	AMT[a], *mcy*E	5′ TTTGGGGTTAACTTTTTTGGCCATAGTC ′3, 5′AATTCTTGAGGCTGTAAATCGGGTTT ′3	52	Jungblut and Neilan (unpublished)

[a] Aminotransferase.

Polymerase buffer (Fischer Biotech), 0.2 mM of dNTPs (Fischer Biotech, Perth, Australia) and 0.5 pM of forward and reverse primers.

The Qmet-PCR reactions were performed as described for the 16S rDNA amplification. The protocol was carried out with an initial 2 min denaturation step at 95 °C followed by 30 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C for 50 s. This was followed by a final annealing step at 72 °C for 7 min. For all PCR reactions ca. 1 ng of chromosomal DNA was used. All PCR products were analyzed on 2% agarose gels with 1× TAE buffer and stained with ethidium bromide (1 μg ml^{-1}) for 10 min. A Gel-DOC Bio-RAD System with Quantity One 4.1R software (BIO-RAD, USA) was used for photographic documentation.

2.4. Sequencing and sequence analysis

Sequencing of the PCR products was carried out using primers HepF, 809R, QmetR and PCβF. Automated sequencing was performed using the PRISM Big Dye cycle sequencing system and ABI 3730 Capillary Applied Biosystem (Foster City, CA). Sequence data was analyzed using the Applied Biosystem Auto-Assembler computer program. The identities of the sequenced amplicons were determined using a BLAST search on GenBank. All sequences were checked manually for quality of the data.

2.5. Analysis of microcystins using ELISA

The presence of microcystins in health foods obtained from various sources (Table 1) was determined using the Envirogard® Microcystin Plate Kit (Strategic Diagnostic, Inc., Newark, USA). This assay uses antibodies against microcystin-LR, the most common microcystin (Chu et al., 1990). Briefly, 20 mg of material was extracted in 5 ml of milli-Q ultrapure water with sonication on ice. The solution was then centrifuged at 10,000*g* for 5 min and the supernatant filtered (0.2 μm) and applied to the assay kit. The absorbance was measured using a Well-Scan ELISA plate reader (Denley Corp., UK) at a wavelength of 450 nm.

3. Results

The cyanobacterial specific 16S rDNA PCR resulted in a product for all of the samples, indicating that all of the food supplements investigated in this study contained cyanobacterial DNA (Fig. 1). The 16S rDNA PCR products were purified and sequenced. A 782-bp partial sequence was generated between positions 27 and 809 for all of the samples, corresponding to the *E. coli* 16S rDNA sequence numbering. The assembled sequences were analyzed using the BLAST program in GenBank (Altschul et al., 1990) for alignment to database sequences and to confirm that the origin of the generated sequence was cyanobacterial.

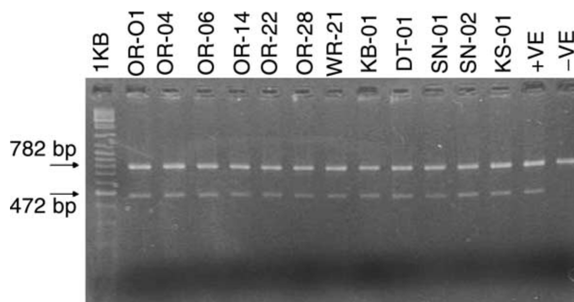

Fig. 1. The cyanobacterial specific 16S rDNA PCR amplified a 782-bp fragment from all of the health supplement samples analyzed. The Hep primer set amplified a ca. 472-bp product from all food supplements containing cyclic peptide of toxigenic cyanobacteria. *Microcystis aeruginosa* PCC7806 was used as the positive control and a non-toxic *Anabaena* strain (NIES 19) was used as a negative control. A 1 KB molecular ladder is also shown.

The 16S rDNA gene sequences were then compared to each other and to other sequences available in GenBank.

The 16S rDNA sequences obtained from the food supplements showed more than 97% similarity to *Aphanizomenon* sp. (Table 3). All samples except OR-14 showed the same high level of similarity to sequences of *A. flos-aquae* PMC9707 (accession no: AFL293130), *A. gracile* PMC9402 (AGR2931270), *A. flos-aquae* PMC9401 (AFL293126), *A. flos-aquae* var. Klebahnii 218 (AFL293123), *A flos-aquae* var. Klebahnii 83 (AFL293122) and *Aphanizomenon* sp. 326 (AFL293121). In addition, samples OR-28, DT-01 and SN-01 showed high sequence similarity to *A. flos-aquae* str. 'Aph Zayi' (AY196082.1) and *A. flos-aquae* str. 'Aph Ku' (AY196085.1). Sample OR-14 also displayed a high level of similarity to *Aphanizomenon* sp., although the highest similarity was detected with *A. flos-aquae* str. 'Aph Inba' (AY196083.1). Despite the strong similarity between the samples in terms of their 16S rDNA sequences, a manual check showed that all of the sequences were contaminated with other 16s rDNA PCR products.

To further characterize the species present in the cyanobacterial-containing food supplements, the phycocyanin operon was amplified and sequenced, using the primers shown in Table 2. The products obtained from this PCR reaction were analyzed on a 2% gel, then purified and sequenced and found to consist of a 650-bp fragment from the phycocyanin operon. Analysis of the sequence data using GenBank showed highest similarity (94%) to the phycocyanin operon of *Aphanizomenon* sp. and *Anabaena* sp. As was the case with the cyanobacterial specific 16S DNA sequences, all of the phycocyanin gene sequences were found to be contaminated with another phycocyanin gene product.

The Hep PCR reactions resulted in amplification of a 472-bp fragment for all twelve samples analyzed in this study. The PCR-products were sequenced in order to

Table 3
Highest similarities of the sequence analysis in GenBank by BLASTN or BLASTX of the investigated food supplements using cyanobacterial specific 16S rDNA primers, Hep- and Qmet primers. NT, not tested

Sample	%	16S rDNA Highest match NCBI	%	Hep highest match NCBI	%	Qmet highest match NCBI
OR-01	98	*A. flos-aquae* PMC9707 (AFL293130), *A. gracile* PMC9402 (AGR2931270), *A. flos-aquae* PMC9401 (AFL293126), *A flos-aquae* var. Klebahnii 218 (AFL293123), *A flos-aquae* var. Klebahnii 83 (AFL293122), *A* sp. 326 (AFL293121)	96	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)		NT
OR-04	97	See sample OR-01	97	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)		NT
OR-06	98	See sample OR-01	96	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)		NT
OR-14	98	*A. flos-aquae* str. 'Aph Inba' (AY196083.1)	97	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)		NT
OR-22	97	See sample OR-01	96	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)	100	*Mcy*A *M. aeruginosa* PCC7806 (AAF17323.1)
OR-28	98	*A. flos-aquae* PMC9707 (AFL293130), *A. gracile* PMC9402 (AGR2931270), *A. flos-aquae* PMC9401 (AFL293126), *A flos-aquae* var. Klebahnii 218 (AFL293123), *A flos-aquae* var. Klebahnii 83 (AFL293122), *A* sp. 326 (AFL293121), *A. flos-aquae* str. 'Aph Zayi' (AY196082.1), *A. flos-aquae* str. 'Aph Ku' (AY196085.1)	97	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)		NT
WR-21	98	See sample OR-01	97	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)	100	*Mcy*A *M. aeruginosa* PCC7806 (AAF17323.1)
KB-01	98	See sample OR-01	97	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)		NT
DT-01	97	See sample OR-28	92	McyE, *M. aeruginosa* K-139 (BAB12211.1)		NT
SN-01	96	See sample OR-28	97	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)	97	*Mcy*A *M. aeruginosa* PCC7806 (AAF17323.1)
SN-02	98	See sample OR-01	96	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)		NT
KS-01	97	See sample1 OR-01	97	McyE, *M. aeruginosa* PCC7806 (AAF00958.1), McyE, *M. aeruginosa* K-139 (BAB12211.1)	100	*Mcy*A *M. aeruginosa* PCC7806 (AAF17323.1)

confirm the identity of the amplified fragments. Sequence analysis of the amplicons showed that all conformed to the expected gene fragment of the aminotransferase (AMT) found in *mcy*E from the cyanobacterial multi-enzyme complex of microcystin synthetase. The DNA sequences were then compared with other published sequence data on GenBank and found to show highest similarity (at least 97%) to *mcy*E of the microcystin synthetase gene from *M. aeruginosa* PCC7806 (AAF00958.1) and *mcy*E from *M. aeruginosa* K-139 (BAB12211.1) (Table 3). The samples were clearly different to the published sequences of the microcystin synthetase gene of other hepatotoxic species such as *Planktothrix* sp. CYA 126/8 and *Anabaena* sp. strain 90. Both of these strains yielded similarities of less than 88%. As an additional confirmation, PCR was carried out on all samples using the Qmet primer set (Table 2). These primers were designed to target the *mcy*A module of the microcystin synthetase of *M. aeruginosa*. For all samples, a 200-bp PCR-product was amplified and representative PCR-amplicons from samples OR-22, WR-21, SN-01 and KS-01 were sequenced in order to confirm the source of the product. In all cases, the PCR products showed greatest similarity (97 to 100% in the GenBank BLASTX search) to the *mcy*A region of the non-ribosomal peptide synthetase of microcystin synthetase from *M. aeruginosa* PCC7806 (AAF17323.1; Table 3).

The presence of microcystins in all of the food supplements was confirmed by ELISA (Table 1), however, there was considerable variation in the concentrations detected within the samples, which ranged from 0.10 to 4.72 $\mu g\ g^{-1}$. In general, the microcystin content of the samples included in the Lawrence et al. (2001) study yielded higher concentrations than those that were purchased from internet distributors in May 2004 (Table 1). Furthermore, the samples that were included in the Lawrence et al. (2001) study and re-analyzed in this study yielded microcystin concentrations that were 50–75% lower than the values that were originally reported.

4. Discussion

The presence of hepatotoxic compounds such as microcystins, in products marketed for their health properties is a cause for concern. While other reports have highlighted the presence of microcystins in these supplements (Lawrence et al., 2001; Gilroy et al., 2000), this is the first report showing the identity of the contaminating organism. In this study we were unable to identify the contaminating species using conventional techniques such as microscopy, or characterization of the 16S rDNA or phycocyanin operon, due to the presence of more than one species or DNA type in the DNA extracts which were used as the template for PCR. However, analysis of the PCR products obtained using primers that were specifically designed to target the genes responsible for microcystin production suggested a clear origin of the toxigenic contaminants. The nucleotide sequences obtained for the Hep-PCR products all twelve samples showed a very high similarity (>96%) to fragments of the microcystin synthetase gene reported for *Microcystis* species. Much lower similarities (<88%) were obtained when the sequences were compared with the microcystin synthetases genes reported for other toxigenic genera including *Planktothrix* and *Anabaena*. This result indicates that *Microcystis* sp. was the source of contamination in all of the food supplement samples. This result was confirmed by sequencing of the PCR-products obtained using the Qmet primers, which provided nucleotide sequence similarity in the range of 97–100%.

The ability to identify toxigenic species such as *M. aeruginosa*, based on sequence analysis of the microcystin synthetase genes has been discussed in other studies and suggests the congruent evolution of cyanobacterial speciation and microcystin synthetases (Rantala et al., 2004; Jungblut and Neilan, unpublished). Such an evolution indicates that the microcystin synthetase genes carried by microcystin producing species demonstrate a close phylogenetic relationship between microcystin producing cyanobacteria. *Microcystis* is one of the many genera known to produce microcystins and it is likely that the techniques used in this study are equally as useful for detecting the presence of other known microcystin-producing genera including *Anabaena*, *Planktothrix*, *Nostoc*, *Hapalosiphon* and *Anabaenopsis*. Studies of the phytoplankton at Klamath Lake have shown that several other genera of toxin producing cyanobacteria are present in this water body. However, whilst the contaminated sequences obtained for the 16s rDNA and phycocyanin operon indicated the presence of more than one type of cyanobacterial DNA, there was no evidence of contamination of either the Hep or Qmet sequecnces. This result indicates that among the samples tested in this study, *M. aeruginosa* was the only cyanobacterium potentially capable of producing cyclic peptides. The possible presence of other cyanotoxins cannot be discounted without further detailed analyses. To date, it remains unclear whether strains of *Aphanizomenon flos-aquae* are capable of producing compounds that can effect human health (Li et al., 2003) however, there are no published reports of microcystin production by strains of *A. flos-aquae* and it should also be noted that neurotoxins have never been detected in the phytoplankton from Klamath Lake. Another species belonging to the Genus *Aphanizomenon*, *A. ovalisporum* has been shown to produce a potent hepatotoxin known as cylindrospermopsin (Banker et al., 1997).

Based on previous studies investigating the potential health related effects of microcystins in food supplements (Gilroy et al., 2000), a value of 1 $\mu g\ g^{-1}$ was considered to be an appropriate safety level for food products used for human consumption. It has also been reported that this level

is highly conservative and that based on the results of a mouse assay, could justifiably be increased to 10 μg g^{-1} (Schaeffer et al., 1999). The results of this study have confirmed that despite the high concentrations obtained for some of the samples collected in the period from 2000 to 2001 (up to 4.72 μg g^{-1}), the samples collected in May 2004 were within an acceptable range from 0.02 to 0.38 μg g^{-1}, and demonstrate the vigilance of the health food companies for controlling the concentrations within their products.

The re-analysis of samples collected in the study conducted by Health Canada (Lawrence et al., 2001) resulted in concentrations much lower than those originally reported (Table 1). This might have been caused by the different methodologies used in the two studies. It is well known that different ELISA techniques can potentially provide different results (Metcalf et al., 2000; Metcalf and Codd, 2003). While the earlier study used the Envirologix kit, here we employed the Envirogard kit. It is also possible that microcystins may have been degraded in the samples over the 4-year period since their original collection. While some of the products, according to the label, consisted of 100% freeze–dried cyanobacteria others were diluted with various binding agents and preservatives such as gelatin, modified cellulose gum, magnesium stearate, stearic acid, silicon dioxide, fluctoo-ligosaccaride, soy lecithin, sesame meal and *Acacia*. Some were also supplemented with vitamins such as Vitamin C and niacin. While the addition of these compounds may have affected the concentration of microcystins (expressed on a gravimetric basis), they did not appear to influence the efficacy of DNA extraction or inhibit the various PCR reactions employed in this study.

The techniques used in this study were found to be highly sensitive for detecting the presence of microcystin-producing contaminating microorganisms in health food products. Increasing interest in the use of molecular techniques have lead to the identification of the genes responsible for the production of some other cyanobacterial toxins such as cylindrospermopsin (Schembri et al., 2001) and nodularin (Moffit and Neilan, 2001). If used in combination with other chemical and biochemical techniques such as HPLC, ELISA and the protein phosphatase assay, the molecular techniques could be useful for the rapid, sensitive and routine monitoring and quality control of products containing cyanobacteria that are used for human consumption.

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