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# *Cyanobacterial Toxins in Drinking Water*

Document for Public Consultation

Prepared by the Federal-Provincial-Territorial Committee on Drinking Water

Review period ends
April 15, 2016

Canada

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January 2016

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## Purpose of consultation

The Federal-Provincial-Territorial Committee on Drinking Water (CDW) has assessed the available information on cyanobacterial toxins with the intent of establishing a drinking water guideline. The purpose of this consultation is to solicit comments on the proposed guideline, on the approach used for its development and on the potential economic costs of implementing it, as well as to determine the availability of additional exposure data.

The existing guideline on cyanobacterial toxins, last updated in 2002, based its maximum acceptable concentration (MAC) of 0.0015 mg/L (1.5 µg/L) for microcystin-LR on liver changes in mice, taking into consideration limitations in analytical methodology and treatment technology. This new document takes into consideration newer scientific studies and finds that weight of evidence continues to identify liver changes as the key end-point for the most common and researched cyanotoxin, microcystin-LR. It provides updated data and information related to exposure to cyanobacterial toxins in Canada, to analytical methods and to treatment considerations at the municipal and residential scales. Based on these considerations, the document proposes to reaffirm a MAC of 0.0015 mg/L (1.5 µg/L) for total microcystins in drinking water.

The CDW has requested that this document be made available to the public and open for comment. Comments are appreciated, with accompanying rationale, where required. Comments can be sent to the CDW Secretariat via email at water_eau@hc-sc.gc.ca. If this is not feasible, comments may be sent by mail to the CDW Secretariat, Water and Air Quality Bureau, Health Canada, 3rd Floor, 269 Laurier Avenue West, A.L. 4903D, Ottawa, Ontario K1A 0K9. All comments must be received before April 15, 2016.

Comments received as part of this consultation will be shared with the appropriate CDW member, along with the name and affiliation of their author. Authors who do not want their name and affiliation shared with their CDW member should provide a statement to this effect along with their comments.

It should be noted that this Guideline Technical Document on cyanobacterial toxins in drinking water will be revised following evaluation of comments received, and a drinking water guideline will be established, if required. This document should be considered as a draft for comment only.

January 2016

# Cyanobacterial Toxins

## Part I. Overview and Application

## 1.0 Proposed guideline

*A seasonal maximum acceptable concentration of 0.0015 mg/L (1.5 µg/L) is proposed for total microcystins in drinking water. This guideline is considered to be protective of the general population, including young children. Because of the increased exposure of infants relative to body weight, as a precautionary approach during a cyanobacterial bloom, or when microcystins are detected in finished water, drinking water authorities should consider informing the public in the affected area that an alternate suitable source of drinking water (such as bottled water) should be used to reconstitute infant formula.*

## 2.0 Executive summary

Under the right environmental conditions, microcystins and other cyanobacterial toxins are naturally formed in water in the environment. They are produced and stored in the cells of cyanobacteria, and released when the cell rupture or die. Most scientific studies on cyanobacterial toxins focus on microcystins, which are generally regarded as the most important of the freshwater cyanotoxins.

This guideline technical document reviews and assesses the identified health risks associated with cyanobacterial toxins in drinking water. It assesses new studies and approaches and takes into consideration limitations in analytical methods and treatment technologies. Based on this review, the proposed guideline for total microcystins in drinking water is a maximum acceptable concentration of 0.0015 mg/L (1.5 µg/L). Guidelines are not proposed for other cyanotoxins, including anatoxin-a and cylindrospermopsin, as health and other data on these toxins are limited.

During its May 2015 meeting, the Federal-Provincial-Territorial Committee on Drinking Water reviewed the guideline technical document for cyanobacterial toxins and gave approval for this document to undergo public consultations.

### 2.1 Health effects

Studies on the health effects of cyanobacterial toxins focus primarily on microcystin-LR. A short-term MAC is proposed for total microcystins as studies have found liver toxicity in rats following short-term exposure to microcystin-LR. The proposed MAC is based on health effects in adults and considered protective of all Canadians.

Currently available science suggests that adults are more sensitive to the effects of microcystins than younger age groups. However, infants can ingest a significantly larger volume of water per body weight, up to 5 times more drinking water per kilogram of body weight than an adult. Consequently, in areas where microcystins could be present in drinking water (i.e., current or recent presence of a bloom potentially affecting a drinking water intake), an alternate suitable source of drinking water (such as bottled water) should be used to reconstitute infant formula.

### 2.2 Exposure

Exposure to microcystins is primarily through ingestion of drinking water and can also occur during recreational water activities such as swimming. Some exposure may occur through foods affected by contaminated water (primarily fish and shellfish, some crops) and natural health products that have been contaminated (including algal supplements).

Cyanobacterial blooms have been observed in drinking water sources across Canada, except in Prince Edward Island, where all drinking water originates from groundwater sources, and in the territories. In the rest of Canada, exposure to microcystins generally occurs over short periods (less than 30 days), as the conditions required for bloom formation are seasonal, requiring warm temperatures.

### 2.3 Analysis and treatment

There are many analytical methods capable of measuring total microcystins in drinking water to levels well below the proposed MAC. Analysis should be conducted for all of the measurable microcystin variants that are both dissolved in the water and bound within the cyanobacterial cells using a laboratory validated method. Similarly, water should be treated to remove both intracellular (cell-bound) and dissolved microcystins. The most effective way to remove microcystins is to remove the cyanobacterial cells intact, without damaging them, to prevent the release of additional microcystins into the water. Treatment for intracellular microcystin removal may consist of conventional or membrane filtration, while removal of dissolved microcystins would require activated carbon, oxidation, biodegradation or small pore membrane processes. Well operated and optimized drinking water treatment plants can remove total microcystins to concentrations well below the proposed MAC.

Options available for individual households obtaining their drinking water from a surface water source affected by a cyanobacterial bloom would include switching to an alternative water supply, changing the location of the water intake pipe and installing a drinking water treatment system. However, the treatment of water supplies for the removal of cyanobacteria and microcystins at the residential scale is complex, and there are no drinking water treatment systems available that are certified for the removal of microcystins.

## 3.0 Application of the guideline

*Note: Specific guidance related to the implementation of drinking water guidelines should be obtained from the appropriate drinking water authority in the affected jurisdiction*

### 3.1 Municipal scale drinking water supply systems

Responsible authorities and water supply managers should develop an appropriate monitoring and response strategy for their systems. Appendix B provides information on the steps to take in preparation for a possible cyanobacterial bloom, as well as a flowchart for utilities and decision-makers.

#### *3.1.1 Monitoring drinking water sources*

Drinking water supplies known or suspected to be susceptible to blooms should be monitored routinely for the presence of cyanobacteria. The recommended approach is visual monitoring of source waters for evidence of increasing cyanobacterial cell density or bloom development, followed by increased vigilance and additional steps if such evidence is detected.

Visual monitoring of source water is a straightforward approach that provides useful information about the relative hazard posed by the development of cyanobacterial blooms. Water bodies that have exhibited blooms should be visually monitored during the peak season (usually late May to early October, but this may deviate depending on local conditions). Key locations for inspection include near intakes and along banks and shorelines. Early visual signs of a bloom may include water that appears unusually cloudy or the presence of what appears to be fine grass clippings or small clumps that may be well spaced. Colours can range from grey or tan to olive to blue-green to bright blue to red ("wine" coloured). As the bloom develops, the water may take on a "pea soup" or "spilled paint" appearance. A bloom may form a scum at the water surface (often overnight, if conditions are calm), which may then be pushed to the shoreline by the water current or wind. When planning visual monitoring, it is important to consider that cyanobacteria may be mixed back into the water column during the day.

Monitoring cell counts or biovolume estimates in source waters, when integrated with toxin information, can contribute to a better understanding of treatment requirements, potential optimization needs for plant operations and removal efficiencies. There is no direct relationship between the number of cells found in the source water and the levels of cyanotoxins in the water. A cyanobacterial bloom can consist of a mix of species, each of which may or may not produce toxins. Cell numbers should only be considered indicative of the possibility of bloom development, and by extension, the potential for the presence of toxins. Any decisions concerning triggers for possible action based on cell counts or biovolume values are made at the local or provincial/territorial levels, taking into account site-specific knowledge. For health risk assessment it is necessary to determine the levels of total microcystins (intracellular and extracellular) present.

As part of a total management plan or water safety plan, it is beneficial for drinking water authorities to be aware of the types of cyanobacteria that may affect their source waters. For example, some types of cyanobacteria may develop at depths below the surface or at the bottom of the water where light can still penetrate, and would not show strong signs of a bloom. The visual detection of mats of cyanobacterial material being washed up on shore or of evidence of fouling of intakes may provide an indication of their presence. Other potential indicators that can help detect the growth of a cyanobacterial bloom include changes in TOC, turbidity, and taste and odour of the water. Additional tools available to aid in the detection of blooms include in-place or portable pigment analyzers for phycocyanin or chlorophyll-a and remote satellite sensing (based on changes in the optical properties of the water column) for those on large water sources.

Samples should be collected for analysis if a bloom is detected or suspected by visual inspection, and may also be collected as part of routine surveillance. It is recommended that authorities analyze samples during, and after the collapse of, the bloom to better characterize the extent of the risk posed by the cyanobacteria (see Appendix B).

In the source water, if a bloom develops, it should be sampled to establish the extent of its spread and the variability in the population. Sampling from a water body should be done as close to the inlet/shore and/or the bloom formation as possible. Source samples can be collected and processed as individual grab samples from different sites and depths to assess the extent of the bloom (depth, width), or a composite sample integrated across several depth zones can be analyzed to provide an estimate of the average toxicity across the affected water.

Raw water samples should be collected at the intake to the treatment plant prior to any pretreatment, including prechlorination or filtration to determine the concentration of microcystins entering the treatment plant.

Treated samples should be taken at the treated water tap of the treatment plant after the final treatment step. Samples of raw and treated supplies (if applicable) can be collected at the same time in order to be efficient. If treatment performance is being assessed, then samples of raw and treated water need to be staggered to account for the residence time of water in the treatment plant. Sampling within the treatment plant after each treatment step (e.g., clarification, filtration) may also be needed to assess treatment plant performance.

In order to determine an appropriate sampling frequency, the following factors should be considered:

- past frequency of occurrence of blooms and cyanotoxins in the water source or nearby water bodies;
- characteristics of the water body (size, depth, current/flow, thermal stratification) and changes in these characteristics (e.g., prolonged periods of stagnation or decreased water levels);
- source water quality (nutrient levels, turbidity, clarity);
- seasonal and weather influences (inside/outside peak bloom season, temperature, prevailing wind strength and direction, rainfall) and changes to these influences;
- noted changes (increases/decreases) in monitoring program elements (cell counts, pigments, such as phycocyanin and chlorophyll-a, toxins); and
- adequacy of treatment and capacity of the treatment plant to deal with cyanotoxins.

The timing of sampling for cyanobacteria can also be important during calm, stratified periods. Buoyant cyanobacteria tend to accumulate near or at the water surface overnight. Temporary surface scums may be observed early in the morning, but can disperse as winds increase and may even be mixed back into the water column during the day.

Toxin analysis should be performed on the raw and treated water supply if the responsible authority determines that the drinking water intake is vulnerable to contamination by a cyanobacterial bloom. Analysis may be advised based purely on bloom confirmation in the source water if the authority deems it necessary. Although the guideline is based on the toxicity of microcystin-LR (MC-LR), it is important to measure total microcystins, which includes all of the measurable microcystin variants, not just MC-LR, that are free in the water as well as bound within cyanobacterial cells.

In general, laboratory enzyme-linked immunosorbent assay (ELISA), protein phosphatase inhibition assays (PPIA) or a physicochemical method such as liquid chromatography with tandem mass spectrometry (LC-MS/MS) should be used to measure total microcystins in treated water. It is recommended that when microcystins are detected in source or drinking water using ELISA or PPIA, a portion of subsequent samples should be analyzed using a physico-chemical method (i.e., LC-MS/MS) so that the predominant variants in a bloom can be identified and quantified and potential low level microcystin concentrations can be measured. Depending on the variants present in the sample, results from ELISA and PPIA may not be directly comparable to results from LC-MS/MS. However, data obtained from the different methods is considered to be complementary and can be used to better understand the nature of a bloom. In addition, both intracellular and dissolved microcystins can be present in raw and treated water, therefore it is important for samples to be processed so that a total microcystin concentration can be measured or calculated (sum of individual concentrations of intracellular and dissolved microcystins). Field test kits can be used as a qualitative (presence/absence) tool for determining if a bloom is toxic or if treatment plant operations need to be adjusted during a bloom event but do not provide

quantitative analyses that can be used to determine if treated drinking water concentrations meet the guideline value.

### *3.1.2 Notification*

If results for treated water exceed the proposed MAC, the sampling authority should follow standard protocol for notifying communities and other appropriate authorities and agencies. Dialysis treatment providers at all levels (e.g., large facilities/hospitals, small community facilities, mobile units, providers for independent/home dialysis) should also be notified, especially if it is a first-time occurrence for blooms on this supply.

Authorities also may choose to issue precautionary advice, such as the following:

- In cases where blue-green algal blooms are present near drinking water intakes, inform parents to use another suitable source of drinking water to reconstitute infant formula.
- For small systems that lack the necessary knowledge or treatment capabilities, the use of a do not consume advisory should be considered.

### *3.1.3 Corrective actions*

The degree of response to the presence of total microcystins should be discussed with the appropriate authorities (e.g., for system ownership, regulation and health) and will depend on a risk-based assessment of the significance and extent of the problem, taking into account the history and variability of the quality of the raw water supply and the documented effectiveness of the treatment process.

If results for treated water exceed the proposed MAC, the responsible authorities may wish to:

- resample the treated water supply and perform additional toxin analysis;
- initiate risk assessment consultation and decision-making. During the discussion process, possible treatment adjustments and strategy for continued monitoring of the treated water supply should be reviewed.

If results for the confirmation sample exceed the MAC, the responsible authorities may wish to:

- notify communities and other appropriate authorities;
- issue a do not consume advisory;
- consider treatment improvements, intake adjustments or use of an alternative supply as part of ongoing consultation and decision-making;
- communicate that lysing of the bloom by the addition of algicides may be harmful and that boiling is not effective in reducing or removing microcystins. Because of this, boil water advisories are not recommended as a response to the detection of microcystins in water supplies;
- continue monitoring the raw water and treated water supply.

If results for the confirmation sample do not exceed the MAC, results should be sent to the communities and other appropriate authorities and agencies. Authorities and water providers should continue to visually monitor the source water for intensification of the existing bloom or bloom recurrence if still in the season where cyanobacterial blooms could develop and repeat the steps discussed above, as necessary.

## 3.2 Residential scale and private drinking water systems

Surface water drinking water supplies known or suspected to be susceptible to blooms should be monitored routinely for the presence of cyanobacteria. The recommended approach for

residential scale and private supplies is for owners or authorities to visually monitor the source waters during the peak season for evidence of increasing cyanobacterial cell density or bloom development. The peak season is usually from late May to early October, but it may be longer, depending on local conditions. Taste and odour problems of natural origin (often described as musty, earthy, sulphurous or tobacco-like) can also be indicative of increased biological activity. However, the absence of taste and odour should not be equated with the absence of cyanobacteria, as not all types of cyanobacteria produce taste- and odour-causing compounds.

### *3.2.1 Notification*

Owners of residential scale and private drinking water systems who suspect that their water has been contaminated with cyanobacteria should consult their local health authority as to whether a concern exists and what actions should be taken.

### *3.2.2 Corrective actions*

In cases where an individual household obtains its drinking water from a surface water supply where there is a cyanobacterial bloom present and microcystins may be present, consumers need to consider the best option for obtaining a safe drinking water supply. These options may include switching to an alternative water supply, changing the location (distance and depth) of the water supply pipe or installing a drinking water treatment system. Microcystins are not destroyed by boiling, and therefore boil water advisories are not recommended as a response to the detection of microcystins in water supplies. However, consumers should be aware that treating water supplies for the removal of cyanobacteria and microcystins is complex, and it is difficult to assess the performance of treatment devices for microcystin removal. It is recommended that a qualified person be consulted prior to selecting and installing a residential scale treatment system. It is also important to note that it may be very difficult to treat source water that is significantly impacted by cyanobacteria (e.g., a large bloom).

# Part II. Science and Technical Considerations

## 4.0 Identity of cyanobacteria and their toxins

The cyanobacteria are bacteria that have features in common with algae (they obtain energy through photosynthesis and are of similar size). They are commonly, but mistakenly, called blue-green algae because of these similar features, as well as the blue-green colour that is native to many species and blooms. They are hardy organisms that can be found naturally in all types of water environments, in habitats ranging from the tropics to the Arctic (AWWA, 2010). Cyanobacteria are a normal component of aquatic phytoplankton, with many species occurring in fresh waters. More than 20 different genera have been identified to date as having the genetic capacity to produce cyanotoxins which can cause negative human health effects (Funari and Testai, 2008). The problems posed by cyanotoxins for drinking water utilities have traditionally been associated with planktonic cyanobacteria (Chorus and Bartram, 1999; Quiblier et al., 2013). In Canada the most troublesome planktonic toxic cyanobacteria genera are also those most frequently encountered worldwide: *Anabaena* (now called *Dolichospermum*), *Aphanizomenon*, *Gloeotrichia*, *Microcystis*, *Planktothrix*, *Pseudoanabaena* and *Woronichinia* (Winter et al., 2011; Ministère du Développement durable, de l'Environnement et des Parcs du Québec, 2012; Ontario Ministry of the Environment, 2012). Benthic toxic genera such as *Oscillatoria*, *Phormidium* and *Lyngbya* can also be found in freshwater habitats in Canada (Vis et al., 2008; Lajeunesse et al., 2012; Quiblier et al., 2013). The information database for benthic cyanobacterial populations is comparatively sparse (Quiblier et al., 2013); more study of their ecology and the potential problems they may pose for drinking water utilities is needed. The types of toxins that can be produced by cyanobacteria include hepatotoxins or liver toxins (e.g., microcystins, cylindrospermopsin), neurotoxins or nervous system toxins (e.g., anatoxins, saxitoxins), dermatotoxins or skin toxins (e.g., lyngbyatoxins) and irritant toxins or toxins that affect the skin and mucous membranes (e.g., lipopolysaccharides) (Chorus and Bartram, 1999; AWWA, 2010; Žegura et al., 2011; Merel et al., 2013).

### 4.1 Microcystins

Microcystins (MC) are hepatotoxins that are generally regarded as the most important of the freshwater cyanotoxins owing to their stability and resilience to biological and chemical breakdown, their widespread occurrence and their potential to reach high concentrations in blooms and scums (Boyer, 2007; Williams et al., 2007; Winter et al., 2011). Functionally, they are inhibitors of the enzyme protein phosphatase, acting by disrupting the balance of phosphorylation and dephosphorylation in the structures that give liver cells their shape. This causes the cells to collapse and separate from one another, which can lead to hemorrhaging, tissue damage, liver failure and, ultimately, death. A number of cyanobacterial genera have been identified as microcystin producers (Kotak and Zurawell, 2007; Funari and Testai, 2008; Pearson et al., 2010; Martins and Vasconcelos, 2011). The ones most commonly observed in North America are *Dolichospermum* (*Anabaena*), *Microcystis*, *Planktothrix* and *Pseudoanabaena* (Williams et al., 2007; Winter et al., 2011). Although *Aphanizomenon* is not generally reported as a major microcystin producer, blooms containing this taxon have been reported with high microcystin concentrations (Robert et al., 2005; Mooney et al., 2011).

Structurally, microcystins belong to the cyclic peptide group of toxins. They have seven amino acids joined to form a circular peptide that contains a unique amino acid side chain (known as the ADDA group) and two variable amino acids at the end of the molecule, which determine the identity of each microcystin variant. For example, the variant MC-LR has leucine (L) and

arginine (R) in the variable amino acid position, whereas MC-YA contains tyrosine (Y) and alanine (A) (Carmichael, 1992).

Although estimates vary slightly, it is currently believed that there are more than 80–90 microcystin variants (Meriluoto and Spoof, 2008; Pearson et al., 2010). MC-LR is the most commonly encountered and toxic variant worldwide (Graham et al., 2010). However, accounts of MC-LA, MC-RR or MC-YR dominating blooms and/or co-occurring with MC-LR have been documented (Kemp and John, 2006; Graham et al., 2010; Li et al., 2010; Sabart et al., 2010; B.C. Ministry of Health, 2012; Ministère du Développement durable, de l'Environnement et des Parcs du Québec, 2012; Srivastava et al., 2012). It is suspected that microcystin variants other than MC-LR are more common than previously thought.

### 4.2 Anatoxins and saxitoxins

Neurotoxic blooms are in general less common than hepatotoxic blooms; however, they are encountered in Australia and certain parts of Europe and North America (Kotak and Zurawell, 2007; Watson et al., 2008; Rantala-Ylinen et al., 2011). As a result of their detection in numerous countries and known association with deaths of domestic and wild animals (Boyer, 2007; Jacoby and Kann, 2007), neurotoxins are relevant to water supplies, second only to hepatotoxins from a management standpoint (Boyer, 2007). Structurally, the cyanobacterial neurotoxins are alkaloids. These are a broad group of low to moderate molecular weight (< 1000 Da) compounds that contain ring structures and at least one carbon–nitrogen bond.

The anatoxins (anatoxin-a, anatoxin-a(s), homoanatoxin-a) are neurotoxins that interfere with the activity of the nerve transmitter acetylcholine. This, in turn, affects the functioning of the nervous system by disrupting communication between nerves and muscle cells. Anatoxins can be produced by species of *Dolichospermum* (*Anabaena*) (anatoxin-a, anatoxin a(s)), *Aphanizomenon* (anatoxin-a), *Microcystis* (anatoxin-a) and *Oscillatoria* (anatoxin-a, homoanatoxin-a) (Chorus and Bartram, 1999; Funari and Testai, 2008). *Cuspidothrix issatschenkoi* (formerly *Aphanizomenon issatschenkoi*) is also a recognized anatoxin-a producer in Europe and Japan (Hodoki et al., 2012).

Saxitoxins are a group of related toxins that include saxitoxin, neosaxitoxin, the gonyautoxins and C-toxins (Codd et al., 1999; Wiese et al., 2010). These toxins act by blocking sodium channels in nerves and muscle cells, preventing the transmission of electrical impulses. They are also called paralytic shellfish poisoning toxins because they have largely been associated with marine shellfish accumulating the toxins by feeding on blooms of the marine plankton *Alexandrium* (Codd et al., 1999). Some members of the freshwater cyanobacteria *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis* and *Lyngbya* have also been reported to produce saxitoxins (Codd et al., 1999; Aráoz et al., 2010).

In Canada, a low detection frequency and limitations of the analytical methods mean that there are few data available on the levels of anatoxins and saxitoxins in natural waters affected by cyanobacterial blooms. Thus, although it is difficult to assess how widespread these toxins may actually be, to date, neurotoxic blooms have not come to light as a major health concern in Canadian drinking water supplies. An area of cyanobacterial research receiving increased focus is the ecology of benthic cyanobacteria. Studies have documented the detection of anatoxins and saxitoxins (as well as most of the other known cytotoxins) in benthic cyanobacterial populations (Vis et al., 2008; Lajeunesse et al., 2012; Quiblier et al., 2013). Further studies on this subject are needed to close some of the knowledge gaps in terms of potential impacts for drinking water resources.

### 4.3 Cylindrospermopsin

Cylindrospermopsin is a toxic alkaloid with a molecular weight of approximately 400 Da and a tricyclic ring structure as one of its identifying features. It is primarily categorized as a hepatotoxin, but the toxin has also demonstrated evidence of cytotoxic properties against other organs, such as the spleen, thymus, heart and gastrointestinal tract (Chorus and Bartram, 1999; Chong et al., 2002; Falconer, 2005; Funari and Testai, 2008).

Cylindrospermopsin is more commonly encountered in tropical and subtropical regions of the globe (Williams et al., 2007). Australia and the state of Florida in particular have reported multiple instances of cylindrospermopsin detection in lakes, rivers and drinking water reservoirs (Falconer and Humpage, 2006; de la Cruz et al., 2013). In recent years, there have been increasing reports of potential toxin-producing species in temperate fresh waters, suggesting that the geographical range of these species may be expanding (Graham et al., 2010; Xie et al., 2011; Sinha et al., 2012).

Important cyanobacterial species capable of producing cylindrospermopsin are *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum* and *Aphanizomenon flos-aquae*. *Cylindrospermopsis raciborskii* has long been recognized as the most widely distributed species capable of producing cylindrospermopsin. Yet, at present, only strains of *C. raciborskii* found in Australia, New Zealand and Asia, and possibly more recently in Europe, have been shown to produce the toxin (Burford and Davis, 2011). No North American strains have yet been found to possess the ability to produce cylindrospermopsin (Burford and Davis, 2011; Yilmaz and Phlips, 2011). *Cylindrospermopsis raciborskii* blooms are routinely reported in the state of Florida, and the organism has also been detected in the northern United States and Canada: in Ohio (Conroy et al., 2007), Michigan (Hong et al., 2006), Minnesota (Sinha et al., 2012), Manitoba (Kling, 2009) and Ontario (Hamilton et al., 2005). Despite the detection of the organism, neither cylindrospermopsin nor any *C. raciborskii* strains possessing the toxin-producing genes have been documented in these settings. It has been proposed that *Aphanizomenon ovalisporum* is the cylindrospermopsin-producing species of cyanobacteria most likely to be found in the United States (Yilmaz and Phlips, 2011).

### 4.4 Dermatotoxins and other irritant toxins

Certain marine and freshwater cyanobacteria can produce toxins called aplysiatoxins and lyngbyatoxins, which may cause dermatitis among people who come into contact with the cyanobacterial filaments (Chorus and Bartram, 1999; Lajeunesse et al., 2012). Similarly, it is thought that the lipopolysaccharide component of cyanobacterial cell walls can produce an irritant or allergenic response in humans (Chorus and Bartram, 1999). These are not considered a concern for exposure through ingestion of drinking water, but are addressed in the Guidelines for Canadian Recreational Water Quality (Health Canada, 2012).

### 4.5 β-Methylamino-L-alanine

An emerging compound of interest is the unusual amino acid β-methylamino-L-alanine (BMAA). BMAA was first identified during exploratory studies into the high rate of amyotrophic lateral sclerosis/parkinsonism–dementia complex (ALS-PDC) observed among the Chamorro people of Guam. ALS-PDC is a neurodegenerative disease with symptoms similar to Parkinson's disease and Alzheimer's disease. Researchers investigating the issue reported that BMAA could be detected in brain tissues of ALS-PDC patients in Guam and Canada (Cox et al., 2003) and that BMAA could be found in virtually all groups of cyanobacteria (Cox et al., 2005; Metcalf et al., 2008; Brand et al., 2010). The further observation that BMAA could be detected in flying foxes

(bats) led the researchers to hypothesize that BMAA could be subject to magnification up the food chain.

These findings and their implications are currently the subject of debate (Water Quality Research Australia, 2005). Some researchers have provided conflicting evidence regarding BMAA detection in brain tissue of ALS-PDC patients (Montine et al., 2005), whereas others have questioned the data relating to neurotoxicity and food chain magnification (Duncan and Marini, 2006).

Further investigation is needed before a cause and effect relationship between BMAA and neurological disease can be established or discounted (Water Quality Research Australia, 2005). The present evidence does not suggest that BMAA is a water quality hazard of human health concern, but developments on this topic will continue to be monitored.

## 4.6 Cyanobacterial growth and the development of blooms

Blooms are formed when conditions permit cyanobacteria to proliferate intensively to form a large population. It is the blooms that are of human health concern, particularly where these aggregate as surface scums and concentrate any cell-bound toxins. Many buoyancy-controlling cyanobacteria can position themselves in the water column at depths that correspond to optimal conditions of light and nutrients. They may be visible to the naked eye depending on their size and depth and may have the appearance of paint streaks or spills, foam, mats or grass clippings. Genera, such as *Microcystis*, that favour or can tolerate high sunlight intensities can occur very close to the surface (Chorus and Bartram, 1999; Falconer, 2005). Others, such as *Dolichospermum* (*Anabaena*), *Woronochinia* or *Aphanizomenon*, can form bands just below the surface in response to different light and nutrient limitations (Falconer, 2005). *Cylindrospermopsis* blooms can locate themselves at even greater depths and have a distinctly different appearance from the other genera mentioned (Falconer, 2005). Benthic, potentially toxic cyanobacteria, such as *Lyngbya* or *Oscillatoria*, can form mats on various substrates at the bottom of water bodies (Izaguirre et al., 2007; Quiblier et al., 2013).

The colours of bloom or scum masses are most frequently blue-green to light green, although some can have a reddish colour as a result of additional pigments (Chorus and Bartram, 1999; Falconer, 2005).

Surface scums can form when there is a sudden disruption of the stability of the water column and the cells cannot adjust their buoyancy fast enough and gather at the water's surface. Offshore winds can then drive the scums towards shore, where they can accumulate and their density may increase by more than 1000-fold (Chorus et al., 2000). In this way, blooms can appear to form almost overnight. Within benthic populations, bubbles of oxygen created through photosynthetic growth can become trapped within the population, causing mats of material to detach from the substrate (Quiblier et al., 2013). These dislodged mats may then wash up on shorelines.

The growth of cyanobacteria and the formation of blooms and scums are affected by a variety of chemical, physical and biological factors, and these can be influenced by both natural and human activities. As a result of the interplay of these factors, there may be large year-to-year fluctuations in the levels of cyanobacteria, even in the same body of water.

### *4.6.1 Chemical factors*

Phosphorus and nitrogen are the most notable macronutrients, and it is their availability and limitation that control the growth of cyanobacteria (Downing et al., 2001; Merel et al., 2013). Important sources of both nutrients are raw and treated sewage and septic waste, agriculture

(fertilizers, soil erosion, animal wastes), industry and pesticides (Falconer, 2005). Cyanobacteria are effective at surplus uptake of soluble phosphorus (as polyphosphates) and storage of phosphorus, when it is abundant (AWWA, 2010). *Cylindrospermopsis raciborskii* in particular is thought to have an extremely adaptable nutrient acquisition strategy and can dominate under low-nutrient conditions (Falconer, 2005; Burford and Davis, 2011).

Some cyanobacterial species preferentially use ammonia, but most are able to use several forms of dissolved nitrogen, including nitrite and nitrate (Burford and Davis, 2011; W. Chen et al., 2011). Many cyanobacterial taxa (e.g., *Dolichospermum* (*Anabaena*), *Aphanizomenon* and *Cylindrospermopsis raciborskii*) can also fix dissolved atmospheric nitrogen to usable ammonium ions (Falconer, 2005). There are differing views among the scientific community as to the relative importance of phosphorus and nitrogen in microcystin production (Srivastava et al., 2012). The body of evidence supports the following: increased nutrient loading will increase the risk, and biomass, of cyanobacterial blooms and hence the risk of microcystin production; and short-term changes in the availability of nitrogen and phosphorus can influence the bloom composition (e.g., a shift towards favouring nitrogen-fixing species) and/or the expression of toxicity (Kotak et al., 2000; Oh et al., 2000; Vézie et al., 2002; Davis et al., 2009; Krüger et al., 2012). Similar findings have been observed related to *Cylindrospermopsis* and the production of cylindrospermopsin (Burford and Davis, 2011).

Iron is also an important micronutrient for cyanobacteria because of its direct involvement in photosynthesis and nitrogen fixation. Researchers have linked the risk of cyanobacterial blooms with the availability of ferrous iron (Molot et al., 2014). There may also be a yet unresolved role in toxin production (Fujii et al., 2011; Wood et al., 2011); studies have shown increased microcystin levels under conditions of both iron surplus (Amé and Wunderlin, 2005) as well as limitation (Sevilla et al., 2008; Fujii et al., 2011). Eutrophic waters with high nutrient loading are important in cyanobacterial bloom development, but they are not an absolute requirement. Several authors have reported bloom and microcystin detection in oligotrophic and mesotrophic lakes (Boyer, 2007; Watson et al., 2008; Winter et al., 2011; Carey et al., 2012b). Given the extreme variability in data on nutrient availability and toxin production, investigators have proposed that nutrients may have a more indirect action by influencing the growth of individual cyanobacterial variants, as opposed to directly inducing toxin production (Kotak et al., 2000; Giani et al., 2005; Kotak and Zurawell, 2007).

Alkalinity and pH largely determine the chemical speciation of inorganic carbon, such as carbonate, bicarbonate and carbon dioxide, and, together with oxygen, can affect nutrient mobilization from sediments. Cyanobacteria are found more commonly in alkaline waters, likely because of their ability to use bicarbonate as a source of inorganic carbon (AWWA, 2010; Sinha et al., 2012).

Optimal values for individual environmental parameters exist for each cyanobacterial species, and it is these that influence the success of species in the phytoplankton community. The only general conclusion that can be made at this time is that strains that are better able to compete for the nutrients that are in limited supply have a competitive advantage that facilitates their dominance (Falconer, 2005; AWWA, 2010; Burford and Davis, 2011).

### *4.6.2 Physical factors*

The hydrodynamic properties of a water body can influence the potential for bloom development. Slow-moving or still waters are more conducive to bloom stability; therefore, waters with faster flows, greater mixing or higher turnover rates have a lower likelihood for

bloom development. Timing and duration of cyanobacterial blooms are also influenced by climatic conditions, along with other factors, such as the inoculum size and location.

Temperature increases and changes in climate variability affecting the intensity and duration of drought periods can have a strong effect on cyanobacterial growth rates and bloom formation (Dupuis and Hann, 2009; Paerl and Paul, 2012). Rising temperatures can lead to increased water temperatures, a longer ice-free season and increased thermal stratification (Winter et al., 2011; Salmaso and Cerasino, 2012). In contrast, low winter temperatures combined with a cold spring can bring about a greater degree of lake overturn and resuspension of sediment nutrients (Dupuis and Hann, 2009; Salmaso and Cerasino, 2012).

Higher frequency and intensity of rainfall followed by longer drought periods can create greater nutrient mobilization and longer periods of high temperatures without mixing (Chen et al., 2009; Reichwaldt and Ghadouani, 2012). Cyanobacteria are able to rapidly take advantage of the nutrient inputs provided by rainfall events (Reichwaldt and Ghadouani, 2012). Strong winds can also influence populations by forcing scum material towards shorelines, where it accumulates (AWWA, 2010).

Climate factors can also act to counter the development of cyanobacteria populations—for example, through winds and rainfall contributing to water body mixing, flushing and bloom destabilization and dispersal (AWWA, 2010; Reichwaldt and Ghadouani, 2012).

In temperate zones such as Canada, bloom occurrences are most prominent during the late summer and early fall. However, the seasonal trends that have historically been observed with respect to bloom development are no longer as clear-cut (Jacoby and Kann, 2007). Canadian data demonstrate that blooms are appearing earlier in the spring and extending later into the year. Blooms in Ontario lakes that persisted into the month of November during several years have been reported (Winter et al., 2011; Ontario Ministry of the Environment, 2012). A review of provincial reports showed that blooms were found in several provinces as early as March and April and, in one case, could be detected year-round in Victoria, British Columbia, where winter temperatures can stay above freezing (B.C. Ministry of Health, 2012). An additional notable finding by researchers has been the detection of a bloom of *Aphanizomenon flos-aquae* (a potential cyanotoxin producer) under ice on a German lake (Bertilson et al., 2013; Üveges et al., 2013).

A general concern is that increases in global temperatures brought on by climate change may contribute to continued geographic expansion of toxin-producing cyanobacterial blooms into temperate regions as well as an overall global increase in the frequency and severity of bloom events (Paerl and Paul, 2012; Sinha et al., 2012). Some cyanobacteria have optimal temperatures for growth above 25°C (Chorus and Bartram, 1999; Davis et al., 2009), which are higher than for other phytoplankton (Fujimoto et al., 1997; Chorus and Bartram, 1999). *Cylindrospermopsis* can thrive at temperatures from 20° to 35°C (Saker and Griffiths, 2000; Briand et al., 2004), with maximum growth occurring at 30°C (Saker and Griffiths, 2000; Burford and Davis, 2011). It has been proposed that tolerance to this temperature range helps to explain the occurrence of *C. raciborskii* in temperate regions in the summer months (Hamilton et al., 2005; Hong et al., 2006; Sinha et al., 2012) and year-long bloom persistence in some tropical and subtropical locations (Sinha et al., 2012). At the other end of the spectrum, *Microcystis* and many other taxa are also capable of surviving in sediments and can endure for longer than one overwintering period (Misson et al., 2012). This has important consequences, as overwintering populations of toxin-producing cells can then reinoculate the water body during spring thaw or throughout the growing season during resuspension events (Brunberg and Blomqvist, 2003; Ye et al., 2009). Little information is available on the relative survival of toxic versus non-toxic strains under these

conditions. Misson et al. (2012) concluded that the toxic potential of *Microcystis* cells was well preserved during overwintering, but that toxic cells were not likely to have a competitive advantage. *Cylindrospermopsis raciborskii* can form resting stages called akinetes that can provide protection for the organism in unfavourable temperatures (Paerl and Paul, 2012; Sinha et al., 2012). It has been suggested that rising global temperatures are at least partially responsible for the spread of this species outside of tropical/subtropical climates (Burford and Davis, 2011; Sinha et al., 2012). Cold adaptation of this organism has been suggested based on detection at temperatures below 20°C (Falconer, 2005; Bonilla et al., 2012). There are conflicting opinions among researchers on the effect of higher temperatures on cyanobacteria relative to other phytoplankton, and more research in this area is needed (Carey et al., 2012a; Paerl and Paul, 2012). Some authors maintain that temperature increases confer a direct advantage to cyanobacteria because of their preference for elevated growth temperatures (Jöhnk et al., 2008; Carey et al., 2012a). Others suggest that cyanobacteria benefit indirectly from the temperature increases by way of increases in water column stratification and stability and extended growing season (Paerl and Huisman, 2008; Wagner and Adrian, 2009). In many cases, it is likely that all of these factors may facilitate blooms.

Many cyanobacteria possess photoadaptive characteristics that allow them to outcompete other phytoplankton for light resources. They have multiple photosynthetic pigments that enable them to use light wavelengths that are not favourable for many competing phototrophic species (Chorus and Bartram, 1999). They also function at extreme light levels and can thus outgrow other phytoplankton at high irradiances (e.g., at the surface), in deeper or more turbid waters or in benthic mats (Chorus and Bartram, 1999; Falconer, 2005; Burford and Davis, 2011; Quiblier et al., 2013). As noted above, some cyanobacteria are buoyancy regulating and can optimize their position in the water column in response to available light or move (glide) towards more illuminated areas of a bottom substrate (Falconer, 2005; AWWA, 2010; Quiblier et al., 2013). Light requirements vary among individual cyanobacterial species. For example, *Microcystis* species have a preference for higher-light environments, whereas others, such as *Planktothrix agardhii* and *Cylindrospermopsis raciborskii*, prefer low light intensities (Chorus and Bartram, 1999; AWWA, 2010). *Cylindrospermopsis* is known for having less buoyancy than other cyanobacteria, and reservoirs with deep mixing can favour its dominance (O'Brien et al., 2009; Burford and Davis, 2011). This and other cyanobacteria, such as *Gloeotrichia*, can acclimatize to and exploit nutrient-rich deeper layers or benthic areas under low light (Eiler et al., 2006; O'Brien et al., 2009). These taxa can then increase their primary production once they mix back into the upper, more light-rich zones (Eiler et al., 2006; O'Brien et al., 2009). For benthic populations, having sufficient light able to penetrate to the water bottom is an important consideration for the depth at which cyanobacterial growth can occur (Quiblier et al., 2013).

### *4.6.3 Biological factors*

Biological mechanisms affecting cyanobacterial growth and toxin production in aquatic food webs are multiple and complex. Zooplankton are primary grazers of phytoplankton (Agrawal and Agrawal, 2011). Relative to other phytoplankton groups, cyanobacteria are poor food sources for zooplankton grazers, as they are low in (or lack) essential elements, such as unsaturated fatty acids and sterols (de Bernardi and Giussani, 1990; Agrawal and Agrawal, 2011). Ingestion and digestion by zooplankton can also be affected by the size, shape and biochemical properties of cyanobacteria, with filamentous species, colonies and strains associated with toxic effects being more difficult to digest for some species (de Bernardi and Giussani, 1990; Wojtal-Frankiewicz et al., 2010). Where cyanobacteria are not easily digested by zooplankton, their populations may

increase in relation to other, more easily digestible phytoplankton. From the opposite perspective, aquatic plants can compete with cyanobacteria and other phytoplankton for nutrients and light; and aquatic bacteria can also compete with cyanobacteria for nutrients (de Bernard and Guissani, 1990). Dreissenid mussels (zebra and quagga mussels) are also active grazers of phytoplankton (Fishman et al., 2010). Some researchers have suggested that these mussels can selectively filter feed and reject toxic cyanobacteria, although this capacity to differentiate among toxic and non-toxic strains is debated (Vanderploeg et al., 2001; Knoll et al., 2008). Nevertheless, the resulting adjustments to clarity, nutrient availability and phytoplankton population by zebra mussels in aquatic systems may provide a competitive advantage to some cyanobacteria (Vanderploeg et al., 2001; Knoll et al., 2008; Fishman et al., 2010).

Overall, however, the relationship between cyanobacterial growth and toxin production and grazing by zooplankton and dreissenid mussels is unresolved, and drawing broad-ranging conclusions that are applicable to all species or strains is not possible. Indeed, such responses may be habitat specific (Knoll et al., 2008).

### 4.7 Cyanotoxin production and stability

Concentrations of cyanotoxins present within a bloom can vary considerably, over both distance and time (Hotto et al., 2008; Briand et al., 2009). The mechanisms affecting cyanotoxin production and the relationship between cyanotoxin production and the growth of toxin-producing species are not well understood (Orr et al., 2010; Martins and Vasconcelos, 2011). Toxin levels do not necessarily coincide with maximum cyanobacterial biomass (Kotak and Zurawell, 2007; Dyble et al., 2008; Burford and Davis, 2011). The abundance of potentially toxic *Microcystis* cells within blooms ranges from less than 1% to 100%; thus, it is very difficult to use population size to predict toxin levels (Rinta-Kanto et al., 2005; Watson et al., 2008; Davis et al., 2009).

Both biological and environmental factors affect the potential for toxin production by cyanobacterial populations. It is thought that differences in the proportion of toxin-producing and non-producing strains and differences in toxin gene expression account for the variability in toxin concentrations within blooms (Kurmayer et al., 2003; Briand et al., 2008; Martins and Vasconcelos, 2011). Davis et al. (2009) observed that at Lake Champlain (Vermont) study sites, densities of potentially toxic *Microcystis* cells were significantly correlated with microcystin levels, often at a very high level of significance. In contrast, Hotto et al. (2008) reported that only roughly 50% of the variations in microcystin concentrations in Oneida Lake (New York) samples were attributable to the occurrence of *Microcystis* containing *mcyD* genes (which are part of the gene cluster coding for microcystin production). Ye et al. (2009) noted that peaks in toxin concentration and the amount of microcystin-producing genotypes (copies/mL) did not co-occur within blooms in Lake Taihu, China, and suggested that microcystin production may be dominated by a few highly productive species or strains.

Variability in the levels of toxin produced by different strains can span 3 orders of magnitude or more (Chorus and Bartram, 1999; Carrillo et al., 2003; Kotak and Zurawell, 2007). For example, Kurmayer et al. (2003) showed that within a natural *Microcystis* sp. population, larger size classes of *Microcystis* colonies had a greater proportion of microcystin-producing genotypes and average microcystin cell quotas 10–30 times greater than the cell quotas of the smallest size class of colonies. Microcystin cell quotas ranged from 10 to 350 fg/cell (mean: 140 fg/cell) in late summer samples collected from Saginaw Bay, Lake Huron (Fahnenstiel et al., 2008). The microcystin toxin quotas for 26 *M. aeruginosa* strains isolated from a Spanish reservoir ranged from 200 fg/cell to greater than $1.5 \times 10^5$ fg/cell (Carrillo et al., 2003). Similar findings have been reported for cylindrospermopsin. Saker and Griffiths (2000) noted a difference

in cylindrospermopsin concentration of greater than 4 orders of magnitude between the most toxic and least toxic isolates of *C. raciborskii*. Orr et al. (2010) reported nearly a 10-fold difference in cylindrospermopsin quotas between the most toxic and least toxic isolates.

Environmental factors are thought to have a smaller influence on total bloom toxicity, in general accounting for less than a 10-fold difference in toxin production (Chorus and Bartram, 1999; Carrillo et al., 2003). Numerous studies have examined potential correlations between microcystin concentration and parameters such as nutrients, temperature, pH and light, but the results have been contradictory (Sivonen, 1990; Orr and Jones, 1998; Kaebernick et al., 2000; Lee et al., 2000; Oh et al., 2000; Vézie et al., 2002; Yoshida et al., 2007; Orihel et al., 2012). Studies examining cylindrospermopsin, although more limited in number, have also demonstrated conflicting results (Saker and Griffiths, 2000; Falconer, 2005; Orr et al., 2010; Burford and Davis, 2011).

A few studies have taken advantage of advances in molecular methods to better understand the factors that influence microcystin gene expression. Kaebernick et al. (2000) provided evidence of microcystin gene expression tied to light quality and intensity, and Sevilla et al. (2008) observed that iron deficiency could induce microcystin synthesis. In a later study, Sevilla et al. (2010) concluded that microcystin gene transcription was not linked to nitrate concentrations. As a result of the conflicting information, theories have been put forth as to the role of environmental factors in microcystin production. It has been proposed that optimal growth occurs under conditions of light, temperature and nutrients that are different from those related to optimal toxin production (Kotak et al., 2000; Orr et al., 2010). Further, others suggest that the relative influence of environmental factors can be different depending on the species/strain and the environment (Rinta-Kanto et al., 2005; Martins and Vasconcelos, 2011). Briand et al. (2009) suggested that under suitable growth conditions, the energy costs of producing cyanotoxins outweigh the benefits, but that it is advantageous for cyanobacteria to produce toxins when conditions for growth are limiting. In the absence of clear evidence that directly ties environmental factors to cyanotoxin gene function, it seems that there is at least an indirect impact of environmental factors on cyanotoxin production by influencing growth and cell division (Orr and Jones, 1998; Kurmayer et al., 2003; Briand et al., 2008; Orr et al., 2010). It has been suggested that because of the complexity of the factors involved in microcystin production, the toxin may have multiple functions, including roles in cell-to-cell signalling and defence against predatory microorganisms (Briand et al., 2008).

### *4.7.1 Degradation in the environment*

Current information indicates that some cyanotoxins, such as microcystins, show some stability in the environment, and toxicity can persist from a few days to a few weeks after the bloom has disappeared (Jones and Orr, 1994; Ho et al., 2012). The persistence of cyanotoxins in natural waters can be affected by numerous factors, including dilution, adsorption (e.g., to sediments or plant material), photolysis and biodegradation by aquatic organisms (Stevens and Krieger, 1991a; Jones et al., 1994; Smith et al., 2008).

As noted above, microcystins have a chemical structure that is stable in water, and they are relatively persistent in the aquatic environment (Jones and Orr, 1994; Harada et al., 1996). They are resistant to chemical hydrolysis and oxidation and are not destroyed by boiling (Chorus and Bartram, 1999). Studies in Australia showed that MC-LR persisted up to 3 weeks following treatment of a *Microcystis aeruginosa* bloom with a copper sulphate algicide (Jones and Orr, 1994). Microcystins can be degraded by some types of aquatic bacteria that naturally occur in a

variety of different water bodies (Bourne et al., 1996; Christoffersen et al., 2002; Edwards et al., 2008; Giaramida et al., 2012; Ho et al., 2012).

Researchers have observed that lag periods in microcystin biodegradation (the time between when microorganisms encounter the toxin and when breakdown begins) could be substantially reduced, and in some circumstances eliminated, when bacteria were pre-exposed to microcystin (Christoffersen et al., 2002; Giaramida et al., 2012). Although it appears to reduce the lag, previous exposure to microcystin is not a prerequisite for degradation, as removal of microcystin occurs in water bodies that have no prior history of contamination with the toxin (Edwards et al., 2008). Thus, no general relationship can be discerned, and the microbial communities in different habitats will have variable capacities for degradation (Christoffersen et al., 2002; Holst et al., 2003; Ho et al., 2006b, 2007; Edwards et al., 2008).

In experiments on the effects of photodegradation of microcystins at the water surface, 78.7% of the initial concentration was degraded after 22 days (Wörmer et al., 2010). Photodegradation observed at depths of 1 and 4 m was significantly less, with only 24.4% and 9.9% of the microcystin having disappeared after 22 days. The authors suggested that photodegradation may play an important role in the fate of microcystins only under specific situations, such as in shallow systems (Wörmer et al., 2010).

Anatoxins are not stable in the environment compared with other cyanotoxins (Stevens and Krieger, 1991a; Bownik, 2010). Sunlight photolysis is thought to be an important means of anatoxin inactivation (Stevens and Krieger, 1991a). Early stability studies showed that anatoxin-a was rapidly degraded by sunlight, with a half-life of 1–2 hours under light and pH conditions similar to those encountered during a late summer bloom in northern temperate climates (Stevens and Krieger, 1991a). Decay under dark conditions was much slower, with anatoxin-a showing a half-life of 4–10 days (Stevens and Kreiger, 1991a).

In biodegradability studies of anatoxin-a, the toxin decayed faster in samples containing inocula from lake water or sediment (Rapala et al., 1994). The toxin could be detected at 4 days following incubation in inoculum-supplemented samples under dark conditions. Anatoxin-a response to light exposure was not investigated (Rapala et al., 1994).

Cylindrospermopsin is also stable under wide ranges of pH and temperature and is resistant to boiling (Chiswell et al., 1999). Degradation of cylindrospermopsin by sunlight is reported to be low under natural conditions where low quantities of photosensitizing pigments are present (Chiswell et al., 1999; Wörmer et al., 2010). Smith et al. (2008) reported that biodegradation of cylindrospermopsin occurred only in waters having a previous history of toxic *C. raciborskii* blooms. Lag periods were reduced following repeated exposures, and it was suggested that a threshold toxin concentration may be required to start degradation (Smith et al., 2008). In contrast, in laboratory experiments, Wörmer et al. (2008) observed no significant cylindrospermopsin degradation over a 40-day period in water samples containing bacterial populations that had previous exposure to the toxin. Chiswell et al. (1999) reported that in laboratory solutions (where pigment levels were presumed high), the cylindrospermopsin half-life was 1.5 hours, whereas in natural water samples (where plant pigment levels were presumed low), the half-life was 11–15 days.

Data on biodegradation of the saxitoxins are extremely limited. Ho et al. (2012) recorded half-life values for saxitoxin (obtained from *Dolichospermum* (*Anabaena*) *circinalis*) ranging from 5 to 24 days. Saxitoxin was more slowly degraded than either microcystin or cylindrospermopsin during this study (Ho et al., 2012). Marine bacteria isolated from shellfish were capable of reducing the toxicity of a saxitoxin mixture by 90% within 3 days (Donovan et al., 2008).

The effects of temperature on biodegradation in water have been studied. For example, the degradation of MC-LR and MC-RR was highest at 30°C and fairly rapid between 10°C and 30°C, with 4 days required for the concentration to decline from 20 mg/L to below the limits of detection in both instances (Park et al., 2001). At 5°C, degradation was very slow, and over 30% of the initial toxin concentration remained after 7 days (Park et al., 2001). Wang et al. (2007) reported no difference in MC-LR biodegradation rates at 25°C and 30°C, with complete removal of a 5 µg/L concentration within 3 days. Degradation was significantly slower at 22°C, with 6 days required for removal (Wang et al., 2007). Ho et al. (2012) noted that more rapid biodegradation of MC-LR was evident at 24°C compared with 14°C in autumn and winter samples taken from an Australian reservoir. Harada and Tsuji (1998) observed little MC-LR breakdown in lake water at 5°C and 20°C.

Smith et al. (2008) observed that temperature effects on cylindrospermopsin degradation followed the following rankings (fastest to slowest rate): 25°C > 35°C > 20°C. At 25°C and 35°C, disappearance of 35 µg/L was complete in just under 25 days, whereas at 20°C, roughly 40% of the toxin still remained at day 44 (Smith et al., 2008). Mohamed and Alamri (2012) noted the highest rates of cylindrospermopsin biodegradation at 25°C and 30°C, with complete removal of concentrations of 10 µg/L and 100 µg/L occurring within 7 and 8 days, respectively. Degradation rates at 10°C and 20°C were slower and incomplete, with 77% and 25% of the toxin still present at day 8. Temperature may contribute less to biodegradation compared with factors such as anoxic conditions and organic matter content (Klitzke and Fastner, 2012).

# 5.0 Exposure

## 5.1 Water

### *5.1.1 Provincial/territorial drinking water monitoring program data*

Ingestion of drinking water is the major pathway for potential human exposure to cyanobacterial toxins. Data collected through monitoring programs in the provinces and territories have characterized the occurrence of cyanobacteria and cyanotoxins in Canadian drinking water supplies. This unpublished information from the provinces and territories provides a valuable perspective on where and when blooms are encountered in Canada and the concentrations of cyanobacterial toxins, when detected (Giddings et al., 2012).

In 2011–2012, British Columbia reported an unconventional *Aphanizomenon* bloom on an inland lake. Toxin analysis found MC-YR to be the dominant toxin variant in the raw water supply, which reached a concentration greater than 25 µg/L at two municipal drinking water intakes. MC-LR almost always accompanied MC-YR in the raw water samples, but reached concentrations only as high as 3–6 µg/L. Treated water samples contained microcystin on 10 occasions; all other samples were below the limits of detection. All 10 samples that were positive for microcystin contained MC-YR; nine of these had toxin concentrations below 1.5 µg/L. One sample had an MC-YR concentration of 7 µg/L. Five of the samples also contained MC-LR at concentrations below 0.51 µg/L. The bloom continued into 2012, with intake sample toxin concentrations up to 62.40 µg/L for MC-YR and 7 µg/L for MC-LR. Only two treated water samples were found to contain microcystins; total microcystins were below the B.C. drinking water guideline value of 1.5 µg/L. In the two treated water samples that contained microcystin, *Aphanizomenon* counts did not exceed 1700 cells/mL, yet no toxin was detected in treated samples when cell counts were at their highest levels, ranging from 15 000 to 40 000 cells/mL (B.C. Ministry of Health, 2012).

In 2005, Alberta initiated routine monitoring for microcystin in lakes and reservoirs. Microcystin is highly prevalent in Alberta, with toxin typically occurring in 75%—and as many as 96%—of water bodies monitored annually. Although microcystin concentrations are typically low (i.e., up to 0.5 µg/L), elevated concentrations greater than 10 µg/L do occur periodically. Research indicates MC-LR to be the most common variant, but others, including MC-RR and MC-YR, have occurred. Research also suggests that conventional water treatment is effective at removing most microcystin from drinking water. In 2011, water treatment facilities detected microcystin in the source water at nine of the 23 locations submitting data. Concentrations of microcystin observed in positive samples ranged from 0.1 to 0.6 µg/L, all below the Alberta guideline value of 1.5 µg/L (Alberta Environment, 2012).

Saskatchewan drinking water utilities have observed recurring cyanobacterial blooms on a lake serving one of their drinking water treatment plants. Nevertheless, microcystins have not been detected in raw or treated municipal drinking water samples reported by the province. Flow rates of surface water sources and inflow positioning have been cited as contributing to these observations. Microcystins have been detected in bloom samples affecting recreational lakes elsewhere in the province (Saskatchewan Environment, 2012).

In the summer and fall of 2010, a bloom was observed in raw water samples in Manitoba at one of its drinking water treatment plants. The cyanobacterial population was dominated by *Aphanizomenon*, but also included *Microcystis*. Raw water cell counts and microcystin concentrations were shown to reach values as high as 340 000 cells/mL and 21.8 µg/L, respectively. In treated water samples collected over the same period, toxin levels were all below the limits of detection, and cell counts never exceeded 1500 cells/mL. In August 2011, the raw water supply was again impacted by an *Aphanizomenon*-dominated bloom. Cell counts reached 180 000 cells/mL, but microcystin levels remained below 1 µg/L in the raw water and were undetectable in treated water samples (Manitoba Water Stewardship, 2012).

In Ontario, data from the Ministry of the Environment's surveillance programs from 2009 to 2012 showed that cyanobacterial blooms were detected in the province as early as May and as late as the end of November. Cyanobacteria were detected at 24–29 sites per year (in various water bodies, such as lakes, rivers, creeks, marshes and reservoirs). Detectable levels of microcystins were found at 20–73% of those sites sampled in any given year. The highest microcystin concentration encountered was 2800 µg/L, although the vast majority of positive samples were below 20 µg/L. *Dolichospermum* (*Anabaena*) was most frequently recorded as the dominant genus (either on its own or in mixed populations), followed by *Microcystis* and *Aphanizomenon*. Blooms of *Gloeotrichia*, a recognized but infrequently encountered microcystin producer, were also occasionally reported, with microcystin concentrations ranging from below the limit of detection to 96 µg/L (Ontario Ministry of the Environment, 2012).

In Quebec, concentrations of cyanobacteria and cyanotoxins have been documented in raw and treated water of various drinking water treatment plants since 2001. Concentrations of cyanobacteria above 20 000 cells/mL were measured regularly in affected raw waters (maximum concentration above $2 \times 10^6$ cells/mL), whereas microcystins (MC-LR, MC-RR, MC-YR, MC-LA) were regularly detected but only occasionally had total concentrations above 1.5 µg/L (maximum concentration in raw water of 97 µg/L for total microcystins in MC-LR equivalent toxicity). In treated waters (after filtration and disinfection, including in most cases either ozone or powdered activated carbon [PAC]), concentrations of cyanobacteria were generally around or below 200 cells/mL, and concentrations of microcystins were generally lower than 0.1 µg/L. In July to September 2011, the province reported a bloom that impacted several local drinking water treatment plants. *Aphanizomenon* dominated the population in July and August, reaching counts

as high as $2 \times 10^6$ cells/mL. Over the bloom's duration, a succession of cyanobacterial species was observed, and *Microcystis* took over as the principal genus in late August. The most prominent impact of this bloom was that microcystins were detected in the clarifiers of three treatment plants. Several microcystin variants could be detected within the water samples of these plants: MC-LA, MC-LR, MC-RR and MC-YR. Total microcystin concentrations exceeded 1.5 µg/L at several times at one location. The highest total microcystin concentration encountered was 6.0 µg/L in a clarifier surface sample. Despite these findings, microcystins were not detected in any finished drinking water sample. It is notable that during this period, there were several instances in which total microcystin concentrations did not match very well with cell count readings—for example, the toxin was detectable at very low cell counts and non-detectable at very high cell counts. Also of interest during that period was the detection of a *Woronichinia* population (10 000–20 000 cells/mL) at one treatment plant, which was accompanied by an MC-LA concentration of 0.3 µg/L (Ministère du Développement durable, de l'Environnement et des Parcs du Québec, 2012).

New Brunswick's first report of a bloom affecting one of its drinking water treatment plants occurred in August 2010. The species *Gloeotrichia echinulata* was identified as the dominant cyanobacterial species. A similar bloom returned in June 2011, persisting to October. Samples of the raw water and water at the end of the treatment chain collected during both bloom occurrences were negative for microcystins (New Brunswick Department of Health, 2012).

Nova Scotia reported issues with cyanobacterial blooms at multiple drinking water treatment plants in the spring and fall of 2006 and spring of 2007. Microcystins were detected at multiple locations at concentrations ranging from 0.1 to 0.6 µg/L, but all concentrations were below the provincial guideline value of 1.5 µg/L. The cyanobacterial species was not reported. Notable locations at which microcystins were reported included an infiltration gallery, treated surface water (both filtered and non-filtered supplies) and groundwater considered not to be under the influence of surface water. Treatment upgrades were applied to the individual systems encountering problems and included technologies such as enhanced coagulation, dual media filtration and membrane filtration. Following the upgrades, microcystin was not detected at these locations (Nova Scotia Department of Environment and Labour, 2012).

Prince Edward Island water utilities have not reported being impacted by cyanobacterial blooms, as 100% of the province's drinking water is supplied by groundwater resources (P.E.I. Department of Environment, Energy and Forestry, 2012).

In 2007, Newfoundland and Labrador experienced its first cyanobacterial bloom (Government of Newfoundland and Labrador, 2008; Newfoundland and Labrador Department of Energy and Conservation, 2012). The affected bodies of water were a chain of ponds that were not a source of drinking water, but were used by the public for recreational activities. *Dolichospermum* (*Anabaena*) was the genus responsible for the bloom, and toxin analysis found MC-LR at three out of four ponds at low concentrations, ranging from less than 0.22 to 0.36 µg/L. MC-LR was again detected in May 2008 in all ponds at concentrations ranging from 0.28 to 0.61 µg/L. No cyanobacterial cells were detected, however, and MC-LR was not observed in any other sample from June to September. It was speculated that the toxin persisted over the winter during low flow and ice conditions and then was released into the water during the spring thaw (Newfoundland and Labrador Department of Energy and Conservation, 2012).

Yukon, Northwest Territories and Nunavut have not noted problems with cyanobacteria and microcystins in drinking water supplies in their jurisdictions (Northwest Territories Department of Health and Social Services, 2012; Nunavut Health and Social Services, 2012; Yukon Environmental Health Services, 2012).

### *5.1.2 Published literature—North America*

Microcystins can be detected at low levels across a large number of surface waters, but many fewer show concentrations that exceed the current Canadian drinking water or recreational water guidelines (1.5 µg/L and 20 µg/L, respectively). For example, a large, multiyear survey of microcystin concentrations in phytoplankton samples from Alberta lakes found that the highest mean volumetric MC-LR concentration was 2.48 µg/L (Kotak and Zurawell, 2007). In the two Alberta lakes known to experience blooms and where the highest microcystin concentrations had been measured, concentrations of microcystin in the water rarely exceeded 1.0 µg/L (Kotak and Zurawell, 2007). Data from the Quebec Ministry of the Environment surveillance program indicated that as of 2007, the highest historical concentration of microcystin encountered in raw water was 5.35 µg/L (Carrière et al., 2010). Winter et al. (2011) reported that in 2009, 67% of blooms reported in Ontario inland lakes tested positive for these toxins. Microcystins were detected in 62% of phytoplankton samples collected from Alberta water bodies from 1990 to 1995 (Kotak and Zurawell, 2007). They were also detectable in all phytoplankton samples (concentration range 0.006–4.29 µg/L) in a 2001 study of four eutrophic lakes prone to bloom development in the province of Quebec (Rolland et al., 2005).

Large water bodies across Canada are increasingly at risk of developing blooms, especially those with inputs from highly developed watersheds, such as Lake Winnipeg and some of the lower Great Lakes. However, despite severe blooms, most of these lakes do not exhibit high lake-wide toxin levels. In Lake Winnipeg, which now sees annual and extensive blooms of *Aphanizomenon flos-aquae* and *Dolichospermum* (*Anabaena*) spp., toxins have been detected in some surface blooms, and erratic peaks of up to 200 µg/L total microcystin have been reported (Kling et al., 2011). In the Great Lakes, offshore summer surveys during 2000–2004 detected low levels of microcystin in 40% of the more than 300 Lake Erie samples and 20% of the more than 700 Lake Ontario samples, but a few samples (12% and 1%, respectively) showed concentrations above 1 µg/L (Boyer, 2007). High microcystin levels have been documented at inshore areas or within more eutrophic embayments of water bodies (e.g., Murphy et al., 2003; Watson et al., 2008). For example, in profiling a microcystin bloom in Hamilton Harbour on Lake Ontario, Murphy et al. (2003) noted that high concentrations of microcystin (up to 400 µg/L) were found in the wind-blown scums, whereas much of the harbour had low toxin concentrations. In a recent study, Davis et al. (2014) demonstrated genetic connectivity between the *Microcystis* strains in Lake St. Clair and the lower Great Lakes. These findings highlight the potential for established bloom habitats to contribute to spread of problematic strains through interconnected systems; however, the authors noted that further experiments need to be conducted in these systems to identify any common environmental drivers (Davis et al., 2014).

In the state of Florida, where toxic cyanobacterial blooms are a common occurrence, Williams et al. (2007) reported that year-round open lake water microcystin concentrations were 0.05–3.6 µg/L, despite occasional bloom events with microcystin concentrations ranging from 5 to 7500 µg/L. Evidence suggests that as long as intakes for drinking water systems are not in close proximity to the borders of a microcystin-producing bloom, they will not take in quantities of cyanobacterial cells or toxins in amounts that would be of human health concern in drinking water. Furthermore, healthy blooms typically do not release a significant amount of dissolved microcystin. Most of the toxin is stored within the cyanobacterial cells; release into the water largely occurs when the cells rupture (e.g., through grazing, sudden environmental stress, treatment with algicides or other disrupting processes) or die (Graham et al., 2010; Merel et al., 2013; Wu et al., 2014). In an analysis of cyanotoxins in blooms in the U.S. Midwest, Graham et

al. (2010) reported that the amount of dissolved microcystins never exceeded 30% of the total microcystin concentration.

Accumulations or scums of toxic blooms contain very high amounts of toxin. Bloom and water sample concentrations of microcystin have reached as high as 10 000 µg/g dry weight and 25 000 µg/L, respectively (Chorus and Bartram, 1999). Murphy et al. (2003) observed microcystin concentrations of 400 µg/L at the height of a bloom in Hamilton Harbour, Lake Ontario, whereas Boyer (2007) reported a shoreline bloom sample from Missisquoi Bay, New York, that contained 5000 µg/L microcystin. It is evident that within the confines of the bloom, very high levels of toxin in water are possible, particularly once the bloom has collapsed. Jones and Orr (1994) demonstrated that dissolved microcystin concentrations reached 1800 µg/L 4 days after treatment of a bloom with algicide.

The U.S. Environmental Protection Agency (EPA) reviewed state cyanobacterial bloom and microcystin monitoring data in developing its draft Health Effects Support Document for Cyanobacterial Toxins. The information showed similarities to the Canadian situation in several respects. Notably, the data are largely unpublished and reside with the respective state and local governments. Further, microcystin was the most commonly occurring cyanotoxin, and toxic blooms were reported in most states, spanning all the major regions of the country: West, Midwest, Northeast and South. Most surface water samples contained microcystin concentrations below 1 µg/L, although there were instances of open-water or non-scum samples with toxin concentrations above 20 µg/L and 50 µg/L, respectively. Very high microcystin concentrations were found in scum-containing samples, reaching as high as 8000 µg/L and 26 000 µg/L. Blooms of *Microcystis* were very common, but populations were periodically encountered in which *Dolichospermum* (*Anabaena*), *Oscillatoria*, *Aphanizomenon* or *Woronichinia* was the dominant genus (U.S. EPA, 2013).

Very few studies have been conducted on cyanobacterial neurotoxins in North American fresh waters, with most of the work being focused on anatoxin-a (Kotak and Zurawell, 2007). In Alberta, Canadian researchers monitoring for anatoxin-a in blooms, lakes and reservoirs found that the toxin was not frequently detected and, when present, was mostly near the limits of detection (Kotak et al., 1993; Kotak and Zurawell, 2007). During a 3-year span of monitoring data from Quebec source waters where blooms have historically been reported, the highest raw water anatoxin-a concentration was 2.3 µg/L (Robert et al., 2005). A large multi-year surveillance study noted that anatoxin-a was present in 92/2307 (4%) samples collected from New York lakes (Boyer, 2007). The authors further reported that of the positive samples, 4% exceeded an anatoxin-a concentration of 0.01 µg/L, and 1% exceeded a concentration of 1.0 µg/L (Boyer, 2007). In a study of cyanobacterial blooms, Graham et al. (2010) reported anatoxin-a in 30% of samples from bloom events in Iowa, northeastern Kansas, southern Minnesota and northwestern Missouri. Anatoxin-a concentrations ranged from 0.05 to 9.5 µg/L (Graham et al., 2010). A survey of cyanotoxins in Florida's water resources for the year 2000 found that 10% of raw water samples and 11/56 (20%) ecological samples were positive for anatoxin-a (Williams et al., 2007). Concentrations ranged from 0 to 0.11 µg/L among the raw water samples and from undetectable to 156 µg/L for the ecological samples. The maximum concentration of anatoxin-a identified in published reports was a bloom in Finnish waters with a concentration of 4400 µg/g dry weight (Sivonen et al., 1989).

For the saxitoxin group of toxins, North American occurrence data are sparse (Boyer, 2007; Watson et al., 2008). Graham et al. (2010) included saxitoxins among the compounds monitored in their survey of blooms in the U.S. Midwest. The authors reported detection of the toxin at very low concentrations (0.2 µg/L) in 4/23 blooms (17%). It was speculated that this was

the first published report of saxitoxin occurrence in this region (Graham et al., 2010). In other countries, a Danish survey indicated that members of the saxitoxin group could be detected at 11/96 lake and pond locations (Kaas and Henriksen, 2000). Saxitoxin was the most frequently detected toxin (8/11 locations), at concentrations ranging from 5.9 to 224.1 µg/g dry weight (Kaas and Henriksen, 2000). *Dolichospermum* (*Anabaena*) *lemmermannii* was the most dominant cyanobacterial species encountered in saxitoxin-positive lakes. A similar study of blooms in Finland detected saxitoxin at concentrations of 33–1070 µg/L at 7/11 bloom locations (Rapala et al., 2005). Again, *D. lemmermannii* was the predominant species in toxin-positive samples (Rapala et al., 2005). A 70% detection frequency for cyanobacterial saxitoxins has been reported in Australian coastal areas (Velzeboer et al., 2000).

Cylindrospermopsin has rarely been detected in North America. In the previously mentioned surveillance study of New York lakes, only 8/517 samples (2%) tested positive for the toxin. Of those positive samples, 5 (63%), were taken from the same lake and had concentrations between 0.1 and 0.25 µg/L (Boyer, 2007). Using enzyme-linked immunosorbent assay (ELISA), cylindrospermopsin was detected at low concentrations (< 0.*2* µg/L) in 9% of blooms in the U.S. Midwest. The toxin was detected only in blooms dominated by *Aphanizomenon*. However, follow-up testing with liquid chromatography–tandem mass spectrometry (LC-MS/MS) showed that the ELISA response was not due to the presence of cylindrospermopsin; rather, it was due to the presence of some other cylindrospermopsin-like compound (Graham et al., 2010).

A notable difference between cylindrospermopsin- and microcystin-producing blooms is that with the former, a substantial amount of toxin is released into the water column during bloom growth, whereas microcystins are largely cell-bound until death (Shaw et al., 1999; Falconer, 2005). Reasons proposed for this cylindrospermopsin release include its high solubility in water, significant release from the producing species, the ability to pass through membranes and limited degradation in the environment (Wörmer et al., 2008, 2009). Shaw et al. (1999) reported that at least 85% of the cylindrospermopsin was released to the water during a study of a bloom of *Aphanizomenon ovalisporum*. Others have shown that the proportion of dissolved cylindrospermopsin can be high and ranges widely, from 6% to 100% (Wörmer et al., 2009; Cirés et al., 2011; Yen et al., 2011).

### *5.1.3 Detection of toxin genes*

The genes responsible for the synthesis of the major cyanobacterial toxins—microcystins (*mcy* genes), cylindrospermopsin (*cyr* genes), anatoxin-a (*ana* genes) and saxitoxin (*sxt* genes)—have been identified and sequenced (Tillett et al., 2000; Shalev-Alon et al., 2002; Preußel et al., 2006; Kellmann et al., 2008; Rantala-Ylinen et al., 2011). Polymerase chain reaction (PCR) primers that target these gene sequences have been used by researchers to improve the understanding of the toxicity potential of cyanobacterial populations (Rinta-Kanto et al., 2005; Al-Tebrineh et al., 2010; Ballot et al., 2010). With microcystins, primers directed at various genes in the peptide synthetase gene cluster (*mcyA–J*) in microcystin have been developed and used (Kurmayer et al., 2003; Rinta-Kanto et al., 2005; Hotto et al., 2008). In an investigation of Lake Erie blooms occurring between 2003 and 2004, the *mcyD* gene was detected at 15/17 (88%) of the sample locations; microcystin was also detected at 13 of these *mcyD* gene–positive locations at concentrations ranging from 0.04 to 15.4 µg/L (MC-LR equivalents) (Rinta-Kanto et al., 2005). Only one sample was reported in which the toxin was detected and the gene was not (Rinta-Kanto et al., 2005). In a study of four diverse U.S. Northwest lakes, purportedly toxic *Microcystis* cells (measured as *mcyD* cell equivalents) comprised 12–100% of the *Microcystis* bloom population from one lake system and 0.01–6.0% of the populations from three other systems (Davis et al.,

2009). Another study reported the mean proportion of *mcyB*-positive *Microcystis* cells to range between 67% and 97% (Kurmayer et al., 2003). Briand et al. (2008) documented the proportions of *Planktothrix* strains having *mcyA* genes to be in the range of 30–80% during a 2-year survey of a perennial bloom. Hotto et al. (2008) observed that *mcyD* gene detection was better correlated with microcystin presence than with *mcyB*, *mcyA* or a total *Microcystis* primer. More recently, Davis et al. (2014) discovered that *mcy*A sequences isolated from a toxic *Microcystis* bloom in Lake St. Clair were genetically related to *mcyA* sequences isolated from *Microcystis* in Lake Ontario and Lake Erie, suggesting a strong genetic connectivity between the three lakes. Overall, detection of *mcy* genes is regarded as a useful indicator of potential toxicity, but there is still some uncertainty as to their usefulness as indicators of microcystin production (Kurmayer et al., 2003; Hotto et al., 2008). There is less information available for anatoxins and saxitoxins. In an investigation of saxitoxin toxicity of blooms in Australian lakes and rivers, one study found a positive correlation between saxitoxin concentration and blooms having high *sxt* gene copy numbers (Al-Tebrineh et al., 2010).

## 5.2 Food

Microcystins and other cyanobacterial toxins have been found in some foods. Algal dietary supplements and fish or seafood (mussels, shrimp, clams, crabs, etc.) taken from waters contaminated with cyanobacterial blooms have the potential to contain toxins. Fish can be exposed to microcystins directly by feeding (on phytoplankton or via the food web) or passively when the toxins pass through the gills or skin (Malbrouck and Kestemont, 2006; Ibelings and Chorus, 2007). Similarly, mussels, shrimp and crabs can accumulate the toxins by filtering contaminated waters, feeding on phytoplankton or having cyanotoxins dissolved in water enter their body and accumulate in their tissues (Ibelings and Chorus, 2007).

Elevated tissue levels of microcystins have been reported in crab (0.82 mg/kg, hepatopancreas), prawn (1.2 mg/kg, hepatopancreas), shrimp (55 mg/kg, hepatopancreas), snail (2.3 mg/kg visceral sac), carp (137 mg/kg, intestine) and tilapia (32 mg/kg) (Xie et al., 2005; Chen et al., 2006, 2007; Zimba et al., 2006; Song et al., 2007). Wilson et al. (2008) recorded a maximum liver microcystin concentration of 1.18 mg/kg dry weight in Lake Erie yellow perch collected during the occurrence of a large, toxic *Microcystis* bloom.

Numerous authors have found that microcystins accumulate to a lesser extent in the edible parts of aquatic organisms, such as muscle (Xie et al., 2005; Zimba et al., 2006; Song et al., 2007; Wilson et al., 2008; Deblois et al., 2011; Vareli et al., 2012; Gutiérrez-Praena et al., 2013). In a survey of microcystins in water and fish in two temperate Great Lakes (Erie and Ontario), the highest microcystin concentrations in fish muscle observed for Lake Erie were for alewives (20.0–37.5 µg/kg) and northern pike (1.6–25.8 µg/kg) and for Lake Ontario were walleye (5.3–41.2 µg/kg), white bass (4.2–27.1 µg/kg) and smallmouth bass (1.5–43.6 µg/kg) (Poste et al., 2011). Muscle tissue microcystin concentrations in yellow perch collected during a toxic bloom were lower in comparison (0.12–0.02 ng toxin/g dry weight [= µg/kg]) (Wilson et al., 2008). Nevertheless, concentrations of microcystins in edible tissues have exceeded 0.1 µg/g for fish, crab, mussels and shrimp (Freitas de Magalhães et al., 2001; Mohamed et al., 2003; Xie et al., 2005; Vareli et al., 2012).

Xie et al. (2005) studied the distribution of MC-LR and MC-RR in various fish in a lake prone to heavy blooms of *Microcystis* and *Dolichospermum* (*Anabaena*). The total microcystin concentration in blooms in Lake Chaohu, China, was 240 µg/g dry weight, and the maximum concentrations detected in fish were 137 µg/g dry weight in the gut of silver carp (104 µg/g of MC-RR + 33.3 µg/g of MC-LR) and the muscle of goldfish (0.97 µg/g of MC-RR + 2.29 µg/g of

MC-LR). In general, the microcystin content in the liver and muscle was highest in carnivorous fish, followed by omnivorous fish, and was lowest in planktivorous and herbivorous fish (Xie et al., 2005).

In another example, sea silverside fish were fed *Microcystis aeruginosa* through their diet (MC-LR:1.3 µg/g body mass, incorporated in standard fish food). The toxin accumulated rapidly in the intestine and liver, up to 22.9 µg/g and 9.4 µg/g body mass, respectively. Accumulation of the toxin was high in both tissues at the beginning of the experiment and then decreased over time (Bieczynski et al., 2013).

After fish are exposed, microcystin concentrations in their tissues decrease with time as a result of detoxification and depuration processes (Tencalla and Dietrich, 1997; Xie et al., 2004; Mohamed and Hussein, 2006; Wood et al., 2006; Gutiérrez-Praena et al., 2013). Numerous authors have found that bioaccumulation (uptake and concentration of the toxins from the water) occurs in the food web, but that there was not strong evidence for biomagnification (uptake and concentration in organisms at higher trophic levels via the food) (Ibelings et al., 2005; Xie et al., 2005; Ibelings and Havens, 2008; Papadimitriou et al., 2012). Researchers have also suggested that biodilution may be occurring given the observations of depuration and toxin elimination within organisms (Ibelings and Havens, 2008; Poste et al., 2011). It has also been suggested that biotransformation of microcystin by aquatic organisms to covalently bound forms may complicate the complete measurement of total microcystin content in tissues (Williams et al., 1997; Wilson et al., 2008; Dyble et al., 2011). However, it has been recognized that bound or conjugated forms are created though detoxification processes and are not readily absorbed by higher organisms (Wilson et al., 2008).

Data relative to the detection of other cyanotoxins in foods are very limited. In the muscle tissue of finfish that were collected from a tropical lake system characterized by a year-round dominance of a toxic *Cylindrospermopsis* bloom, cylindrospermopsin was detected at low concentrations of 0.09–1.26 µg/kg (Berry et al., 2012). The maximum concentration of cylindrospermopsin accumulated in tissues of freshwater mussels exposed to 14–90 mg/L for 16 days was 61.6 mg/g dry weight (hemolymph), 5.9 mg/g dry weight (viscera) and 2.9 mg/g dry weight (whole-body extracts) (Saker et al., 2004). No cylindrospermopsin was detected in the gills or adductor muscle. Following a 2-week depuration period, approximately 50% of the toxin remained in the tissues (Saker et al., 2004).

It is difficult to estimate the exposure of humans to microcystins in fish and shellfish. There are no widely accepted estimates of the average daily consumption of these foods for adults and children. The length of food web exposures to blooms can also vary greatly between geographical regions. In temperate lakes, microcystins, if present, would be most prevalent in the warmer weather, thus mitigating year-round exposure in humans that consume aquatic organisms from water bodies susceptible to cyanobacterial blooms. Populations having access to food sources taken from different water bodies would also have a lower likelihood of exposure (Poste et al., 2011). Similarly, populations in communities in tropical locations with persistent year-round cyanobacterial blooms, which rely on local fish for subsistence and frequently consume the entire fish, would be considered at greater risk for microcystin exposure (Dyble et al., 2011; Poste et al., 2011). Any guidance values for microcystin contamination of foods are best established through consultation with jurisdictional authorities and should include the consideration of local data, as available, on the frequency and magnitude of contamination and local fish and seafood consumption habits.

With regard to food preparation, there has been conflicting evidence on the effects of cooking techniques on the removal of microcystins from contaminated food. Microcystins are

chemically stable at high temperatures and can withstand several hours of boiling (Van Apeldoorn et al., 2007). D. Zhang et al. (2010) observed that microcystin levels were higher in boiled muscle than in freeze-dried muscle. It was suggested that boiling may have sped up the release of the toxin from phosphatase enzymes (Gutiérrez-Praena et al., 2013). D. Zhang et al. (2010) also detected microcystins in the boiled water. Morais et al. (2008) demonstrated that microwaving mussels for 1 and 5 minutes significantly reduced the microcystin content in the tissues, whereas boiling for 5–30 minutes did not. In contrast, Guzmán-Guillén et al. (2011) observed that boiling, continuous boiling and microwave oven cooking were able to reduce unconjugated (not bound to organism tissue) microcystin levels in muscle tissue from tilapia. No information was provided for other techniques, such as grilling, roasting or frying (Guzmán-Guillén et al., 2011). More studies are needed to confirm the effectiveness of various cooking procedures in reducing microcystin residues in aquatic organisms.

Crop plants irrigated with water containing microcystin-producing cyanobacteria have been investigated as a potential indirect route of human exposure to cyanobacterial toxins. Accumulation of microcystins in plants and vegetables following spray irrigation with contaminated water has been reported. Hereman and Bittencourt-Oliveira (2012) detected microcystin levels in lettuce leaf tissue of 8.31–177.8 µg/kg after being sprayed once a day for 15 days with solutions containing the toxin at concentrations ranging from 0.62 to 12.5 µg/L. Microcystins were also found in the roots of rapeseed, ryegrass, lettuce and clover (mean range: 0.12–1.45 mg/kg) and in shoots of clover and lettuce (mean range: 0.20–0.79 mg/kg) following treatment with lake water having a high total microcystin concentration (1.6 mg/L) and containing a mixture of microcystin variants (Crush et al., 2008). J. Chen et al. (2012) observed MC-LR accumulation in rice grains harvested from fields bordering a lake known to be repeatedly contaminated by cyanobacterial blooms. Data showed that 11/21 positive samples had MC-LR values above 1.0 µg/kg (J. Chen et al., 2012). MC-LR was detected in root samples of broccoli and mustard at low levels (0.9–2.9 ng/g) following watering with 10 µg MC-LR/L over a 19- to 20-day period (Järvenpää et al., 2007). Toxin accumulation varied greatly among durum wheat, peas, lentils and corn after 30 days of irrigation with surface water containing microcystins (Saqrane et al., 2009). Root toxin levels ranged from not detected (durum) to a high of 16.00 µg/g (lentil) after a low-concentration irrigation (0.50 µg MC-LR/mL) and from 16.66 µg/g (durum) to 190.85 µg/g (pea) after a high-concentration treatment (4.20 µg MC-LR/mL) (Saqrane et al., 2009). For the general public, exposure to microcystins from contaminated foodstuffs is expected to be low (Dietrich and Hoeger, 2005). However, further research is needed to assess dietary exposure to microcystins from aquatic food organisms. Certain regions or populations worldwide may have a greater or lesser likelihood of exposure to cyanobacterial toxins, depending on, for example, the susceptibility of nearby water bodies to contamination, the genetic composition of cyanobacterial blooms and consumption patterns of foods collected from these areas (Dietrich and Hoeger, 2005; Wilson et al., 2008; Dyble et al., 2011).

### 5.3 Natural health products

Cyanobacteria taxa (e.g. *Spirulina*, *Aphenizomenon*) used in the commercial manufacture of natural health products can be cultured in large scale in bioreactors, controlled ponds or naturally, in natural lakes. *Aphanizomenon* blooms harvested for supplements come from the natural environment and may be contaminated with cyanobacterial species that produce toxins. Most *Spirulina* samples used in supplements come from culture, and there is little contamination with other toxic species; however, some environmental contamination can occur (Jiang et al., 2008; Heussner et al., 2012). A survey of cyanobacterial supplements available in Canada and

elsewhere in North America showed that microcystins were detected in 83/85 products, with 72% having concentrations above 1 µg/g (Gilroy et al., 2000). Mean microcystin levels in *Aphanizomenon*-based products were between 0.43 and 10.89 µg/g, whereas those in *Spirulina*-based products ranged from 0.15 to 0.52 µg/g, with one sample containing 2.12 µg/g (Gilroy et al., 2000). Ortelli et al. (2008) found that 6/9 samples of *Aphanizomenon flos-aquae* supplements contained MC-LR and MC-LA at up to 4 µg/g, whereas 0/10 samples of *Spirulina* contained microcystins. According to Jiang et al. (2008), 34/36 of *Spirulina* supplements obtained from various retail outlets in China tested positive for microcystins, at concentrations ranging from 2 to 163 ng/g (0.002–0.163 µg/g). In Canada, licensing of natural health products that contain cyanobacteria must adhere to a finished product tolerance limit of 0.02 µg MC-LR/kg body weight (bw) per day or a raw material tolerance limit of 1 part per million (ppm) (Health Canada, 2015).

## 6.0 Analytical methods

Cyanobacteria are a normal component of aquatic phytoplankton, with many species occurring in fresh waters. Cyanobacteria have the capacity to produce toxins which can cause negative health effects in humans (Funari and Testai, 2008). A cyanobacterial bloom can consist of a mix of species, each of which may or may not produce toxins.

There is no direct relationship between the number of cells found in the source water and cyanotoxin levels. Cell amounts should only be considered indicative of the possibility of bloom development, and by extension, the potential for the presence of toxins. For health risk assessment it is necessary to determine the total microcystins (intra and extracellular) present.

Monitoring cell counts or biovolume estimates in source waters, when integrated with toxin information, can contribute to a better understanding of treatment requirements, potential adjustment needs for plant operations and removal efficiencies.

### 6.1 Detection and measurement of cyanobacteria in source water

Traditionally, the most common laboratory methods for monitoring of cyanobacteria involve counting cells and/or biomass measurements, and the measurement of amounts of photosynthetic pigments such as chlorophyll-a (AWWA, 2010; Zamyadi et al., 2012a).

Cell counts can be obtained by collecting a field sample and analyzing a portion of that sample via specialized counting chambers under a microscope. Benefits of cell counting are that it is a direct enumeration method that provides information on the identity of the cyanobacterial species present. A drawback associated with the method that should be noted is that providing accurate counts can be difficult for untrained individuals. Cyanobacteria cells come in different shapes and sizes (from round to filamentous); groups of cells can exist as dense colonies or as long filaments; and populations can be composed of a mixture of these cell types and groups. The use of a trained microscopist with experience in identifying cyanobacteria is favourable when performing cell counts. Standard Methods for the Examination of Water and Wastewater (APHA et al., 2012) contains procedures for the enumeration of cyanobacteria (as phytoplankton) in water. Because cell sizes can vary considerably within and between species, and toxin concentration relates more closely to the amount of cyanobacterial material than to the number of cells, biovolume estimates are often calculated as a more ideal measure of population size or potential toxicity (Chorus and Bartram, 1999). These are calculated by obtaining reference values for cell volume for each of the species present and multiplying these by the corresponding cell numbers present as determined from cell counts (Chorus and Bartram, 1999).

The use of spectral imaging of intracellular photosynthetic pigments for the detection of chlorophyll-containing algae and cyanobacteria has been recognized by fresh and marine water researchers for some time (Chang et al., 2012). Traditional methods of pigment analysis have involved a somewhat laborious process involving sample collection, filtration, pigment extraction and absorbance or fluorescence analysis (Chorus and Bartram, 1999). Recently, methods based on in-place monitoring of fluorescence of photosynthetic pigments have received support as a suitable tool for rapid estimation of cyanobacteria biomass at drinking water treatment plant intakes (Gregor et al., 2007; Izydorczyk et al., 2009; AWWA, 2010; McQuaid et al., 2011).

Chlorophyll-a measurements have long been used as a surrogate indicator of total phytoplankton values. Since cyanobacteria make up a portion of total phytoplankton, measurements of this pigment have also been utilized as a rough indicator of cyanobacteria density. Chlorophyll-a analysis does not permit the distinguishing of cyanobacteria from other phytoplankton in mixed populations, however. Therefore, detection methods based on this pigment are more useful where cyanobacteria dominate total phytoplankton counts, or when the taxonomy of the population is verified alongside the pigment measurements. Izydorczyk et al., (2009) observed a statistically significant correlation between cyanobacterial biovolume and concentration of chlorophyll-a measured by an online fluorescence analyzer at a local drinking water intake when the phytoplankton was dominated by *Microcystis aeruginosa*. Those findings were used by the researchers to establish threshold values for use in an early warning method for cyanobacteria at that facility (Izydorczyk et al., 2009).

Because of the limitations of chlorophyll-a as a diagnostic parameter for cyanobacteria, attention has been focussed on phycocyanin for its standing as the most common photosynthetic accessory pigment to chlorophyll, and the most specific pigment present in cyanobacteria cells (Cagnard et al., 2006).

McQuaid et al (2011) demonstrated the usefulness of a phycocyanin fluorescence probe for monitoring cyanobacterial biovolume at the intake of a drinking water treatment plant intake. Probe-measured phycocyanin fluorescence was significantly correlated with cyanobacterial biovolume ($r = 0.68$, $p < 0.01$), and values provided an appropriate warning of the potential for toxin-containing cells at the plant's intake. Additional work by Zamyadi et al., (2012a) at two Quebec drinking water treatment plants known to be susceptible to blooms, further validated the success of phycocyanin probes for rapid assessment of the occurrence of cyanobacteria in drinking water sources. Other groups have similarly shown successful implementation of phycoyanin fluorescence probes for cyanobacterial assessment at drinking water treatment plant intakes (Gregor et al., 2007; Song et al., 2013). Researchers have identified scenarios that could contribute to interference and/or false readings, include: high proportion of specific phycocyanin-containing algae, high turbidity levels, growth stage and depth of the cyanobacteria (Gregor et al., 2007; McQuaid et al., 2011; Chang et al., 2012). As a result, the importance of site-specific calibration and complementary taxonomic and turbidity monitoring has been emphasized (McQuaid et al., 2011; Chang et al., 2012).

Since online methods for both pigments have shown individual success, naturally it has been suggested that having both phycocyanin and chlorophyll-a probes in place in a drinking water intake would improve the accuracy of monitoring and management response for drinking water sources in which blooms from multiple types of phytoplankton are likely (Cagnard et al., 2006; Zamyadi et al., 2012a).

The use of satellite remote sensing imagery to detect radiance of chlorophyll-a and phycocyanin in mass developments of cyanobacteria has also been employed as a tool for large scale tracking and detection of blooms. The National Oceanic and Atmospheric Administration

(NOAA) began monitoring cyanobacterial blooms in Lake Erie using high resolution satellite imagery since 2008 (Wynne et al., 2013). Imagery from the Medium Resolution Imaging Spectrometer (MERIS) on board the European Space Agency satellite, Envisat-1 provides data on Lake Erie approximately 4–5 times per week. Data from the images are processed through algorithms to produce an index value for cyanobacteria, and forecasts of bloom movement are developed through the use of specifically-developed hydrodynamic models. These forecasts are then distributed as bulletins to key environment and health officials and drinking water utility managers (Wynne et al., 2012). Wheeler et al. (2012) similarly reported on the successful use of Envisat's MERIS imagery in combination with algorithms for data retrieval and atmospheric correction to produce timely assessments of cyanobacterial blooms in the Missisquoi Bay of Lake Champlain, Vermont.

There are obstacles that have so far hindered the integration of remote sensing into current water quality monitoring programs. The number of satellite sensors available for this purpose is small; and because of sensor resolution limits, detection is primarily suited for large blooms in large water bodies. Nevertheless, for some utilities and authorities, this technology can present a valuable early warning method capable of significantly aiding water quality management and public health protection (Wheeler et al., 2012).

## 6.2 Detection and measurement of microcystins

Monitoring drinking water sources to assess the presence of cyanotoxins can be difficult, as cyanobacterial blooms can contain complex mixtures of microcystins as well as other classes of cyanotoxins (Spoof et al., 2003; Kaushik and Balasubramanian, 2013). In addition, there is no single method available that can identify and quantify all of the different types of toxins and their variants simultaneously (Merel et al., 2013). This section focuses on the analytical methods available for the measurement of microcystins. Detailed information on the analysis of other cyanotoxins, including cylindrospermopsin and anatoxin-a, is available in Nicholson and Burch (2001), Meriluoto and Codd (2005), Westrick et al. (2010a), de la Cruz et al. (2013) and Kaushik and Balasubramanian (2013).

Over the last decade, comprehensive research has been conducted on the analysis of microcystins, which has subsequently been reviewed in several reports and papers (Nicholson and Burch, 2001; Spoof, 2005; Westrick et al., 2010a; Kaushik and Balasubramanian, 2013; Merel et al., 2013). However, the analysis of microcystins in drinking water remains an area of ongoing research, and only two methods have been published by standards development or government organizations (ISO, 2005; Shoemaker et al., 2015). Recognizing the need for a standardized approach, standard operating procedures and quality control guidance have been developed (Meriluoto and Codd, 2005; Papageorgiou et al., 2012). The limited availability of certified standards for accurate quantification of microcystins further complicates their analysis (Fastner et al., 2002). For these reasons, microcystin analyses should be conducted by an accredited laboratory and/or water utilities should discuss in detail the methodology and quality assurance/quality control (QA/QC) of the laboratory selected to conduct the analyses.

The analytical methods for microcystins that are currently being used in commercial and research laboratories include 1) physicochemical analysis by chromatographic separation (i.e., liquid chromatographic (LC) methods, such as high-performance liquid chromatography [HPLC] or ultra-high-performance liquid chromatography [UPLC]) and detection by either ultraviolet (UV) absorbance (photodiode array detector) or tandem mass spectrometry (MS/MS); 2) enzyme linked immunosorbent assay (ELISA); and 3) protein phosphatase inhibition assays (PPIA). In general, monitoring of microcystins is a multi-step procedure that involves initial screening of

samples using biochemical methods, such as ELISA and PPIA, in the laboratory, with a portion of the samples analyzed using a physicochemical method (e.g., LC-MS/MS). The use of screening methods can provide cost-effective and more rapid results that can be used to aid in decision-making while cyanobacterial blooms are occurring, but they are not applicable for the identification and quantification of individual microcystins (Spoof, 2005; Nicholson et al., 2007; Triantis et al., 2010; Papageorgiou et al., 2012). When microcystins are detected in source or drinking water, a portion of subsequent samples should be analyzed using a physicochemical method (e.g., LC-MS/MS) so that the predominant variants in a bloom can be identified and quantified and potential low level microcystin concentrations can be measured. As discussed in section 6.8, depending on the type and quantity of variants present in the sample, results from biochemical analyses may not be directly comparable to results from physicochemical analyses (Graham et al., 2010). However, data obtained from the different methods is considered to be complementary and can be used to better understand the nature of a bloom, treatment plant performance and identify when low levels (<0.4 µg/L) of microcystins are present (Spoof et al., 2005, Triantis et al., 2010).

Owing to the lack of standardization for the majority of methods used for microcystin analysis, there are no statistical data available to determine the practical quantitation level (PQL) achievable by a wide variety of laboratories. For reporting purposes, laboratories typically use a limit of quantification (LOQ) or minimum reporting level (MRL) to indicate the lowest concentration of an analyte that can be determined with an acceptable level of accuracy and precision. Method detection limits (MDLs) that represent the minimum concentration of an analyte that can be measured and reported with confidence that the concentration is greater than zero are also often reported by laboratories. LOQs and MDLs will vary by laboratory and by analytical method. However, they can be useful when assessing the feasibility of using a specific analytical method for compliance testing or decision-making.

Where available, LOQs and MDLs for the methods reported in the literature for microcystin analysis are provided in the relevant sections below. With the appropriate sample pretreatment, the LOQs for most of the laboratory methods reported (except HPLC-UV and field test kits) range from 0.05 to 0.4 µg/L. The MDLs range from 0.002 to 0.1 µg/L. Overall, most of the analytical methods are capable of measuring microcystin concentrations of 0.4 µg/L or greater. Therefore, determining whether microcystins are present in drinking water at concentrations equal to the MAC should be feasible for drinking water utilities.

As discussed in Section 5.1.2, dissolved microcystins in the water outside of cyanobacterial cells are defined as extracellular microcystins. Extracellular microcystins typically make up less than 20% of the total microcystin concentration in source water. Most of the toxin is stored within the cyanobacterial cells (intracellular), and release into the water occurs largely when the cells rupture or die. In addition, several different variants of microcystins may be present in a bloom and co-occur with nuisance taste- and odour-causing compounds (Graham et al., 2010). Both intracellular and extracellular microcystins may also be present in treated water, depending on the type of treatment processes in place (Zamyadi et al., 2012a). Therefore, it is important to note that analysis for microcystins should be for total microcystins, which should include all of the measurable microcystin variants, not only MC-LR, that are dissolved in the water as well as bound to or inside cyanobacterial cells.

### 6.3 Sample preparation

The procedures used for sample collection and preservation for microcystin analysis vary depending on the water type (raw or finished drinking water), the type of analytical data required

(total versus dissolved microcystin concentration) and the method used for analysis. Utilities should obtain specific instructions from the laboratory on sample collection, preservation and shipping. General recommendations on sample collection and preservation are provided in Nicholson and Burch (2001), Metcalfe et al., (2005), Graham et al. (2008) and Newcombe (2009). Considerations include how the sample is collected (e.g., grab, composite, discrete depth), sample preservation (i.e., antimicrobial, dechlorinating, buffering or binding inhibition agents) as well as storage and shipping (e.g., temperature, holding times). In general, samples for microcystin analysis should be collected in amber glass bottles as microcystins have been shown to sorb to polypropylene (Graham et al., 2008). Sample preservation is also an important consideration for both raw and finished drinking water samples, as microcystins are degraded both microbially and photochemically. The addition of an antimicrobial preservative may be necessary, along with storage of samples in dark cold conditions. The analysis of finished drinking water also requires sample preservation due to the potential for oxidation of microcystins and/or interferences from disinfectants. Studies have shown that certain microcystins are destroyed in the presence of chlorine and the use of quenchers is needed (Ho et al., 2006a; Froscio et al., 2010). However, care must be taken when adding quenchers to samples as Froscio et al. (2010) found that sodium thiosulphate, sodium sulfite and taurine all affected analysis using ELISA, whereas ascorbic acid did not affect the assay results.

Analysis of microcystins generally requires extensive sample preparation in the laboratory, which will vary depending on the form of the microcystin (total, intracellular or dissolved), the variants to be analyzed and the specific analytical method to be used. In general, sample preparation includes one or more of the following steps: cell concentration, cell lysis, microcystin extraction and concentration and clean-up (Nicholson and Burch, 2001; Westrick et al., 2010a). Current analytical methods are capable of measuring only dissolved microcystins. Therefore, when determining total microcystin concentrations, sample preparation must include cell lysis and extraction so that the intracellular toxins can be quantified. The efficiency of the processes used for sample preparation, including extraction and concentration, has been shown to vary greatly between laboratories and can impact the accurate quantification of microcystins in a sample (Lawton and Edwards, 2001; Fastner et al., 2002). Therefore, it is important for responsible authorities to ensure that laboratories are validating the methods chosen for sample preparation.

As discussed above, analysis of total microcystins is needed for comparison with the guideline. When treatment is needed for removal of microcystins, utilities also need to have an understanding of the amounts that are present in both the intracellular and dissolved (extracellular) forms. This information will help the utility adjust treatment processes or determine whether additional treatment is needed. In this case, two samples should be taken, with one processed for total microcystin analysis and the other filtered in the laboratory or field and then analyzed for dissolved microcystins (extracellular). Samples should be carefully filtered to minimize cell damage so that the microcystin concentration in the filtrate is representative of the dissolved fraction. The intracellular concentration is the difference between the total and dissolved microcystin concentrations.

### *6.3.1 Extraction of intracellular microcystins*

Extraction of intracellular microcystins begins with isolating and concentrating the cells using filtration or centrifugation. The cell walls must then be ruptured or lysed in order to expose the intracellular microcystins. Cell lysis can be achieved by a variety of methods, including sequential freeze-thawing, freeze-drying, mechanical or sonic homogenization, boiling,

autoclaving or chemical/enzymatic extraction (Lawton et al., 1994; Spoof, 2005; Sangolkar et al., 2006; Westrick et al., 2010a). Freeze-drying and freeze-thawing are the most commonly reported methods for cell lysis. However, there is no consensus in the literature on the most effective method (Spoof, 2005; Westrick et al., 2010a). Rapala et al. (2002) observed that a high number of intact cells were present following several freeze-thaw cycles and bath sonication and that heating at 50°C interfered with subsequent analyses using PPIA. The results indicated that probe sonication was the most effective method to disrupt cells and release toxins. Other researchers have also reported that freeze-thawing followed by probe sonication is the most effective cell disruption method for microcystins, but cautioned that prolonged use of sonicators may result in evaporation or degradation of microcystins (Ramanan et al., 2000; Spoof et al., 2003). In addition, a proprietary set of reagents has been developed for rapidly lysing cyanobacterial cells. Loftin et al. (2008) compared the use of a commercial lysing kit and sequential freeze-thaw for use with microplate ELISA and LC-MS/MS analytical methods. The study found that there was no statistically significant difference between the cell lysis techniques for determining microcystin concentrations in natural waters. In contrast, Aranda-Rodriguez and Jin (2011) found that use of a commercial lysing agent on samples analyzed using a PPIA test kit caused interferences and could not be used on natural water samples.

Following cell lysis, microcystins need to be extracted for analysis, with typical solvents used being dilute acid, aqueous methanol, acidified methanol/water mixtures and butanol/methanol/water mixtures (Harada et al., 1988, 1996; Lawton et al., 1994; Fastner et al., 1998; Spoof et al., 2003; Barco et al., 2005). Extraction efficiency has been shown to vary depending on the type of solvent, the hydrophobicity of the variant, the water content of the cells (freeze-dried versus frozen) and differences between field samples and laboratory cultures (Lawton et al., 1994; Fastner et al., 1998; Lawton and Edwards, 2001; Barco et al., 2005). Lawton et al. (1994) noted that extraction efficiencies of MC-LR, MC-LY, MC-LW, MC-LF and MC-RR were good using methanol or a butanol/methanol/water mixture, but that 5% acetic acid was not effective, particularly for the more hydrophobic variants (e.g., MC-LW). Fastner et al. (1998) found that 75% methanol or sequential extractions with methanol followed by water were the most effective for the extraction of lyophilized cyanobacteria from field samples. Spoof et al. (2003) found that 75% methanol was also effective on freeze-thawed samples. More recently, Barco et al. (2005) studied the effectiveness of different solvents, pH, volume and time on the extraction of various microcystins. The results indicated that sonication with acidified methanol (pH 2) was the most rapid and efficient method for the routine analysis of a wide range of intracellular microcystins. Research has also been conducted to optimize intracellular microcystin extraction where analysis is conducted using PPIA. The most effective protocol was filtration with a nylon filter followed by extraction using 80% methanol, 0.1% trifluoroacetic acid and 0.1% Tween 20® (polyoxyethylene sorbitol ester) with 30 minutes of stirring. This study did not evaluate extraction methods for physicochemical methods such as LC-MS/MS (Sevilla et al., 2009).

Variations in extraction efficiency impact the accurate quantitation of total microcystins. Fastner et al. (2002) conducted an international interlaboratory study comparing microcystin analytical methods. The results indicated that the reproducibility of the analysis of field samples was significantly lower than that of the standard solution. In addition, several laboratories found 1.5–3 times more total microcystins than did other laboratories when analyzing field samples. The authors concluded that this variability was partially due to variations in the extraction efficiencies between the laboratories, particularly for variants other than MC-LR (Fastner et al., 2002). Given the variations in cell lysis and extraction efficiency discussed above, responsible authorities

should ensure that the appropriate methods are being used in the laboratory depending on the type of sample, the analytical method selected and the microcystins being investigated (Lawton and Edwards, 2001).

### *6.3.2 Concentration and cleanup*

At low concentrations, the direct determination of dissolved microcystins may not be feasible, and a preconcentration step may be required. In particular, HPLC-UV requires preconcentration in order to achieve limits of detection in the microgram and nanogram per litre range. Typically, dissolved microcystin analysis samples are filtered and/or centrifuged to remove cells and particulates, followed by freeze-drying, solid-phase extraction (SPE) and immunoaffinity chromatography to concentrate the particulate free samples (Westrick et al., 2010a).

In SPE, the sample is passed through a cartridge that adsorbs microcystins, which are subsequently eluted from the cartridge using solvents such as methanol. This process can be used to both concentrate microcystins and selectively eliminate interferences. Alkyl bonded silicas are commonly used in the cartridges, and several are available commercially (Nicholson and Burch, 2001; Westrick et al., 2010a). One of the limitations of the SPE step is that the extraction efficiency varies depending on the cartridge type, sample matrix and microcystins present (ISO, 2005; Spoof, 2005). In general, the extraction efficiency of MC-LR has been reported to be high (75–85%), whereas the more hydrophobic variants, such as MC-LA and MC-LW, are reported to have lower percent recoveries (Lawton et al., 1994; Papageorgiou et al., 2012). Papageorgiou et al. (2012) evaluated the performance of several commercial SPE cartridges for MC-LR concentration and found that $C_{18}$ and polymer-based cartridges were the most effective. Immunoaffinity cartridges have been reported to give much cleaner extracts, free of interferences, and high recovery compared with conventional $C_{18}$ SPE, which enables better detection and identification of microcystins (Kondo et al., 2000; Aranda-Rodriguez et al., 2003). However, these columns are limited by the amounts and stability of the antibodies needed for the columns (Spoof, 2005).

Another consideration is that many of the $C_{18}$ cartridges also extract the natural organic matter (NOM) present in water, which can interfere in the chromatographic separation of microcystins (Nicholson and Burch, 2001). In addition, the presence of NOM can also cause ion suppression or enhancement in LC-MS and other quantitation problems in HPLC-UV (Nicholson and Burch, 2001). Tsuji et al. (1994) developed a two-step method, with SPE on a $C_{18}$ cartridge followed by a silica gel cartridge to minimize interferences. Non-polar polymer sorbents have also been shown to concentrate microcystins from water effectively (Rapala et al., 2002).

To date, there is no consensus on the most effective approach for preparing microcystin samples for analysis (extraction, concentration and separation). This is attributed to different goals in sample analysis, such as analysis of only one or two key microcystins versus characterizing several microcystins present in a sample (Lawton and Edwards, 2001). It is critical for laboratories to validate the methods chosen to ensure that adequate recovery of microcystins is achieved.

## 6.4 Immunological and biochemical assays

Several immunological and biochemical assays have been developed for the determination of microcystins in water. These methods are widely used as screening tools for estimating total microcystin concentrations in drinking water, as they are quick, are relatively inexpensive and do not require extensive analytical expertise (Codd et al., 2001; Metcalf and Codd, 2003; Spoof,

2005; Nicholson et al., 2007; Westrick et al., 2010a). The two most commonly used assays for the determination of microcystins are ELISA and PPIA, which can be purchased as commercial kits. In cases where utilities choose to perform analyses at the treatment plant, it is recommended that the analyses be conducted by a well-trained technician or analyst and that duplicate samples be sent periodically to an accredited laboratory to confirm the on-site analyses.

### *6.4.1 Enzyme-linked immunosorbent assays*

Immunoassays have been developed that detect microcystins in a sample through recognizing and binding of an antibody (isolated against a microcystin) with antigens (e.g., microcystins) present in the sample. A variety of antibodies have been isolated against MC-LR (Chu et al., 1989; Nagata et al., 1995) and MC-RR (Young et al., 2006) as well as recombinant antibody fragments (McElhiney et al., 2000) and antibodies against the amino acid ADDA, which is a common component of microcystins (Fisher et al., 2001; Zeck et al., 2001). These antibodies have been incorporated into various ELISA systems as in-house laboratory methods (Chu et al., 1990; An and Carmichael, 1994; Nagata et al., 1997; Metcalf et al., 2000a; Fisher et al., 2001; Zeck et al., 2001; Kim et al., 2003; Young et al., 2006; Sheng et al., 2007). Commercial ELISA kits that contain all of the reagents needed for analysis have also been developed. Several reports have been published that provide information on the type of ELISA, type of antibody and target antigen, as well as the detection limits of commercial ELISAs (Nicholson et al., 2007; Westrick et al., 2010a; Weller, 2013).

ELISAs have been shown to be highly sensitive for the detection of microcystins; however, they have variable degrees of cross-reactivity towards different variants, and they cannot identify which variants are present in a sample. As individual variants are not identified and quantified, the results of ELISA are reported as MC-LR equivalents ($MC\text{-}LR_{equiv}$). Several authors have suggested that ELISAs should be viewed as producing semi-quantitative results, as their accuracy will depend on the microcystins present, their cross-reactivities relative to the standard used for quantification and susceptibility of the sample to interferences (Nicholson and Burch, 2001; McElhiney and Lawton, 2005).

The ability of ELISAs to successfully screen of microcystins is dependent on the ability of the antibodies used in the system to recognize all or most of the microcystins present in the sample (McElhiney and Lawton, 2005). The cross-reactivity of different variants using various in-house laboratory ELISA systems has been shown to vary greatly (Chu et al., 1990; An and Carmichael, 1994; Nagata et al., 1995; Fisher et al., 2001; Rapala et al., 2002; Nicholson et al., 2007). For example, Young et al. (2006) reported a cross-reactivity of MC-RR of 37% using a polyclonal antibody isolated against MC-LR compared with 96% reported by Zeck et al. (2001) using antibodies targeted against microcystins containing an arginine at position 4. In comparison, Zeck et al. (2001) reported low cross-reactivity for MC-LA, whereas Metcalf et al. (2000a) reported a high cross-reactivity for MC-LA.

Commercial ELISA microplate kits typically provide a cross-reactivity chart for some of the variants (e.g., MC-LR, MC-RR, MC-YR) that are commonly found in water. These range from 50% to 85% for MC-RR, from 35% to 181% for MC-YR and from 10% to 124% for MC-LA. Nicholson et al. (2007) conducted an independent study of the cross-reactivity of commercial ELISA kits. The results were variable, with some kits successfully measuring MC-RR and MC-LA but not MC-YR and others successfully measuring MC-RR and MC-YR but not MC-LA. The authors concluded that given the potential variability of variants between samples and the difference in variant cross-reactivity, ELISA is a less accurate method than instrumental methods such as LC-MS/MS.

The MDLs of MC-LR using in-house laboratory ELISA methods reported in the literature range from 0.01 to 0.5 µg/L (Chu et al., 1990; An and Carmichael, 1994; Metcalf et al., 2000a; Pyo et al., 2005; Young et al., 2006). More sensitive methods with MDLs between 4 and 8 ng/L have been developed by Zeck et al. (2001) and Linder et al. (2004) using a specific monoclonal antibody isolated against microcystins containing arginine in position 4. The MDLs of several commercial laboratory ELISA kits have been reported by Nicholson et al. (2007) and Weller (2013), with values ranging from 0.04 to 0.2 µg/L for MC-LR. Young et al. (2006) reported an LOQ of 0.31 nM (0.31 µg/L) and Fisher et al. (2001) reported an LOQ of 0.05 µg/L for MC-LR using in-house laboratory ELISA methods. Commercial ELISA microplate kits generally have quantitation ranges from 0.2 µg/L (LOQ) to an upper limit of 5 µg/L (Nicholson et al., 2007; Westrick et al., 2010a). Recently, two high-sensitivity ELISA microplate kits have become commercially available for analysis of drinking water. These kits have MDLs that range from 0.04 to 0.05 µg/L (Weller, 2013). It should be noted that most of the commercial laboratory ELISA microplate kits have MDLs between 0.15 and 0.2 µg/L; therefore, if lower detection limits are needed, high-sensitivity kits need to be used for analysis.

Several studies have found that naturally occurring water contaminants such as NOM, metals, and salts as well as chemicals used for sample preservation or preparation can interfere in the results obtained using ELISA (Metcalf et al., 2000a; Rapala et al., 2002; Froscio et al., 2010, de la Cruz et al., 2012). de la Cruz et al. (2012) found that a low pH (<3) or the presence of 250 µg/mL of calcium or magnesium, 0.01% of ascorbic acid or 0.10% EDTA chelating agent inhibited MC-LR detection using microplate ELISA. The presence of these contaminants inhibited the detection of 0.75 ng/mL by 15% to 59%. The authors also reported that different sources of NOM at concentrations between 1- 50 µg/mL did not affect the microplate system; however, it did interfere with the detection of MC-LR. Overall, increasing amounts ( 1 – 50 µg/mL) of NOM caused increased inhibition of MC-LR detection. Metcalf et al. (2000a) found that increasing methanol concentrations (used to extract intracellular microcystins) resulted in the removal of MC-LR from the ELISA plate and interfered with the accurate quantification of microcystins in the sample. Results showed that dilution to 7% methanol prior to analysis was necessary to decrease the interference. Froscio et al. (2010) studied the impact of quenchers used to preserve finished drinking water samples containing chlorine on the results obtained using a commercial ELISA kit. The authors found that sodium thiosulphate, sodium sulphite and taurine all affected the assay and led to an overestimation of the microcystin concentration in the sample. Ascorbic acid did not affect the assay results. The potential for interferences varies depending on the type of ELISA, and therefore utilities need to check with the laboratories conducting their analyses to ensure that the appropriate quencher is being added to drinking water samples.

As many laboratories in Canada are using ELISA methods for the routine analysis of microcystins, it is important for responsible authorities to have an understanding of the cross-reactivity, sensitivity, matrix interferences and detection limits of the type of ELISA system being used so that the limitations of the analytical results can be considered in the decision-making process. In some cases, enhanced-sensitivity ELISA methods with detection limits equal to or below 0.05 µg/L may be necessary when the potential presence of low levels (<0.4 µg/L) of microcystins needs to be determined.

### *6.4.2 Protein phosphatase inhibition assays*

The ability of microcystins to inhibit certain serine/threonine (Ser/Thr) protein phosphatases (protein phosphatase 2A [PP2A] and 1 [PP1]) has led to the development of various assays for the detection and quantification of microcystins (Bouaicha et al., 2002). The basis of

PPIAs is the measurement of phosphate release from phosphorylated protein in the presence of a phosphatase enzyme preparation and an inhibitor such as microcystin (Nicholson and Burch, 2001; McElhiney and Lawton, 2005; Westrick et al., 2010a). A variety of detection methods (radiometric, colorimetric, etc.) and substrates can be used to perform this assay. These include radioactive detection assays using $^{32}$P-labelled substrates (Lambert et al., 1994; Xu et al., 2000a) and colorimetric assays using *p*-nitrophenol phosphate as the substrate (An and Carmichael, 1994; Ward et al., 1997; Wirsing et al., 1999; Heresztyn and Nicholson, 2001). The method has also been adopted for fluorescence measurements using the substrates methylumbelliferyl phosphate (Bouaicha et al., 2002; Mountford et al., 2005) and difluoromethylumbelliferyl phosphate (Fontal et al., 1999).

As with ELISAs, PPIAs are sensitive, rapid and accessible due to the commercial availability of enzymes; in most cases, it is not necessary to preconcentrate samples, regardless of the sample matrix. However, the assays do not show the same sensitivity for all microcystin variants and are not capable of identifying which microcystin variants are present in a sample (Heresztyn and Nicholson, 2001; McElhiney and Lawton, 2005). In addition, it is possible for reactions to occur with non-specific phosphatases in the sample or with endogenous protein phosphatases, resulting in an underestimation of the quantity of toxins (An and Carmichael, 1994). Other limitations of PPIAs include the complexity of preparing the protein substrate and the expense of commercially available enzymes (Kaushik and Balasubramanian, 2013).

The detection limit of total microcystins, reported as MC-LR equivalents (MC-LR$_{equiv}$), using radiometric protein phosphatase assays is approximately 0.1 µg/L or less. Lambert et al. (1994) reported an MDL of 0.1 µg/L for MC-LR using a PP1c radiometric assay. Xu et al. (2000a) achieved a lower MDL of 0.02 µg/L using PP2A. However, radiometric assays are not commonly used, as many laboratories are not equipped to prepare radiolabelled proteins and carry out radioactive measurements (Nicholson and Burch, 2001). Early work using colorimetric PP1 inhibition assays reported relatively high detection limits between 10 and 20 ng/mL (Ward et al., 1997; Wirsing et al., 1999). Rivasseau et al. (1999) developed a colorimetric assay using commercially available materials (PP2A, microcystins, etc.) with an MDL of 0.03 µg/L and a quantitation range of 0.4–10 µg/L. The method includes additional sample preparation to increase the accuracy at lower concentrations, which results in a lower quantitation range of 0.2–0.8 µg/L. Similarly, Heresztyn and Nicholson (2001) developed a colorimetric assay using PP2A with a quantitation range of 0.2–1 µg/L. Wong et al. (1999) found a similar MDL of approximately 0.1 ng/mL (0.1 µg/L). More recently, Bouaicha et al. (2002) employed fluorogenic substrates to detect microcystins in drinking water, resulting in a 2-fold increase in assay sensitivity compared with colorimetric assays. The authors reported an MDL of 0.25 µg/L using the colorimetric method and 0.1 µg/L using the fluorometric method.

Dominguez (2015) reported the development of a commercial colorimetric PPIA kit that contains all of the reagents needed for analysis. The standard available with the kit is for MC-LR and, therefore, results are reported as MC-LR equivalents. The method includes analysis for intracellular (following a cell lysis procedure) and dissolved microcystins. The MDL and LOQ are respectively 0.08 µg/L and 0.19 µg/L, based on testing conducted on natural water. The method was validated by examining the repeatability, intralaboratory reproducibility and accuracy on natural and drinking water samples (James et al., 2011; Dominguez, 2015).

PPIAs are subject to matrix interferences from a variety of parameters, including iron, manganese and other protein phosphatase inhibitors. In addition, careful consideration needs to be given to the minerals added to the assay medium. Wong et al. (1999) noted that the absence of manganese in the assay medium significantly suppressed PP2A activity. Oliveira et al. (2005)

found that drinking water with chlorine, iron and aluminum significantly affected the ability of PPIAs to accurately detect microcystins. The authors reported a significant reduction in the quantitation of a mixture of microcystins (80% MC-LR) due to either decomposition (oxidation) or complexation with the metal ions.

### 6.5 Field test kits

Rapid tests for the identification of the presence of microcystins in water have been developed for use in the field. Field test kits can be used as a presence/absence tool for determining if a bloom is toxic or if treatment plant operations need to be adjusted during a bloom event, but they are not quantitative analyses and cannot be used to determine whether treated drinking water meets the guideline value. Commercially available test kits use a variety of methods, including immunochromatography, ELISA and protein phosphatase inhibition, to estimate the level of microcystins in a water sample. The applicability of field test kits ranges between manufacturer but is generally between 1 and 5 µg/L of microcystins. Several field test kits do not include a lysing agent and therefore determine only the presence of extracellular (dissolved) microcystins. When using these field test kits, users need to purchase a lysing agent and release the intracellular microcystins in order to determine the level of total microcystins. However, some lysing agents may interfere with the test method (Aranda-Rodriguez and Jin, 2011).

Limited studies have been published examining the use of field test kits for determining the presence of microcystins in laboratory and natural water (Humpage et al., 2010; Lawton et al., 2010; Aranda-Rodriguez et al., 2015). In general, available information indicates that test kits are a useful tool for rapidly determining the presence or absence of microcystins in a bloom but are not suitable for compliance based decision-making (Humpage et al., 2010).

Aranda-Rodriguez et al. (2015) evaluated three commercially available field test kits and compared the results with data obtained using laboratory ELISA and LC-MS/MS methods. At a threshold concentration of 1 µg/L, the strip test analysis showed a false positive rate of 38% and a false negative rate 2%, indicating that this test can be used to provide qualitative data on the presence/absence of microcystins at this value. The tube test used in this study was designed to indicate if a sample concentration is less than 0.5 µg/L, between 0.5 and 3.0 µg/L, or above 3.0 µg/L. Analysis of data from this kit indicated a false negative rate of 0% for concentrations less than 0.5 µg/L. In contrast, a false positive rate of 80% for the range between 0.5 and 3 µg/L was reported. The authors concluded that the tube kit may not be suitable for monitoring water supplies at a threshold value of 1 µg/L due to difficulties in interpreting the test results between 0.5 and 3 µg/L. The authors also cautioned that users need to clearly understand the scope of the kit being used, including knowledge of the concentration range, the concentration at which a response is observable, limitations on the quantification, the type of measurement obtained (i.e., total or extracellular) and most importantly, how the results are interpreted.

Humpage et al. (2012) tested the accuracy, precision, cross-reactivity, matrix effects and interoperator variability of two ELISA-based strip tests for microcystin monitoring: one kit designed for recreational water testing (detection range of 1 to 10 µg/L) and the other kit designed for drinking water testing (detection range of 0.5 to 5 µg/L). Testing using both kits indicated that interoperator variability was relatively high, with 78% of strip tests read correctly when operators were trained and only 58% of strips read correctly by untrained operators. Comparison of the drinking water strip test results for reservoir water samples spiked with 1.0, 2.5 and 5.0 µg/L of MC-LR indicated that the test responded correctly around its target range of 1 µg/L ($MC\text{-}LR_{equiv}$). Overall, the authors concluded that strip tests can provide a rapid indication of the presence of

microcystins in a water supply and assist with day-to-day operational decision-making. However, owing to limitations in the accuracy and precision of strip tests, compliance testing or longer-term decision-making should be conducted using accurate quantitative analytical methods.

## 6.6 Physicochemical methods

There are a number of analytical techniques available that can accurately quantify and identify the most common microcystin variants that may be present in water samples (McElhiney and Lawton, 2005). Physicochemical methods for microcystin analysis typically rely on two steps: separation of the compounds present in a sample by chromatography, followed by their quantification using various detectors. To date, the most widely used methods for both research and routine analysis are based on reversed-phase HPLC methods coupled with MS or UV detectors (Meriluoto, 1997; Rapala et al., 2002; McElhiney and Lawton, 2005; Tillmanns et al., 2007). Although these methods can provide detailed information, they often require expensive equipment, highly trained personnel and lengthy analysis times.

### *6.6.1 Liquid chromatography*

Separation techniques using LC are commonly used for microcystin analysis, as they allow the distinction of several co-occurring variants within a single analysis as well as the use of a wide range of detectors, including UV absorbance, fluorescence and MS. Limited gas chromatographic (GC) methods have also been developed. Toxins can be identified by comparing the separation profile of the sample with references obtained by the analysis of standards or purified compounds (Merel et al., 2013).

The separation of microcystin variants is largely dependent on the composition of the mobile phase and the stationary phase used in the analysis. Microcystins can be separated using both gradient elution and isocratic mobile phases, although a wider range of toxin variants can be resolved when a gradient is employed (McElhiney and Lawton, 2005). Detailed reviews of the chromatographic methods available for microcystins are provided in Meriluoto (1997), Spoof et al. (2001) and Spoof (2005).

HPLC is widely used to separate microcystins. A variety of stationary phases have been used, including reversed-phase $C_{18}$ columns, amide $C_{18}$ columns, internal surface reversed-phase columns or ion exchange columns (Gathercole and Thiel, 1987; Meriluoto and Eriksson, 1988; Lawton et al., 1994; Spoof et al., 2001). Mobile phases typically consist of water and methanol or acetonitrile, acidified with trifluoroacetic acid or formic acid (Spoof et al., 2003; Ortea et al., 2004; Barco et al., 2005). Careful selection of the mobile phase is needed to ensure a good resolution of analytes. For example, Lawton et al. (1994) found that MC-LR and MC-YR co-elute with an acetonitrile/ammonium acetate mobile phase, whereas a methanol-based mobile phase was effective for separation of five different microcystin variants.

Spoof (2005) conducted an extensive review of chromatographic systems used for microcystin separation. The author noted that methods reported in the literature could be separated into five categories: 1) neutral mobile phases with ammonium acetate and acetonitrile, reversed-phase chromatography; 2) acidic mobile phases with trifluoroacetic acid and acetonitrile, reversed-phase chromatography; 3) methanol-containing mobile phases with different buffers and pHs, reversed-phase chromatography; 4) other reversed-phase chromatographic systems, such as internal surface reversed-phase separations; and 5) alternative mobile and stationary phases, such as anion exchange chromatography. In general, acidic mobile phases are able to resolve more microcystins than neutral ones. Current HPLC analyses typically use aqueous trifluoroacetic acid–acetonitrile with gradients ranging between 25% and 70% acetonitrile, so that both

hydrophilic and hydrophobic microcystins can be separated (Spoof, 2005). However, other work has found that a neutral phase may be necessary to achieve adequate recoveries of MC-RR and MC-YR when NOM is present in the sample (Tettenhorst and Shoemaker, 2013).

More recently, UPLC has been used to separate MC-LR, MC-RR, MC-YR, MC-LY, MC-LW and MC-LF prior to detection using MS (Beltran et al., 2012). LC separation was performed using a $C_{18}$ column with a mobile phase of water, acetonitrile and 0.025% formic acid. A combination of online SPE combined with the lower chromatographic run time offered by UPLC led to an overall analysis time of a few minutes.

### *6.6.2 Detection with ultraviolet absorbance*

Ultraviolet/visible (UV) absorbance is a commonly used detection technique with HPLC (Nicholson and Burch, 2001; McElhiney and Lawton, 2005). Most microcystins have a UV absorbance maximum at 238 nm, with the exception of variants that contain tryptophan, such as MC-LW, which have absorbance maxima at a lower wavelength (222 nm) (Lawton et al., 1994). The wavelength of the UV detector can be set at these values to record the responses of microcystins in sample extracts separated on the HPLC. The retention time, UV spectra and peak area of commercially available or laboratory standards are the basis of identification and quantification of microcystins using HPLC-UV (Rapala et al., 2002). However, owing to the limited number of commercially available standards, the toxins are often identified by comparison with an MC-LR standard and reported in terms of MC-$LR_{equiv}$ (McElhiney and Lawton, 2005).

The ability of single-wavelength UV detectors to distinguish between several different variants can be limited, as most variants have similar absorption profiles between 200 and 300 nm (Sangolkar et al., 2006). More recent analytical methods have incorporated photodiode array (PDA) to generate a UV spectrum and provide better evidence of the presence of microcystins for which standards do not exist (Codd et al., 2001; Spoof, 2005). Several authors have reported effective HPLC-UV/PDA methods for the analysis of microcystins (Harada et al., 1988; Lawton et al., 1994; Rapala et al., 2002; Ortea et al., 2004; Barco et al., 2005; Tillmanns et al., 2007; Triantis et al., 2010; Spoof et al., 2010).

The advantage of using an HPLC-UV/PDA method is that the instrumentation is available in many laboratories, and its operation is easily automated and relatively inexpensive. The main limitations are that authentic standards that are necessary for accurate quantitation are difficult to obtain, and UV detection is susceptible to interferences (Ortea et al., 2004; Spoof, 2005). Studies have shown that NOM and additives leached from plastic laboratory materials can co-elute with microcystins and cause erroneous results (Nicholson and Burch, 2001; Rapala et al., 2002).

In addition, this technique is significantly less sensitive than other methods, such as ELISA and LC-MS/MS. Detection limits of HPLC-UV/PDA methods will depend partially on the sample volume and concentration factors. The typical detection limit in most HPLC-UV/PDA systems is a few nanograms per injection (20–30 µL injection volume) for pure samples, but several times higher for field samples (Meriluoto, 1997; Spoof, 2005). Therefore, substantial concentration and cleanup of samples with concentrations of 1 µg/L or less are necessary when HPLC-UV/PDA is being used for microcystin analysis.

The International Organization for Standardization (ISO) has validated a method for microcystin analysis in source and finished drinking water based on reversed-phase solid extraction to concentrate and clean up samples followed by quantification by HPLC with UV/PDA detection (ISO, 2005). The method was developed for analysis of MC-LR, MC-RR and MC-YR. The standard requires that the method have an MDL of 0.1 ng/µL and an LOQ of

0.2 ng/µL. Samples must therefore be concentrated prior to analysis to be able to quantify less than 1 µg/L of microcystins.

Papageorgiou et al. (2012) developed an operating protocol for HPLC-PDA analysis of microcystins based on the method developed by Lawton et al. (1994). The protocol includes recommendations on materials for microcystin extraction and concentration, HPLC-PDA operating conditions, injection volume, standard preparation and calibration. The authors determined that using the recommended procedures, the LOQ was 0.1 µg/mL (100 µg/L) for MC-LR and MC-YR and 0.5 µg/mL (500 µg/L) for MC-LA and MC-RR. Samples with concentrations below 1 µg/L were concentrated 500-fold prior to analysis.

### *6.6.3 Detection with mass spectrometry*

The use of LC-MS for the analysis of microcystins has become increasingly common over the last decade because of its high sensitivity and exceptional specificity, particularly when MS/MS techniques are used (Spoof et al., 2003; Merel et al., 2013). In MS, molecules are converted to ions, which are resolved by mass analyzers on the basis of mass and charge. Microcystins in a sample can be identified as long as a mass spectrum of an authentic standard is available. Tentative identification can also be made by comparing the sample mass spectrum with those described in the literature (Barco et al., 2002; Sangolkar et al., 2006). The use of more advanced MS/MS generates a fragmentation pattern that can greatly assist in determining the identities of unknown microcystins. In addition, the selectivity and sensitivity of microcystin analysis are higher using MS/MS; therefore, it is the preferred MS method (Yuan et al., 1999; Zweigenbaum et al., 2000; Zhang et al., 2004; Kaloudis et al., 2013).

There are several advantages to using LC-MS and LC-MS/MS systems for the analysis of microcystins. One of the primary advantages is the ability of LC-MS/MS to measure trace concentrations of individual microcystins. Typical detection limits for microcystins in modern LC-MS/MS systems are in the low picograms per injection range, and many studies have achieved detection limits of less than 0.02 µg/L following SPE (Spoof, 2005; Msgati et al., 2006; Yen et al., 2011; Kaloudis et al., 2013 Shoemaker et al., 2015) and without SPE (Graham et al., 2010). LC-MS/MS also allows the signal-to-noise ratio to be substantially improved in the detection of microcystins and has some potential for the identification and structural elucidation of microcystins when no analytical standards are available or when a new microcystin variant is present in a sample (Diehnelt et al., 2005; Kaloudis et al., 2013). In addition, the degree of LC separation required is less stringent when MS is used (compared with PDA), but it is still desirable to minimize matrix effects, and the ion chromatograms are simpler to interpret. However, as with the other analytical methods, LC-MS/MS is subject to co-eluting substances and mobile-phase interferences, which can cause ion suppression or enhancement effects—collectively termed matrix effects—that can be difficult to control in field samples (Spoof, 2005). Shoemaker et al. (2015) reported that matrix interferences during LC-MS/MS analysis may be caused by contaminants that are co-extracted from the sample. The extent of matrix interferences will vary considerably depending on the water quality characteristics of the sample. Humic and/or fulvic material can be co-extracted during SPE and high levels can cause signal enhancement and/or suppression in an electrospray ionization source. Also, high levels of humic and/or fulvic material can cause low recoveries on the SPE sorbent. Total organic carbon (TOC) is a good indicator of humic content of the sample. Single-point standard addition may be used to minimize the impact of matrix effects on quantitation but this can increase the length of analysis time and is slightly more costly (Graham et al., 2010, Shoemaker et al., 2015).

Various LC-MS and LC-MS/MS systems with different interface and ionization configurations have been reported to successfully identify and quantify microcystins in water (Kondo et al., 1992a; Tsuji et al., 1994; Lawton et al., 1994; Zweigenbaum et al., 2000; Spoof et al., 2003; Meriluoto et al., 2004; Ortea et al., 2004; Zhang et al., 2004; Triantis et al., 2010; Draper et al., 2013; Kaloudis et al., 2013; Shoemaker et al., 2015). Spoof (2005) provided a summary of the LC-MS conditions used for microcystin analysis in a large number of studies.

The use of electrospray ionization (ESI) made LC-MS more convenient to use and significantly increased the analytical sensitivity (Spoof, 2005). Poon et al. (1993) first developed LC-ESI-MS for the quantitative analysis of microcystins. Subsequently, several studies have demonstrated that LC-ESI-MS is a suitable technique for the monitoring and identification of a wide range of microcystins in environmental samples (Zweigenbaum et al., 2000; Barco et al., 2002; Diehnelt et al., 2005). Zweigenbaum et al. (2000) used microbore LC for the separation of microcystins from aqueous methanol extracts followed by ESI ion trap MS. The authors reported that for certain microcystins, an ion trap mass spectrometer provided better detection limits than a triple quadrupole instrument. A full-scan mass spectrum was obtained when a minimum of 250 pg (per 10 µL injection volume) of MC-LR was directly injected (without a concentration step) into the HPLC column. Barco et al. (2002) also successfully analyzed MC-LR, MC-RR and MC-YR using LC-ESI-MS. Samples were concentrated using SPE followed by LC using a 1 mm $C_{18}$ column and a water, acetonitrile and formic acid mobile phase. A quadrupole mass spectrometer with a coaxial electrospray source operated in the positive mode was used for analysis. The limits of detection were 11, 72 and 21 pg (per 5 µL injection) for MC-LR, MC-RR and MC-YR, respectively. Spoof et al. (2003) used LC-ESI-MS to screen environmental water samples for microcystins. Microcystins were separated using a reversed-phase $C_{18}$ column with a mobile phase of 0.5% formic acid and acetonitrile. A triple quadrupole mass spectrometer with ESI operated in the positive single ion recording mode was used for quantification. Microcystin concentrations equal to or greater than 0.2 µg/L were confirmed in water samples taken from a variety of freshwater and brackish locations.

Trace amounts of microcystins in water samples have been measured using LC-ESI-MS by several authors (Meriluoto et al., 2004; Yen et al., 2011). Yen et al. (2011) used a dual SPE and LC-ESI-MS system to successfully detect and quantify low levels of six microcystins in drinking water. The detection limits for MC-LR, MC-RR, MC-YR, MC-LW, MC-LF and MC-LA ranged between 20 and 31 ng/L (0.02–0.031 µg/L) in raw water samples. The authors noted that these detection limits were 3–10 times higher than for samples of pure water spiked with microcystins. More recently, Meriluoto et al. (2004) evaluated the suitability of LC-ESI-MS in the high-throughput screening of microcystins in cyanobacterial extracts. The analysis of over 514 samples per day was achieved by using a short $C_{18}$ column, rapid aqueous formic acid–acetonitrile gradient and MS detection. The detection limit was on the order of 50–100 pg MC-LR per injection (5 µL). UPLC-MS/MS has also been used for the analysis of several microcystins (MC-RR, MC-YR, MC-LR, MC-LA, MC-LY, MC-LW and MC-LF) in a wide variety of matrices because of its sensitivity and selectivity (Oehrle et al., 2010).

MS/MS identifies compounds more accurately than LC-MS. In the absence of mass data, the patterns obtained using in-source or MS/MS fragmentation are invaluable in detecting unknown microcystin variants in complex matrices (Lawton et al., 1995; Hummert et al., 2001; Diehnelt et al., 2005; Frias et al., 2006). Pietsch et al. (2001) developed a method using SPE with reversed-phase LC coupled with ESI-MS/MS using single ion mode and product ion scan for compound identification and multiple reaction monitoring for quantification. The quantitation limit for MC-LR, MC-YR, MC-RR and MC-LA was 50 ng/L (0.05 µg/L). Similarly, SPE-LC-

MS/MS methods have also successfully quantified trace amounts (LOQs < 0.05 µg/L) of microcystins in water samples (Cong et al., 2006; Rodrigues et al., 2013). Zhang et al. (2004) examined the optimal collision-induced dissociation energy for the determination of MC-LR in surface water using LC-MS/MS in full-scan mode. A relative collision energy of 30% achieved excellent sensitivity. With a 1000-fold preconcentration step using SPE, the MDL for this method was 2.6 ng/L (0.0026 µg/L).

The U.S. EPA has recently published a method (U.S. EPA Method 544) for the analysis of MC-LA, -LF,-LR,-LY,-RR,-YR, and nodularin in drinking water by SPE and LC-MS/MS (Shoemaker et al., 2015). The method includes detailed procedures for sample collection, preservation and storage, as well as sample preparation including cell lysis and extraction of intracellular toxins. The method uses divinylbenzene N-vinylpyrolidone copolymer SPE cartridges for extraction of microcystins, followed by elution with methanol containing 10% reagent water. The extract is concentrated to dryness by evaporation with nitrogen in a heated water bath and then adjusted to a 1-mL volume with methanol containing 10% reagent water. A 10 µL injection is made into an LC with a $C_8$ column that is interfaced to a tandem mass spectrometer. The method specifies that the MS must be capable of positive ion electrospray ionization near the suggested LC flow rate of 0.3 mL/min. The system must also be capable of performing MS/MS to produce unique product ions for method analytes within specified retention time segments. A minimum of 10 scans across the chromatographic peak is required to ensure adequate precision. The validation data presented in the method were collected using a triple quadrupole mass spectrometer. The single laboratory lowest concentration MRL (LCMRL) for analytes ranged from 2.9 - 22 ng/L using this method. The method allows for modifications to be made to the separation technique, LC column, mobile phase composition, LC and MS/MS conditions provided that the method meets the method quality control acceptance criteria and the method performance can be verified in a real sample matrix.

Kaloudis et al. (2013) also conducted a validation study for an SPE LC-ESI-MS/MS method for the analysis of MC-LR, -RR, -YR, and -LA in source and drinking water in the low nanogram per litre range. The study included method validation to ISO and European guidelines. The accuracy expressed as mean recovery ranged from 71.4 - 114.1 % and from 70.0 - 106.5% for drinking and surface water respectively at three concentration levels (0.006, 0.1 and 1 µg/L). Precision expressed as relative standard deviation were below 23.9 % for drinking water and 22.3% for surface water The LOQ was 0.006 µg/L for all compounds.

Recently, Draper et al. (2013) investigated the optimization of the (SPE) LC-MS/MS procedure for detecting MC-LR, MC-RR, MC-YR, MC-LA, MC-LF and MC-LW in water. This method was capable of achieving MDLs between 0.03 and 0.04 µg/L and LOQs between 0.1 and 0.2 µg/L, with accuracies between 65% and 70%. Measurement of total microcystins in bloom samples was reproducible, with relative standard deviations of 8.3–12%. However, the authors noted that 16–98% of dissolved microcystin variants were lost to sorption on three types of syringe filter cartridges, including polytetrafluoroethylene, cellulose acetate and polypropylene. Satisfactory recoveries (average of 66%) were obtained only using polypropylene filters after the filtrate was combined with a methanol rinse. The authors also found that dissolved MC-LR and MC-LA were microbially degraded over a period of two weeks while the samples were stored at 4°C indicating that the use of a preservative and/or that processing samples rapidly in the laboratory is important to obtain accurate microcystin concentrations in natural water samples. This study demonstrated that many factors including sample stability (biodegradation), sorption losses during filtration, poor recoveries on reversed phase SPE and inadequate cell lysis procedures contributed to significant error in microcystin determination in natural water samples.

Overall, it was concluded that sample preservation, careful sample workup, and quality control procedures are essential for achieving reliable microcystin measurements using modern LC-MS/MS instruments.

A new approach using laser diode thermal desorption–atmospheric pressure chemical ionization interface coupled to tandem mass spectrometry (LDTD-APCI-MS/MS) has been developed for the analysis of total microcystins in complex environmental matrices (Roy-Lachapelle et al., 2014). The method is based on oxidation of the microcystins in a sample using potassium permanganate under alkaline conditions to produce 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB). MMPB is then extracted and directly injected (no chromatographic separation) into the LDTD-APCI-MS/MS system. This approach results in ultra-fast sample analysis with simple sample preparation, reducing time and material costs associated with chromatographic separation. This method does not require individual microcystin standards, but, similar to ELISA and PPIA, the results do not provide information on the identity of the individual microcystin variants. The MDL and LOQ are 0.2 and 0.9 µg/L, respectively (Roy-Lachapelle et al., 2014).

Microcystins can also be analyzed without preliminary chromatographic separation using time-of-flight mass spectrometers. Welker et al. (2002) demonstrated that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a reliable and rapid tool to identify microcystin variants in small samples, such as single *Microcystis* colonies. This technique is performed directly on dried and solid samples, providing measurements that support the identification of analytes over a wide range of mass. The main advantage of MALDI-TOF-MS compared with other analytical techniques is that it enables analysis of a small amount of sample, without any pre-purification. However, MALDI-TOF-MS cannot be used for determining the concentration of microcystins in a sample and must be used with another technique for quantification (Kaushik and Balasubramanian, 2013).

## 6.7 Microcystin standards

The analytical methods available for microcystins require the use of standards of the variants for identification and quantification. Limited availability of purified and/or certified microcystin standards is a constraint on research and routine analysis. Although many microcystin variants may be present in a water sample, only a few are commercially available, including, but not limited to, MC-LR, dmMC-LR, MC-RR, MC-LA, MC-YR, MC-LW and MC-LF. Therefore, the presence and quantities of other microcystins can only be estimated. In addition, most of the microcystin standards that are available are not certified, and their purity must be verified (Westrick et al., 2010a; Giddings et al., 2012). In Canada, MC-LR and MC-RR are available as certified reference materials from the National Research Council of Canada (NRC). In many cases, laboratories use MC-LR as the surrogate standard in analytical methods such as ELISA and HPLC-PDA, even though the sample may contain variants other than MC-LR (Codd et al., 2001). In cases where commercial and/or certified standards are not available, Papageorgiou et al. (2012) provide a detailed procedure for production of analytical microcystin standards for use in the laboratory. However, preparation of microcystin standards can be expensive and time-consuming.

## 6.8 Comparison of methods

Many studies have been conducted that compare the results obtained using the different methods for microcystins analysis (Pietsch et al., 2001; Fastner et al., 2002; Rapala et al., 2002; Hoeger et al., 2004; Tillmanns et al., 2007, Graham et al., 2010; Triantis et al., 2010; Yen et al., 2011). In general, results obtained using biochemical and physico-chemical methods are

considered complementary and may not be directly comparable (Graham et al., 2010). However, combined data obtained from the different methods can be used to better understand the nature of a bloom and treatment plant performance, and to identify when low levels (<0.4 µg/L) of microcystins are present (Spoof et al., 2005; Triantis et al., 2010). Factors affecting how well different methods compared included the nature of the samples (e.g., type and quantity of different variants, presence of interferences), specific characteristics of the analytical methods (e.g., antibodies utilised in ELISA) and the purity and availability of microcystin standards for LC-MS/MS.

Several studies have compared the results of ELISA methods with physicochemical analytical methods (Rapala et al., 2002; Hoeger et al., 2004; Young et al., 2006; Triantis et al., 2010). In some cases, ELISA results were comparable with those obtained using HPLC-UV (Rapala et al., 2002; Triantis et al., 2010) and LC-MS/MS (Yen et al., 2011). Other studies have found that ELISA underestimates total microcystins (Metcalf et al., 2000a; Young et al., 2006). In particular, Young et al. (2006) found that the results from ELISA using antibodies isolated against MC-LR underestimated the amount of total microcystins when MC-RR made up greater than 70% of the total microcystins in the sample. Rapala et al. (2002) found that ELISA underestimated the concentration of hydrophobic microcystins (MC-LW, MC-LF). Other investigators have found that in environmental samples where many variants are likely present, the concentration of total microcystins measured using ELISA was 1.5–4 times higher than measured using HPLC-UV or LC-MS/MS (Hoeger et al., 2004; Mountford et al., 2005; Tillmanns et al., 2007; Aranda-Rodriguez and Jin, 2011). Similarly, Kaloudis et al. (2013) compared commercial PPIA kit results with LC-MS/MS and found that total microcystin concentrations were higher using PPIA analysis. In all cases, the differences were attributed to the presence of microcystins in the sample that were not identified and quantified using HPLC-UV or LC-MS.

It is important for utilities to be aware that it is not possible to determine if ELISA or PPIA has accurately quantified the microcystins present in a sample without a basic understanding of the variants present in the bloom as well as the characteristics of the ELISA or PPIA method being used (e.g., type of antibodies, cross-reactivity of variants). To ensure accurate quantification and characterization of microcystins, it is recommended that a screening method (ELISA, PPIA) be combined with a physicochemical method (HPLC-UV or LC-MS/MS).

When considering which of the physicochemical methods to use for microcystin analysis, the detection limit required and the potential for matrix interferences should be taken into consideration. Pietsch et al. (2001) compared the ability of LC-UV/PDA and LC-MS/MS to accurately quantify microcystins in environmental samples. Comparison of the chromatograms found that samples containing high amounts of NOM resulted in interferences and inaccurate quantification using LC-UV/PDA. Triantis et al. (2010) compared several analytical methods for the routine monitoring of microcystins. The authors suggested that ELISA or PPIA could be used for the initial screening of samples, followed by HPLC-PDA or LC-MS/MS analysis on samples above the detection limit (approximately > 0.15 µg/L). The investigators also recommended that samples requiring measurement of microcystins at trace levels (i.e., ng/L or less) should be analyzed by LC-MS/MS, as it is more sensitive than HPLC-PDA. Based on the performance of all of the methods tested (ELISA, PPIA, LC-MS/MS and HPLC-PDA), the authors developed an analytical protocol for monitoring of microcystins in surface and drinking water.

When utilities want to determine if low levels (< 0.4 µg/L) of microcystins are present in treated drinking water, enhanced sensitivity ELISA kits or LC-MS/MS methods should be used for analysis. In addition, a portion of raw and treated water samples should be analyzed using a physico-chemical method (LC-MS/MS or HPLC-PDA) so that an understanding of the types and

amount of variants present in the bloom is obtained and treatment plant effectiveness can be more accurately predicted and/or assessed.

### 6.9 Quality assurance and control

As discussed previously, the only two standardized methods for the quantitative analysis of microcystins in drinking water— ISO Method 20179 using SPE-HPLC-UV (ISO, 2005) and EPA Method 544 using SPE LC-MS/MS (Shoemaker et al., 2015) — are not widely used in commercial laboratories in Canada. This makes it difficult for responsible authorities to have confidence in the analytical data and the subsequent decisions made regarding guideline compliance, selection of water treatment technologies and implementing appropriate public health protection measures. To help address these concerns, QA/QC protocols and/or standard operating procedures have been developed for various microcystin analytical methods. These protocols include QC criteria for the analysis of microcystins using HPLC-PDA and ELISA methods (Meriluoto and Codd, 2005; Papageorgiou et al., 2012). It is particularly important for responsible authorities to have an understanding of the methods being used by the laboratory selected for microcystin analysis and to discuss the factors that may affect the interpretation of the results. Examples of factors include the type of separation and extraction methods, cross-reactivity and matrix interferences for ELISA, as well as the verification of the purity of microcystin standards.

## 7.0 Treatment technology

Choosing appropriate technology to control both cyanobacteria and their toxins begins with an understanding of cyanobacterial ecology. Managing risks from cyanobacteria includes control strategies within the watershed and reservoir to prevent bloom growth, together with a monitoring program, reliable analytical methods and effective treatment technology.

### 7.1 Prevention of cyanobacterial growth

The most effective component of a long-term strategy for reducing the incidence of cyanobacterial blooms is to control the input of nutrients (specifically phosphorus and nitrogen) into the water body (Jančula and Maršálek, 2011; Paerl et al., 2011a; Matthijs et al., 2012). Although phosphorus has historically been regarded as making a greater relative contribution than nitrogen to cyanobacterial bloom problems (Scott and McCarthys, 2010), the significance of nitrogen loading is now recognized. Thus, a reduction strategy that addresses only phosphorus is unlikely to be successful (Havens et al., 2000; Howarth and Paerl, 2008; Conley et al., 2009). One way in which nutrient control can be achieved is by limiting inputs to the source water (wastewater and industrial effluent, runoff from urban and agricultural areas). As these inputs can be influenced by rainfall events, climate change is expected to put additional pressure on water supplies and systems. Therefore, a nutrient control strategy that addresses the connection between climate change and nutrient loading will be important (Paerl et al., 2011b; Carey et al., 2012a). Efforts to develop cost-effective tools to assist utilities in their climate change awareness, preparedness and education are continuing (de Boer, 2007; Fries et al., 2012).

Input control measures require the cooperation of stakeholders from multiple sectors and jurisdictions and can be expensive and time-consuming, requiring years for efforts to be successful. Therefore, such measures may not be financially accessible for many areas (Jančula and Maršálek, 2011). However, in a very large watershed, input control measures may not be effective (Watson et al., 2008; Liu et al., 2012).

Adding chemicals to source waters to reduce nutrient availability is another potential control mechanism. This approach involves binding with, precipitating and locking up soluble phosphorus in sediments so that it is not accessible to phytoplankton (Paerl et al., 2011b). The insoluble phosphate–coagulant complexes form a barrier on the sediment surface that prevents phosphate release (Lelková et al., 2008; Paerl et al., 2011b). Direct removal of phytoplankton through coagulation, aggregation and sedimentation is also a possible secondary effect (Jančula and Maršálek, 2011). Challenges associated with the use of chemicals include the expense, dosing concentrations, water chemistry (pH, alkalinity), concentrations of the intended target and possible ecotoxic effects (Jančula and Maršálek, 2011).

Aluminum salts (aluminum sulphate, aluminum chloride) and polymers (polyaluminum chloride) are some of the most frequently used coagulants in drinking water production and wastewater treatment. Aluminum in its pure form can be toxic for aquatic biota and fish in acidic waters; thus, treatment in water bodies with neutral pH and having good buffering capacity is an important consideration. Some studies have reported adverse effects on zooplankton several days after aluminum addition to the water. These were largely related to nutrient and phytoplankton coagulation and removal, as opposed to aluminum toxicity (Jančula and Maršálek, 2011). Use of aluminum coagulants does not result in damage to cyanobacterial cells and the subsequent release of cyanotoxins (Drikas et al., 2001). Treatment with polyaluminum hydrochloride resulted in a significant decrease in *Planktothrix agardhii* abundance in a shallow, eutrophic fish pond (Lelková et al., 2008). Microfloc alum treatment has also been explored, whereby ultra-small aluminum hydroxide particles with lower settling velocities and longer residence times are created and dosed continuously or intermittently, as opposed to whole-lake alum treatments. Researchers conducting a long-term study with the technique have reported improved Secchi disc depth, decreased total phosphorus concentrations and no adverse biological impacts (Moore et al., 2009).

Iron-based coagulants such as polyferric sulphate, ferric sulphate and ferric chloride have also been used. As with alum, cell lysis and/or toxin release from iron use are not considered likely. Iron-based coagulants are more sensitive to oxygen concentrations, however, and increased phosphate release can occur from settled flocs under anoxic conditions (Jančula and Maršálek, 2011).

Calcium application either as slaked lime (calcium hydroxide) or calcite (calcium carbonate) has also been investigated as a possible lake restoration tool (Prepas et al., 2001; Jančula and Maršálek, 2011). A potential disadvantage of lime application is that it can increase water pH. If the water is held within its natural pH range, toxic effects on the ecosystem are not expected (Jančula and Maršálek, 2011). Lime use does not result in cell lysis and cyanotoxin release (Kenefick et al., 1993). Successful reductions in phytoplankton biomass, phosphorus and chlorophyll-a have been documented in studies of lime and calcite application; however, long-term effects have been reported only in lakes receiving multiple doses (Prepas et al., 2001; Zhang et al., 2001).

Other control methods have been suggested to address organic-rich sediments, which can be a significant source of nitrogen and phosphorus loading to the overlying water. Management actions can include sediment capping or physical removal through dredging. There are a number of drawbacks with these strategies, however, and there have been few reports of successful implementation. Sediment capping or removal is costly and can result in the resuspension of nutrients and potentially toxic substances (Paerl et al., 2011b). Much preplanning is necessary when dredging, as a considerable amount of phosphorus can be released when the action takes place (Falconer, 2005). Negative impacts on bottom-dwelling biological communities are also a

concern with these actions. As well, any benefits will be only temporary unless they are combined with efforts to curtail nutrient inputs (Paerl et al., 2011b).

Physical means of altering the hydrology of the water body have also been used to counter cyanobacterial blooms. Artificial mixing with mechanical mixers or bubble plumes created by air nozzles are technologies that have been used for water column destratification. Direct injection of oxygen to the bottommost layer of water that lies above the sediment has also been suggested as a mixing mechanism that would limit nutrient (phosphorus) release (Paerl et al., 2011a, 2011b). Obstacles identified with the use of these methods are that they require significant capital costs and energy inputs; and application will be site-specific—dictated by the hydrology of the water body in question (Falconer, 2005). It is important to note that artificial mixing attempts can actually worsen conditions if destratification is only partially achieved and nutrients are seeded into an area that remains stratified (Falconer, 2005). Destratification attempts have been found to be ineffective against blooms of *Cylindrospermopsis raciborskii*, because the species can adapt to low light conditions when mixed (O'Brien et al., 2009; Burford and Davis, 2011).

Flushing, by increasing the water flow through a water body, can disrupt thermally stratified waters and also reduce the residence time for cyanobacteria, making it difficult for a bloom to become established (Maier et al., 2004; Paerl et al., 2011b). There are two general approaches to flushing: a low-volume steady release to maintain an effective minimum flow speed, or a large-volume release to flush out an establishing bloom. In an Australian river system regulated by upstream lakes, a steady flow release of 300 ML/day was effective in preventing the redevelopment of blooms of *Anabaena circinalis* (Mitrovic et al., 2011). Although this strategy can be effective in bloom reduction, it is also system specific, as not all water systems have the capability to regulate their own flow (Falconer, 2005).

Selective water withdrawal from the lake bottom has also been identified as a means for destabilizing the water column and removing phosphorus-rich waters (Falconer, 2005; Lehman et al., 2009). An advantage is that it addresses the source of eutrophication by removing phosphorus from the system. The drawback, however, is that phosphorus removal through treatment is needed prior to discharging the water to another body (Falconer, 2005).

Biological measures for the prevention or control of cyanobacterial blooms without the use of chemicals have also been explored. Numerous researchers have studied the ecology of zooplankton species capable of grazing cyanobacteria (Chorus and Bartram, 1999; Paerl et al., 2011b). Others have looked at the ecology of freshwater mussels that can consume cyanobacteria, but can also have the unwanted effect of improving the surroundings for cyanobacterial growth by altering the light and nutrient conditions of the aquatic environment. Further strategies investigated have included attempts to modify the fish population to reduce the number of plankton-eating fish species or to introduce cyanophages that are capable of attacking specific cyanobacterial species (Falconer, 2005). The use of barley straw to reduce cyanobacterial populations is another novel measure that has been explored. Decomposing barley straw has been reported to release compounds of yet-unconfirmed identity that have anti-cyanobacterial properties (Chorus and Bartram, 1999). Results regarding the success of this approach have been conflicting. The technique adds a sizable amount of organic material to the water body, which is an important consideration. Although economically favourable, the use of barley straw is not well enough understood to be considered ready for regular use. In general, research into biological control measures is still in the early stages, and further work is needed to fully realize the potential benefits and limitations of individual approaches.

### *7.1.1 Intervention measures for cyanobacterial blooms*

Intervention measures (actions intended to destroy an active bloom) have the potential to create ecological or human health risks; therefore, they are regarded as emergency measures (Chorus and Bartram, 1999). Preventive measures and water body management techniques are the preferred course of action for the control of cyanobacteria and their toxins. A serious consequence of measures that destroy toxin-containing cyanobacterial cells is that they can result in the release of toxins into the water.

Algicides have been used in certain regions to kill cyanobacterial blooms (Chorus and Bartram, 1999; Chorus, 2012). Despite the human health and environmental concerns associated with these agents, they have been used to provide short-term control of cyanobacterial blooms in circumstances where alternative risk management strategies have been deemed not available, not practical or not effective. Many countries have established environmental regulations restricting the use of such products (Chorus and Bartram, 1999). Chemicals that have been investigated for bloom management are addressed here; however, the use of algicides to control blooms in drinking water source waters is discouraged.

Copper (as copper sulphate or in commercial algicides) is the most well-known and widely used algicide (Chorus and Bartram, 1999; Jančula and Maršálek, 2011). The rapid breakdown of a toxic *Microcystis aeruginosa* bloom by copper sulphate treatment in an Australian lake was followed by microcystin concentrations of 1300–1800 µg/L in the water, which persisted for 9 days (Jones and Orr, 1994). Environmental concerns are also frequently cited with the use of copper algicides (Falconer, 2005). Copper is a non-specific poison that can exert harmful effects on a number of non-target organisms in water ecosystems (Falconer, 2005; Jančula and Maršálek, 2011). Copper complexes that are formed can also settle and accumulate in sediments over time to reach levels that are harmful to the resident organisms. The degree to which copper is toxic to aquatic species is influenced by many factors, including water pH and alkalinity, the form of copper used, the dose applied and the individual sensitivities (Chorus and Bartram, 1999; Falconer, 2005).

Oxidants such as chlorine and potassium permanganate have historically been used in drinking water treatment operations, but are not normally applied as algicides as part of whole-lake or reservoir intervention measures (Falconer, 2005). In recent years, hydrogen peroxide has received interest as a potential algicide. Characteristics identified as making hydrogen peroxide attractive are rapid breakdown, environmentally safe reaction products (oxygen and water) and research results suggesting a greater toxicity towards cyanobacteria than towards other phytoplankton (Jančula and Maršálek, 2011; Wang et al., 2012a). The drawbacks (aside from cell lysis) are that breakdown is so rapid that repeated applications are needed; and too high a concentration can be harmful to other organisms. Proper dose control is very important (Jančula and Maršálek, 2011). Application of hydrogen peroxide at a concentration of 2 mg/L resulted in the collapse of a toxic *Planktothrix agardhii* population and decreased total microcystin concentrations (Matthjis et al., 2012). Cyanobacterial numbers remained low for 7 weeks after treatment. Major effects on phytoplankton and zooplankton were not observed at this concentration; however, in treatment experiments, zooplankton decreased at a hydrogen peroxide concentration of 2.5 mg/L and was strongly affected by higher hydrogen peroxide concentrations. Treatment with hydrogen peroxide is expected to vary from site to site, being influenced by species composition, concentrations of dissolved organic carbon (DOC), iron and manganese, redox potential, light conditions and biological activity. Further understanding of this technique is needed (Matthjis et al., 2012).

Herbicides such as diuron, endothall, glyphosphate and simazine have been tested for their potential in controlling cyanobacterial blooms (Falconer, 2005; Jančula and Maršálek, 2011; L. Chen et al., 2012). However, there are numerous concerns with the use of these products as algicides, including accumulation and persistence in the environment, toxicity against other aquatic species at the recommended doses and the possibility for the development of resistance in some cyanobacteria (Falconer, 2005; Jančula and Maršálek, 2011).

Sonication—the use of ultrasonic sound waves to disrupt cyanobacterial cells—has also been investigated as a potential treatment option (Rajasekhar et al., 2012). The application of ultrasonic radiation in water generates millions of bubbles that, upon implosion, result in high temperatures and pressures and the generation of free radicals. Within the cyanobacterial cell, this causes disruption of the cell wall and membrane and interruption of activities such as photosynthesis and cell division (Rajasekhar et al., 2012). A significant benefit of sonication is that it is environmentally friendly compared with chemical treatment strategies. The technique has also been reported to be capable of degrading MC-LR (Song et al., 2005). Drawbacks include that application frequencies are difficult to calculate and are system specific and that applications on a large scale require more powerful—and therefore more expensive—equipment. Sonication shows potential for use in cyanobacterial bloom management, but further study to determine effective operating procedures is needed before it can be considered a feasible approach (Rajasekhar et al., 2012).

## 7.2 Municipal scale

Control of cyanotoxins at the water treatment plant can be achieved using a variety of chemical, physical and biological treatment methods to remove either the intracellular (cell-bound) or extracellular (dissolved) cyanotoxins. In most cases, there will be both intracellular and dissolved toxins present in the source water entering a treatment plant, and utilities should consider treatment of both forms (Newcombe and Nicholson, 2004; Westrick et al., 2010b; Merel et al., 2013). In Canada, when drinking water treatment is needed for cyanotoxins, the focus will most often be on the removal of microcystins. Therefore, this section focuses on the treatment methods that are effective for microcystins. A brief discussion of methods that may be effective for cylindrospermopsin is found in Section 7.2.9.

To remove both intracellular and extracellular microcystins from drinking water, a multi-barrier approach is required, which may consist of conventional or membrane filtration for intracellular microcystin removal and granular activated carbon (GAC), powdered activated carbon (PAC), oxidation, biodegradation or small pore membrane processes for the removal of dissolved microcystins. The first approach, and most effective way to deal with high microcystin concentrations, is to remove the cyanobacterial cells intact, without damaging them, to prevent the release of additional dissolved microcystins into the water. Other treatment technologies can then be used to remove the dissolved microcystins that remain in the water (Newcombe and Nicholson, 2004; Newcombe, 2009; Westrick et al., 2010b). Several detailed reviews of the effectiveness of a variety of treatment methods for intracellular and/or dissolved microcystin removal have been published in recent years (Newcombe and Nicholson, 2004; Svrcek and Smith, 2004; Hoeger et al., 2005; Newcombe, 2009; Warren et al., 2010; Westrick et al., 2010b; Merel et al., 2013; Antoniou et al., 2014).

In the absence of cell damage, conventional filtration employing coagulation, clarification and rapid granular filtration can be effective at removing intact cells and the majority of intracellular toxins. However, it has been reported that accumulation of cyanobacterial cells and microcystins in clarifiers can lead to their breakthrough into filtered water (Zamyadi et al., 2012a,

2013c). In addition, cell lysis can occur during preoxidation or in clarifier sludge, increasing the dissolved concentration of microcystins in the treatment plant. Conventional filtration is generally considered to have limited effectiveness for the removal of the dissolved form of microcystins (Hall et al., 2000; Drikas et al., 2001; Hoeger et al., 2004). Hence, additional processes, such as adsorption, chemical oxidation, biodegradation or reverse osmosis (RO) and nanofiltration (NF), are required to remove dissolved microcystins (Newcombe and Nicholson, 2004; Westrick et al., 2010b).

Adsorption processes, such as GAC and PAC, are effective at removing dissolved microcystins, but are not capable of removing intact cells and intracellular toxins. Removal through adsorption depends on many factors, including the type of activated carbon used, the microcystin variant and water quality conditions (Newcombe, 2002; Alvarez et al., 2010).

Chemical oxidation using chlorine, potassium permanganate or ozonation can be effective at removing dissolved microcystins, but can also cause cellular lysis, resulting in increased concentrations of dissolved microcystins in drinking water. By applying conventional filtration (or other filtration process) first, the majority of intact cells are removed, and the dissolved microcystin concentration is less likely to increase due to cell lysis when filtered water is treated with oxidants (Sharma et al., 2012; Merel et al., 2013). In cases where preoxidation is practised, it may need to be discontinued during a cyanobacterial bloom, or adjustments to the oxidant type and doses may be needed to minimize cell rupture prior to filtration (Newcombe et al., 2015).

Membrane processes can also effectively remove microcystins. Although microfiltration (MF) and ultrafiltration (UF) membranes can remove both cyanobacterial cells and intracellular microcystins, removal of dissolved microcystins using UF is variable (35–70%), and MF is not effective for removal of dissolved microcystins (Gijsbertsen-Abrahamse et al., 2006; Dixon et al., 2011b). NF membranes and RO can achieve high removals of intracellular and dissolved microcystins. As size exclusion is the dominant rejection mechanism, pore size is an important factor in removal efficiency for these processes.

Drinking water treatment plants using a combination of treatment processes, such as conventional filtration with oxidation or activated carbon, to remove both intracellular and dissolved microcystins can typically remove up to 99.99% of total microcystins to achieve concentrations below 0.1 µg/L in treated water (Karner et al., 2001; Lahti et al., 2001; Hoeger et al., 2005; Jurczak et al., 2005; Rapala et al., 2006; Zamyadi et al., 2013c). In addition to the data from the peer-reviewed studies cited above, data provided by members of the Federal-Provincial-Territorial Committee on Drinking Water (CDW) on microcystin concentrations in raw and treated water in Canada also indicate that most treatment plants achieve microcystin concentrations below 0.1 µg/L in treated water (Zurawell, 2002; Robert et al., 2005; Robert, 2008; Manitoba Water Stewardship, 2012; Ontario Ministry of the Environment, 2014). Certain drinking water systems, such as small systems that do not have filtration for particle removal, will not be able to remove intracellular microcystins. A detailed discussion on the effectiveness of combined treatment processes for microcystin removal is provided in Section 7.2.7.

The ability of treatment plants to effectively remove microcystins will depend on the site-specific water quality (e.g., pH, temperature, turbidity, presence of NOM) and the treatment processes in place. Utilities need to have an understanding of the type and concentration of microcystin variants present in the source water and to conduct site-specific evaluations to determine the most effective treatment strategy (Harrington and Swank, 2010). In addition, knowledge of the cyanobacterial species present in a bloom is important for optimizing cell removal in treatment plants (Zamyadi et al., 2013a). Detailed information on the operational

considerations of a variety of treatment methods can be found in Hoeger et al. (2004), Newcombe (2009), Zamyadi et al. (2012a, 2013c) and Newcombe et al. (2015).

### *7.2.1 Conventional filtration*

Conventional filtration consisting of coagulation, clarification (sedimentation or dissolved air flotation) and rapid granular filtration can be effective for removal of cyanobacterial cells and therefore intracellular (cell-bound) microcystins when particulate or NOM removal is optimized (Chow et al., 1998; Newcombe et al., 2015). Rapid sand filtration without pretreatment (coagulation) is not effective for cyanobacterial cell removal (Lepisto et al., 1994). In addition, studies have reported limited removal of extracellular (dissolved) microcystins using these processes (Keijola et al., 1988; Chow et al., 1999; Drikas et al., 2001; Hoeger et al., 2005).

The efficiency of the conventional filtration process to remove cyanobacterial cells and intracellular microcystins varies from 60% to 99.9% and depends on a variety of factors, including the cyanobacterial species and cell density, coagulant type and dose, pH and operational parameters, such as flocculation time, frequency of filter backwashing and clarifier sludge removal (Vlaski et al., 1996; Hitzfeld et al., 2000; Hoeger et al., 2004; Jurczak et al., 2005; Zamyadi et al., 2012a, 2013c; Antoniou et al., 2014; Newcombe et al., 2015).

Removal of cyanobacterial cells and intracellular microcystins occurs at each stage of the conventional filtration process and should be monitored and optimized throughout the treatment plant during bloom events. Typically, 60–95% of cells and intracellular microcystins can be removed during sedimentation, with up to 99.9% removal achieved following the filtration step (Lepisto et al., 1994; Drikas et al., 2001; Hoeger et al., 2004; Newcombe et al., 2015).

#### *7.2.1.1 Cyanobacterial cell removal*

The efficiency of coagulation for cell removal is dependent on pH, coagulant type and dose and the morphological characteristics of the algae. There is no consensus in the literature identifying the most effective coagulant or conditions for cyanobacterial cell removal. However, successful removal of several cyanobacterial species (*Microcystis aeruginosa, Anabaena circinalis, Cylindrospermopsis raciborskii, Anabaena flos-aquae, Asterionella. formosa* and *Pseudanabaena*) has been demonstrated in jar tests using a variety of coagulants, including aluminum sulphate, ferric chloride, polyferric sulphate and aluminum chlorohydrate (Jiang et al., 1993; Chow et al., 1998, 1999; Henderson et al., 2010; Dixon et al., 2011a, 2011b; Gonzalez-Torres et al., 2014; Newcombe et al., 2015). Mohamed et al. (2001) demonstrated in jar testing that lime softening was not effective for cyanobacterial removal, but lime (100 mg/L calcium hydroxide) and alum (75 mg/L) were effective for completely removing approximately 2000 cells/mL. Recent work has demonstrated that, in most cases, optimizing coagulation for NOM removal also optimizes (≥ 90%) cyanobacterial cell removal during clarification (Newcombe et al., 2015). Therefore, it is recommended that the existing coagulation/flocculation processes be optimized before considering a change in coagulants during a cyanobacterial bloom (Newcombe et al., 2010).

The morphological characteristics of algae—in particular, size, shape and surface characteristics—also influence the efficiency of coagulants for the removal of algal cells. Bernhardt and Clasen (1991) suggested that smooth spherical algal cells were removed through charge neutralization mechanisms, compared with filamentous cells, which were removed by sweep coagulation during coagulation and flocculation. Henderson et al. (2008) reported that morphology, motility, extracellular organic mater and surface charge affect coagulation and flocculation, with morphology and motility being more important during sedimentation. The

authors found that cell surface area could be used as a preliminary indicator of the coagulant dose required for optimum cell removal (Henderson et al., 2008, 2010). Variations in cell removal based on cyanobacterial species has also been documented in a full-scale treatment plant (Zamyadi et al., 2013a). Daily monitoring of a conventional filtration plant demonstrated that *Aphanizomenom* cells were significantly less effectively coagulated (54–75% removal) than other cyanobacterial genera, such as *Microcystis* and *Anabaena* (> 99% removal). Furthermore, *Aphanizomenom* cells were not efficiently removed in the sludge and passed through the filter (Zamyadi et al., 2013a). Therefore, identification of the cyanobacterial genera present at the intake of a conventional filtration plant is useful for optimizing or predicting the effectiveness of the treatment processes (Antoniou et al., 2014).

Other factors can affect the efficiency of the clarification process. Bench-scale testing conducted by Henderson et al. (2006) found that achieving steady-state floc size required 25 minutes for *Chlorella vulgaris* cells ($5 \times 10^5$ cells/mL) compared with 4 minutes for NOM. In addition, the authors noted that during the first 6–7 minutes, no agglomeration of cells was observable. This time lag was attributed to the cells' ability to react to changes in the immediate environment, as the surface charge of cells can vary to compensate for the positive charge of the coagulants. In addition, Knappe et al. (2004) found the addition of a flocculant aid (anionic bridging polymer) greatly enhanced floc settleability of cyanobacterial cells provided that coagulation conditions were optimized. The type of clarifier may also impact the efficiency of the process. Mouchet and Bonnelye (1998) reviewed the effectiveness of different sedimentation methods and determined that sludge blanket clarifiers were more effective than static settlers for cyanobacterial cell removal.

Dissolved air flotation (DAF) of coagulated/flocculated water can be more effective than sedimentation for the removal of specific cyanobacteria with gas vacuoles that exhibit diurnal buoyancy patterns. Detailed reviews of DAF for cyanobacteria removal are found in Knappe et al. (2004), Teixeira and Rosa (2006a, 2007) , Teixera et al. (2010), Henderson et al. (2008) and Antoniou et al. (2014). Bench-scale testing conducted by Vlaski et al. (1996) demonstrated that when coagulation/flocculation processes were optimized and a low-dose coagulant aid was used, DAF achieved up to 99% removal of *Microcystis aeruginosa* cells. Similarly, Henderson et al. (2010) found that algal cells with varying morphology, including *M. aeruginosa*, were successfully removed (94–99%) using coagulation/flocculation and DAF. Teixeira et al. (2010) demonstrated that removals between 92% and 98% of both *M. aeruginosa* cells and *Planktothrix rubescens* filaments as well as intracellular microcystins could be achieved using DAF when coagulant doses were optimized for chlorophyll-a removal.

Microsand ballasted clarification processes have also been investigated for the removal of cyanobacterial cells. The microsand acts as a ballast, accelerating the settling of flocs; therefore, settling basins can be smaller than conventional sedimentation basins. Robinson and Fowler (2007) conducted pilot-scale testing of a microsand ballasted clarification process and found that 95% of cyanobacteria were removed following clarification. Jar tests conducted by Knappe et al. (2004) demonstrated that microsand-enhanced flocculation achieved the same removal (97–98%) of *M. aeruginosa* cells (50 000 cells/mL) spiked into lake water.

Filter operations have also been found to affect the passage of cyanobacteria into filtered water. Pilot testing conducted by Dugan and Williams (2006) demonstrated that increasing the hydraulic loading rate to the filters by 50% resulted in 1.6–48 times greater filter effluent chlorophyll-a concentrations, indicating the presence of more cyanobacteria in the filtered water. The chlorophyll-a peaks were also noted to be larger for tests conducted with *M. aeruginosa* than for *Anabaena flos-aquae*. The study also found that the chlorophyll-a concentration in filtered

water was higher for an initial filter loading rate of 10 m/h compared with an initial filter loading rate of 7 m/h.

Hoeger et al. (2004) conducted detailed monitoring of cyanobacterial cell removal in two full-scale drinking water treatment plants. During a period of high cyanobacterial (*M. aeruginosa* and *A. circinalis*) cell densities in the raw water ($1.26 \times 10^6$ cells/mL) at one treatment plant, the efficiency of each treatment step for cell removal was investigated. Cells were reduced by 99% through coagulation, flocculation and sedimentation without differences between the cyanobacterial species. However, because of the high raw water cell counts, up to 12 400 cells/mL were still present in the clarified water. Rapid sand filtration removed the remaining 99% of *A. circinalis* and 85% of *M. aeruginosa* cells, resulting in an overall removal of greater than 99.9% for the conventional filtration process. However, the authors noted that more than 2000 cells/mL remained in the treated water, even with 99.9% removal, when high cell densities ($2 \times 10^6$ cells/mL) were present in the raw water.

*7.2.1.2 Intracellular and dissolved microcystin removal*

In addition to the many studies discussed above that have examined specific aspects of cyanobacterial cell removal, there have also been multiple full-scale studies examining the removal of intracellular and dissolved microcystins using conventional filtration processes (Karner et al., 2001; Lahti et al., 2001; Tarczynska et al., 2001; Hoeger et al., 2004, 2005; Jurczak et al., 2005; Rapala et al., 2006; Bogialli et al., 2012). These studies have demonstrated that up to 99.9% of intracellular microcystins can be removed when conventional filtration processes are optimized. These studies also showed that source water concentrations of intracellular microcystins up to 10 µg/L can be effectively removed to below 0.5 µg/L using conventional filtration processes. Only low or negligible (< 0.2 µg/L) dissolved microcystin removal has been documented in full-scale applications (Jurczak et al., 2005). As with any contaminant, the removals achieved in these plants are site specific; however, they provide an indication of the potential achievability of microcystin removal in conventional filtration plants. Selected studies are discussed in greater detail below.

A study conducted by Jurczak et al. (2005) at two full-scale treatment plants found that coagulation, sedimentation and filtration were effective in removing intracellular microcystins. Weekly monitoring was conducted after each treatment step in the plants over a 3-month bloom event. At one of the plants, *Microcystis aeruginosa* was the dominant cyanobacterial species in the raw water, with a peak cyanobacterial biomass of 20.9 mg/L and a chlorophyll-a concentration of 65 mg/L. The raw water total microcystin concentration (sum of variants MC-LR, MC-RR and MC-YR) ranged from 0.19 to 4.6 µg/L, and the microcystins were predominantly intracellular. A ferric-based coagulant (PIX) was dosed at 80–120 g/m$^3$ along with 15–25 g/m$^3$ of hydrated lime followed by flocculation and sedimentation. During the peak microcystin concentration (4.6 µg/L), the sedimentation process was capable of removing approximately 98% of the intracellular microcystins down to a concentration of 0.08 µg/L. The remaining microcystins were removed in the filtration step. In another sampling event, 0.25 µg/L of dissolved microcystins was lowered to less than the MDL of 0.01 µg/L; however, the authors did not indicate if these concentrations were significantly different.

Rapala et al. (2006) conducted monitoring of cyanobacteria and microcystins at a full-scale treatment plant during several cyanobacterial blooms that occurred over a 4-year period. Microcystin concentrations correlated strongly with the occurrence of *Planktothrix agardhii*, which was the predominant species present during the blooms with the highest microcystin concentrations (approximately 10 µg MC-LR$_{equiv}$/L). Monitoring conducted following coagulation

with aluminum salts, clarification and sand filtration demonstrated that a mean reduction of 1.6 log was achieved at microcystin concentrations greater than 1 µg MC-$LR_{equiv}$/L in the raw water. The range of log removals over the 4-year period was 1.2–2.4. Peak raw water microcystin concentrations of 10 µg MC-$LR_{equiv}$/L were lowered to 0.3 µg MC-$LR_{equiv}$/L or less. Ozonation was required to remove the remaining dissolved microcystins (0.3 µg MC-$LR_{equiv}$/L) that were present in the filtered water to achieve finished water concentrations below 0.04 µg MC-$LR_{equiv}$/L. The authors noted that although treated water microcystin concentrations were below the detection limit (0.02 µg MC-$LR_{equiv}$/L), unidentified filaments of oscillatorialean cyanobacteria were commonly detected (300–28 000 cells/mL) in the treated water.

Although conventional filtration can be effective in removing cyanobacterial cells and intracellular microcystins, there are several challenges that treatment plants may need to consider during a cyanobacterial bloom. These include potential increases in dissolved microcystin concentrations due to cell lysis during preoxidation or release from clarifier sludge and cell accumulation and breakthrough within the treatment plant (Schmidt et al., 2002; Hoeger et al., 2004; Zamyadi et al., 2012a, 2013c).

In most cases, data presented in the literature indicate that under optimal conditions, coagulation, flocculation and filtration do not cause cell lysis or increases in the dissolved microcystin concentrations in treated water (Chow et al., 1998, 1999; Drikas et al., 2001; Sun et al., 2012). Under optimal coagulation conditions, Chow et al. (1999) and Drikas et al. (2001) found that cultured *M. aeruginosa* cells spiked into natural water were removed intact using conventional filtration and did not result in an increase in the amount of MC-LR in treated water. The experiments used surface water spiked with $3.5 \times 10^5 – 4.5 \times 10^5$ cells/mL, aluminum sulphate doses (expressed as aluminum) between 4.8 and 5.8 mg/L, a pH of 6.2 and a filtration rate of 600 mL/min. Additional data from these experiments presented by Drikas et al. (2001) indicated that cells in the filter backwash water showed only minor damage (> 93% cell viability). Similar laboratory experiments showed that 30 mg/L of ferric chloride did not cause cell lysis of *M. aeruginosa* (Chow et al., 1998). In addition, Sun et al. (2012) demonstrated that shear stress caused by mixing at between 0 and 500 revolutions per minute did not cause any damage to *M. aeruginosa* cells or release of MC-LR.

In contrast, other studies have found that cyanobacterial cell lysis can occur depending on the cyanobacterial cell growth phase or coagulation conditions (Pietsch et al., 2001; Schmidt et al. 2002; Qian et al., 2014). Schmidt et al. (2002) found a small increase (0.2 µg/L) in the dissolved microcystin concentration during pilot-scale testing of a conventional filtration process for the removal of *Planktothrix rubescens* from reservoir water. A maximum total microcystin concentration of 7 µg/L (MC-LR, MC-RR, MC-LA) was flocculated with 2.6 mg/L Al (aluminum sulfate) followed by sand filtration at a rate of 363 L/h. The authors hypothesized that, depending on the stability of the algal cells (growth phase), toxin release was possible during flocculation/filtration due to hydraulic stress and pressure gradients in pipes and filters. Qian et al. (2014) conducted jar testing to determine whether a decrease in pH caused by the addition of metal hydroxide coagulants can cause lysis of *M. aeruginosa*, *Cylindrospermopsis* and *Anabaena circinalis* cells. The results indicated that low pH stress (pH < 5) can cause substantial cell lysis and metabolite release for *A. circinalis* and *Cylindrospermopsis*, whereas *M. aeruginosa* was more tolerant to acidic conditions. At pH 4, the majority of intracellular cylindrospermopsin (10.3 µg/L) was released within 30 minutes. For *M. aeruginosa*, up to 6% of MC-LR (equivalent to 1 µg/L of the initial intracellular MC-LR) was released at pH 4 and 30 minutes of exposure. In contrast, high pH conditions (pH = 11) had only a minor impact on cells. It was concluded that

increasing the pH prior to the application of coagulants has the potential to minimize cell damage and toxin release for cyanobacterial species that are not acid tolerant (Qian et al., 2014).

*7.2.1.3 Accumulation and breakthrough of cells and microcystins into treated water*

The accumulation and breakthrough of cyanobacterial cells and microcystins following coagulation, flocculation and filtration have been reported in full-scale treatment plants (Zamyadi et al., 2012a, 2013c). During a large cyanobacterial bloom in 2008, Zamyadi et al. (2012a) reported an influx of high cyanobacterial (*Microcystis aeruginosa* and *Anabaena* sp.) cell numbers ($2.01 \times 10^5 – 7.7 \times 10^5$ cells/mL) into a conventional filtration plant over a 2-day period. This cumulative flux of coagulated cells to the clarifier resulted in a large accumulation of cells ($4.7 \times 10^6$ cells/mL) and microcystins (40 µg MC-$LR_{equiv}$/L) in the sludge bed. Although the sludge blanket clarifier removed 86% of the cyanobacterial cells and reduced the total microcystin concentration from 6.1 to 2.1 µg MC-$LR_{equiv}$/L, 26 000 cells/mL remained in the water post-sedimentation (on the surface of the clarifier). Filtration further reduced the cyanobacteria (predominantly *Anabaena flos-aquae*) concentration to 4300 cells/mL and microcystin concentration to 0.3 µg MC-$LR_{equiv}$/L. Additional monitoring conducted in 2010 found the presence of a green scum over the clarifier that contained 10 300 µg/L of total microcystins (sum of MC-LR, MC-LF, MC-RR, MC-YR, MC-LY, MC-LW, MC-dm-LR) as well as cylindrospermopsin. This confirmed breakthrough of microcystins from the sludge bed and their movement and accumulation over the settled water collection pipes. A scum containing over 100 µg/L of total microcystins was also observed on the surface of the filter, resulting in breakthrough of microcystins (2.47 µg/L) into the treated water. The authors suggested that use of coagulant aids may be needed during large cyanobacterial blooms to improve settling of the flocs containing cyanobacteria. Newcombe et al. (2015) also noted that although cell removals of up to 99% can be achieved through sedimentation processes, 90–95% removal is considered optimum. Therefore, if high cell concentrations are present in raw water, this could result in an accumulation of cyanobacteria in clarifiers and filters.

In another study conducted by Zamyadi et al. (2013c), the accumulation of cyanobacterial cells in the sludge bed and on the surface of the sedimentation and filtration basins was also reported in two conventional filtration plants considered low risk because of low (< 400 cells/mL) cyanobacterial cell concentrations in the raw water. The concentrations of cyanobacterial cells (*Microcystis*, *Anabaena*, *Aphanizomenom*, *Pseudanabaena* and *Gloeotrichia*) were as high as $1.5 \times 10^6$ cells/mL in the scum above the clarifier at one treatment plant. The corresponding concentration of total microcystins was 9.3 µg MC-$LR_{equiv}$/L (0.9 µg/L dissolved). High cell concentrations (> 100 000 cells/mL) and microcystin concentrations (5.5 µg MC-$LR_{equiv}$/L) over the filtration basin were also documented. It should be noted that this occurred when microcystin concentrations were below the detection limit (0.15 µg MC-$LR_{equiv}$/L) in all raw water samples collected biweekly over a period of 2 months. The operators of the treatment plant began collecting the scum off the top of the clarifier using a polyvinyl chloride pipe as a collection trough to try to minimize breakthrough of cells into the clarified water. The authors concluded that the use of intervention threshold values based on raw water cell or microcystin concentrations does not take into consideration the major long-term accumulation of potentially toxic cells on the surface of the sedimentation and filtration basins and in the sludge of treatment plants and the risk of toxin release into treated water (Zamyadi et al., 2013c). In addition, monitoring of cyanobacterial cell densities (counts) and microcystin removal within a conventional filtration plant aids in determining when treatment adjustments are needed (Hoeger et al., 2004; Tarczynska et al., 2001; Zamyadi et al., 2012a, 2013c).

Further investigation of the behaviour of cyanobacteria and microcystins following coagulation/sedimentation and during water treatment sludge management processes has found that cells can lyse and release dissolved microcystins into the supernatant (Drikas et al., 2001; Ho et al., 2012a, 2013; Sun et al., 2012). Drikas et al. (2001) found that sludge collected during jar testing contained up to $8 \times 10^6$ cells/mL of cultured *M. aeruginosa* cells. These cells lysed within 2 days and released dissolved MC-LR at concentrations up to 2000 µg/L. After 5 days, the dissolved microcystin concentration began to decrease owing to the onset of biodegradation. Sun et al. (2012) also observed cell lysis and MC-LR release when flocs containing *M. aeruginosa* were stacked over 6 days. Ho et al. (2013) found that sludge supernatant contained high concentrations of microcystins (90 µg/L) as a result of the lysis of cells from natural bloom material. The rate of release and biodegradation varied between microcystin variants, with MC-RR release starting immediately and peaking (30 µg/L) after 10 days, followed by complete biodegradation after 17 days. In contrast, MC-YR concentrations did not increase until after 13 days and peaked at 22 days. No biodegradation of MC-YR was observed over the 30-day study period. The authors noted that recycling sludge supernatant (from wastewater ponds) to the head of the treatment plant should not be conducted during cyanobacterial blooms.

As discussed above, process operations in conventional filtration plants have an impact on the successful removal of cyanobacterial cells and microcystins and can have an impact on the breakthrough of microcystins into treated water (Hoeger et al., 2004; Dugan and Williams, 2006; Zamyadi et al., 2012a, 2013c; Ho et al., 2013). Detailed operational guidance for optimal removal of cyanobacterial cells using conventional treatment processes can be found in Newcombe (2009) and Newcombe et al. (2015). Operational considerations for removing cyanobacterial cells using coagulation and flocculation are similar to those considered for achieving effective particle removal. The appropriate coagulant and coagulation pH should be determined through jar testing, and tests should be carried out on waters with high algal concentrations to maximize cell removal. Sufficient mixing must be provided at the point of chemical addition to ensure rapid contact, and an appropriate mixing speed must be determined to optimize the flocculation process (Newcombe, 2009). Optimized coagulation/flocculation is important to minimize the potential for the accumulation of cyanobacterial cells as scums at the surface of sedimentation basins and filters (Zamyadi et al., 2012a, 2013c).

Effective sludge removal from sedimentation/clarification processes is important to minimize the release of intracellular and dissolved microcystins into the surrounding waters, as significant cell numbers can accumulate within the sludge, and cells contained within the sludge can break down rapidly (Drikas et al., 2001; Zamyadi et al., 2012a; Ho et al., 2013). During a cyanobacterial bloom, sludge and sludge supernatant should be isolated from the water treatment plant. Additionally, backwash water from the filters may contain cyanobacterial cells and/or dissolved microcystins; hence, filter-to-waste should be practised to prevent the reintroduction of cells and toxins into the treatment train. Although longer filter run times are typically desirable between backwashing, during periods of high algal concentrations, cells can accumulate in the filter, which can potentially lead to a significant amount of dissolved microcystins released into the filtered water. Frequent backwashing has been recommended to minimize the risk of breakthrough of cells into filtered water (Newcombe, 2009). However, Zamyadi et al. (2013a) demonstrated that more frequent backwashing may not be effective, depending on the species of cyanobacteria present in the bloom (Zamyadi et al., 2013a). Additional filter operation considerations may also be necessary.

### *7.2.2 Adsorption*

Removal of dissolved (extracellular) microcystins can be achieved using either GAC or PAC (Lambert et al., 1996; Newcombe, 2002; Newcombe et al., 2003, 2009). In general, mesoporous carbons (e.g., chemically activated wood-based carbons) are the most effective for the removal of microcystins (Newcombe et al., 2009). Other factors, such as the type of microcystin variant present, the raw water quality (e.g., NOM and pH) and contact time, affect microcystin removal efficiency when using activated carbon processes. Water treatment plants should conduct testing to determine the most effective activated carbon process for their water quality. Detailed guidance on the optimization, operational and monitoring considerations of activated carbon processes can be found in Newcombe et al. (2009) and Newcombe (2009).

#### *7.2.2.1 Granular activated carbon*

The performance of GAC adsorption for extracellular microcystin removal depends upon the empty bed contact time (EBCT), carbon age, carbon pore size and raw water quality characteristics (e.g., NOM and pH), as well as the microcystin variant (Newcombe, 2002; Newcombe et al., 2003; Ho and Newcombe, 2007; Wang et al., 2007). The presence of biodegradation has also been shown to be an important factor in determining the effectiveness of GAC adsorption for microcystin removal (Newcombe, 2002; Wang et al., 2007). The removal of low levels of extracellular microcystins (< 1 µg/L) in source water to below 0.25 µg/L has been documented in full-scale treatment plants; however, the operational data available for these studies were limited (Lambert et al., 1996; Bogialli et al., 2012). Additional bench-scale and pilot-scale data suggest that higher concentrations of microcystins may be removed, but the GAC filter bed life may be limited depending on the source water quality characteristics and the degree of biodegradation (Bruchet et al., 1998; Hart et al., 1998; Newcombe, 2002; Newcombe et al., 2003; Wang et al., 2007; Alvarez et al., 2010).

Lambert et al. (1996) evaluated the removal of microcystins in drinking water at a full-scale water treatment plant using conventional filtration followed by GAC adsorption. The influent concentrations of total microcystins to the GAC process were 1.2 and 0.6 µg MC-$LR_{equiv}$/L during two separate monitoring events. Removals of 60% and 43% to achieve effluent concentrations of 0.5 and 0.3 µg MC-$LR_{equiv}$/L, respectively, were observed. The raw water was characterized by a high total organic carbon (TOC) concentration (25–30 mg/L), and the authors noted that the low levels of microcystin removal were likely due to competition from NOM adsorption. Similarly, Bogialli et al. (2013) reported that a conventional treatment plant that installed post-filtration GAC units was operated for 12 months, achieving approximately 75% removal of total microcystins (predominantly (d-Asp3)-MR-RR and MC-RR), from an influent concentration of 1.1 µg/L down to less than 0.25 µg/L. No information on the characteristics of the activated carbon used in these studies was provided.

Several bench-scale and pilot-scale studies have shown that loading of NOM onto GAC filters reduces the adsorption capacity for microcystins and results in more frequent replacement/regeneration of GAC than typically practised by water treatment plants (Craig and Bailey, 1995; Bruchet et al., 1998; Newcombe, 2002; Huang et al., 2007a; Wang et al., 2007). Newcombe (2002) observed the removal of two microcystin variants (MC-LR and MC-LA) from treated water (prior to chlorination) in multiple pilot-scale studies using GAC filter adsorbers. The systems consisted of a chemically activated wood-based GAC filter with a column depth of 1.5 m, a diameter of 8 cm, a flow rate of 25 L/h and an EBCT of 15 minutes. The systems demonstrated that influent concentrations of dissolved MC-LR and MC-LA up to 13 and 16 µg/L, respectively, were reduced to less than the MDL of 0.1 µg/L for periods ranging between 1 and 6 months. The

authors attributed the early breakthrough of microcystins (1 month) in the system with a DOC concentration of 6 mg/L to adsorption of NOM onto the GAC surface, reducing the adsorption capacity available for toxin removal. In addition, the authors noted that in all of the trials, MC-LA was adsorbed less effectively than MC-LR. Ho et al. (2011) suggested that differences in the charge, molecular size and conformation of microcystin molecules may influence their adsorption. Huang et al. (2007a) found similar results to Newcombe (2002) in bench-scale GAC experiments. The results indicated that high DOC (7.8 mg/L) in the influent water resulted in competitive adsorption and reduced the removal of MC-LR from 65% to 12% (initial concentration of 250 µg/L).

Alvarez et al. (2010) demonstrated that, in most cases, the EBCT for GAC filter adsorbers needs to be greater than 10 minutes to achieve adequate MC-LR removal when TOC concentrations between 1.5 and 5.1 mg/L are present in the source water. The authors estimated that source waters with greater than 4 mg/L TOC would have to replace a GAC bed with an EBCT of 10 minutes every 18 months in order to treat water with an influent MC-LR concentration of 2 µg/L down to less than 1 µg/L. It was noted that this frequency of replacement would be greater than the intervals typically practised by utilities.

Several authors have demonstrated that biodegradation of microcystins within a biologically active GAC filter increases the effectiveness and lifetime of this process for microcystin removal (Newcombe, 2002; Ho and Newcombe, 2007; Wang et al., 2007). In the Newcombe (2002) studies previously discussed, the authors noted that following 6 months of GAC filter operation, biodegradation began to occur in one of the filters, resulting in significantly improved microcystin removal. MC-LR and MC-LA concentrations of approximately 20 µg/L were removed to below 0.1 µg/L under these conditions. In similar studies, Wang et al. (2007) found that sterile GAC columns (autoclaved weekly) with a bed depth of 15 cm, a flow rate of 0.6 m/h and an EBCT of 15 minutes were capable of only 70% and 40% removal of 5 µg/L of MC-LR and MC-LA, respectively, following 6 months of operation. In contrast, the column with adsorption and biodegradation removal mechanisms demonstrated 90% and 70% removals for MC-LR and MC-LA, respectively, under the same conditions in the first 38 days of operation. After this period, removal increased to 100% because of the onset of biodegradation.

The type of GAC (pore size) has an impact on the performance of GAC filters for microcystin removal, and several studies have demonstrated that mesoporous carbons (e.g., chemically activated wood-based carbons) are the most effective for the removal of microcystins (Pendleton et al., 2001; Huang et al., 2007a; Newcombe et al., 2009; Sorlini and Collivignarelli, 2011). Pendleton et al. (2001) investigated the effectiveness of wood-based and coconut-based carbons on the removal of MC-LR spiked into pure water (bench scale). The authors found that wood-based carbons, containing both mesopores (> 1.6 nm to < 50 nm) and micropores (≤ 1.6 nm), adsorbed more microcystins than did the coconut-based carbons, which contained only micropores. MC-LR exhibits dimensions in the range of secondary micropores (0.7–1.6 nm). As such, both secondary micropore and mesopore volumes were thought to be important factors influencing the adsorption of MC-LR. Similarly, Huang et al. (2007a) and Sorlini and Collivignarelli (2011) also reported higher adsorption capacities of MC-LR using activated carbon with a high mesopore volume in natural water. Huang et al. (2007a) reported adsorption capacities of 73.7 mg/g for wood-based GAC, 16.6 mg/g for coal-based GAC and 14.5 mg/g for coconut-based GAC. Given the variability in adsorption of microcystins depending on the variant and carbon type, utilities should conduct testing to select the most effective carbon prior to installing GAC.

Pendleton et al. (2001) also examined the effect of solution chemistry on MC-LR adsorption onto GAC. Enhanced removal of MC-LR was observed at a lower pH (2.5 versus 6.5) owing to either a precipitation effect or the reduced solvency effect. Similarly, Huang et al. (2007a) reported that the adsorption of MC-LR, by three different types of activated carbon, increased as the solution pH was lowered from pH 8.0 to 3.0 (quantitative data not shown in paper). The authors hypothesized that at a lower pH, MC-LR molecules become smaller in size, and hydrogen bonds are more likely to form between the molecules and carbon surface; hence, adsorption capacity increases.

*7.2.2.2 Powdered activated carbon*

Removal of dissolved microcystins by PAC can be highly effective (up to 95%) depending on the microcystin variant and concentration, the PAC type and dose, the contact time and the water quality characteristics, such as TOC (Newcombe et al., 2003; Cook and Newcombe, 2008; Ho et al., 2011). Although a variety of treatment plants have used PAC for removal of microcystins in full-scale applications, data are available only for the combined PAC, coagulation, sedimentation and filtration processes (Lambert et al., 1996; Nasri et al., 2007; Zamyadi et al., 2012a). However, several authors have conducted bench-scale studies to determine the optimal contact time and PAC dose for removal of a variety of microcystin variants (Newcombe et al., 2003; Alvarez et al., 2010).

Alvarez et al. (2010) conducted bench-scale studies to determine the PAC doses needed to remove MC-LR under a range of typical water quality conditions. The results indicated that removal efficiencies greater than 60% could be obtained at PAC doses of 10 mg/L and a contact time of 30 minutes when a source water with a TOC concentration up to 5.1 mg/L and an MC-LR concentration of approximately 1 µg/L was treated. The authors concluded that a PAC dose of 10 mg/L and a contact time of between 30 and 120 minutes would be sufficient to decrease an MC-LR concentration between 2 and 3 µg/L to below 1 µg/L in most source waters. Based on knowledge of PAC efficiency from a variety studies, Newcombe et al. (2009) made a general recommendation that a PAC dose of 20 mg/L and a contact time of at least 45 minutes should be considered for removal of most dissolved microcystins (with the exception of MC-LA).

Cook and Newcombe (2008) investigated the removal of MC-LR and MC-LA by a wood-based PAC in two different Australian surface waters at bench scale. The Hope Valley water had a higher concentration of DOC (9.9 mg/L versus 6.7 mg/L), a higher specific UV absorbance (3.2 L/m·mg versus 2.2 L/m·mg) and higher colour (58 Hazen units [HU] versus 12 HU) compared with the River Murray water. PAC doses ranging from 2 to 15 mg/L were added to both surface waters that were spiked with MC-LR and MC-LA, with contact times tested ranging from 10 to 60 minutes. With initial MC-LR concentrations of 17.4 µg/L (Hope Valley) and 9.2 µg/L (River Murray), a PAC dose of 15 mg/L and 60 minutes of contact time, approximately 40% and 67% removal were observed from the Hope Valley and River Murray waters, respectively. Lower removals were seen for MC-LA with a higher PAC dose, as it is less readily adsorbed than MC-LR. With initial MC-LA concentrations of 19.2 µg/L (Hope Valley) and 9.9 µg/L (River Murray), a PAC dose of 25 mg/L and 60 minutes of contact time, approximately 10% and 25% of the toxin were removed from the Hope Valley and River Murray waters, respectively. The authors noted that the lower adsorption of both variants from the Hope Valley water was likely a result of greater competition for adsorption sites on the activated carbon between NOM and the toxins, as the DOC concentration was higher in the Hope Valley water.

Ho et al. (2011) studied the adsorption of four microcystin variants (MC-RR, MC-YR, MC-LR and MC-LA) in three different surface waters using two different PACs in bench-scale

experiments. Unfiltered raw water was collected at the inlet of three different water treatment plants (DOC 3.9–5.0 mg/L; pH 7.5–7.7) and spiked with 4 µg/L of each of MC-RR, MC-YR and MC-LR and 10 µg/L of MC-LA. Two steam-activated coal-based PACs were added to the raw waters at doses of 5, 10, 25, 50 and 100 mg/L for contact times of 30, 45 and 60 minutes. PAC-A had an effective size of 20–25 µm and a surface area of 1289 $m^2$/g, whereas PAC-B had an effective size of 10 µm and a surface area of 1105 $m^2$/g. For all surface waters and contact times, with both PACs, a dose of 100 mg/L could achieve a 95% reduction in total microcystins (with an effluent concentration of approximately 1.0 µg/L). Increased contact time (in excess of 30 minutes) did not appear to enhance microcystin adsorption on either PAC. The authors noted that the higher PAC doses (50 and 100 mg/L) are generally not practical at most conventional water plants, owing to the need for residual management facilities to remove the PAC prior to discharge to the distribution system. Hence, removal performance was also investigated for lower PAC doses. At 25 mg/L of PAC-A, with a contact time of 30 minutes, a 60% reduction in total microcystins was achieved for both the Waikerie and Warragamba surface waters. However, when PAC-B was applied under the same dosing conditions, an 80–95% reduction was achieved. The superior removal of total microcystins observed with PAC-B was likely due to its lower effective size, as particle size can influence adsorption kinetics, with more rapid adsorption onto smaller particles. Differences in water quality did not have as much of an effect on the adsorption of microcystins as did the PAC type. At most, the removal of total microcystins achieved in the Waikerie water, with both PACs, was approximately 15% higher than the removals achieved in the Warragamba water. Removals were likely lower in the Warragamba water due to a higher DOC concentration (5.0 mg/L) contributing to competitive adsorption. In all waters using the respective PACs, the order of the ease of removal of the microcystin variants followed the trend: MC-RR > MC-YR > MC-LR > MC-LA.

Newcombe et al. (2003) observed the removal of four microcystin variants (MC-LR, MC-LA, MC-RR and MC-YR) from raw water (prior to chlorination) in bench-scale experiments with 2 mg/L of PAC (applied over 5 days). The raw water had a DOC concentration of 10 mg/L, a pH of 7.8 and an alkalinity of 77 mg/L as calcium carbonate. For each microcystin variant, an initial concentration of 10 µg/L was spiked into the raw water. Removals for MC-RR, MC-YR, MC-LR and MC-LA were 68%, 38%, 15% and 2%, respectively. The authors hypothesized that the net charges of the compounds were responsible for the trend observed, as the adsorption of MC-LA was improved (17% removal) with the addition of salt. A preliminary cost analysis indicated that the use of PAC to treat a bloom where MC-LA was a major component would not be cost effective.

### *7.2.3 Chemical oxidation*

There are a variety of chemical oxidants that can be effective for the destruction of dissolved microcystins, including chlorine, ozone, permanganate and, in some cases, chlorine dioxide. The effectiveness of chemical oxidation for the removal of microcystins depends on the type of oxidant, dose, contact time, microcystin variant and water quality characteristics, such as pH and DOC (Newcombe, 2009; Sharma et al., 2012). Laboratory-scale experiments have demonstrated the general trend for the effectiveness of the oxidation of intracellular and dissolved microcystins to be ozone > permanganate > chlorine >>> chlorine-based oxidants (Ding et al., 2010; Sharma et al., 2012). The oxidation rate constants reported in the literature are $4.1 \times 10^5$ $M^{-1} \cdot s^{-1}$ for ozone; 375 $M^{-1} \cdot s^{-1}$ for permanganate; 33 $M^{-1} \cdot s^{-1}$ for chlorine; and 1.24 $M^{-1} \cdot s^{-1}$ chlorine dioxide (Acero et al., 2005; Rodriguez et al., 2007a, 2007b). However, selection of the

most appropriate oxidant for microcystins should be based on the characteristics of each source water (Sharma et al., 2012).

Although chemical oxidation can decrease total microcystin concentrations (intracellular and dissolved microcystins), in some cases, the concentration of dissolved microcystins will increase following oxidation owing to lysing of cells and subsequent release of microcystins into the water. Therefore, it is a general recommendation that chemical oxidation be conducted following the removal of cyanobacterial cells to help minimize the concentration of dissolved microcystins (Newcombe et al., 2010, 2015).

It is also important to recognize that the use of oxidants may result in the formation of microcystin by-products as well as other disinfection by-products (DBPs), which should be considered when selecting a strategy for oxidizing microcystins (Merel et al., 2010a; Zamyadi et al., 2012b; Wert et al., 2014). For example, ozone and chlorine dioxide can result in the formation of inorganic DBPs, such as bromate and chlorite/chlorate, respectively. The issues of DBPs are further discussed in the Guideline Technical Documents on trihalomethanes (THMs) (Health Canada, 2006), haloacetic acids (HAAs) (Health Canada, 2008a), bromate (Health Canada, 1998) and chlorite and chlorate (Health Canada, 2008b).

#### *7.2.3.1 Chlorine*

The oxidation of microcystins by chlorine has been found to be highly effective (> 90% removal) under experimental conditions (Ho et al., 2006a; Acero et al., 2008; Merel et al., 2009; Sorlini and Collivignarelli, 2011). However, full-scale treatment plants have demonstrated variable effectiveness for microcystin removal (range of 26–97% removal) (Tarczynska et al., 2001; Jia et al., 2003). The effectiveness of chlorination for the oxidation of microcystins depends upon the chlorine dose, contact time, pH, temperature and other water quality characteristics (Sharma et al., 2012). Several studies have found that microcystins are efficiently oxidized if pH is maintained below 8, the chlorine dose is greater than 3 mg/L and 0.5–1.5 mg/L of free chlorine residual is present after 30 minutes of contact time (Nicholson et al., 1994; Hitzfeld et al., 2000; Acero et al., 2005; Ho et al., 2006a; Xagoraraki et al., 2006; Newcombe et al., 2010). However, much higher chlorine doses (2–10 mg/L) are required to release and oxidize cell-bound microcystins (Zamyadi et al., 2013b).

Tarczynska et al. (2001) examined the efficiency of microcystin removal in each stage of a full-scale treatment plant employing conventional filtration followed by ozonation and chlorination. The removal of total dissolved microcystins in the chlorination step varied between 26% and 97%, lowering the concentration from 0.260–0.109 µg/L to 0.008–0.080 µg/L. The chlorine dose ranged between 2.7 and 3.1 mg/L, with a contact time of 12 hours. Jia et al. (2003) studied the removal of dissolved microcystins in a full-scale surface water treatment plant. Treatment included preoxidation with chlorine dioxide and conventional filtration followed by disinfection with chlorine. The authors found that approximately 57% of the total dissolved microcystins were removed by the chlorination process (1–2 mg/L dose; residual chlorine 0.5 mg/L) alone, from a filtered water concentration of 0.82 µg/L down to 0.35 µg/L, following chlorination.

The kinetics and factors that affect the oxidation efficiency of chlorine towards microcystins have been studied in detail at bench scale. Overall, the kinetics follow a second-order reaction, but first order with respect to the concentration of both free chlorine and microcystins (Acero et al., 2005; Ho et al., 2006a). The apparent second-order rate constants were determined by Acero et al. (2005) for MC-LR and found to be highly dependent on pH, with values decreasing from $4.75 \times 10^2$ $M^{-1} \cdot s^{-1}$ at pH 4.8 to 9.8 $M^{-1} \cdot s^{-1}$ at pH 8.8. Half-life times

ranged from minutes at pH 6 to hours above pH 8 for a constant chlorine dose between 0.5 and 1.0 mg/L. Xagoraraki et al. (2006) also studied the effects of pH, chlorine dose, initial toxin concentration and temperature on the rate of oxidation of MC-LR in carbonate-buffered laboratory water. Three different initial MC-LR concentrations (1, 2 and 8 µg/L), chlorine doses (1, 3 and 9 mg/L), pH values (6.0, 7.5 and 9.0) and temperatures were tested (11°C, 20°C and 29°C). The rate of oxidation was found to increase with an increase in chlorine dose and temperature and decrease with an increase in pH. Higher oxidation was observed at pH 6.0 (> 90% removal) after 100 minutes of contact time with 1.1 mg/L free chlorine, compared with pH 9.0 (< 30% removal). The initial toxin concentration had no effect on oxidation efficiency.

Sorlini and Collivignarelli (2011) conducted bench-scale oxidation experiments in raw surface water to compare the efficiencies of chlorine (sodium hypochlorite) and chlorine dioxide for the removal of MC-LR under natural water conditions. The water quality characteristics included low turbidity (< 1 nephelometric turbidity unit [NTU]), high alkalinity (80–110 mg/L as calcium carbonate) and low TOC (2.7–3.4 mg/L). MC-LR was spiked into the raw water at a concentration of 10 µg/L. A chlorine dose of 3 mg/L was required to achieve an 80% removal, resulting in a final MC-LR concentration of 2 µg/L. The authors noted that to reduce the concentration below 1 µg/L, chlorine doses higher than 3 mg/L would be required.

Ho et al. (2006a) studied the differences in chlorine reactivity for four different microcystin variants in treated water (DOC = 2.9–5.0 mg/L) and found that oxidation efficiency decreased in the following order: MC-YR > MC-RR > MC-LR > MC-LA. Each microcystin variant was spiked into the treated water at a concentration of 20 µg/L. For all microcystins, 90% oxidation was achieved with a chlorine dose of 1.5 mg/L (total chlorine) and a reaction time of 30 minutes. The authors noted that for waters with a DOC of 5 mg/L and a microcystin concentration of 5 µg/L or less, concentration × time (CT) values between 15 and 25 mg·min/L should be sufficient to achieve a treated water concentration below 1.0 µg/L. The authors noted that water suppliers should be aware of which microcystin variants are present in their water source, as variants are oxidized to a different degree, and oxidant doses and contact times may need to be adjusted accordingly.

NOM has been found to interfere with the oxidation of microcystins by chlorine. Acero et al. (2008) investigated the impact of naturally occurring DOC and ammonia in a surface water on the oxidation of microcystins by chlorination. Experiments were performed at a pH of 7.3 and 20°C with an initial chlorine dose of 3 mg/L (total chlorine) and an initial MC-LR concentration of 5 µg/L. Under these baseline conditions, the treated water MC-LR concentration was less than 0.5 µg/L (> 90% removal) after 60 minutes of contact time. An increase in DOC concentration from 5.4 to 10 mg/L was found to decrease MC-LR degradation from 100% to 57% (removals were observed following the complete depletion of chlorine). Similarly, an increase in ammonia concentration from 49 to 133 µg/L decreased MC-LR degradation from 100% to 85% (removals were observed following the complete depletion of chlorine).

By-products of the oxidation of microcystins using chlorine have been reported in the literature. Tsuji et al. (1997) treated MC-LR with a chlorine to MC-LR molar ratio of 2:1 and subsequently measured four isomers, which were identified as dihydroxymicrocystins. Merel et al. (2009) identified monochlorohydroxymicrocystin, dihydroxymicrocystin and monochloromicrocystin as intermediate by-products of the chlorination of microcystins, formed within 2 minutes of the reaction time. Trichlorohydroxymicrocystin was also found to be an intermediate formed within 10 minutes of reaction time. Monochlorodihydroxymicrocystin and dichlorodihydroxymicrocystin were identified as final by-products.

Research has also been conducted examining the effects of chlorine on the integrity of cyanobacterial cells and the potential for release of intracellular microcystins (Daly et al., 2007; Lin et al., 2009; Zamyadi et al., 2013b; Wert et al., 2014). During the oxidation of 50 000 cells/mL of *Microcystis aeruginosa*, there was no net increase in the MC-LR concentration after exposure to chlorine (doses 0.63–5 mg/L). However, at higher cell concentrations (200 000 cells/mL), release of intracellular MC-LR increased to 2.02 µg/L when using a chlorine dose of 0.63 mg/L (CT = 40 mg·min/L). At higher chlorine exposures (CT > 413 mg·min/L), the rate of MC-LR oxidation was sufficient to remove any released toxin, resulting in no net increase in the microcystin concentration (Wert et al., 2014). Daly et al. (2007) found that cell lysis occurred at chlorine exposures between 7 and 29 mg·min/L and that intracellular toxin released from damaged cells accumulated at a rate 3 times faster than it was degraded by chlorine (up to 36 µg/L of MC-LR). Similarly, Zamyadi et al. (2013b) found that chlorine exposures between 100 and 296 mg·min/L were required to obtain 76% cell lysis (*M. aeruginosa*) and oxidation of released cell-bound MC-LR to below 1 µg/L. The authors also estimated that the cellular chlorine (as $Cl_2$) demand of *M. aeruginosa* was 5.6 pg $Cl_2$/cell, and this information could be used to adjust the chlorination dose of treatment plants to satisfy the total chlorine demand associated with the presence of cells. However, increasing the chlorine dose in the presence of cyanobacterial cells can also result in release of organic matter that can contribute to the creation of DBPs (Zamyadi et al., 2012b; Wert and Rosario-Ortiz, 2013), and treatment plants will need to carefully consider and monitor potential DBP formation.

#### *7.2.3.2 Chlorine dioxide*

There is conflicting information in the literature regarding the effectiveness of chlorine dioxide ($ClO_2$) for oxidizing microcystins. Most laboratory studies have found that $ClO_2$ is not effective for oxidizing dissolved microcystins (Kull et al., 2004, 2006; Ding et al., 2010; Sorlini and Collivignarelli, 2011) or cyanobacterial cells and intracellular microcystins (Ding et al., 2010; Wert et al., 2014) at dosages (1–2 mg/L) and contact times typically applied to drinking water. In contrast, full-scale treatment plant data show evidence that removal of 1–2 µg/L of total microcystins may be feasible with $ClO_2$. Performance depends upon the $ClO_2$ dose, contact time and the initial concentration of microcystins present in the water. To a lesser extent, pH and temperature can also have an impact on the removal of microcystins by $ClO_2$ (Kull et al., 2004; Wu et al., 2012). In addition, utilities need to limit the $ClO_2$ dose applied to limit the chlorite and chlorate in the finished water. Chlorite and chlorate are not easily removed once formed, and their levels must be controlled by limiting the $ClO_2$ dose in order to meet the guidelines (Health Canada, 2008b).

$ClO_2$ is mainly used in drinking water treatment as a preoxidant prior to conventional filtration or other treatment processes. As the NOM concentration prior to the preoxidation step may be as high as several milligrams per litre, the ability of $ClO_2$ to oxidize micropollutants such as microcystins may be limited. Data from several full-scale treatment plants using $ClO_2$ as a preoxidant have shown that some oxidation of microcystins is possible using this treatment process (Jia et al., 2003; Jurczak et al., 2005; Bogialli et al., 2012). Jurczak et al. (2005) reported that preoxidation using $ClO_2$ doses of 1.6–2.8 mg/L and a contact time of 30 minutes resulted in an average reduction of total microcystins of 40.4%; however, most of the decrease (78%) was due to oxidation of intracellular microcystins, whereas there was a small increase (17%) for the dissolved form. The reduction was variable over a 3-month period, but typically removals of 1–2 µg/L from influent concentrations between 3 and 7 µg/L were achieved. Bogialli et al. (2012) also reported that preoxidation with a $ClO_2$ dose between 1 and 2 mg/L was effective at reducing a

total microcystin concentration up to 2.5 µg/L in the raw water to less than 0.5 µg/L post-oxidation. In contrast, Tarczynska et al. (2001) observed a 4–38% increase in dissolved microcystin concentrations with a 2.5 mg/L dose of $ClO_2$ and a 32-hour contact time. A decrease in the intracellular microcystin concentration from 1.7 to 0.8 µg/L was observed during the same sampling events.

Laboratory-scale studies have been conducted to obtain information on the kinetics of the oxidation of microcystins with $ClO_2$ and factors that affect the reaction. Kull et al. (2004) found that the reaction between MC-LR and $ClO_2$ in pure water was relatively slow. At a pH of 5.65 and a temperature of 20°C, the second-order rate constant for the oxidation of MC-LR in pure water with 1 mg/L of $ClO_2$ was 1.24 $M^{-1} \cdot s^{-1}$. Under these conditions, the theoretical half-life of MC-LR is 10.5 hours. Experiments to determine the impact of the presence of NOM on the oxidation of MC-LR found that in the presence of 1.0 mg/L of fulvic acid, an initial MC-LR concentration of 10 µg/L was reduced to only 7.44 µg/L with 1 mg/L $ClO_2$ and 10 hours of reaction time. The authors concluded that under the doses typically applied in drinking water treatment (1 mg/L), $ClO_2$ is not an effective oxidant for the complete removal of dissolved MC-LR. Additionally, the high reaction rate between $ClO_2$ and NOM is expected to significantly impair the removal of microcystins in real waters (Kull et al., 2006). Similarly, Sorlini and Collivignarelli (2011) conducted bench-scale oxidation experiments in raw surface water to compare the efficiencies of chlorine (sodium hypochlorite) and $ClO_2$ for the removal of MC-LR. The water characteristics were turbidity below 1 NTU, alkalinity of 80–110 mg/L as $CaCO_3$ and TOC of 2.7–3.4 mg/L. MC-LR was spiked into the raw water at a concentration of 10 µg/L. Even when a $ClO_2$ dose of 4.5 mg/L was applied for 60 minutes, only a 20% reduction in MC-LR could be achieved, resulting in a final concentration of approximately 8 µg/L.

Limited data are available on the effectiveness of the oxidation of microcystin variants other than MC-LR using $ClO_2$. Ying et al. (2008) determined that the second-order rate constant for MC-RR in pure water (bench scale) spiked with 100 µg/L increased with increasing temperature and decreasing pH. At a temperature of 10°C, the rate constant ranged from 10.2 to 8.8 $M^{-1} \cdot s^{-1}$ at pH 3.44 and 10.41, respectively. The authors concluded that $ClO_2$ could be effective for the removal of MC-RR from drinking water; however, the doses of $ClO_2$ applied in this study (8–20 mg/L) were much higher than those used in the study completed by Kull et al. (2004). More recently, Wu et al. (2012) investigated the removal of MC-LR, MC-RR and MC-YR using $ClO_2$. The results indicated that pure water (pH 6.8; 22°C) spiked with a total 6 µg/L of microcystins (MC-LR, MC-RR and MC-YR) and treated with 0.6 mg/L of $ClO_2$ for 30 minutes could achieve a treated water total microcystin concentration of less than 1 µg/L.

Recently, studies have been conducted on the effect of $ClO_2$ on cyanobacterial cell integrity, toxin degradation and DBP formation (Wert et al., 2014; Zhou et al., 2014). Wert et al. (2014) conducted batch oxidation experiments by adding *Microcystis aeruginosa* cells to natural water (DOC = 2.65 mg/L; pH = 8.0; alkalinity = 138 mg/L) and varying $ClO_2$ doses (0.63–5.0 mg/L). No net increase in the MC-LR concentration (< 0.5 µg/L) was observed for CTs between 610 and 4500 mg·min/L in the presence of 50 000 cells/mL. At higher cell concentrations (200 000 cells/mL), the MC-LR concentration increased to 3.3 µg/L when applying a dose of 0.63 mg/L (CT = 560 mg·min/L). At doses greater than 0.63 mg/L (CT > 690 mg·min/L), MC-LR was concurrently released and oxidized, resulting in a concentration below 0.5 µg/L in the water at a CT value above 890 mg·min/L. The results illustrated the potential to release MC-LR from cyanobacterial cells under exposure conditions ($ClO_2$ < 1.88 mg/L) that may occur in a drinking water treatment plant. Ding et al. (2010) also demonstrated that at CTs for chlorine dioxide commonly used for disinfection (11–347 mg·min/L), $ClO_2$ resulted in cell lysis, release and

accumulation of MC-LR. In contrast, Zhou et al. (2014) found that oxidation of a cyanobacterial suspension containing 100 000 cells/mL of *M. aeruginosa* with a $ClO_2$ dose of 1.0 mg/L for 10 minutes resulted in the presence of almost no integral cells. Under these conditions, up to 10 µg/L of intracellular MC-LR was released and completely degraded. A DOC concentration up to 1.25 mg/L was also measured in this experiment, and up to 20 and 60 µg/L of total THMs and HAAs were produced.

*7.2.3.3 Permanganate*

The oxidation of microcystins in water by permanganate addition is one of the more effective processes for removal of dissolved microcystins from water (Sharma et al., 2012). Rodriguez et al. (2007b) studied the oxidation of MC-LR, MC-RR and MC-YR with potassium permanganate. Results of the bench-scale experiments conducted in ultrapure water found second-order rate constants of 357 $M^{-1}\cdot s^{-1}$ for MC-LR, 418 $M^{-1}\cdot s^{-1}$ for MC-RR and 405 $M^{-1}\cdot s^{-1}$ for MC-YR at pH 7 and 20°C. The influence of pH on the oxidation process was not appreciable; however, the rate constants increased by a factor of 1.5 with a 10°C temperature increase. The authors also investigated the oxidation of MC-LR, MC-RR and MC-YR in natural water (DOC = 6.7 mg/L) at pH 7 and 20°C. The initial concentrations of MC-LR, MC-RR and MC-YR were 3.2, 7.1 and 1.1 µg/L, respectively. A dose of 1.25 mg/L of permanganate was required to oxidize the total microcystin concentration to below 1 µg/L (> 90% removal). In other bench-scale studies conducted by Rodriguez et al. (2007a), the oxidation of 1 mg/L of MC-LR in natural water (DOC = 3.6 mg/L) with permanganate was studied. The results indicated greater than 90% removal of MC-LR at a dose of 1.0 mg/L, a contact time of 60 minutes, a pH of 8 and a temperature of 20°C. Complete removal occurred at a dose of 1.5 mg/L (Rodriguez et al., 2007a).

X. Chen et al. (2005) investigated the reaction kinetics of the oxidation of MC-RR by permanganate in aqueous solutions (bench scale). A second-order rate constant of 469 $M^{-1}\cdot s^{-1}$ was reported at pH 7.0 and 20°C. The reaction rate was slightly faster under acidic conditions (low pH). At a pH of 6.7, a temperature of 25°C and a permanganate dose of 5 mg/L, the half-life was 0.671 minutes for an initial MC-RR concentration of 4.38 mg/L; hence, more than 99.5% of MC-RR was degraded within 10 minutes.

More recently, Ding et al. (2010) studied the inactivation of cells and the release and removal of microcystins from *Microcystis aeruginosa* using different oxidants. A second-order rate constant of 67.7 $M^{-1}\cdot s^{-1}$ was determined for the bacterial cell inactivation of *M. aeruginosa* at pH 7.6 and 22°C. The calculated half-life was 35 minutes based on a permanganate concentration of 0.5 mg/L. The results also showed that permanganate was highly effective at oxidizing dissolved MC-LR (20 µg/L), with exposures of less than 25 mg·min/L being needed for complete removal. The authors noted the absence of MC-LR accumulation (from cell lysis and release) in the experiments and attributed it to sufficiently fast oxidation rates.

There are limited publications on the full-scale application of permanganate for microcystin removal. Karner et al. (2001) studied the effectiveness of full-scale treatment plants in Wisconsin for microcystin removal, including two plants using permanganate preoxidation. One plant injected 2.7 mg/L of potassium permanganate at the inlet to a pretreatment basin with a retention time of 5.9 days followed by another injection of 3.5 mg/L before spray aeration. The average total microcystin concentration decreased by 54% following the first injection and by an additional 33% following the second injection. Typical influent concentrations to the plant were 1 µg $MC\text{-}LR_{equiv}$/L, but were as high as 5.5 µg $MC\text{-}LR_{equiv}$/L at times. The second plant dosed 1.3 mg/L of potassium permanganate at the intake pipe with a residence time of 21 minutes followed by 33 minutes in a detention basin. The average total microcystin concentration was reduced by

52% in the detention basin with influent concentrations ranging between 0.5 and 6.5 µg MC-$LR_{equiv}$/L. Treatment plants considering potassium permanganate for oxidation of microcystins should be aware that permanganate can discolour water when it is present at concentrations in excess of 0.05 mg/L. Therefore, dosage control is important to avoid consumer complaints (MWH, 2012).

*7.2.3.4 Ozone*

The oxidation of microcystins in water by ozone has been shown to be highly effective (> 90% removal) in laboratory-scale studies (Rositano et al., 2001; Shawwa and Smith, 2001; Brooke et al., 2006). The efficacy depends on temperature, pH, ozone dose, contact time and other water quality characteristics, such as DOC and alkalinity (Sharma et al., 2012). Full-scale treatment plant data have demonstrated variable effectiveness of ozone for microcystin removal (Tarczynska et al., 2001; Hoeger et al., 2005; Jurczak et al., 2005; Rapala et al., 2006). Utilities should also be aware that the use of ozone may result in the formation of bromate and other DBPs. As such, characterization of the source water needs to be undertaken to ascertain the potential for bromate formation (Health Canada, 1998).

In a full-scale study, Hoeger et al. (2005) reported that preozonation with 1.0 mg/L of ozone reduced 7.05 µg/L of total microcystins to 0.19 µg/L in the treated water (no contact time provided). The authors noted that although ozonation damaged the cells and resulted in the release of intracellular microcystins, the overall total microcystin concentration decreased. Jurczak et al. (2005) reported a reduction in microcystins (MC-LR, MC-RR and MC-YR) using ozone doses between 1.2 and 3.5 mg/L and a contact time of 1 hour. Monitoring on selected days in the plant found that residual microcystins were present following filtration (0.12 µg/L), but concentrations were lowered to 0.03 µg/L following ozonation. Another full-scale study found that ozone doses ranging between 1.7 and 2.0 mg/L and a contact time of 18 hours were capable of reducing dissolved microcystin concentrations as high as 0.508 µg/L by 29–56%, but were not always effective at removing intracellular microcystins (Tarczynska et al., 2001).

Laboratory studies have demonstrated that ozone reacts quickly with MC-LR, MC-LA and MC-RR (Rositano et al., 2001; Shawwa and Smith, 2001; Newcombe, 2002; Brooke et al., 2006). In general, the reaction kinetics of ozone with MC-LR improve with decreasing pH as well as increasing ozone dose and temperature (Shawwa and Smith, 2001; Al Momani and Jarah, 2010). Shawwa and Smith (2001) studied the kinetics of the oxidation of MC-LR by ozonation in pure water (bench scale) and found that the oxidation potential was lower under alkaline conditions (i.e., higher pH) due to the rapid decomposition of ozone by the hydroxyl ions. At 20°C, the average rate constant increased from $3.4 \times 10^4$ to $1.0 \times 10^5$ $M^{-1} \cdot s^{-1}$ as the pH was decreased from 7 to 2. As the temperature was increased from 10°C to 30°C at pH 2, the average rate constant increased from $5.8 \times 10^4$ to $1.6 \times 10^5$ $M^{-1} \cdot s^{-1}$. The authors also studied the effect of DOC on the oxidation of 500 µg/L of MC-LR. Humic acid was dissolved in pure water to simulate DOC concentrations of 1, 3 and 5 mg/L. For the solution with 1 mg/L DOC, an ozone dose of 0.3 mg/L (0.3 mg ozone/mg DOC) was sufficient to oxidize more than 95% of the microcystins after 2 minutes of contact time. However, as the DOC concentration was increased to 5 mg/L, more than 1.0 mg/L (0.2 mg ozone/mg DOC) of ozone was required to completely oxidize MC-LR. Onstad et al. (2007) determined the reaction kinetics of both ozone and hydroxyl radicals with MC-LR. The authors reported a second-order rate constant for the reaction with hydroxyl radicals at pH 7 to be $1.1 \times 10^{10}$ $M^{-1} \cdot s^{-1}$ for MC-LR.

Several studies have been conducted examining the effects of water quality on oxidation of microcystins using ozone. Rositano et al. (2001) studied the oxidation of MC-LR and MC-LA

at bench scale with ozone using a range of doses and four treated waters with differing water quality. DOC and alkalinity in the treated waters ranged from 4.6 to 15.5 mg/L and from 30 to 133 mg/L as calcium carbonate, respectively. The influent concentration of total microcystins was 40 µg/L (20 µg/L each). Ozone doses ranging from 0.4 to 0.8 mg/L, with a 5-minute contact time, resulted in greater than 90% removal of microcystins in the treated waters (effluent concentration < 4 µg/L). An ozone dose of 0.8 mg/L (0.05 mg ozone/mg DOC) was required for the water with the highest DOC concentration (15.5 mg/L) and alkalinity (133 mg/L as calcium carbonate). DOC was thought to be the main factor influencing microcystin oxidation.

Similarly, Brooke et al. (2006) conducted bench-scale experiments to observe the removal of MC-LA and MC-LR by oxidation with ozone in two treated waters with DOC concentrations of 5.3 and 19.9 mg/L. Initial concentrations of 20 µg/L of MC-LA and MC-LR were spiked into the treated waters prior to ozonation. Experiments were completed at a pH of approximately 7.5 and 20°C. In the water with high DOC, a lower ozone residual was observed after 5 minutes of contact time; hence, less ozone was available to oxidize microcystins. Regardless of the differences in water quality, the authors found that at ozone doses where a significant residual was present after 5 minutes of contact, microcystins could be completely removed. For both waters, a dose of 2 mg/L of ozone resulted in 100% removal.

The impact of ozonation as a preoxidant on cyanobacterial cells and the potential release of intracellular microcystins and DOC has also been studied (Miao and Tao, 2009; Ding et al., 2010; Coral et al., 2013). Coral et al. (2013) conducted laboratory studies with *Microcystis aeruginosa* and *Anabaena flos-aquae* suspensions at concentrations of 250 000 cells/mL and 1.5 × $10^6$ cells/mL and ozone doses of 0.5, 2.0 and 4.0 mg/L at pH 6 and 8. A quick and complete loss of cell viability occurred for both species after exposure to ozone at a CT of less than 0.2 mg·min/L. Although no cell lysis was observed for *M. aeruginosa* after 10 minutes of contact time and 4.0 mg/L of ozone at pH 5, a visible alteration in cell wall morphology was observed. *Anabaena flos-aquae* cells were impacted by ozone after only 30 seconds of contact time with 2 mg/L of ozone. As a result of cell damage, DOC concentrations up to 0.96 and 1.63 mg/L resulted after ozonation of 250 000 cells/mL of *M. aeruginosa* and *A. flos-aquae*, respectively. In addition, preozonation of *A. flos-aquae* cells increased the formation of THMs and HAAs by 174% and 65%, respectively, when chlorine was dosed to obtain a free chlorine residual of 1.0 mg/L after 24 hours of contact time. The authors recommended that utilities using ozone for oxidation of cyanobacterial cells consider the benefit of cyanobacterial control with the potential increased formation of chlorinated DBPs. Wert et al. (2014) and Ding et al. (2010) studied the impact of ozonation of *M. aeruginosa* cells on the release of MC-LR. An MC-LR concentration of 1.2 µg/L was detected after oxidation of 200 000 cells using 0.63 mg/L of ozone (CT = 0 mg·min/L). As the ozone dose increased (> 1.25 mg/L, CT > 0.22 mg·min/L), MC-LR concentrations were found to be below the MDL (0.5 µg/L).

#### *7.2.3.5 Monochloramine*

Monochloramine is a weaker oxidant than chlorine and is not an effective treatment barrier for microcystins (Westrick et al., 2010b). Nicholson et al. (1994) studied the oxidation of total microcystins in distilled water (bench scale) with monochloramine and found that at a dose of 20 mg/L, with a contact time of 5 days, only a 17% removal was achieved at room temperature. A 10% removal was achieved after 30 minutes. The initial concentration of microcystins was not described. The dose and contact times used in the study exceeded typical in-plant conditions when operating with monochloramine, although detention times on the order of days are available in the distribution system.

### *7.2.4 Ultraviolet irradiation*

Studies have indicated that UV irradiation may be effective for the oxidation of microcystins; however, exposure times and/or UV doses tested in the bench-scale experiments were greater than those typically applied in drinking water treatment. The efficacy of UV photolysis depends upon UV dose, exposure time and the UV spectrum of the microcystin (Sharma et al., 2012; Merel et al., 2013). Tsuji et al. (1995) studied the effect of UV light photolysis on the degradation of MC-LR in distilled water (bench scale) with an initial concentration of 10 mg/L. Removal of MC-LR increased with UV light intensity and contact time. After 10 minutes of contact time, the removal increased from 50% to 100%, as the UV intensity was increased from 0.147 to 2.55 mW/cm$^2$ (low-pressure UV lamp; spectral output: 254 nm). An intensity of 2.55 mW/cm$^2$, with a contact time of 10 minutes, equates to a UV dose of 1530 mJ/cm$^2$, which is far greater than the typical UV doses used for pathogen control (10–40 mJ/cm$^2$; Westrick et al., 2010b). Qiao et al. (2005) studied the degradation of MC-RR with UV radiation (low-pressure UV lamp; spectral output: 254 nm) in pure water at bench scale. Degradation increased with an increase in reaction time and UV light intensity. After 60 minutes, 70% of MC-RR could be removed with an initial concentration of 0.72 mg/L, a pH of 6.8 and a UV light intensity of 3.66 mW/cm$^2$ (UV dose = 13 176 mJ/cm$^2$).

### *7.2.5 Biological filtration*

Biological filtration using either biologically active sand or activated carbon has been shown to be effective for the removal of dissolved microcystins in bench-scale and pilot-scale studies (Keijola et al., 1998; Bourne et al., 2006; Ho et al., 2006b, 2008, 2012) and in limited full-scale studies (Grutzmacher et al., 2002; Rapala et al., 2006). The removal of intracellular microcystins through physical straining in slow sand filters has also been documented (Grutzmacher et al., 2002; Pereira et al., 2012). Bank filtration may also be effective for the removal of microcystins (Lahti et al., 1998; Schijven et al., 2002). A detailed review of biological treatment options for cyanotoxin removal conducted by Ho et al. (2012) identified the type and concentration of microcystin-degrading bacteria, concentration of microcystins and temperature as key factors that influence the efficiency of biological filtration for the removal of microcystins. In addition, the concentration of other organic matter within the source water may inhibit biodegradation, as microcystins may be a secondary substrate in the presence of NOM. Particle size, chemical composition and roughness or topography of the surface of the media used for filtration have also been identified as important factors for biofilm growth and ultimately the biodegradation of microcystins (Wang et al., 2007; Ho et al., 2012).

Several studies have identified the bacterial strains as well as the genes responsible for the degradation of microcystins (Bourne et al., 1996, 2001, 2006; Park et al., 2001). Bacterial strains of the genus *Sphingomonas* have been reported to fully degrade several microcystin variants (MC-LF, MC-LW, MC-LR, MC-RR and MC-YR) after 4–5 days in laboratory experiments (Park et al., 2001; Ishii et al., 2004). Park et al. (2001) demonstrated that a bench-scale bioreactor inoculated with $2.5 \times 10^6$ cells/mL of *Sphingomonas* and spiked with 20 mg/L of MC-RR or MC-LR achieved degradation rates of 13 and 5.4 mg/L per day, respectively. The microcystins were completely degraded in 4 days. In addition, increasing temperature between 5°C and 30°C also increased degradation rates, with the maximum rate of 7 mg/L per day occurring at 30°C (Park et al., 2001). Similar experiments conducted by Ishii et al. (2004) found that an isolated strain (7CY) of *Sphingomonas* was capable of completely degrading 6 µg/mL of MC-LW, MC-LF and MC-LR in 4 days. Other bacteria, such as *Arthrobacter* sp., *Brevibacterium* sp., *Rhodococcus* and

probiotic bacteria, were also found to have the capacity to degrade microcystins (Ho et al., 2012). Ho et al. (2006b) investigated the bacterial degradation at bench scale of microcystins in a biologically active sand filter under slow and rapid filtration conditions. MC-LR and MC-LA were spiked at concentrations of 20 µg/L into surface water with a pH of 6.7 and a DOC concentration of 11.8 mg/L. EBCTs of 4.0, 7.5, 10, 15 and 30 minutes were tested at filtration rates of 2.4, 1.2, 0.9, 0.6 and 0.3 m/h, respectively. Even under rapid sand filtration conditions, complete removal of the two microcystin variants was possible. The removal of microcystins in the sand filters was found to occur primarily through biological degradation, rather than physical processes. An initial breakthrough occurred for the first 3 days, followed by complete (100%) removal for the remainder of the experiment, suggesting that a period of 4 days is required to establish a sufficient biofilm.

Bourne et al. (2006) studied the degradation of MC-LR spiked into river water (50 µg/L) in six biologically active slow sand pilot-scale filter columns. The hydraulic loading rate on the columns was 0.0165 m/h, corresponding to an EBCT of 30 hours. Filters were inoculated with $5 \times 10^8$ cells/mL of a strain (MJ-PV) of *Sphingomonas.* Complete removal (100%) of MC-LR was obtained in all sand filters within 6 days, with no breakthrough detected after 15 days. Grutzmacher et al. (2002) conducted tests with full-scale slow sand filters to assess the efficiency of biological filtration for the removal of intracellular and dissolved microcystins. Slow sand filters with a bed depth of 80 cm, an average flow rate of 2.47 $m^3$/h and a filtration velocity of 0.8 m/day were fed raw water from a reservoir with a concentration of 11.8 µg $MC\text{-}LR_{equiv}$/L (dissolved). Greater than 95% removal of dissolved microcystins was obtained over a 30-hour filter operation period, with a maximum treated water concentration of 0.42 µg $MC\text{-}LR_{equiv}$/L. A second test was conducted to evaluate the removal of intracellular microcystins. A high density of *Planktothrix agardhii* (40 $mm^3$/L) and 43–58 µg $MC\text{-}LR_{equiv}$/L (92% intracellular) were present in the reservoir at the beginning of the 26-day experiment. The total microcystin concentration measured in the effluent ranged from 0.2 to 0.93 µg $MC\text{-}LR_{equiv}$/L (67–99% removal). It was determined that removal of the intracellular microcystins was through physical filtration. The higher microcystin concentrations (> 0.2 µg/L) observed over the 26-day period were attributed to the presence of increased dissolved microcystins in the raw water and temperatures that were too low (4°C) for complete biodegradation. Pereira et al. (2012) found that a pilot-scale slow sand filter was capable of removing 80% of phytoplankton with an average concentration of $5 \times 10^5$ cells/mL of predominantly *P. agardhii* in the raw water. Removal increased up to 96.8% following a filter maturation period of 3 days. The authors noted that, because of the high influent cell concentration, the filtered water still contained $9.03 \times 10^3$ cells/mL.

Schijven et al. (2002) summarized several studies indicating that riverbank filtration can be effective for the removal of cyanobacterial cells and microcystins. Lahti et al. (1998) reported that 98–99% of cyanobacterial cells were removed using riverbank filtration, but that traces of microcystins (< 0.1 µg/L) and single cells of cyanobacteria were found in filtered water even with a subsurface filtration path length as long as 100 m (Lahti et al., 1998).

As discussed in Section 7.2.2, biologically active GAC filters can effectively remove dissolved microcystins (Newcombe, 2002; Ho and Newcombe, 2007; Wang et al., 2007). Wang et al. (2007) studied the biodegradation of microcystins in bioreactors using bacteria sourced from a conventional GAC column. Treated water was spiked with 5 µg/L of MC-LR, inoculated with $7.6 \times 10^7$ active bacteria/mL and incubated aerobically. Degradation was found to be dependent on temperature and initial bacteria inoculum concentration. As the temperature was increased from 22°C to 33°C, a higher degradation rate was observed. At 33°C, 100% removal was observed after 3 days, whereas a period of 6 days was required to reach complete degradation at

22°C. Additionally, a higher initial bacterial inoculum concentration led to a faster degradation of MC-LR.

### *7.2.6 Membrane filtration*

Membrane filtration, including MF and UF, can achieve greater than 98% removal of cyanobacterial cells and intracellular microcystins (Chow et al., 1997; Gijsbertsen-Abrahamse et al., 2006; Campinas and Rosa, 2010; Sorlini et al., 2013), whereas NF, RO and, to a lesser extent, UF can be used for both intracellular and dissolved microcystin removal (Neumann and Weckesser, 1998; Lee and Walker, 2008; Dixon et al., 2011a, 2011b). However, in practice, cyanobacterial cells would likely be removed prior to RO to prevent membrane clogging and fouling. The performance of membrane filtration for microcystin removal depends on characteristics of the membrane, such as molecular weight cut-off (MWCO) and hydrophobicity, initial concentration, size and molecular weight of the microcystins, and operating parameters such as flux, recoveries and degree of fouling (Antoniou et al., 2014).

#### *7.2.6.1 Microfiltration/ultrafiltration*

Laboratory and pilot-scale studies have demonstrated that MF and UF can remove more than 98% of cyanobacterial cells (Chow et al., 1997; Gijsbertsen-Abrahamse et al., 2006; Campinas and Rosa, 2010; Sorlini et al., 2013), and UF can be moderately effective (35–70%) for the removal of dissolved microcystins (Lee and Walker, 2008). Several studies have also demonstrated that release of dissolved microcystins from the shear stress on cyanobacterial cells during MF and UF is possible, although it generally results in permeate microcystin concentration increases of less than 12% (Gijsbertsen-Abrahamse et al., 2006; Campinas and Rosa, 2010).

A study of a hollow fibre MF pilot plant demonstrated greater than 98% removal of a large number of cyanobacterial species using a membrane with an MWCO of 200 kDa, a flow rate of 2–4 $m^3$/h and a transmembrane pressure of 0.5–2.5. Feed water containing $6.3 \times 10^5$ – $10.8 \times 10^6$ cells/L of 17 species of cyanobacteria, including *Anabaena lemmermannii*, *Chroococcus* sp. and *Planktothrix* sp., achieved between 98% and 100% removal depending on the cyanobacterial species, with *Aphanothece* spp. being the least well removed (Sorlini et al., 2013).

Gijsbertsen-Abrahamse et al. (2006) studied the removal of intracellular microcystins from surface water in bench-scale experiments by a hollow fibre UF membrane with an MWCO of 100 kDa. The experiments were conducted using a dense cell culture of *Planktothrix agardhii* and a natural bloom of *P. rubescens*. A centrifugal pump with a shear rate of 1000 $s^{-1}$ was used to simulate the potential stress on cells due to pumping. The UF was run at a constant flux of 50 $L/m^2 \cdot h$ and a backwash at 15-minute intervals over a filter operating time of 4 hours. Almost complete removal of between 26 and 37 µg/L of intracellular microcystins was achieved (98%) in the experiments using cultured *P. agardhii*. In addition, the release of intracellular microcystins was less than 2%, even when cells were subjected to shear stress from the pump. Similarly, experiments conducted with *P. rubescens* demonstrated that dissolved microcystin concentrations were equal to or lower than the feed concentration (0.1 µg/L). Chow et al. (1997) observed similar results using MF and UF for removal of *Microcystis aeruginosa* cells. Similarly, Campinas and Rosa (2010) found complete removal of laboratory cultures of *M. aeruginosa* single cells using a hollow fibre cellulose acetate membrane (MWCO = 100 kDa) operated in cross-flow filtration mode. Feed water contained between 17 and 36 µg/L of chlorophyll-a. The authors also reported that cell lysis occurred during the UF runs, especially for older cell cultures. The permeate concentration remained equal to or lower than the feed concentration owing to the interaction between microcystins and algal organic matter and adsorption onto the membrane.

A decrease in flux (Campinas and Rosa, 2010) and an increase in transmembrane pressure (Gijsbertsen-Abrahamse et al., 2006) attributed to fouling of UF membranes with algal organic matter were observed in the studies discussed above, although Gijsbertsen-Abrahamse et al. (2006) noted that the fouling was reversible and could be removed with backwash. In contrast, Qu et al. (2012) found that cyanobacterial cells and cell fragments caused both reversible and irreversible fouling of UF membranes. The presence of $4 \times 10^6$ cells/mL of *M. aeruginosa* resulted in up to 30% irreversible membrane fouling. In addition, extracellular organic matter was identified as a major cause of the flux decline as macromolecular organics such as proteins and polysaccharides were deposited on the membrane.

Lee and Walker (2008) investigated the factors influencing the removal of dissolved MC-LR by UF membranes in bench-scale experiments. Factors examined included membrane characteristics, initial concentrations of MC-LR, water recovery and operating pressure. Seven membranes were studied, with MWCOs ranging from 1000 to 30 000 Da and differing hydrophobicities. Membrane hydrophobicity and porosity were both found to influence the removal of MC-LR by UF. In the early stages of filtration, the dominant rejection mechanism was adsorption. High adsorption of MC-LR (initial concentration of 50 µg/L) was observed for hydrophobic membranes, such as polysulphone (91%) and polyethersulphone (67%), as MC-LR is composed of amino acids possessing hydrophobic properties. Low adsorption was observed for the hydrophilic cellulose acetate membrane (> 5%). For tight UF membranes (MWCO = 1000–4000 Da), size exclusion was found to be the dominant rejection mechanism after adsorption reached equilibrium. Removal of microcystins via size exclusion (after 9 hours) was found to increase from 34.8% to 69.5% as the MWCO decreased from 4000 to 1000 Da. A higher permeate flux, resulting from increasing water recovery or operating pressure, led to an increase in microcystin removal by adsorption and a decrease in removal by size exclusion.

Dixon et al. (2011a) studied the effectiveness of coagulation, PAC and UF for removing microcystins in two Australian surface waters (bench scale). Experiments were conducted with UF only using lake water during a *Microcystis flos-aquae* bloom in March 2010. The total concentration of microcystins in the feed water to the UF membrane was 5.9 µg/L. A 70% removal of total microcystins was achieved by the UF membrane alone. In another study conducted by Dixon et al. (2011b), a laboratory culture of *Anabaena circinalis* (100 000 cells/mL) spiked into reservoir water was completely removed using a hollow fibre UF membrane.

#### *7.2.6.2 Nanofiltration/reverse osmosis*

The removal of dissolved microcystins by NF and RO is very effective (> 90%). Removal depends predominantly on the membrane pore size (MWCO), as the rejection of microcystins occurs via size exclusion. Gijsbertsen-Abrahamse et al. (2006) investigated the removal of MC-RR, MC-LR, MC-YR and MC-LA in tap water with a spiral wound NF membrane (MWCO not reported). Influent concentrations of the microcystins ranged from 1.3 to 9.4 µg/L, and permeate concentrations after 72 hours were typically less than the detection limit (0.08 µg/L). In almost all cases, greater than 99% removal was achieved with the NF membrane. Based on these results, the authors concluded that at least 90% removal of total microcystins should be achievable at full scale, even at high dissolved concentrations of 10 µg/L.

Similarly, Teixeira and Rosa (2006b) found that NF membranes were an effective barrier against microcystins in drinking water. Bench-scale experiments were conducted with 10 µg/L of MC-LR in decanted water from the Tavira water treatment plant (Algarve, Portugal). Greater than 94% of MC-LR was removed, regardless of the feed water quality (NOM range 4.6–10.2 mg/L as

carbon and pH 4.1–7.7) and the recovery rate. The removal mechanism was predominately size exclusion, as the MWCO of the NF membrane (150 g/mol) was small compared with the molecular weight of microcystins (approximately 1000 g/mol).

Dixon et al. (2011c) performed bench-scale experiments to determine the removal of microcystins (MC-RR, MC-LR, MC-YR and MC-LA) in two treated waters by four different spiral wound NF membranes. The membranes had MWCOs ranging from 100 to 800 Da, and the treated waters had DOC concentrations of 3.1 mg/L (low DOC) and 5.3 mg/L (high DOC, as defined by the study authors). The treated waters were spiked with 10 µg/L of each microcystin variant. The three NF membranes with lower MWCOs (100–300 Da) had similar removals for all four microcystins (> 90%) in both waters, even though two of the membranes were considered to be hydrophilic (one polyamide/polysulphone and one polyamide membrane). The hydrophobic membrane (sulphonated polyethersulphone) with a higher MWCO (600–800 Da) showed lower removals of MC-RR, MC-LR and MC-YR in both waters (40–90%). Different concentrations and NOM characteristics in the influent water had a negligible effect on the removal of microcystins.

RO membranes will retain a high percentage of microcystins present in the feed water, as the MWCO for microcystins is typically around 1000 g/mol (Lawton and Robertson, 1999). Neumann and Weckesser (1998) studied the removal of MC-LR and MC-RR by three different RO membranes in tap water and seawater (3000 mg/L sodium chloride). Initial toxin concentrations were in the range of 70–130 µg/L. The removal of MC-LR and MC-RR from tap water and seawater was greater than 96% and greater than 98%, respectively, for all three membranes tested.

### *7.2.7 Combined treatment technologies*

In practice, full-scale treatment plants use a combination of treatment technologies (i.e., conventional filtration and chemical oxidation) in order to remove both intracellular and dissolved microcystins. Data from operational treatment plants indicate that by combining treatment methods, utilities can effectively remove both forms of microcystins to achieve concentrations below 0.1 µg/L (Lahti et al., 2001; Boyd and Clevenger, 2002; Zurawell, 2002; Hoeger et al., 2005; Jurczak et al., 2005; Robert et al., 2005; Rapala et al., 2006; Haddix et al., 2007; Nasri et al., 2007; Robert, 2008; Zamyadi et al., 2013c). However, some studies have shown that the presence of high concentrations of cells ($10^5$ cells/mL) and/or microcystins in raw water (100 µg/L) may be challenging for treatment plants to remove to below 0.1 µg/L (Tarczynska et al., 2001; Hoeger et al., 2004; Zamyadi et al., 2012a).

Jurczak et al. (2005) monitored intracellular and dissolved microcystin concentrations in two full-scale drinking water treatment plants. The main cyanobacterial species present was *Microcystis aeruginosa*. Processes in the first plant included preoxidation with chlorine dioxide (1.6–2.8 mg/L) and conventional filtration, followed by ozonation (1.2–3.5 mg/L) and chlorination (1.32–3.88 mg/L). The results indicated that the overall treatment process was capable of removing greater than 99% of the microcystins present in the raw water (MC-LR, MC-RR and MC-YR) from a maximum influent concentration of 6.69 µg/L down to a treated water concentration below the detection limit of 0.01 µg/L. The second treatment plant employed conventional filtration followed by ozonation (1.5–5.5 mg/L) and chlorination (0.43–1.15 mg/L). This plant was capable of treating a maximum raw water microcystin concentration of 4.05 µg/L (MC-LR, MC-RR and MC-YR) down to a finished water concentration below 0.01 µg/L. The authors noted that the most effective step of the treatment processes in the removal of microcystins was the conventional filtration process, which reduced microcystins by greater than 75%.

Microcystin concentrations in raw and treated water at five drinking water systems in the United States were monitored to determine the effectiveness of each treatment step for removing both intracellular and extracellular microcystins (Karner et al., 2001). In general, the treatment plants used a combination of potassium permanganate (as a preoxidant), PAC, coagulation, clarification, rapid granular filtration and chlorination. All of the treatment plants were effective in removing total microcystin concentrations up to 7 µg MC-$LR_{equiv}$/L down to less than 0.017 µg MC-$LR_{equiv}$/L. The data from this study demonstrated that microcystin removal occurred incrementally at several points in the drinking water treatment process, with the largest removals (average of 61%) occurring with the use of potassium permanganate or PAC, and was further enhanced by coagulation and sedimentation (approximate removal of 43%).

A similar study was conducted by Lahti et al. (2001), examining the removal of cyanobacterial cells and microcystins in full-scale drinking water treatment plants with a variety of treatment process combinations. The treatment plant with the highest raw water microcystin concentration (9.4 µg MC-$LR_{equiv}$/L) was capable of achieving a finished water concentration of 0.03 µg/L. The treatment process included conventional treatment followed by ozonation, slow sand filtration and chlorination. A second plant with a maximum raw water microcystin concentration of 8.3 µg/L (MC-$LR_{equiv}$) reported finished water concentrations below 0.02 µg/L using conventional treatment, activated carbon filtration and chlorination.

Hoeger et al. (2005) reported that a drinking water treatment plant with preozonation (1.0 mg/L), rapid sand filtration, intermediate ozonation (0.5 mg/L), activated carbon filtration and slow sand filtration effectively removed both cyanobacterial cells and toxins. Raw water containing approximately 8.05 µg/L of total microcystins was effectively treated to achieve a finished water concentration of 0.240 µg/L.

The data reported above are supported by data provided by CDW and from provincial reports on the performance of nine full-scale drinking water treatment plants in Canada (Zurawell, 2002; Robert et al., 2005; Robert, 2008; Manitoba Water Stewardship, 2012; Ontario Ministry of the Environment, 2014). Robert et al. (2005) and Robert (2008) reported microcystin concentrations in raw and treated water during bloom events from six plants in Quebec over a period of 6 years. All of the treatment plants used a minimum of conventional filtration followed by chlorination, with some plants also using preoxidation (ozone or potassium permanganate) or PAC. Raw water concentrations up to 8 µg/L were effectively treated to below the MDL of 0.05 µg/L in all of the plants. Similar treatment plant effectiveness was observed during bloom events in Manitoba and Ontario (Manitoba Water Stewardship, 2012; Ontario Ministry of the Environment, 2014).

Zamyadi et al. (2012a) studied the breakthrough and accumulation of cyanobacterial cells and microcystins over three bloom seasons in a full-scale treatment plant. The treatment process included PAC (7–9 mg/L) and conventional filtration, followed by chlorination (1.2–2.0 mg/L). The process was capable of removing 6.3 µg/L total microcystins to below the MDL of 0.1 µg/L. The results indicated that chlorination of filtered water with an estimated CT of 117 mg·min/L was not efficient at lysing the remaining cyanobacterial cells, but that it was effective for the removal of the remaining dissolved microcystins. However, during a different monitoring event, the total microcystin concentration reached 118.70 µg/L in the raw water and was lowered to only 2.7 µg/L in the chlorinated water. Although this represented a 97.7% removal of toxins, the high influent concentration of cyanobacterial cells and microcystins made it difficult to achieve a low treated water concentration without treatment adjustments. The authors noted that the doses of PAC and chlorine used in the treatment plant were insufficient to effectively remove the high total microcystin concentration. The study also found that cells were not effectively settled in the

sedimentation tank and that 49 000 cells/mL and 10 300 µg/L of microcystins were present in scums over the clarifier. The authors also noted that a constant influx of cyanobacteria into a plant can accumulate, and ultimately both cyanobacteria and dissolved microcystins can be released from the clarifier sludge into the clarified water.

In a similar study, Tarczynska et al. (2001) monitored intracellular and extracellular microcystin concentrations in a full-scale drinking water treatment plant that used preoxidation with chlorine dioxide (2.5 mg/L), PAC (15 mg/L) and conventional filtration, followed by ozonation (1.7 mg/L) and chlorination (2.7 mg/L). The results indicated that the overall treatment process was capable of removing 77% of total microcystins from an influent concentration of 2.3 µg/L down to a treated water concentration of 0.52 µg/L. The authors noted that the ozonation/chlorination oxidation process was responsible for 98% of the removal of dissolved microcystins and that the microcystins that remained at the end of the treatment process were predominantly intracellular.

Maatouk et al. (2002) also found that even low levels of dissolved microcystins (63 ng/L) could not be effectively removed using prechlorination (0.42 mg/L), conventional filtration and PAC (20 mg/L). The authors suggested that the doses of chlorine and PAC were not optimized for the water quality of the system. Effective removal was observed when the system increased the PAC dose to 40 mg/L (Maatouk et al., 2002).

In most cases, utilities will be able to effectively remove intracellular microcystins with processes that are already in place (conventional filtration) when they are optimized for cyanobacterial cell or NOM removal. Dissolved microcystins may also be removed by many treatment plants by using existing treatment, such as chlorination, or by the addition of simple processes, such as PAC (Carrière et al., 2010). Although it is possible to remove both intracellular and dissolved microcystins effectively using a combination of treatment processes, the results of the studies discussed above indicate that the removal efficiency can vary considerably. Utilities need to ensure that the treatment processes in place have been optimized for removal of both cyanobacterial cells and dissolved microcystins and that the appropriate monitoring is being conducted to ensure that adequate removal is occurring at each step in the treatment process. Several of the studies discussed above and in Section 7.2.1 indicate that cyanobacterial cells can be present in filtered water (Rapala et al., 2002; Hoeger et al., 2005; Zamyadi et al., 2012a); therefore, it is important that analysis of treated water include cell lysis so that the total microcystin concentration is determined. In addition, the final treatment barrier, such as oxidation, may need to be adjusted to account for the presence of intracellular microcystins (i.e., oxidation of cells and dissolved microcystins).

### *7.2.8 Emerging technologies*

Several emerging technologies (advanced oxidation processes [AOPs] and ultrasonic degradation) may offer viable alternative treatments to remove microcystins; however, evidence of their efficacy is limited to bench-scale studies. AOP-based degradation is complex, as it must take into account competition for reactive sites, the interference of particles with UV transmittance, and the role of pH and temperature in determining reaction rates. The formation and possible toxicity of microcystin degradation by-products as a result of treatment must also be considered. Further full-scale and pilot-scale studies are required to evaluate the degradation efficacy of multiple microcystins at typical influent concentrations under various water quality conditions.

*7.2.8.1 Ultraviolet and hydrogen peroxide*

The effectiveness of UV/hydrogen peroxide treatment was examined by He et al. (2012) on MC-LR and by Qiao et al. (2005) on MC-RR. Under optimal conditions, He et al. (2012) observed 93.9% degradation with an initial MC-LR concentration of 1 µmol/L (UV = 80 mJ/cm$^2$; 882 µmol/L hydrogen peroxide). Qiao et al. (2005) observed, under optimal conditions, a 94.83% degradation with an initial MC-RR concentration of 0.72 mg/L (UV = 3.66 mW/cm$^2$; 1 mmol hydrogen peroxide; pH 6.8).

Qiao et al. (2005) found that there was a synergistic effect with the combination of UV and hydrogen peroxide, compared with either UV or hydrogen peroxide treatment alone. Increasing the hydrogen peroxide dose above the optimal dose resulted in a decreased degradation rate for MC-LR (He et al., 2012) and MC-RR (Qiao et al., 2005), as the excess hydrogen peroxide likely competed with the microcystins for hydroxyl radicals. The efficacy of the UV/hydrogen peroxide treatment was observed to decrease as the initial concentration of MC-LC (He et al., 2012) and MC-RR (Qiao et al., 2005) increased. Qiao et al. (2005) observed experimentally that at higher pH (pH 11), the degradation rate of MC-RR decreased. Similarly, He et al. (2012) observed a decreased degradation rate of MC-LR above pH 10 and suggested that as pH increases, the presence of $HO^-$ competes with MC-LR for reactions with hydroxyl radicals. He et al. (2012) also used natural water in the experiments and observed that alkalinity tested in the range of 89.6–117.8 mg/L as calcium carbonate and NOM in the range of 2–9.6 mg/L as TOC significantly decreased the destruction rate. The work by both He et al. (2012) and Qiao et al. (2005) highlights that future investigations need to assess a wide range of influent values for site-specific water quality.

*7.2.8.2 Ozone and ultraviolet*

The sequential application of a UV treatment followed by ozone has been demonstrated as an effective treatment at the bench scale using deionized water (Liu et al., 2010). Liu et al. (2010) observed that with an initial MC-LR concentration of 100 µg/L, a 5-minute UV (2.6 mW/cm$^2$) treatment followed by ozone at 0.2 mg/L could decrease the MC-LR concentration to less than 1 µg/L, and ozone at 0.5 mg/L decreased the MC-LR concentration to less than 0.1 µg/L. However, NOM was observed to negatively impact the performance of ozone.

*7.2.8.3 Ozone and hydrogen peroxide*

The application of ozone and hydrogen peroxide has also been observed to be effective in bench-scale studies using deionized water (Rositano et al., 1998; Al Momani et al., 2008). At a peroxide/ozone weight ratio of 0.5, Rositano et al. (1998) observed that in deionized water, the combination of UV with hydrogen peroxide at an ozone dose of 0.02 mg/L provided 54% destruction of MC-LR after 15 seconds, and UV with hydrogen peroxide at an ozone dose of 0.2 mg/L provided 100% destruction after 15 seconds. Al Momani et al. (2008) experimented with different ratios of ozone to hydrogen peroxide. After an 80-second reaction, 98% of MC-LR was degraded by dosing 0.5 mg/L ozone and 0.001 mg/L hydrogen peroxide, and total degradation was observed using doses of 0.1 mg/L ozone and 0.01 mg/L hydrogen peroxide. Al Momani et al. (2008) observed that NOM interfered with the degradation of MC-LR, as it provided competing reactions. Temperature and pH were also observed to affect the degradation kinetics of ozone (Al Momani et al., 2008).

#### *7.2.8.4 Titanium dioxide photocatalysis*

Titanium dioxide photocatalysis has been successfully used in the destruction of microcystins in bench-scale studies. Lawton et al. (1999) examined the destruction of MC-LR with an initial concentration of 200 µg/mL at pH 4 and 306 K. A 50% decline in toxin was observed as a result of adsorption of MC-LR to titanium dioxide (1% m/v). Only a small MC-LR peak was detected by HPLC after 20 minutes of UV exposure (280 W); however, degradation by-products were detected. Cornish et al. (2000) used the same water type as Lawton et al. (1999) (pH 4, 306 K) to examine the impact of adding hydrogen peroxide to titanium dioxide photocatalysis. Cornish et al. (2000) found that when there was addition of hydrogen peroxide at an optimal dose of 0.005–0.1%, the titanium dioxide/UV system performed more effectively. However, when an excess of hydrogen peroxide was added, competition between MC-LR and hydrogen peroxide likely occurred for surface sites of the titanium dioxide. Cornish et al. (2000) did not detect any by-products from the reaction; however, the incomplete mineralization of MC-LR suggested that undetectable by-products had been formed. B. Yuan et al. (2006) demonstrated that low doses of iron(VI) further enhanced degradation using a combination of titanium dioxide (2 g/L) and UV (40 W/m$^2$). At the optimal pH of 6, a 0.08 mmol/L ferrate dose provided 100% degradation after 30 minutes. The degradation rate was negatively impacted by both an excess of ferrate addition and non-optimal pH (B. Yuan et al., 2006).

#### *7.2.8.5 Ultrasonic degradation*

Ultrasonic irradiation is known to inhibit cyanobacterial growth (Ahn et al., 2003). It involves the growth and collapse of gas bubbles, leading to the pyrolysis of water and the production of hydroxyl radicals. Ultrasonic irradiation has several advantages over other AOPs, as it does not require the addition of chemicals, and efficacy is not affected by turbidity. However, there have been concerns regarding the release of microcystins into the water from intact cells (Song et al., 2005). Song et al. (2005) studied the effect of ultrasonic irradiation on the degradation of microcystins in an aqueous solution. Bench-scale tests were conducted with a 640 kHz ultrasonic transducer on solutions containing 2.7 µmol MC-LR/L. Within 3 minutes of contact time, less than 50% of the initial toxin concentration remained (1.1 µmol/L), and after 6 minutes, the concentration was reduced to approximately 0.4 µmol/L (85% removal). Additional experiments were conducted to evaluate the effectiveness of ultrasonic irradiation on the degradation of microcystins in the presence of cellular extracts (dissolved organic matter). After 3 minutes, 58% of MC-LR was degraded in pure water, compared with 35% in the presence of cellular extracts.

### *7.2.9 Cylindrospermopsin*

Treatment for the removal of cylindrospermopsin from drinking water follows the same principles as for microcystins: removal of intact cyanobacterial cells followed by removal of dissolved cylindrospermopsin. However, cylindrospermopsin tends to be present in source water in the dissolved form to a greater degree than microcystins, and this factor needs to be taken into consideration in the treatment process (Ho et al., 2011). De la Cruz et al. (2013) recently conducted a comprehensive review of treatment methods that are effective for the removal of cylindrospermopsin. Several treatment methods are effective for the removal of cylindrospermopsin, but limited full-scale treatment plant data have been reported. The available information includes studies on conventional and membrane filtration for cell removal and activated carbon and oxidation using chlorine, ozone and RO for dissolved cylindrospermopsin removal (Westrick et al., 2010b; de la Cruz et al., 2013). Factors that affect the efficacy of the

treatment processes for the removal of cylindrospermopsin are similar to those for microcystins discussed in Section 7.2.1.

Hoeger et al. (2004) studied the removal of cyanotoxins in full-scale drinking water treatment plants in Australia. Greater than 99% of cylindrospermopsin was removed using conventional filtration followed by chlorination, achieving a treated water concentration below the detection limit of 0.2 µg/L from an initial concentration of 1.17 µg/L.

Ho et al. (2008, 2011) conducted bench-scale and modelling studies on the use of PAC for the adsorption of cylindrospermopsin. The results demonstrated that to remove 5 µg/L of cylindrospermopsin to less than 1 µg/L, a PAC dose of 33 mg/L and a contact time of 30 minutes would be required. Similarly, modelling results suggested that PAC can be effective, but that relatively high doses are required. In addition, comparison of the removals of different cyanotoxins using PAC found that cylindrospermopsin is removed to a similar extent as for MC-RR.

A number of studies have been conducted examining the removal of cylindrospermopsin using oxidation (Rodriguez et al., 2007a, 2007b; Merel et al., 2010a; de la Cruz et al., 2013). Several studies have demonstrated that cylindrospermopsin is rapidly oxidized using chlorine. Rodriguez et al. (2007a) found that maintaining a chlorine residual of 0.5 mg/L with a 30-minute reaction time could completely degrade 60 µg/L of cylindrospermopsin. Ozone has also been shown to effectively oxidize cylindrospermopsin in laboratory-scale studies. At pH 8, approximately 95% of cylindrospermopsin (initial concentration of 415 µg/L) was oxidized using 0.38 mg/L ozone and an unlimited reaction time. In contrast, the oxidation of cylindrospermopsin with permanganate and chlorine dioxide was slow, corresponding to second-order rate constants of 0.3 and 0.9 $M^{-1} \cdot s^{-1}$, respectively. The authors concluded that permanganate and chlorine dioxide would not be effective under typical treatment plant conditions. As with microcystins, consideration should be given to potential DBP formation from the addition of oxidants to the water. This is discussed in greater detail in Section 7.2.3.

Limited information is available on the use of membrane filtration for cylindrospermopsin removal. Dixon et al. (2011b) studied the removal of cyanobacterial metabolites by NF. The results indicated that average removals between 90% and 100% could be achieved for cylindrospermopsin using membranes with a low MWCO (< 300 Da). Dixon et al. (2012) also conducted laboratory-scale testing of integrated membrane systems for cyanotoxin removal. The results showed that a UF system with pretreatment using 2.2 mg/L of alum and 20 mg/L of PAC resulted in 97% removal of intracellular and extracellular cylindrospermopsin to achieve a treated water concentration of less than 0.1 µg/L (Dixon et al., 2012).

## 7.3 Residential-scale treatment

Municipal treatment of drinking water is designed to reduce contaminants to levels at or below guideline values. As a result, the use of residential-scale treatment devices on municipally treated water is generally not necessary, but primarily based on individual choice. In cases where an individual household obtains its drinking water from a surface water supply where a cyanobacterial bloom is present and microcystins may be present in the water, consumers need to assess the best option for obtaining safe drinking water. These options may include switching to an alternative water supply, changing the location (distance and depth) of the water supply pipe or installing a drinking water treatment system. However, consumers should be aware that treating water supplies for the removal of cyanobacteria and microcystins is very complex. It is also difficult to assess if the treatment system for microcystin removal is functioning properly, as there are currently no standardized analytical methods for microcystins, and the analysis of

microcystins is not routinely conducted by many laboratories. In addition, it may be very difficult to treat a source water that is significantly impacted (large bloom) by cyanobacteria.

Limited information is available on residential-scale treatment technologies for the removal of cyanobacterial cells and microcystins. The few studies that have been conducted support the use of a series of specific filter types for the reduction of microcystins in drinking water (Lawton et al., 1998; Horman et al., 2004; Pawlowicz et al., 2006; Colling et al., 2014). This is supported by the data available for large-scale treatment systems. It is recommended that a qualified person be consulted prior to selecting and installing a residential-scale treatment system.

The most effective way to treat water containing microcystins is to remove the cyanobacterial cells first, followed by removal of dissolved microcystins that may be present in the water. The first step should be the installation of a filtration system at the point of entry (POE) to the home to reduce the number of cyanobacterial cells that are present in water used for bathing and washing dishes. This POE filtration system should also be installed upstream of any disinfection that may already be present in the home treatment system (e.g., UV or chlorination). The filtration system should consist of a prefilter for removal of large particles (e.g., sand, sediment) followed by a filter with a smaller pore size for removal of cyanobacteria. Based on the size of cyanobacteria, a filter with an absolute pore size of 1 µm or less will likely remove most cells, but this will depend on the type of cyanobacteria present in the water.

The final filtration step should be a filter system installed at the point of use (POU) of drinking water—for example, before the kitchen faucet. This POU filtration system should include an activated carbon filter followed by an RO filter. These types of filter systems have been shown to remove dissolved microcystins (Horman et al., 2004; Pawlowicz et al., 2006). RO systems should include an activated carbon filter within the unit, or a separate activated carbon device can be purchased and installed before (upstream of) the RO unit. RO systems are intended for POU installation, as larger quantities of influent (incoming) water are needed to obtain the required volume of treated water, and this is generally not practical for residential-scale POE systems. In addition, RO systems are certified only for POU installation, as the treated water may be corrosive to internal plumbing components.

Health Canada does not recommend specific brands of drinking water treatment devices, but it strongly recommends that consumers use devices that have been certified by an accredited certification body as meeting the appropriate NSF International (NSF)/American National Standards Institute (ANSI) drinking water treatment unit standards. These standards have been designed to safeguard drinking water by helping to ensure the material safety and performance of products that come into contact with drinking water. No drinking water treatment devices are certified specifically for microcystin removal at this time, as microcystins are not currently included in any of the NSF/ANSI drinking water treatment unit standards. Consumers should consider devices that have been certified under the standards that cover those technologies that are effective for microcystins (e.g., activated carbon, RO): NSF/ANSI Standard 58: Reverse Osmosis Drinking Water Treatment Systems; and NSF/ANSI Standard 53: Drinking Water Treatment Units—Health Effects. No performance testing is available for microcystins under these standards.

Certification organizations provide assurance that a product conforms to applicable standards and must be accredited by the Standards Council of Canada (SCC). In Canada, the following organizations have been accredited by the SCC to certify drinking water devices and materials as meeting NSF/ANSI standards (SCC, 2015):

- CSA Group (www.csagroup.org);
- NSF International (www.nsf.org);

- Water Quality Association (www.wqa.org);
- UL LLC (www.ul.com);
- Bureau de normalisation du Québec (www.bnq.qc.ca – available in French only); and
- International Association of Plumbing & Mechanical Officials (www.iapmo.org).

An up-to-date list of accredited certification organizations can be obtained from the SCC (2015).

Generally, before a treatment system is installed, the water should be tested to determine general water chemistry and to provide an estimate of the concentration of the contaminant to be removed. In the case of microcystins, the ability to obtain useful testing results will be limited, as microcystin concentrations in source water are continually changing depending on a variety of factors, including the type of cyanobacteria present and the stage of growth of the bloom. In addition, analysis of microcystins is not routinely conducted in many laboratories. Despite these limitations, periodic testing should be conducted by an accredited laboratory on both the water entering the home and the finished water (at the POU) to verify that the treatment devices are effective. Consumers should request that the laboratory analyze for total microcystins, which includes both the microcystins that are within the cells of the cyanobacteria and the dissolved microcystins in the water.

In addition, consumers should be aware that both the activated carbon treatment device and RO system can lose removal capacity through usage and time. As such, these systems need to be maintained and/or replaced. The adsorption medium (i.e., carbon) is crucial to the proper functioning of the RO system, and consumers should verify its expected longevity and replace the medium and service the system when required, as per the manufacturer's recommendations. It should also be noted that the treatment devices discussed above are not designed to disinfect drinking water, and additional treatment is required for water that requires disinfection.

# 8.0 Kinetics and metabolism

## 8.1 Absorption

### *8.1.1 Microcystins*

No data were available that quantified the intestinal, respiratory or dermal absorption of microcystins. Most of the available evidence indicates that absorption from the intestinal tract and transport into liver, brain and other tissues require facilitated transport using receptors belonging to the organic acid transporter polypeptide (OATP[1]) family. The OATP family transporters are part of a large family of membrane receptors that facilitate cellular, sodium-independent uptake and export of a wide variety of amphipathic compounds, including cyanobacterial toxins (Meriluoto et al., 1990; Cheng et al., 2005; Fischer et al., 2005, 2010).

Ito et al. (1997a) and Ito and Nagai (2000) qualitatively studied the oral absorption of MC-LR (purified from an cyanobacterial bloom sample) in male ICR mice following a single gavage dose of 500 μg/kg bw. Targeted immunostaining indicated that MC-LR was absorbed primarily in the small intestine, although some absorption did occur in the stomach (Ito and Nagai, 2000). Erosion of the surface epithelial cells of the small intestinal villi was observed, which might facilitate uptake of the toxin into the bloodstream (Ito et al., 1997a; Ito and Nagai, 2000).

[1] For this document, the abbreviation for the organic acid transporter polypeptides will be written as OATP rather than differentiating the animal versions from the human versions by using lowercase letters for the animal versions and uppercase letters for the human versions.

The oral bioavailability of MC-LR was indirectly studied *in situ* using isolated intestinal loops of rats (Dahlem et al., 1989). Rats given an infusion of MC-LR (> 95% pure) into the ileum showed clinical symptoms, such as laboured breathing and circulatory shock, plus evidence of liver toxicity within 6 hours of a single 5 mg/kg bw dose. Infusion of a similar dose into a jejunal loop produced a lower degree of liver toxicity. These results suggest that there could be site specificity in intestinal absorption of MC-LR; however, differences in absorptive surface area were not considered.

Oral absorption of $^{3}$H-labelled dihydromicrocystin (75 µg/kg bw) was also demonstrated in swine using ileal loop exposure (Stotts et al., 1997a, 1997b). In the exposed swine, the maximum blood concentration of the toxin occurred 90 minutes after dosing.

Microcystins are not volatile and are not likely to be present in gaseous form in air at ambient temperatures. However, they can be present as aerosols generated by wind and during showering or swimming, thereby providing contact with the respiratory epithelium. Pulmonary absorption of MC-LR (purified from an cyanobacterial bloom sample) was demonstrated by intratracheal instillation of a sublethal dose of 50 µg/kg bw or a lethal dose of 100 µg/kg bw in mice (Ito et al., 2001). Immunostaining of the lung occurred within 5 minutes. A lag period of 60 minutes occurred after the lethal dose and 7 hours after the sublethal dose before staining was observed in the liver. These data demonstrate that uptake from the lungs into systemic circulation can occur.

No *in vivo* or *in vitro* dermal absorption studies for microcystins were identified**.**

### *8.1.2 Anatoxin-a*

No quantitative data were located regarding the rate or extent of absorption of anatoxin-a in humans or experimental animals. However, acute oral toxicity studies in animals indicate that anatoxin-a is rapidly absorbed, as shown by the occurrence of clinical signs of neurotoxicity, including loss of coordination, muscular twitching and death from respiratory paralysis, within several minutes of exposure (Stevens and Krieger, 1991a; Fitzgeorge et al., 1994).

### *8.1.3 Cylindrospermopsin*

No quantitative data were located regarding the rate or extent of absorption of cylindrospermopsin in humans or experimental animals following oral, inhalation or dermal exposure. Absorption of cylindrospermopsin from the gastrointestinal tract of mice was demonstrated by hepatic and other systemic effects observed in 14-day and 11-week oral toxicity studies of pure cylindrospermopsin (Shaw et al., 1999, 2000, 2001; Humpage and Falconer, 2003).

Uptake of purified cylindrospermopsin into a kidney cell line (Vero cells) was found to be slow and progressive and not energy dependent (Froscio et al., 2009). The cylindrospermopsin-induced effects on cellular protein synthesis in the Vero cells could not be reversed when the toxin was removed. The strong interaction of the toxin with its target to inhibit protein synthesis indicates that cylindrospermopsin remains in the intracellular environment for extended periods. Although dilution of the cell media increased the uptake of cylindrospermopsin, no mechanism could be identified that explained this result.

## 8.2 Distribution

### *8.2.1 Microcystins*

The distribution of microcystins is limited due to the poor ability of these toxins to cross cell membranes (Puiseux-Dao and Edery, 2006). Facilitated transport is necessary for uptake of

microcystins into organs and tissues as well as for their export. Many studies have demonstrated that inhibition of microcystin uptake by its OATP reduces or eliminates the liver toxicity observed following *in vitro* or *in vivo* exposures (Runnegar et al., 1981, 1995a; Runnegar and Falconer, 1982; Hermansky et al., 1990a, 1990b; Thompson and Pace, 1992). OATPs are located in the liver, brain, testes, lungs, kidneys, placenta and other tissues of rodents and humans (Cheng et al., 2005). Only a few of the OATPs have been characterized at their functional, structural and regulation levels. In mice, males often express OATPs in tissues to a greater extent than females (Cheng et al., 2005).

Human OATP1B1, OATP1B3 and OATP1A2 were shown to mediate the transport of $^{3}$H-labelled dihydromicrocystin-LR in *Xenopus laevis* oocytes, and its uptake was inhibited by sulphobromophthalein and taurocholate (Fischer et al., 2005). Additionally, a number of *in vitro* studies have indicated that cells lacking microcystin-competent OATP do not absorb microcystins and that introduction of OATPs to these cells enables them to absorb microcystins (Komatsu et al., 2007; Feurstein et al., 2010; Fischer et al., 2010; Jasionek et al., 2010). One study indicated that the role of OATP in microcystin uptake varies by variant, with greater uptake of MC-LW and MC-LF than of MC-LR and MC-RR (Fischer et al., 2010).

Lu et al. (2008) used OATP1B2-null mice to demonstrate the importance of the OATP system for transporting MC-LR into the liver. OATP1B2-knockout mice did not experience the severe hepatotoxicity and death caused by 120 µg MC-LR/kg bw (administered intraperitoneally) in wild-type mice. Fischer et al. (2010) demonstrated the necessity for microcystin-competent OATP for transport of microcystin across the cellular membrane using primary human hepatocytes and OATP-transfected HEK293 cells compared with control vector HEK293 cells, which were resistant to microcystin cytotoxicity. Primary human hepatocytes were an order of magnitude more sensitive than the OATP-transfected HEK293 cells. The study authors suggested that this was because the transfected HEK293 cells contained only OATP1B1 and OATP1B3, whereas the primary human hepatocytes may contain other OATPs that contribute to the uptake of the microcystin variants. Komatsu et al. (2007) observed similar results, but also found that MC-LR accumulation in OATP-transfected HEK293 cells increased in a dose-dependent manner, which was not observed in the control vector HEK293 cells.

The distribution of MC-LR (purified from an cyanobacterial bloom sample) following oral gavage administration to mice (500 µg/kg bw) was investigated using immunostaining methods (Ito and Nagai, 2000). MC-LR was detected in large amounts in the villi of the small intestine. Erosion of the villi was observed, which may have enhanced absorption of the toxin into the bloodstream. MC-LR was also present in the blood plasma, liver, lungs and kidneys.

Once inside the cell, these toxins covalently bind to cytosolic proteins, resulting in their retention in the liver. The hepatic cytosolic proteins that bind microcystin have been identified as the protein phosphatase enzymes PP1 and PP2A. Covalent adducts of MC-LR, MC-LA and MC-LL with both enzymes were identified by reversed-phase LC. In contrast, the dihydromicrocystin-LA analogue did not form covalent bonds with PP1 and PP2A, which suggests a role for the double bonds of ADDA in the covalent binding. However, the dihydromicrocystin analogue was able to inhibit the enzyme activity, supporting a role for electrostatic interactions in the mode of action for enzyme inhibition as well as covalent binding; the median inhibitory concentration ($IC_{50}$) was similar for MC-LR and the dihydro analogue (Craig et al., 1996).

The distribution of $^{3}$H-labelled dihydromicrocystin-LR in mice was shown to differ for the oral and intraperitoneal injection routes of exposure (Nishiwaki et al., 1994). Intraperitoneal injection of $^{3}$H-labelled dihydromicrocystin-LR resulted in rapid and continuous uptake by the liver, with approximately 72% of the administered dose present in the liver after 1 hour. Small

amounts of radiolabel were found in the small intestine (1.4%), kidney and gallbladder (0.5%), lungs (0.4%) and stomach (0.3%). Oral administration of $^{3}$H-labelled dihydromicrocystin-LR resulted in much lower concentrations in the liver, with less than 1% of the administered dose found in the liver at either 6 hours or 6 days post-administration and about 38% of the dose found in the gastrointestinal tract contents. The distribution of $^{3}$H-labelled dihydromicrocystin-LR (> 95% pure) was evaluated following intraperitoneal injection of a sublethal (45 µg/kg bw) or lethal (101 µg/kg bw) dose in mice (Robinson et al., 1989). The tissue distribution of radiolabel was similar after injection of either the lethal or sublethal dose. Liver accumulation reached a maximal value of 60% by 60 minutes. For the 101 µg/kg bw dose, the liver, intestine and kidney contained 56%, 7% and 0.9% of the radiolabel, respectively. Heart, spleen, lung and skeletal muscle each contained less than 1% of the radiolabel. Within 1 minute of a sublethal intravenous injection (35 µg/kg bw) in mice, MC-LR was distributed to the liver, kidneys, intestines, carcass (body minus the heart, lung, liver, gut, kidney and spleen) and plasma (Robinson et al., 1991). After 60 minutes, the liver contained about 67% of the dose, which did not change across the 6 days of the study. Sixty minutes after the intravenous exposure, the intestines had 8.6%, the carcass 6% and the kidneys 0.8%, with only trace amounts left in the plasma. Levels in the lung were highest within 3 minutes, then fell to non-detectable levels within 10 minutes. There was measurable radiolabel in the spleen.

MC-LR was not found in the milk of dairy cattle that were exposed to *Microcystis aeruginosa* cells via drinking water (Orr et al., 2001; limit of detection 2 ng/L) or ingestion of gelatine capsules containing the cells (Feitz et al., 2002; limit of detection 0.2 ng/L).

Immunostaining methods were used to evaluate the organ distribution following intratracheal instillation of MC-LR purified from an cyanobacterial bloom sample (Ito et al., 2001). Following instillation of a lethal dose (100 µg/kg bw), the lung, liver, small intestine and kidney were positively stained for MC-LR. Intense staining was observed in the lung by 5 minutes post-instillation, followed by the kidney (10 minutes), small intestine (45 minutes) and liver (60 minutes). After approximately 90 minutes, bleeding began around the hepatic central vein. The authors described the pathological changes in the liver as essentially the same as those seen following oral or intraperitoneal injection exposure routes. Intratracheal instillation of a sublethal dose (50 µg/kg bw) resulted in immunostaining of the lung, liver, kidney, cecum and large intestine (Ito et al., 2001). No discernible pathological changes were observed at this dose level.

Data from humans accidentally exposed to microcystin from dialysis water during two different events (1996 and 2001) in Brazil indicate that a large proportion of microcystin in the serum and liver is bound to protein (M. Yuan et al., 2006). Three methods were compared to detect microcystins in stored serum and liver samples from the exposed dialysis patients: 1) direct competitive ELISA using a polyclonal antibody against microcystin, which detects free microcystin in a supernatant fraction; 2) LC-MS after oxidation and SPE to detect bound microcystin in a protein pellet fraction; and 3) GC-MS after oxidation and SPE to detect total microcystin in a serum or liver homogenate. The GC-MS method resulted in a higher concentration of microcystin compared with ELISA and LC-MS, which was hypothesized to be due to better detection of the covalently bound form of microcystin. Levels of free microcystin (free plus protein bound) in serum following the two outbreaks in hemodialyzed patients were reported to be as high as 112.9 ng/mL (Hilborn et al., 2007); serum microcystin concentrations from the 2001 event were reported to range from 0.16 to 0.96 ng/mL during the 57 days after exposure (Soares et al., 2006).

Tissue distribution was evaluated in mice given an intravenous injection of a sublethal dose of $^{3}$H-labelled dihydromicrocystin-LR by Robinson et al. (1991). The liver contained approximately 67% of the radiolabel by 60 minutes, and the amount of hepatic radioactivity did not change throughout the 6-day study period, despite urinary and fecal elimination of 24% of the administered dose. The subcellular distribution of radioactivity in the liver demonstrated that approximately 70% of the hepatic radiolabel was present in the cytosol. *In vitro* experiments showed that radiolabelled microcystin in the liver was bound to high molecular weight cytosolic proteins (Robinson et al., 1991). The nature of the binding was demonstrated to be covalent, saturable and specific for a protein with a relative molecular weight of approximately 40 000. Binding was inhibited by okadaic acid (a potent inhibitor of PP1 and PP2A), suggesting that the target protein is PP1 or PP2A. Binding proteins for MC-LR were found in cytosol derived from several different organs, suggesting that liver specificity is not due to limited distribution of target proteins. Covalent binding to hepatic proteins may be responsible for the long retention of microcystin in the liver.

Brooks and Codd (1987) reported extensive liver uptake following intraperitoneal injection of 125 µg/kg bw of a $^{14}$C-labelled toxin extracted from *M. aeruginosa* strain 7820 (assumed to be a microcystin variant) in mice. Seventy percent of the radiolabel was found in the liver after 1 minute, increasing to almost 90% after 3 hours. The radiolabel was also found in the lungs, kidneys, heart, large intestine, ileum and spleen.

Lin and Chu (1994) evaluated the kinetics of MC-LR distribution in serum and liver cytosol derived from 24 mice. Uptake of pure MC-LR into the serum, as analyzed by direct competitive ELISA, was shown to be rapid following an intraperitoneal injection of 35 µg/kg bw (sublethal dose). The toxin reached a maximum concentration in the serum by 2 hours and in liver cytosol by 12 hours post-injection. MC-LR was shown to be bound to liver cytosolic proteins, and the kinetics of binding was correlated with inhibition of PP2A activity. The maximum decrease in enzyme activity was observed 6–12 hours following injection.

The organ distribution of a $^{125}$I-labelled heptapeptide toxin (relative molecular weight 1019) isolated from *M. aeruginosa* was investigated in female rats following intravenous administration of 2 µg of the peptide (Falconer et al., 1986; Runnegar et al., 1986). The heptapeptide toxin was purified by HPLC prior to reaction with $^{125}$I in the presence of sodium iodide and lactoperoxidase. The highest tissue concentrations of labelled peptide were detected in the liver and kidney. After 30 minutes, 21.7% of the administered dose was present in the liver, 5.6% was present in the kidneys, 7% remained in the gut contents and 0.9% was cleared in the urine (Falconer et al., 1986). The authors reported that no significant accumulation was observed in other organs or tissues.

Most of the early research on the toxicity of microcystins identified the liver as the most severely impacted organ. As a result, many researchers have examined the distribution to the liver using cell cultures. Pace et al. (1991) demonstrated significant accumulation of $^{3}$H-labelled dihydromicrocystin-LR in isolated perfused liver despite a low overall extraction ratio (16% in liver, 79% in perfusate). In the liver, radiolabel corresponding to MC-LR (15%) and a more polar metabolite (85%) was primarily found in the cytosolic fraction.

The cellular uptake of $^{3}$H-labelled dihydromicrocystin-LR was evaluated using primary rat hepatocytes in suspension and in isolated perfused rat liver (Eriksson et al., 1990a; Hooser et al., 1991a). The uptake (as measured by scintillation counting of washed cells) of a mixture of unlabelled MC-LR and $^{3}$H-labelled dihydromicrocystin-LR was shown to be specific for freshly isolated rat hepatocytes (Eriksson et al., 1990a). The uptake of $^{3}$H-labelled dihydromicrocystin-

LR was shown to be inhibited by bile acid transport inhibitors, such as antamanide, sulphobromophthalein and rifampicin, and by the bile salts cholate and taurocholate.

Hooser et al. (1991a) found that the uptake of $^{3}$H-labelled dihydromicrocystin-LR was rapid for the first 5–10 minutes, followed by a plateau, in both rat hepatocyte suspensions (four replicates; two from each of two rats) and the isolated perfused rat liver ($n$ = 2). Uptake was measured as radioactivity in fractionated cells compared with radioactivity in medium. The uptake of $^{3}$H-labelled dihydromicrocystin-LR was inhibited by incubation of suspended rat hepatocytes at 0°C, suggesting the involvement of an energy-dependent process. Uptake was also inhibited by preincubation of hepatocytes with rifampicin, a competitive inhibitor of the bile acid transporter (member of OATP family).

Runnegar et al. (1991) studied the influence of dose level and exposure time on the uptake of $^{125}$I-labelled MC-YM in isolated rat hepatocytes (measured as radioactivity in centrifuged cell pellet). Hepatocyte uptake was initially rapid, with a plateau in the uptake rate observed after 10 minutes. The initial uptake rate (in the first minute of exposure) increased with increasing concentration, but cumulative uptake ceased at a dose that resulted in plasma membrane blebbing.

MC-YM uptake by isolated rat hepatocytes, as measured by cell-associated radioactivity and assays for protein phosphatase inhibition in cell lysates, was temperature dependent and was inhibited approximately 20–60% by *in vitro* preincubation with bile acids or bile acid transport inhibitors (taurocholate, trypan blue, cholate, sulphobromophthalein, cyclosporine A, trypan red and rifamycin) (Runnegar et al., 1995a). This provides evidence to indicate that microcystin uptake occurs by carrier-mediated transport. Pretreatment with protein phosphatase inhibitors (i.e., okadaic acid and calyculin A) resulted in the inhibition of both MC-YM uptake and protein phosphatase inhibition, suggesting that the OATP is itself regulated by Ser/Thr phosphorylation of protein phosphatases.

Primary cultures of liver cells cease to express these OATPs after 2–3 days of being maintained in culture. Therefore, established liver cell lines are generally not useful for evaluating microcystin toxicity (Eriksson and Golman, 1993; Heinze et al., 2001).

The subcellular distribution of $^{3}$H-labelled dihydromicrocystin-LR was evaluated using primary rat hepatocytes in suspension and the isolated perfused rat liver (Hooser et al., 1991a). $^{3}$H-labelled dihydromicrocystin-LR was primarily localized in the cytosolic fraction in both the hepatocytes and liver. In the hepatocytes, protein precipitation with trichloroacetic acid indicated that approximately 50% of the $^{3}$H-labelled dihydromicrocystin-LR was found as free toxin, whereas the remaining 50% was bound to cytosolic proteins. As little of the radiolabel was in the insoluble pellet containing insoluble actin and other elements, the authors suggested that $^{3}$H-labelled dihydromicrocystin-LR did not bind significantly to actin or other cytoskeletal proteins (Hooser et al., 1991a).

The subcellular protein binding of $^{3}$H-labelled dihydromicrocystin-LR was evaluated in rat liver homogenates (Toivola et al., 1994). Most of the radiolabelled toxin (80%) was bound to cytosolic proteins. $^{3}$H-labelled dihydromicrocystin-LR was shown to bind both PP1 and PP2A; however, PP2A was detected primarily in the cytosol, whereas PP1 was found in the mitochondrial and post-mitochondrial particulate fraction (membrane proteins).

### *8.2.2 Anatoxin-a*

No information regarding the tissue distribution of anatoxin-a was identified in the materials reviewed for this assessment. However, anatoxin-a(s) inhibits acetylcholinesterase and can act as a natural organophosphate insecticide (Puiseux-Dao and Edery, 2006). The rapid appearance of symptoms following exposure is consistent with rapid uptake from the

gastrointestinal tract and serum distribution. In a study by Fitzgeorge et al. (1994), deaths occurred within 2 minutes of gavage administration of anatoxin-a at doses greater than 5 mg/kg bw as a result of respiratory paralysis.

### *8.2.3 Cylindrospermopsin*

No information was located regarding the tissue distribution of cylindrospermopsin following oral, inhalation or dermal exposure. The distribution and elimination of intraperitoneally administered $^{14}$C-labelled cylindrospermopsin (> 95% pure; extracted and purified from lyophilized *C. raciborskii* cells) in normal saline were studied in male Quackenbush mice in a series of experiments using sublethal and lethal doses of the chemical (Norris et al., 2001). Analysis of liver, kidneys and spleen at 48 hours showed mean $^{14}$C recovery of 13.1% of the dose in the liver and less than 1% in the other tissues. Total recovery of radiolabel from tissues and excreta was 85–90% of the administered dose in each of the four mice.

In a second experiment reported by Norris et al. (2001), 12 mice were administered a single 0.2 mg/kg bw dose of $^{14}$C-labelled cylindrospermopsin, which is the approximate median lethal intraperitoneal dose. $^{14}$C content was determined in the urine and feces in all animals after 12 and 24 hours; in the liver, kidneys and spleen in five mice that were euthanized after 5–6 days due to toxicity (effects not specified); and after 7 days in the surviving seven mice that had no signs of toxicity. The overall mean (standard deviation) recoveries of $^{14}$C in the liver, kidneys and spleen after 5–7 days were 2.1 ± 2.1%, 0.15 ± 0.14% and < 0.1% (no standard deviation provided) of the dose, respectively. The broad standard deviations are indicative of considerable interindividual differences in response. Comparison of data from four mice with signs of toxicity and four mice without signs of toxicity showed no clear relationship between toxicity and patterns of tissue distribution, although there was a trend towards decreased liver retention in the surviving mice.

A third experiment, in which excretion and tissue distribution were assessed in four mice that were given a 0.2 mg/kg bw intraperitoneal dose of $^{14}$C-labelled cylindrospermopsin and evaluated after 6 hours, was also reported by Norris et al. (2001). $^{14}$C label was detected in all tissues that were examined (liver, kidney, heart, lung, spleen, blood and bile), but occurred predominantly in the liver (20.6% of the dose; range: 14.6–27.9) and kidneys (4.3% of the dose; range: 3.7–4.7). A week after dosing, about 2% of the label was still detectable in the liver.

## 8.3 Metabolism

### *8.3.1 Microcystins*

Limited data are available on the metabolism of microcystins. Microcystins can be conjugated with glutathione (GSH) and cysteine to increase their solubility and facilitate excretion (Kondo et al., 1996; Ito et al., 2002); however, it is not clear whether cytochrome P450 (CYP)–facilitated oxidation precedes conjugation. $^{3}$H-labelled dihydromicrocystin-LR was found not to be extensively metabolized in swine liver after intravenous injection or ileal loop exposure, as it was measured in hepatic tissues primarily as the parent compound (Stotts et al., 1997a, 1997b).

Some metabolism of MC-LR was shown to occur in mice and in isolated perfused rat liver (Pace et al., 1991; Robinson et al., 1991). Male CD-1 mice were administered $^{3}$H-labelled dihydromicrocystin-LR as an intravenous dose of 35 µg/kg bw and monitored for up to 6 days. Over the 6-day interval, 9.2% and 14.5% of the dose were excreted in the urine and feces, respectively, of which approximately 60% was in the form of the parent compound. HPLC analysis for urinary and fecal metabolites revealed several minor peaks of lower retention times.

Analysis of liver cytosol preparations revealed that 83% of the radiolabel was bound to a high molecular weight cytosolic protein after 6 hours, which then decreased to 42% by day 6 (Robinson et al., 1991). In isolated perfused rat liver, binding of both the parent toxin ($^3$H-labelled dihydromicrocystin-LR) and a more polar metabolite to cytosolic proteins was also demonstrated by Pace et al. (1991). Polar metabolites accounted for 65–85% of the hepatic cytosol radiolabel. Metabolites of MC-LR were not further characterized in these studies.

Administration of 125 µg/kg bw of toxin from *Microcystis aeruginosa* (PCC 7820) to mice resulted in decreased amounts of cytochrome $b_5$ and CYP in the liver (Brooks and Codd, 1987). Pretreatment of mice with microsomal enzyme (mixed-function oxidase) inducers (β-naphthoflavone, 3-methylcholanthrene and phenobarbital) was shown to eliminate this effect on hepatic cytochromes and to extend survival and reduce liver toxicity (i.e., changes in liver weight). Cote et al. (1986), however, reported no change in CYP-associated enzyme activity (i.e., metabolism of aminopyrene and *p*-nitrophenol) in microsomes isolated from mouse liver after animals were injected with an extract of *M. aeruginosa*.

GSH and cysteine conjugates have been identified in the liver after intraperitoneal injection of 10 or 20 µg MC-RR to mice or 4 µg MC-LR to rats (both microcystins purified from blooms) (Kondo et al., 1992b, 1996). The conjugates were isolated and compared with chemically prepared standards, which indicated structural modification of the ADDA and N-methyl-dehydroalanine (MDHA) moieties of the microcystin toxins. The authors postulated that these moieties could be the sites of CYP oxidation and subsequent conjugation with GSH or cysteine.

GSH conjugates of MC-LR are formed by glutathione *S*-transferase (GST) enzymes found in both rat liver cytosol and microsomes (Takenaka, 2001). GSH conjugation of MC-LR (> 95% pure isolated from *M. aeruginosa*) by five recombinant human GSTs (A1-1, A3-3, M1-1, P101 and T1-1) has been characterized (Buratti et al., 2011). All five GSTs catalyzed the conjugation, but with different dose–response relationships. The study report also determined that the spontaneous reaction for MC-LR conjugation with GSH was dependent on GSH concentration, pH and temperature. More recently, Buratti et al. (2013) characterized the GSH conjugation of MC-RR by five recombinant human GSTs and human liver cytosol, which appeared to be more efficient than MC-LR conjugation. In the human liver cytosol, however, the spontaneous conjugation reaction is favoured over the enzymatic one in a ratio of 3:1 at physiological GSH content. At low MC-RR concentrations (representing repeated oral exposures) and low GSH content, however, the enzymatic reaction progressively increases, becoming the predominant pathway for MC-RR conjugation (Buratti et al., 2013). In rats and mice, at physiological GSH content, the enzymatic reaction reportedly accounts for half of the total conjugate formation (Buratti and Testai, 2015); rat and mouse GST showed similar MC-LR and MC-RR GSH conjugation. However, compared with humans, rodent catalytic efficiency was found to be 2-fold higher, which can be attributed to higher substrate affinity. More pronounced differences in the conjugation of MC-LR and MC-RR were also observed in rodents compared with humans (Buratti and Testai, 2015).

GSH and cysteine conjugates of MC-LR and MC-YR were demonstrated to be less toxic than the parent compounds based on median lethal dose ($LD_{50}$) estimates; however, toxicity was still significant, with $LD_{50}$ values ranging from 217 to 630 µg/kg bw in mice (Kondo et al., 1992b). Metcalf et al. (2000b) also demonstrated that microcystin conjugates with GSH, cysteine–glycine and cysteine were less toxic in the mouse bioassay than the parent compounds; these conjugates were also shown to be weaker inhibitors of PP1 and PP2A *in vitro*. GSH and cysteine conjugates of MC-LR were primarily distributed to the kidney and intestine following intratracheal instillation in mice (Ito et al., 2002); this suggests that the lower toxicity of GSH and

cysteine conjugates may be related to their distribution to excretory organs and elimination of metabolites *in vivo*.

Ito et al. (2002) synthesized GSH and cysteine conjugates of MC-LR and administered them by intratracheal instillation in mice. The metabolites were demonstrated to be less toxic than the parent compound, as shown by lethal doses about 12-fold higher than the MC-LR lethal dose. The metabolites were distributed primarily to the kidney and intestine, as opposed to the liver (Ito et al., 2002).

### *8.3.2 Anatoxin-a*

No information regarding the metabolism of anatoxin-a was identified in the scientific literature.

### *8.3.3 Cylindrospermopsin*

There is evidence indicating that the hepatic CYP enzyme system is involved in the metabolism and toxicity of cylindrospermopsin. Pretreatment of hepatocytes with known inhibitors of CYP (50 µm proadifen or ketoconazole) diminished the *in vitro* cytotoxicity of cylindrospermopsin (Froscio et al., 2003). Similarly, pretreatment of mice with the CYP inhibitor piperonyl butoxide protected against the acute lethality of cylindrospermopsin in male Quackenbush mice (Norris et al., 2002). Support for the involvement of the CYPs is provided by Shaw et al. (2000, 2001), who demonstrated that a main target of cylindrospermopsin toxicity is the periacinar region of the liver, an area where substantial CYP-mediated xenobiotic metabolism occurs.

Runnegar et al. (1995c) investigated the decrease in cellular GSH and its role in the metabolism and toxicity of cylindrospermopsin in primary cultures of rat hepatocytes. To ascertain whether the decrease in GSH was due to decreased GSH synthesis or increased GSH consumption, total GSH was measured after treatment with 5 mmol/L buthionine sulphoximine (an irreversible inhibitor of GSH synthesis). The rates of decrease in total GSH (in nmol/$10^6$ cells per hour) were 8.2 (±2.5), 6.0 (±1.7) and 5.9 (±1.3) for controls, 2.5 µmol/L and 5 µmol/L cylindrospermopsin pretreated cells, respectively. This suggests that the decrease in GSH induced by the toxin was due to the inhibition of GSH synthesis rather than increased consumption, because the rate of decrease in GSH in the latter case would have been accelerated by toxin pretreatment. Furthermore, excess GSH precursor (20 mmol/L *N*-acetylcysteine), which supported GSH synthesis in control cells, did not prevent the decrease in GSH or toxicity induced by cylindrospermopsin. Addition of CYP inhibitors α-naphthoflavone, SKF525A and cimetidine partially prevented the decrease in cell GSH induced by cylindrospermopsin. Results suggest the formation of an oxidized and possibly GSH-conjugated derivative that may be a more potent inhibitor of GSH synthesis than the parent cylindrospermopsin.

$^{14}$C-labelled cylindrospermopsin (> 95% pure; extracted and purified from lyophilized *C. raciborskii* cells) was studied in a series of mouse experiments that utilized the intraperitoneal route of exposure (Norris et al., 2001). A single non-lethal dose of 0.1 mg/kg bw was administered to four male Quackenbush mice (6 weeks old), then at 12 hour intervals after dosing, urine and fecal samples were collected and weights were recorded. The four mice were sacrificed 48 hours after dosing. Urine, fecal, liver and kidney samples were extracted with methanol to precipitate proteins, and the $^{14}$C in the supernatant was fractionated using HPLC for the detection of metabolites. No attempt was made to fractionate or otherwise identify the $^{14}$C in the protein precipitate.

Analysis of methanol extracts of urine samples suggested that a large part (72%) of the excreted $^{14}C$ was present as cylindrospermopsin. Some (approximately 23.5%) of the urinary $^{14}C$ was detected in protein precipitated by methanol, suggesting the presence of a protein-bound metabolite. The authors did not indicate whether the level of protein in the urine was normal or abnormal. Analysis of liver tissue showed the presence of $^{14}C$ in both a methanol extract and protein precipitate. When fractionated by HPLC, the methanol-extracted $^{14}C$ from the liver had the same elution characteristics seen in some of the urine methanol extracts, suggesting the presence of the same metabolite. No methanol-extractable metabolite was found in kidney tissue.

The authors could not rule out the possibility that the non-extractable $^{14}C$ in the liver was protein-bound cylindrospermopsin, although the evidence from Runnegar et al. (1995c) and Shaw et al. (2000) suggested that it might be a metabolite. Runnegar et al. (1995c) and Shaw et al. (2000) provided evidence of the need for activation of cylindrospermopsin for toxicity, suggesting the presence of one or more metabolites. Although no identification of metabolites was performed, results indicate either that the metabolite is more polar than cylindrospermopsin or that the parent compound is fragmented during metabolism.

## 8.4 Excretion

### *8.4.1 Microcystins*

Limited information on the elimination of microcystins from the human body is available from follow-up of dialysis patients exposed unintentionally to microcystins in dialysis water. More than 50 days after documented exposure, microcystins were detected in patients' serum by ELISA using polyclonal antibodies against MC-LR with cross-reactivity against several microcystin analogues (Soares et al., 2006; Hilborn et al., 2007).

Biliary excretion has been shown in both *in vivo* and *in vitro* studies. Falconer et al. (1986) administered an intravenous dose of 2 µg microcystin in saline extracted from *Microcystis aeruginosa* to female albino rats. After 120 minutes, 9.4% of the administered dose was present in the intestinal contents and 1.9% was present in the urine, suggesting that biliary excretion plays a significant role in its elimination. Similarly, in isolated perfused rat liver, 1.7% of radiolabelled MC-LR was recovered in the bile by the end of the 60-minute perfusion (Pace et al., 1991). In the bile collected during the perfusion, 78% of the radiolabel was associated with the parent toxin, whereas the remaining radiolabel was associated with more polar metabolites.

In a study by Robinson et al. (1991), male VAF/plus CD-1 mice were administered an intravenous dose of 35 µg/kg bw of radiolabelled MC-LR. A total of approximately 24% of the administered dose was eliminated in the urine (9%) and feces (15%) during the 6-day study monitoring period. Approximately 60% of the excreted microcystin, measured at 6 and 12 hours following injection, was present in the urine and feces as the parent compound.

Elimination in swine was evaluated following intravenous injection or ileal loop exposure (Stotts et al., 1997b). $^{3}H$-labelled dihydromicrocystin-LR was detected in the bile as early as 30 minutes after intravenous injection of 75 µg/kg bw. Following ileal loop exposure to the same dose, the concentration of toxin was consistently higher in the portal venous blood compared with peripheral blood. This suggests that first-pass metabolism may play a role in the clearance of MC-LR.

The blood half-life was measured following intravenous administration of a $^{125}I$-labelled heptapeptide toxin extracted from *M. aeruginosa* (relative molecular weight 1019, assumed to be microcystin) (Falconer et al., 1986). A biphasic blood elimination curve was demonstrated, with the first component having a half-life of 2.1 minutes and the second component having a half-life of 42 minutes.

MC-LR excretion was also evaluated in mice (Robinson et al., 1991). A biexponential plasma elimination curve was observed following intravenous injection of a sublethal dose of 35 µg/kg bw of $^{3}$H-labelled MC-LR. Plasma half-lives of 0.8 and 6.9 minutes were reported for the first and second phases of elimination, respectively.

Stotts et al. (1997a, 1997b) evaluated the toxicokinetics of $^{3}$H-labaelled dihydromicrocystin-LR in swine following intravenous injection and ileal loop exposure. Elimination of labelled MC-LR was rapid and followed a biphasic pattern, suggesting that the liver rapidly removes the toxin from the blood. Clearance from the blood is slower at higher dose levels, presumably due to the liver toxicity and circulatory shock observed at high doses. It is important to take into consideration that $^{3}$H radiolabelling may alter the microcystin molecule's ability to bind with protein phosphatases, thus altering the microcystin protein binding and tissue distribution profile (Hilborn et al., 2007).

### *8.4.2 Anatoxin-a*

No information regarding the elimination of anatoxin-a was identified in the scientific literature.

### *8.4.3 Cylindrospermopsin*

No information was located regarding the elimination of cylindrospermopsin following oral, inhalation or dermal exposure. The elimination of intraperitoneally administered $^{14}$C-labelled cylindrospermopsin (> 95% pure; extracted and purified from lyophilized *C. raciborskii* cells) in saline was studied in male Quackenbush mice in a series of experiments using sublethal and lethal dose levels of the chemical (Norris et al., 2001).

In one experiment, four mice were given a single sublethal dose of 0.1 mg/kg bw, and urine and feces were collected at 12-hour intervals for the following 48 hours. The mean cumulative excretion of $^{14}$C in the first 12 hours after dosing was 62.8% of the administered dose in the urine and 15.5% in the feces. There was little additional excretion of $^{14}$C in either the urine or feces following 12 additional hours. The 15.5% mean fecal excretion value reflects a very high fecal excretion in one of the four animals (nearly 60% of the dose compared with < 5% in the other mice); the authors considered the possibility that the high value in the one animal resulted from the injection entering the upper gastrointestinal tract, but concluded that this possibility was unlikely given the injection technique used. Total mean recovery in the urine, feces, liver, kidneys and spleen was 85–90% of the $^{14}$C dose in each of the four mice.

The second experiment reported by Norris et al. (2001) included 12 mice administered a single 0.2 mg/kg bw dose of $^{14}$C-labelled cylindrospermopsin, which is the approximate median lethal intraperitoneal dose (Norris et al., 2001). $^{14}$C content was determined in the urine and feces in all animals after 12 and 24 hours. Results were similar to those obtained with a sublethal dose (reported above), except that there was some continued urinary and fecal excretion over the second 12 hours of the monitoring period. The mean cumulative urinary and fecal excretion of $^{14}$C was 66.0% and 5.7% of the dose within 12 hours and 68.4% and 8.5% of the dose within 24 hours, respectively. The mean total recovery in the urine and feces after 24 hours was 76.9% of the administered dose. The overall mean recoveries of $^{14}$C in the liver, kidneys and spleen after 5–7 days were 2.1%, 0.15% and < 0.1% of the administered dose, respectively. Comparison of data from four mice with signs of toxicity and four mice without signs of toxicity showed no clear relationship between toxicity and patterns of excretion, although trends towards increased urinary excretion and decreased fecal excretion in surviving mice were suggested.

In a third experiment, four mice were given a 0.2 mg/kg bw intraperitoneal dose of $^{14}C$-labelled cylindrospermopsin, and tissue distribution (urine, feces, blood, gallbladder, liver, heart, kidney, lung and spleen) was evaluated after 6 hours (Norris et al., 2001). The mean cumulative urinary and fecal excretion of $^{14}C$ after 6 hours was 48.2% and 11.9% of the administered dose, respectively. One of the four mice eliminated more than 40% of the dose in the feces (additional data and data on metabolites not reported).

# 9.0 Health effects

## 9.1 Effects in humans

### *9.1.1 Short-term case studies and case reports*

Existing case reports and epidemiological studies on microcystins, anatoxin-a and cylindrospermopsin following various exposure scenarios report numerous symptoms, including acute gastrointestinal disorders (e.g., nausea, vomiting and diarrhea), headache and muscle weakness, and liver effects (Dillenberg and Dehnel, 1960; Schwimmer and Schwimmer, 1968; Blyth, 1980; Billings, 1981; Falconer et al., 1983; Turner et al., 1990; el Saadi and Cameron, 1993; Teixeira et al., 1993; el Saadi et al., 1995; Pouria et al., 1998; Carmichael et al., 2001; Griffiths and Saker, 2003; Giannuzzi et al., 2011; Hilborn et al., 2014). A number of these cases were documented by the detection of *Anabaena*, either alone or with *Microcystis*, in the feces. The only case involving deaths associated with cyanotoxin exposure involved intravenous exposure of dialysis patients to microcystins (Carmichael et al., 2001).

### *9.1.2 Long-term and epidemiological studies*

#### *9.1.2.1 Microcystins*

A number of epidemiological studies (published in Chinese) conducted in an area of southeast China that is endemic for hepatocellular carcinoma have been summarized by IARC (2010). Overall, a positive association was found between the risk for hepatocellular carcinoma and water source from surface waters, with estimates of relative risk ranging from 1.5 to 4. Consumption of pond or ditch water was associated with an 8-fold increase in liver cancer incidence when compared with well water consumption. Microcystin-producing cyanobacteria are abundant in surface waters in this area, with significant amounts of microcystins detected in pond and ditch waters, whereas no detectable levels were found in deep well water; this suggests that microcystins in drinking water may have been partially responsible for the higher incidence of hepatocellular carcinomas. Confounders such as hepatitis B infection and aflatoxin exposure were not generally considered in most studies. Although many of the studies reported positive associations with consumption of surface waters, their quality does not allow for any conclusions regarding an increased risk of cancer with exposure to microcystins (IARC, 2010).

Fleming et al. (2002) conducted an ecological epidemiological investigation of the relationship between drinking water source and incidence of primary liver cancer in Florida, as cyanobacteria and toxins, especially microcystins, were detected in surface drinking water sources in Florida. The study population consisted of all cases of primary hepatocellular carcinoma reported to the Florida state cancer registry between 1981 and 1988 and was divided into comparison groups consisting of those served by surface water and those using other sources of drinking water. Evaluation of the individual incidence rates in the 18 surface water service areas compared with the groundwater service areas did not reveal any statistically significant differences among the individual incidence rates. When the service areas were pooled, residence

in a surface water service area was associated with a statistically significant reduced risk of hepatocellular carcinoma compared with either groundwater service areas (standardized rate ratios [SRRs] ranged from 0.8 to 0.98 for the four groundwater comparison groups) or the general Florida population (SRR = 0.8). When comparisons were made between residence in the actual (i.e., not estimated as above) surface water service areas and residence in the buffer areas (areas not serviced by surface water treatment plants) surrounding the service areas, a statistically significant increase in the incidence of hepatocellular carcinoma was observed for those residing within the surface water service area (SRR = 1.39; 95% confidence interval [CI] = 1.38–1.4). Analyses of 1990 census data suggested that the ethnic and socioeconomic backgrounds of the residents in the service areas and buffer areas were similar, although the authors did not report these data. Interestingly, the incidence of hepatocellular carcinoma in the buffer areas was significantly lower than that in the general Florida population (SRR = 0.59).

Li et al. (2011) conducted a cross-sectional study assessing the relationship between liver damage in children and microcystin levels in drinking water and aquatic food (carp and duck) in China. Microcystin levels were measured in three sources of drinking water used by local residents in the Three Gorges Reservoir region of China: a community well rarely contaminated with microcystin; a lake with an occasional cyanobacterial bloom (Lake 1); and a lake that had regular cyanobacterial blooms over the previous 5 years (Lake 2). Participants were considered to have no exposure if they drank water from the wells for more than 5 years and rarely ate fish or duck from the lakes (145 participants), to have low exposure if they drank water from Lake 1 (183 participants) and to have high exposure if they drank water from Lake 2 (994 participants). Samples were collected for the measurement of microcystin levels, participants were administered a questionnaire, blood was obtained for analysis and microcystin levels in blood were measured in approximately 50 participants per exposure group. Microcystin levels in the well water were below the detection limit in all but 1 of the 6 years tested. In the only year in which microcystins were detected, a one time detect of 0.1 µg MC-$LR_{equiv}$/L was reported. The average measurements over the 6 years in Lakes 1 and 2 were 0.24 and 2.58 µg MC-$LR_{equiv}$/L, respectively. MC-LR was found in the aquatic food (fish and ducks), with concentrations higher in the aquatic food obtained from Lake 2 compared with Lake 1. The study authors estimated that children in the low-exposure group consumed 0.36 µg/day, whereas the high-exposure children consumed 2.03 µg/day, based on consumption of drinking water and aquatic food. Mean serum levels of microcystins in the groups were below the detection limit, 0.4 and 1.3 µg MC-$LR_{equiv}$ in the no, low- and high-exposure groups, with detection rates of 1.9%, 84.2% and 91.9%, respectively. Microcystin was associated with increases in aspartate aminotransferase (AST) and alkaline phosphatase (ALP), but there were no increases in alanine aminotransferase (ALT) or gamma-glutamyltransferase (GGT). The odds ratio (OR) for liver damage (based on two or more abnormally elevated liver enzyme assays) associated with microcystin was 1.72 (95% CI: 1.05–2.76). Hepatitis B infection (based on serum measurements of antigens and/or antibodies) was a greater risk for liver damage in children.

In summary, long-term epidemiological studies indicate elevated liver enzyme levels in humans exposed to drinking water contaminated with microcystins and in children consuming high levels of microcystins through contaminated water and food. Numerous studies in China provide contradictory results for the association between microcystin exposure (via consumption of surface water versus groundwater) and hepatocellular carcinoma.

## 9.2 Effects on experimental animals

### *9.2.1 Acute toxicity*

#### *9.2.1.1 Microcystins*

The studies reported in this section implemented routes relevant to exposure via drinking water (i.e., oral and inhalation routes). Although much of the science on the acute toxicity of microcystins in animals has been conducted via administration by intraperitoneal injection, these studies are not discussed here, as this route of exposure is not relevant to drinking water. It is worth noting, however, that $LD_{50}$s generated from these studies are much lower than those reported following oral exposure, indicating greater potency when introduced via this exposure route. See Section 9.4 for a discussion of some of these studies in the context of toxic equivalency among microcystin variants.

Fawell et al. (1999a) conducted acute, subchronic and developmental toxicity studies of MC-LR in mice and/or rats. In the acute portion of the study, single oral gavage doses of MC-LR (commercial product; purity not specified) in aqueous solution were given to male and female CR1:CD-1(ICR)BR(VAF plus) mice and CR1:CD(SD)BR(VAF plus) rats (five of each sex per species). Doses of 500, 1580 and 5000 µg/kg bw were administered. Untreated control groups were not included. The animals were observed for up to 14 days prior to sacrifice and necropsy. Oral $LD_{50}$ values were estimated to be about 5000 µg/kg bw for mice and greater than 5000 µg/kg bw for rats. Effects on the liver, kidneys, spleen and adrenals were reported; the incidence and severity of liver effects increased in a dose-dependent fashion.

Yoshida et al. (1997) assessed the acute oral toxicity of purified MC-LR (> 95% pure by HPLC) in female BALB/c mice. Preliminary experiments using doses of 16.8 and 20 mg/kg bw resulted in death within 160 minutes in two mice; therefore, doses of 8.0, 10.0 and 12.5 mg/kg bw were chosen for $LD_{50}$ determination. MC-LR in saline solution was administered via gavage to a total of five 6-week-old mice. Two control mice received saline via gavage. Mortality was observed over a 24-hour period, and dead animals, including those in the preliminary study, were immediately necropsied. One surviving mouse was sacrificed and necropsied 24 hours after treatment; the remainder were sacrificed and necropsied after a week. Mortality within 24 hours was 0/1 at 8 mg/kg bw, 0/2 at 10 mg/kg bw and 2/2 at 12.5 mg/kg bw. The oral $LD_{50}$ was calculated to be 10.9 mg/kg bw. The only organs affected were liver and kidneys; no kidney effects were reported in animals that survived treatment for at least 24 hours.

Ito et al. (1997a) compared the acute effects of MC-LR on the livers and gastrointestinal tracts of young and aged mice. Single doses of 500 µg/kg bw of MC-LR (purity not specified) dissolved in ethanol and diluted in saline were administered via oral gavage to aged (29 mice aged 32 weeks) and young (12 mice aged 5 weeks) male ICR mice. Three aged and three young untreated mice served as controls. Twenty-two aged mice were sacrificed at 2 hours, five mice at 5 hours and two mice at 19 hours after treatment; four young mice were sacrificed at each time point. Liver damage and gastrointestinal erosion were evaluated, with marked differences in both liver damage and gastrointestinal effects reported between young and aged mice. In young mice, no liver pathology or gastrointestinal changes were reported. In contrast, 18/29 aged mice treated with the same dose developed pathological changes of the liver to varying degrees. The authors indicated that the degree of liver injury was related to the severity of gastrointestinal effects. This study identified liver and gastrointestinal effects in aged mice only at the single dose administered of 500 µg/kg bw.

Rao et al. (2005) evaluated the age-dependent acute hepatotoxic effects of MC-LR in mice following i.p. and oral exposure. In the i.p. study, a single $LD_{50}$ dose of MC-LR (43.0 µg/kg bw) administered to 6- to 36-week-old mice resulted revealed: time to death in the toxin treated

animals decreased with the age of mice; treated animals of all age groups showed significant increases in liver body mass index and increases in serum enzymes (lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, g-glutamyl transpeptidase, sorbitol dehydrogenase) compared to controls. In the acute oral toxicity studies, a single $LD_{50}$ of MC-LR containing extracts (3.5 g of MC extract/kg bw) was administered to 6- and 36-week-old mice. The effects on biochemical variables were similar to the i.p route of exposure. Both groups of mice had increased relative liver weight and DNA fragmentation compared to the control group, but there was no difference between the age groups. In contrast, glutathione depletion and lipid peroxidation were significantly greater in the aged mice when compared with young mice. Further, while most serum enzymes were increased over controls in both groups, GGT was increased to a greater extent in aged mice than in young mice.

Groups of three female Swiss albino mice were administered a single gavage dose of 0, 2 or 4 mg/kg bw of MC-LR and sacrificed 3 or 24 hours after treatment (Gaudin et al., 2008). DNA damage was assessed in blood, liver, kidney, colon and intestine using the comet assay. Clinical observations were not reported. In blood, a statistically significant dose-dependent increase in DNA damage was observed at 3 hours, but not at 24 hours. No effects were seen in the other tissues assayed.

Ito et al. (2001) evaluated the distribution of purified MC-LR after intratracheal instillation of lethal doses in male ICR mice and included a limited description of toxic effects. MC-LR in saline solution was instilled at doses of 50, 75, 100, 150 and 200 μg/kg bw into 34 mice; three mice were sham-exposed as controls. Mortality was 100% in 12 mice receiving doses of 100 μg/kg bw and greater. At 75 μg/kg bw, two of the four exposed mice died, whereas no deaths occurred in 18 mice given 50 μg/kg bw intratracheally. The time course of hepatotoxicity was further evaluated in eight mice given an intratracheal dose of 100 μg/kg bw. One mouse was sacrificed at each of 5, 10, 20, 30, 45, 60, 90 and 120 minutes. Immunostaining for MC-LR showed the toxin in the lungs within 5 minutes and in the liver after 60 minutes. Hemorrhage in the liver was observed after 90 minutes and became severe by 120 minutes.

In a poorly described study, Fitzgeorge et al. (1994) administered MC-LR via gavage to newly weaned CBA/BALB/c mice. The commercially obtained compound was described only as "suitably purified." The $LD_{50}$ was estimated to be 3000 μg/kg bw, and increases in liver (43%) and kidney (5.9%) weights were reported. The same study also evaluated exposure via intranasal instillation and inhalation. A single experiment with mice (number unspecified) inhaling a fine aerosol (particle size 3–5 μm) of 50 μg MC-LR/L for an unspecified duration of time did not result in any deaths, clinical signs of toxicity or histopathological changes (the nature of the examinations was not reported). The authors estimated the delivered dose of MC-LR to be approximately 0.0005 μg/kg bw. The $LD_{50}$ for intranasal instillation of MC-LR was equal to 250 μg/kg bw. Liver and kidney weights were increased by 41.6% and 7.5%, respectively, in the animals receiving MC-LR intranasally. Further evaluation of intranasal instillation exposure to single doses of 31.3, 62.5, 125, 250 and 500 μg/kg bw resulted in liver weight increases of 0%, 1.5%, 24.4%, 37.4% and 87%, respectively. Seven daily intranasal doses of 31.3 μg/kg bw resulted in a liver weight increase of 75%. Effects on the respiratory system, liver and adrenal gland were reported for both the single- and multiple-dose experiments.

In summary, $LD_{50}$ values for mice reportedly range from 3 to 10 mg/kg bw via the oral route of exposure and from 43 to 250 μg/kg bw via inhalation of aerosolized microcystins. Following acute oral exposure, effects on the liver and kidney (increased weights as well as lesions), spleen, adrenals and gastrointestinal tract have been reported, as well as damage to DNA

in liver and blood and impacts on serum enzyme levels. Acute inhalation exposure also reportedly impacted the liver, kidney and adrenal gland, as well as the respiratory system.

*9.2.1.2 Anatoxin-a*

An acute oral (single dose gavage) $LD_{50}$ value of 16.2 mg/kg bw (95% CI: 15.4–17.0) was determined for synthetic (+)-anatoxin-a hydrochloride (commercial product; ≥ 98% purity) in male Swiss Webster ND-4 mice (Stevens and Krieger, 1991b); this $LD_{50}$ is equivalent to 13.3 mg anatoxin-a/kg bw (95% CI: 12.8–14.1). The same authors determined a single-dose gavage $LD_{50}$ value of 6.7 mg/kg bw for male Swiss Webster ND-4 mice administered the toxin as a lysate solution of lyophilized *Anabaena flos-aquae* (NRC-44-1) cells. The same study also determined an acute (single dose) intraperitoneal $LD_{50}$ value of 0.25 mg/kg bw (95% CI: 0.24–0.28) for (+)-anatoxin-a hydrochloride (commercial product; > 98% purity) (0.21 mg anatoxin-a/kg bw) in mice.

A single gavage dose $LD_{50}$ of greater than 5 mg/kg bw was determined for anatoxin-a in newly weaned CBA/BALB/c mice of unspecified sex (Fitzgeorge et al., 1994); the anatoxin-a in this study was a commercial product in a "suitably purified" but unspecified form. Deaths from neurotoxicity occurred within 2 minutes of gavage administration. An acute (single dose) intraperitoneal $LD_{50}$ value of 0.375 mg/kg bw was also determined by the study authors.

Anatoxin-a has been implicated in case reports of poisonings and deaths in dogs, livestock and waterfowl that consumed water containing blooms of toxin-producing cyanobacteria (Carmichael and Gorham, 1978; Pybus et al., 1986; Edwards et al., 1992; Gunn et al., 1992; Puschner et al., 2008). Signs of toxicity were predominantly neurological, with deaths due to respiratory paralysis. Quantitative exposure data were not reported.

Although intraperitoneal injection is not considered to be a relevant route of exposure for assessing the risks from chemicals in drinking water, the following study is included for the purpose of hazard identification. Acute $LD_{50}$ values for anatoxin-a following exposure of male BALB/c mice to single intraperitoneal injections of (+)-, racemic or (−)-anatoxin-a hydrochloride (all > 95% pure) were reported as 0.386 mg/kg bw (95% CI: 0.365–0.408) for (+)-anatoxin-a hydrochloride (0.32 mg anatoxin-a/kg bw) and 0.913 mg/kg bw (95% CI: 0.846–0.985) for racemic anatoxin-a hydrochloride (0.76 mg anatoxin-a/kg bw) 30 minutes post-dosing. No deaths or clinical signs occurred in mice treated with doses of (−)-anatoxin-a hydrochloride as high as 73 mg/kg bw (Valentine et al., 1991). The differences in potency are consistent with mechanistic data indicating that (+)-anatoxin-a is the biologically active enantiomer.

In summary, oral $LD_{50}$ values for anatoxin-a range from 6.7 to 13.3 mg/kg bw, depending on the enantiomer administered and whether a lysate solution or synthetic form was used. Effects reported are limited, with the only reported toxic effects being on the neurological and respiratory systems.

*9.2.1.3 Cylindrospermopsin*

Twelve male MF1 mice were administered a saline suspension of freeze-dried *Cylindrospermopsis raciborskii* cells (strain PHAWT/M or PHAWT/1) by gavage in single doses ranging from 4.4 to 8.3 mg/kg bw (cylindrospermopsin-equivalent) and observed for the following 8 days. The following dose levels were tested (one mouse per level except as noted): 4.4, 5.3, 5.7 (two mice), 5.8, 6.2, 6.5, 6.7, 6.8, 6.9, 8.0 and 8.3 mg/kg bw; there was no control group. Eight of the 12 mice died. The lowest lethal dose was 4.4 mg/kg bw, the highest non-lethal dose was 6.9 mg/kg bw and the average lethal dose was approximately 6 mg/kg bw. Deaths occurred 2–6 days after treatment, and histological examinations showed effects on the liver,

spleen, thymus, heart, esophagus and gastric mucosa; some animals also developed lesions in one or both eye orbits (Seawright et al., 1999).

An aqueous suspension of a cell-free extract of freeze-dried and sonicated *C. raciborskii* (strain AWT 205) cells was administered to an unspecified number of male Swiss mice in a single gavage dose of 1400 mg extract/kg bw. The cylindrospermopsin content of the extract was not specified, but ranged from 1.3 to 5.4 mg/g extract in concurrent intraperitoneal experiments, indicating that the cylindrospermopsin-equivalent gavage dose was likely in the range of 1.8–7.6 mg/kg bw. This dose level was not fatal over the course of the experiment, but the authors observed severe liver and kidney pathology. Additional information on the design and results of the oral study was not provided (Falconer et al., 1999).

Another gavage study reported that the minimum lethal dose of a saline extract of freeze-dried *C. raciborskii* (strain AWT 205) cells in Swiss mice was 2500 mg extract/kg bw. Based on a reported cylindrospermopsin content of 5.5 mg/g extract, the equivalent dose of cylindrospermopsin was 13.8 mg/kg bw (Falconer and Humpage, 2001).

Groups of four Quackenbush mice were administered a cell-free extract of freeze-dried and sonicated *C. raciborskii* (strain AWT 205) cells in water in a single gavage dose of 0, 1, 2, 4, 6 or 8 mg cylindrospermopsin/kg bw and observed for the following 7 days. Hepatic effects were observed at all dose levels, and mortality occurred in 2/4 mice at 6 mg/kg bw (in 5 days) and 4/4 mice at 8 mg/kg bw (in 24–48 hours). Additional information on the experimental design and results was not reported (Shaw et al., 2000, 2001).

Although intraperitoneal injection is not considered to be a relevant route of exposure for assessing the risks from chemicals in drinking water, a brief summary of the results from several intraperitoneal studies is provided for the purpose of hazard identification. Studies by Ohtani et al. (1992), Terao et al. (1994) and Shaw et al. (2000, 2001) administering cylindrospermopsin (purified from extracts of cultured *C. raciborskii* or *Umezakia natans* cells) reported $LD_{50}$ values ranging from 0.2 to 2.1 mg/kg bw (24 hours to 7 days); pathological effects included lesions in the liver, kidney, adrenal gland, lung, intestine, thymus and heart. Falconer et al. (1999) reported cylindrospermopsin-equivalent $LD_{50}$ values of 0.07–0.6 mg/kg bw (24 hours) and 0.03–0.4 mg/kg bw (7 days), with liver and kidney damage in male Swiss albino mice; cylindrospermopsin was acquired from cell-free extracts of sonicated freeze-dried *C. raciborskii* cells. Hawkins et al. (1985) reported a 24-hour $LD_{50}$ value of 64 mg freeze-dried culture/kg bw in mice and in a later study reported cylindrospermopsin-equivalent $LD_{50}$ values of 0.29 mg/kg bw (24 hours) and 0.18 mg/kg bw (7 days) in male Swiss mice (Hawkins et al., 1997); the liver was the main target organ, with lesions also reported in the kidney, adrenal gland, lung and intestine.

In summary, reported oral acute lethal doses for cylindrospermopsin are quite variable and are dependent on the mode of administration. Oral minimum lethal doses range from 4.4 to 13.8 mg/kg bw in mice; administration by intraperitoneal injection yielded much lower $LD_{50}$ values, indicating higher potency via this route of exposure. Acute effects include effects on the liver, kidneys, spleen, thymus, heart, gastrointestinal tract, adrenal gland and lungs.

### *9.2.2 Short-term studies*

#### *9.2.2.1 Microcystins*

Huang et al. (2011) evaluated the effects of orally administered MC-RR on apoptosis in the liver of adult male ICR mice. Groups of five rats were administered a dose of 0, 4.6, 23, 46, 93 or 186 μg/kg bw per day of MC-RR (commercial product; purity not reported) via gavage for 7 days, after which animals were sacrificed and livers were analyzed. A statistically significant dose-dependent increase in the percentage of apoptotic cells in the liver at doses of 46 μg/kg bw

per day and higher was reported. A significant increase in Bax protein expression was found at 46 and 93 µg/kg bw per day, and in p53 protein expression at 93 µg/kg bw per day. Bcl-2 was significantly decreased at doses of 23 µg/kg bw per day and higher. The Bax/Bcl-2 ratio was significantly increased at doses of 23 µg/kg bw per day and higher. No significant changes were found in CHOP protein expression. GRP78 protein expression was significantly increased at 93 µg/kg bw per day, but expression at all of the other doses (including the high dose) was not different from that of the controls. No changes in PP2A activity or alterations in PP2A A subunit messenger ribonucleic acid (mRNA) expression were seen.

Heinze (1999) evaluated the effects of MC-LR (commercial product; purity not reported) in drinking water on 11-week-old male hybrid rats ($F_1$ generation of female WELS/Fohm × male BDIX). Groups of 10 rats were given a dose of 0, 50 or 150 µg/kg bw per day for 28 days in drinking water. Water consumption was measured daily, and rats were weighed at weekly intervals. Dose estimates provided by the authors were not adjusted to account for incomplete drinking water consumption. After 28 days of exposure, rats were sacrificed, organ weights were recorded and hematology, serum biochemistry and histopathology of liver and kidneys were examined. An increased number of leukocytes (38%) in rats was found in the highest dose group; significantly increased mean levels of lactate dehydrogenase (LDH) and ALP in both treatment groups (84% and 100% increase in LDH, 34% and 33% increase in ALP at low and high doses, respectively) was reported, with no changes in mean levels of ALT or AST observed. Dose-dependent increases in relative liver weights (17% and 26% at the low and high doses, respectively) and absolute liver weights (data not provided) were reported. No statistically significant changes in other organ weights or body weights were observed. Liver lesions were observed in both treatment groups, but the severity of the damage was increased in the 150 µg/kg bw per day dose group; the lowest-observed-adverse-effect level (LOAEL) for this study is 50 µg/kg bw per day. No effects on the kidneys were observed.

Schaeffer et al. (1999) administered *Aphanizomenon flos-aquae* to mice via their diet for 64 days. Analysis of the *A. flos-aquae* samples showed an average concentration of 20 ± 5 µg MC-LR per gram of *A. flos-aquae*. Estimated daily exposure to MC-LR in the exposed mice ranged from 43.3 to 333.3 µg/kg bw per day. No clinical signs of toxicity were reported, and no effects on mortality, body weight, organ weights or histology were observed in the treated mice. In addition, no effects on reproductive parameters were reported in five treated mice from the highest dose group that were allowed to breed. Thus, the no-observed-adverse-effect level (NOAEL) for this study is identified as 43.3 µg/kg bw per day.

Benson et al. (2005) exposed groups of six male BALB/c mice to monodispersed submicrometre aerosols of MC-LR via nose-only inhalation for 30, 60 or 120 minutes each day for 7 consecutive days. The concentration of MC-LR was 260–265 µg/m$^3$, and doses deposited in the respiratory tract were estimated to be 3, 6 and 12.5 µg/kg bw per day. Control mice were exposed to the aerosolized vehicle (20% ethanol in water). Animals were sacrificed the day after the last exposure. Blood and serum were subjected to clinical chemistry analysis, organ weights were recorded and histopathological examination of the liver, respiratory tract tissues, adrenals, kidney, spleen, thymus, gastrointestinal tract and testes was conducted. No clinical signs or effects on body weight or organ weights were observed. Treatment-related lesions were observed in the nasal cavity only, with the incidence and severity increasing with length of the daily exposure period.

In summary, short-term oral exposure studies confirm the liver as a major target organ for microcystins. Oral exposure to MC-LR at doses as low as 50 µg/kg bw per day (identified as the lowest available LOAEL) resulted in increased liver weight, liver lesions (with hemorrhages) and

increased ALP and LDH in rats; oral exposure to MC-RR at 46 µg/kg bw per day and above resulted in a statistically significant higher percentage of apoptotic cells in the livers of adult mice. Inhalation exposure to aerosolized MC-LR has been reported to induce lesions in the nasal cavity of mice.

*9.2.2.2 Anatoxin-a*

A 5-day oral toxicity study by Fawell and James (1994) and Fawell et al. (1999b) administered aqueous (+)-anatoxin-a hydrochloride (commercial product; purity not reported) by gavage in daily doses of 1.5, 3, 7.5 or 15 mg/kg bw (equivalent to 1.2, 2.5, 6.2 and 12.3 mg anatoxin-a/kg bw) to groups of two male and two female Crl:CD-1(ICR)BR mice in order to determine the maximum tolerated dose (MTD) for a 28-day study summarized below. All high-dose mice and one female mouse in the 6.2 mg/kg bw per day group died within 5 minutes of dosing. Males in the 6.2 mg/kg bw per day dose group were hyperactive following the third dose; no other adverse effects were observed in this treatment group. The dose of 2.5 mg/kg bw per day (3 mg (+)-anatoxin-a hydrochloride/kg bw per day) was identified as the MTD for the 28-day main study.

In the main study, groups of 10 male and 10 female Crl:CD-1(ICR)BR mice were administered the same aqueous (+)-anatoxin-a hydrochloride by gavage in daily doses of 0 (vehicle control), 0.12, 0.6 or 3 mg/kg bw per day (0, 0.1, 0.5 and 2.5 mg anatoxin-a/kg bw per day) for 28 days. There were three deaths during the course of the study; one death was not treatment related (one male was humanely sacrificed after being attacked by its cage mates), and one male (0.5 mg/kg bw per day) and one female (2.5 mg/kg bw per day) died (cause of death undetermined) within 2.5 hours of dosing on days 10 and 14 of treatment, respectively. The only other effects reported in treated animals were several minor hematology and blood chemistry changes not considered to be toxicologically significant. The study authors concluded that the NOAEL was 0.1 mg/kg bw per day, based on the two deaths that occurred at the higher dose levels. However, the authors stated that the NOAEL may actually be 2.5 mg/kg bw per day, owing to the low incidences of mortality that showed no dose–response relationship or sex consistency (1/10 males at 0.5 mg/kg bw per day and 1/10 females at 2.5 mg/kg bw per day), the lack of characteristic clinical signs of acute neurotoxicity in the two animals that died, the absence of toxicologically significant effects in the surviving mice and the lack of effects at 2.5 mg/kg bw per day in mice as reported in the 5-day study discussed above and a developmental toxicity study discussed below.

*9.2.2.3 Cylindrospermopsin*

Groups of four Quackenbush mice were administered purified cylindrospermopsin by daily gavage for 14 days. The cylindrospermopsin was purified (purity not reported) from an extract of freeze-dried *Cylindrospermopsis raciborskii* (strain AWT 205) cells, with doses ranging from 0 to 0.3 mg/kg bw per day. All animals were evaluated for gross pathological and histological changes. The authors identified a NOAEL of 0.05 mg/kg bw per day and a LOAEL of 0.15 mg/kg bw per day for lipid infiltration in the liver; lymphophagocytosis in the spleen was observed at 0.3 mg/kg bw per day (highest dose tested). Additional information on the experimental design and results was not reported (Shaw et al., 2001).

In an additional study, six Quackenbush mice and two Wistar rats were exposed for 21 days to drinking water containing 800 µg/L cylindrospermopsin. The water was "sourced" from a dammed impoundment. The reported approximate daily dose based on water consumption was 0.2 mg cylindrospermopsin/kg bw in both species. Gross pathological and histological

examinations showed no effects at this dose in the rats and mice. Additional information on the experimental design and results was not reported (Shaw et al., 2001).

Reisner et al. (2004) reported significant increases in liver and testes weights following exposure to purified cylindrospermopsin from *Aphanizomenon ovalisporum* (isolated from Lake Kinneret, Israel, during a 1994 bloom). Groups of eight 4-week-old ICR mice were exposed to plain water containing 0.6 mg/L cylindrospermopsin (estimated dose was 66 µg/kg bw per day) for 3 weeks. Animals were sacrificed at the end of the exposure period, and the liver, kidney and spleen were analysed. Body weight was significantly increased across the duration of the study for the controls and treated animals; however, body weights did not differ significantly across groups at 21 days. Significant ($p < 0.05$) increases in relative liver and testes weights were observed when compared with controls. At the end of the 3-week exposure period, urine excretion rate and urinary orotic acid (a pyrimidine precursor) concentration were significantly altered ($p < 0.05$) in the treated animals compared with the controls. Liver cholesterol levels were significantly ($p < 0.05$) lower in the treated animals compared with the controls.

### *9.2.3 Subchronic studies*

#### *9.2.3.1 Microcystins*

Fawell et al. (1999a) conducted a subchronic toxicity study of MC-LR given via gavage to Cr1:CD-1(ICR)BR(VAF plus) mice (age not specified). MC-LR was obtained commercially (purity not reported) and administered in distilled water. The concentration in the dosing solution was verified by HPLC with UV detection. Daily oral gavage doses of 0, 40, 200 or 1000 µg/kg bw were given to groups of 15 male and 15 female mice for 13 weeks. Comprehensive analyses of clinical signs, body weight and food consumption, hematology and serum biochemistry were performed. All tissues were examined in the control and high-dose animals, whereas only lungs, liver and kidney were examined in the other treated animals. No treatment-related clinical signs of toxicity were observed throughout the study. Mean body weight gain was decreased approximately 15% in all treated male groups. Mean terminal body weights differed from controls by about 7% in these groups. No dose-related trends were evident for body weight gain or body weight in males. The only body weight change observed in females was an increase in body weight gain in the 200 µg/kg bw per day group. Slight (10–12%) increases in mean hemoglobin concentration, red blood cell count and packed cell volume were observed among females receiving 1000 µg/kg bw per day. ALP, ALT and AST levels were significantly elevated (2- to 6-fold higher) in the high-dose males, and ALP and ALT were likewise elevated (2- and 6-fold higher, respectively) in the high-dose females. ALT and AST were also elevated (2-fold) in the mid-dose males. GGT was slightly decreased in some treatment groups. Serum albumin and protein were significantly reduced (13%) in males of the mid- and high-dose groups. Histopathological changes in the liver were reported in the males and females of the mid- and high-dose groups, with a dose-related increase in incidence and intensity. Liver lesions consisted of multifocal inflammation with deposits of hemosiderin and hepatocyte degeneration throughout the liver lobule. Sex-related differences in liver pathology were not apparent. No lesions were found in other tissues. A NOAEL of 40 µg/kg bw per day can be identified from this study.

Falconer et al. (1994) administered dried bloom materials in the drinking water of pigs ($n = 5$ per group) for 44 days. The extract contained at least seven microcystin variants, with the major peak tentatively identified as MC-YR; no peak could specifically be identified as MC-LR. Animals were administered 0, 80, 227 or 374 mg dried cyanobacteria/kg bw per day added to the drinking water. Pigs in the highest-dose group had reduced body weight, likely due to reduced food and/or water consumption at this dose. Plasma samples collected over 56 days showed dose-

and time-dependent increases in GGT, ALP and total bilirubin, as well as a decrease in plasma albumin. Dose-related changes in the incidence and severity of histopathological changes of the liver were also observed.

In summary, evidence from subchronic studies continues to identify liver as an important target organ for microcystins. MC-LR induced mild liver lesions as well as increased serum ALT and AST in male mice following an oral dose of 200 µg/kg bw per day for 13 weeks; no adverse effects were observed at 40 µg/kg bw per day (NOAEL). Exposure to an extract of microcystin variants (mainly MC-YR) at various doses also impacted the livers in pigs exposed for 44 days.

*9.2.3.2 Anatoxin-a*

Groups of 20 female Sprague-Dawley rats were administered anatoxin-a in the drinking water at a concentration of 0, 0.51 or 5.1 mg/L for 7 weeks. The anatoxin-a used in this study was extracted from the culture medium of *Anabaena. flos-aquae* (NRC-44-1) cells and partially purified; purity was not quantified, but the toxin had a UV absorbance spectrum that qualitatively indicated that anatoxin-a was the principal UV-absorbing component. The authors assumed a 0.1 mL/g bw per day consumption, leading to estimated daily intakes of 0.05 and 0.5 mg/kg bw per day for the low- and high-dose groups, respectively. Endpoints evaluated throughout the study included clinical signs, food consumption, body weight, hematology and serum chemistry. Endpoints assessed at the end of the exposure period included hepatic mixed-function oxidase activity (aldrin epoxidation *in vitro*), organ (liver, kidneys, spleen) weights, gross pathology and histology (liver, kidneys, spleen, adrenals, heart, lungs and brain). Additional information regarding the design of this study was not reported. No treatment-related effects were observed in either dose group (Astrachan et al., 1980; Astrachan and Archer, 1981).

*9.2.3.3 Cylindrospermopsin*

Groups of male Swiss albino mice (10 per dose, six in the highest-dose group) were administered purified cylindrospermopsin in water by gavage at a dose of 0, 30, 60, 120 or 240 µg/kg bw per day for 11 weeks (Humpage and Falconer, 2003). The cylindrospermopsin was purified (purity not reported) from an extract of freeze-dried *Cylindrospermopsis raciborskii* (strain AWT 205) cells. No deaths were reported. The mean final body weight was 7–15% higher than in controls in all dose groups, but the increases were not dose related and were statistically significant only at 30 and 60 µg/kg bw per day. There were no significant changes in food consumption; however, water intake was significantly reduced in all dose groups (data not reported). Relative kidney weight was increased in a significant, dose-related manner beginning at 60 µg/kg bw per day (12–23% higher than in controls), and relative liver weight was significantly increased only at the high dose of 240 µg/kg bw per day (13% higher than in controls). Information on absolute kidney and liver weights was not reported. Absolute testis weights were significantly increased at ≥ 60 µg/kg bw per day (data not reported), but these changes were not significant when normalized to body weight. The hematology, serum chemistry and urine evaluations showed no clear exposure-related changes in any endpoint (including serum indicators of liver injury), except for significant decreases in urine protein concentrations (g/mmol creatinine) at 120 µg/kg bw per day and urine specific gravity at 240 µg/kg bw per day (data presented graphically). The postmortem examinations showed "minor increases in histopathological damage to the liver" at ≥ 120 µg/kg bw per day and proximal renal tubular damage at 240 µg/kg bw per day, but additional information regarding the type, severity and incidences of the liver and kidney lesions was not reported. Although cylindrospermopsin is known to inhibit protein synthesis in the liver, serum albumin, a major product of liver protein

synthesis, was not decreased in this study. The most sensitive effects observed were related to kidney damage (increased relative kidney weight at ≥ 60 µg/kg bw per day and decreased urinary protein at ≥ 120 µg/kg bw per day). This study identifies a NOAEL and LOAEL of 30 and 60 µg/kg bw per day, respectively.

The potential effects of cylindrospermopsin were investigated in a 42-week mouse drinking water study by Sukenik et al. (2006). Four-week weaned male and female ICR mice were supplied with food and water *ad libitum* for an experimental period of up to 42 weeks. Animals were divided into two groups consisting of 20 males and 20 females in each group. The control group received freshly prepared cyanobacterial medium (without cylindrospermopsin) as drinking water, whereas the experimental group received spent medium containing variable concentrations of cylindrospermopsin. The concentration of cylindrospermopsin in drinking water for the experimental group was increased gradually from 100 to 550 µg/L. Daily intakes were approximated as follows: 10 µg/kg bw for weeks 0–8; 20 µg/kg bw for weeks 8–16; 30 µg/kg bw for weeks 16–24; and 55 µg/kg bw for weeks 24–42. There were no significant changes in body weight; relative kidney weights were significantly increased in males and females at 20 weeks and 42 weeks. Relative liver weights were increased only at 42 weeks, and relative testes weights were increased in males at 42 weeks. The authors proposed a LOAEL of 20 µg/kg bw per day for increased relative kidney weight in males and females at 20 weeks of exposure; the identified LOAEL also applies to observed structural changes in red blood cells at 20 weeks (see Section 9.2.9.2). It should be noted, however, that confidence in the proposed LOAEL is weak because of the design of the experiment (i.e., changing dose over the duration of the experiment).

Another study exposed groups of male Swiss albino mice (10 per dose except 12 controls and five mice at high dose) to a cell-free extract of sonicated and frozen *C. raciborskii* (strain AWT 205) cells in drinking water at a cylindrospermopsin dose of 0, 216, 432 or 657 µg/kg bw per day for 10 weeks (doses based on actual water consumption). Final body weights were significantly reduced at 432 and 657 µg/kg bw per day (9% and 7% less than in controls, respectively), and relative liver and kidney weights were significantly increased in a dose-related manner at 216–657 µg/kg bw per day (27–47% and 30–43% greater than in controls, respectively). Other statistically significant effects included increased serum total bilirubin at ≥ 216 µg/kg bw per day, decreased serum total bile acids at ≥ 216 µg/kg bw per day and decreased urine protein concentration (g/mmol creatinine) at ≥ 432 µg/kg bw per day. The lowest dose of 216 µg/kg bw per day represents a LOAEL for this study (Humpage and Falconer, 2003).

Shaw et al. (2000, 2001) exposed Quackenbush mice to drinking water containing a cell-free extract of freeze-dried and sonicated *C. raciborskii* (strain AWT 205) cells for 90 days. Gross pathological and histological (liver, kidney, spleen, heart, lungs and thymus) examinations showed no effects at dose levels as high as 0.15 mg/kg bw per day (the highest dose tested). Additional information on the experimental design and results was not reported.

In summary, available subchronic studies indicate that the liver and kidneys are major target organs for cylindrospermopsin-induced toxicity in mice; structural changes in red blood cells have also been shown to be an endpoint of concern. The kidney appears to be the most sensitive target, with the lowest NOAEL for increased relative kidney weight identified as 30 µg/kg bw per day. Decreased urinary protein excretion has also been reported following cylindrospermopsin administration, suggesting a specific effect on the nephron.

### *9.2.4 Neurotoxicity*

#### *9.2.4.1 Microcystins*

Maidana et al. (2006) reported that long-term memory retrieval, as assessed by step-down inhibitory avoidance task, was impaired in rats receiving an intrahippocampal injection (note that this means of administration is considered for hazard identification only and is not representative of exposure via drinking water) of 0.01 or 20 µg/L of a microcystin extract from *Microcystis* strain RST 9501. Exposure to 0.01 µg/L also impaired spatial learning in the radial arm maze, but exposure at the higher concentration did not. The authors indicated that the primary microcystin produced by this strain is [D-Leu$^1$]MC-LR, a variant of MC-LR with a similar potency with regard to phosphatase inhibition. Oxidative damage, as measured by lipid peroxides and DNA damage, was increased in tissue homogenates of the hippocampus from treated animals.

In an *in vitro* study by Feurstein et al. (2010), the expression of murine organic anion–transporting polypeptides (mOATPs) and the uptake of MC-LR, MC-LW and MC-LF in primary murine neurons were investigated in order to determine whether microcystins can cross neuronal cell membranes. Protein levels for two mOATPs (mOATP1A5 and the known MC-LR transporter mOATP1B2) were detected in the membranes of murine neuronal cells, and the transport of all three microcystin variants into neurons was confirmed using a colorimetric PPIA. At low microcystin concentrations (0.31–1.25 µmol/L), the inhibitory effect of all variants was comparable, showing a 20% reduction in protein phosphatase activity compared with the control group. At 2.5 µmol MC-LR, MC-LW or MC-LF per litre, total protein phosphatase activity was reduced by 25%, 30% and 60%, respectively, whereas 5 µmol MC-LF/L reduced total protein phosphatase activity by 65%. Although this study provides *in vitro* evidence of neuronal uptake of microcystins in mice, it has yet to be determined whether oral exposure to microcystins leads to neurotoxicity *in vivo*.

In a follow up study, Feurstein et al. (2011) examined the effects of MC-LR, MC-LF and MC-LW on cytotoxicity, caspase activity, chromatin condensation, and hyperphosphorylation of microtubule-associated Tau protein in isolated murine cerebellar granule neurons (CGN). MC-induced apoptosis was significantly increased by MC-LF and MC-LW, however, only at high concentrations (≥ 3mM); apoptosis was not significantly increased by MC-LR at any concentration tested. 0.5 µM MC-LF led to significantly reduced neurite lengths, whereas higher MC-LW (> 1 µM) and MC-LR (≥ 3 µM) concentrations were required to significantly reduce average neurite lengths; significant neurite degeneration was also observed at 0.5 mM MC-LF. Serine/threonine-specific PP inhibition and sustained hyperphosphorylation of Tau was observed for all MC congeners which may explain the observed disintegration of the neurite network following exposure to MCs. In assessing cytotoxicity, 0.8 µM MC-LF significantly reduced CGN viability (81% of control) with 5 µM MC-LF reducing viability to 8% of control. Conversely, MC-LW concentrations of 3 and 5 µM significantly reduced cell viability to 63 and 50% of control, whereas MC-LR provided for a minor but significant reduction of cell viability (70% of control) only at the highest concentration (5 µM).

Li et al. (2014) also reported impaired memory function, assessed by the Morris water maze, in male rats receiving an intrahippocampal injection of 1 or 10 µg/L of MC-LR (≥ 98% pure). Both concentrations of MC-LR caused increased latency to find the platform. Histology of the brain revealed neuronal damage in the CA1 region of the hippocampus at 10 µg/L only. A significant decrease in the total number of cells and the density of cells, but not in the cell volume, was seen in the CA1 region of high-dose animals. Malondialdehyde levels and catalase activity in the hippocampal CA1 region were increased at both concentrations, but superoxide dismutase and GSH peroxidase activity were significantly increased only at 10 µg/L.

The effects of progestational exposure of female rats to MC-LR on postnatal development in offspring were recently studied by Li et al. (2015). Female Sprague-Dawley rats (28 days old) were exposed to 1.0, 5.0 and 20.0 µg MC-LR/kg by gavage once every 48 h for 8 weeks; controls were exposed to pure water. At the end of the 8 week exposure period, each female rat was mated with an unexposed adult male. Motor and behavioural development of pups were assessed using surface righting reflex, negative geotaxis, and cliff avoidance tests on postnatal day 7; learning ability was assessed by open-field and Morris water maze tests which were performed on postnatal day 28 and day 60. Pups from all of the MC-LR-treated groups revealed significantly lower scores than controls in the cliff avoidance test ($p < 0.05$). Results of the Morris water maze test (assessment of cognitive impairment) revealed significantly lower frequencies of entering the platform zone for male offspring from all treatment groups, significantly lower frequencies of entering the enlarged platform zone for the females (5.0 and 20.0 µg MC-LR/kg), and significantly decreased swimming speed in the female offspring from the group treated with 20.0 mg MC-LR/kg. Regarding indicators of oxidative stress in the rat hippocampus, malondialdehyde contents were significantly increased in the male offspring from the 5.0 mg MC-LR/kg treatment group and in both male and female offspring from the 20.0 mg MC-LR/kg treatment group; total superoxide dismutase activities were significantly increased in the hippocampus of the male and female offspring from the 20.0 mg MC-LR/kg treatment group.

*9.2.4.2 Anatoxin-a*

The only studies located investigating the neurotoxicity of anatoxin-a implemented injection (intraperitoneal, intravenous or subcutaneous) as the means of exposure. As injection is not considered to be a relevant route of exposure in assessing the risks from chemicals in drinking water, results from these studies are only briefly summarized for the purpose of hazard identification.

A 2-day range-finding study by Rogers et al. (2005) exposed female CD-1 mice to anatoxin-a by intraperitoneal injection at a dose of 0.008, 0.08, 0.17, 0.21, 0.25 or 0.33 mg anatoxin-a/kg bw per day. After one dose, mortality increased in a dose-dependent manner up to 100% in the two highest dose groups; all mice that received a second dose survived. Doses of 0.21 mg/kg bw per day and higher caused decreased motor activity, altered gait, difficulty breathing and convulsions 5–6 minutes post-exposure, with death occurring within 10 minutes. Less severe clinical signs were observed in animals that survived in the 0.17 and 0.21 mg/kg bw per day groups, with recovery after 15–20 minutes. A NOAEL of 0.08 mg/kg bw per day can be identified from this study.

In a neurodevelopmental study, Rogers et al. (2005) exposed groups of 8–11 time-pregnant CD-1 mice to 0, 0.10 or 0.17 mg anatoxin-a/kg bw per day by intraperitoneal injection on gestation days (GDs) 8–12 or 13–17; mice were allowed to give birth, and no significant postnatal neurotoxicity was reported, suggesting a NOAEL for neurodevelopmental effects of 0.17 mg/kg bw per day. The mouse pups that were exposed on GDs 13–17 showed no signs of neurotoxicity as adults.

Mice administered a single dose of 8–83 or 25–50 µg anatoxin-a/kg bw showed no exposure-related neurological effects, although the highest doses caused clinical signs of neurotoxicity and death within 1 minute of exposure (Fawell and James, 1994; Fawell et al., 1999b).

Rats receiving a single dose of 0.06 mg anatoxin-a/kg bw by subcutaneous injection showed decreased locomotor activity and partial nicotine-like discriminative stimulus effect (Stolerman et al., 1992).

Anatoxin-a has also been reported to decrease response and reinforcement rates in multiple-schedule operant performance tests in rats treated by subcutaneous injection, although substantial tolerance was developed with repeated administration (Jarema and MacPhail, 2003).

In summary, the acute neurotoxicity of anatoxin-a in mice following a lethal intraperitoneal dose of 0.21 mg/kg bw per day and above includes tremors, altered gait, convulsions and death by respiratory paralysis; no significant effects have been observed following injection exposure to doses of 0.08 mg/kg bw per day (NOAEL) and lower. Acute intravenous exposure to anatoxin-a at doses up to 0.083 mg/kg bw in mice reportedly resulted in no effects on motor activity, coordination, sensory/motor reflexes or other central nervous system responses. In rats, subcutaneous injection of 0.06 mg/kg bw of anatoxin-a resulted in changes in locomotor activity and behaviour.

#### *9.2.4.3 Cylindrospermopsin*

No studies investigating the neurotoxicity of cylindrospermopsin were identified.

### *9.2.5 Reproductive and developmental toxicity*

#### *9.2.5.1 Microcystins*

Falconer et al. (1988) conducted a limited study of reproductive effects using an extract from a *Microcystis aeruginosa* bloom sample. Eight female Quakenbush mice that had been given 1/4th dilution of the extract as drinking water (estimated to contain 14 µg/mL (or approximately 2700 µg/kg bw) of unspecified microcystin toxin) since weaning were mated with similarly treated males. No difference in number of litters, number of pups per litter, sex ratio or litter weight were observed. Reduced brain size was reported to occur in 7/73 pups from treated parents and in 0/67 pups from controls. The litter distribution of the affected pups was not reported by the authors. One of the small brains was examined histologically, revealing extensive damage to the hippocampus.

Fawell et al. (1999a) (as described in Section 9.2.3.1) conducted a developmental toxicity study of MC-LR (commercial product; purity not stated) given via gavage to Cr1:CD-1(ICR)BR (VAF plus) mice (age not specified). MC-LR (0, 200, 600 or 2000 µg/kg bw) was administered to groups of 26 mice on days 6-15 of pregnancy. The mice were sacrificed on day 18 and necropsied. Seven of 26 dams receiving 2000 µg/kg bw died and 2 others were sacrificed moribund. Altered liver appearance was noted during gross examination of these animals. Surviving dams in this group did not display any clinical signs of toxicity or differences in body weight or food consumption. Fetal body weight was significantly lower than controls and there was delayed skeletal ossification at the highest dose; however, the data were not presented in the publication. No evidence of either maternal toxicity or of any other form of developmental toxicity was observed at the lower doses. No effects on resorptions or litter size were observed, nor were there increases in external, visceral or skeletal abnormalities in fetuses of any treatment group.

Sperm quality and testicular function were assessed in male SPF mice administered MC-LR (commercial product; purity not reported) in the drinking water at a concentration of 0, 1, 3.2 or 10 µg/L (approximately 0, 0.25, 0.79 and 2.5 µg/kg bw per day) for 3 or 6 months (Y. Chen et al., 2011). Although body weight and amount of water consumed were measured, these data were not presented, and doses to the animals were not calculated. No clinical signs of toxicity were observed, and body weight, testes weight and water consumption were not affected by treatment. No changes in any sperm or hormone parameters were noted at 1 µg/L. At 3.2 and 10 µg/L, sperm counts were significantly decreased and sperm motility was reduced at 3 and 6 months, with

severity increasing with longer duration of exposure. Animals in the mid- and high-dose groups had a trend towards lower serum testosterone and higher luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels after 3 months, which reached statistical significance by 6 months. Histopathological evaluation of the testes showed a slightly loosened appearance of the organization of the epithelium in the seminiferous tubules at 10 µg/L after 3 months. After 6 months, slight testicular atrophy associated with sparse appearance of the seminiferous tubules was found at 3.2 and 10 µg/L, with dose-related increased severity. The animals given 10 µg/L also showed loss and derangement of spermatogenic cells, enlargement of the lumen of the seminiferous tubules, thinning of the spermatogenic epithelium and depopulation of Leydig cells, Sertoli cells and mature sperm. The number of apoptotic cells in the testes was increased at 10 µg/L after 3 months and at 3.2 and 10 µg/L after 6 months. A NOAEL of 1 µg/L (approximately 0.25 µg/kg bw per day) for impacts on sperm count and motility can be identified from this study. Several concerns with the methodology and reporting of this study call into question the quality of the data reported. These concerns include the use of methanol as a vehicle for MC-LR administration, as well as the lack of methanol administration to the control group (thus rendering the controls an insufficient negative control comparison); effects in the controls (e.g., increasing testosterone levels and decreasing sperm motility over the experimental duration); a steep dose–response curve over small dose increments; poorly described and inadequately conducted sperm and histological analyses; a lack of reporting on the purity of MC-LR and species of mouse used (although later verified through correspondence with the authors); and no validation of the final concentrations of MC-LR administered in drinking water.

The *in vitro* toxicity of microcystins to Leydig cells and Sertoli cells, demonstrated by decreased cell viability (Li et al., 2008; H.Z. Zhang et al., 2011; Li and Han, 2012), suggests that microcystin transport may occur in the testes as it does in the liver. OATPs are active in the testes (Svoboda et al., 2011); however, no studies to date have been located addressing their role in the toxicity of microcystins to the cells of the testes.

Zhou et al. (2012) investigated whether MC-LR could enter rat spermatogonia and exert toxic effects *in vitro*. Following exposure of isolated spermatogonia to 0, 0.5, 5, 50 or 500 nmol MC-LR/L for 6 hours, cell viability was significantly decreased (5 and 50 nmol/L [$p < 0.05$]; 500 nmol/L [$p < 0.01$], as was total antioxidant capacity (5 and 500 nmol/L [$p < 0.01$]; 50 nmol/L [$p < 0.05$]); increases in the ratio of apoptotic cells, production of reactive oxygen species (ROS), mitochondrial membrane potential and intracellular free $Ca^{2+}$ occurred following exposure to 5 nmol/L and higher concentrations of MC-LR. The authors detected at least five OATPs (OATP1A5, OATP3A1, OATP6B1, OATP6C1 and OATP6D1) at the mRNA level in spermatogonia and found that the expression of these OATPs, especially OATP3A1, was affected by MC-LR. The study demonstrated that MC-LR can be transported into spermatogonia, leading to cytotoxicity.

Male reproductive effects similar to those observed in oral exposure studies have also been observed in mice, rats and rabbits intraperitoneally exposed to microcystins. As intraperitoneal injection is not considered to be a relevant route of exposure in assessing the risks from chemicals in drinking water, only a brief summary of the results from these studies is provided for the purpose of hazard identification. Following exposure to microcystin equivalent doses ranging from 3.75 to 110 µg/kg bw per day (Ding et al., 2006; Li et al., 2008; Liu et al., 2009; D. Li et al., 2011; Wang et al., 2012b; Chen et al., 2013), effects reported included histopathological changes in the testes and changes in testes and epididymis; impacts on sperm (count, motility and morphology); oxidative stress and histological changes in spermatogonia and

Sertoli cells; and changes in circulating LH, FSH and testosterone levels, as well as *Lhβ*, *Fshβ* and *GnRH* gene expression.

In summary, reported impacts on the male reproductive system in multiple species following oral and intraperitoneal exposure to MC-LR include decreased testis and epididymis weights, decreased epididymal sperm concentration, viability and motility, as well as increased percent immobile and abnormal sperm; some of these effects were seen following oral exposure to as low as 3.2 µg/L (approximately 0.25 µg/kg bw per day) MC-LR. Other reported effects include atrophy and obstruction of the seminiferous tubules, decreased number of interstitial cells, Sertoli cells and mature sperm in the seminiferous tubule, lipid peroxidation and apoptosis. *In vitro* studies of rat spermatogonia (precursors for spermatocytes) demonstrate uptake of MC-LR with resultant cellular apoptosis and oxidative stress. Further research is required in order to confirm or validate the toxicity of MC-LR to the male reproductive system via the oral route of exposure as well as to determine the effects of MC-LR on the female reproductive tissues and those of offspring following oral exposure.

*9.2.5.2 Anatoxin-a*

A developmental toxicity screening study was conducted in which groups of 10 and 12 pregnant Crl:CD-1(ICR)BR mice were administered aqueous (+)-anatoxin-a hydrochloride (commercial product; purity not reported) by gavage in doses of 0 (vehicle control) or 3 mg/kg bw per day (0 or 2.5 mg anatoxin-a/kg bw per day), respectively, on GDs 6–15. Clinical signs and body weight were recorded until day 18 of gestation, at which time the maternal animals were sacrificed and necropsied. No treatment-related maternal or fetal effects were observed, although it was noted that mean fetal weight (male, female and total) in the treated group was marginally lower than in controls (data not reported). A NOAEL of 2.5 mg/kg bw per day for a lack of adverse effects in dams and fetuses was identified from this study (Fawell and James, 1994; Fawell et al., 1999b).

Although intraperitoneal injection is not considered to be a relevant route of exposure for humans, results from these studies are briefly summarized for the purpose of hazard identification. Rogers et al. (2005) administered anatoxin-a (90% purity) by intraperitoneal injection to 8–11 pregnant females at either 125 or 200 µg/kg bw for 5 days, on either GDs 8–12 or GDs 13–17. A significant dose-related trend towards lower pup weight on postnatal day (PND) 1 in litters treated on GDs 13–17 was reported, with the trend becoming no longer statistically significant on PND 6. No effects were observed on viability or average pup weight in the GDs 8–12 exposed litters or in neurological testing. Both the *in vivo* screen and mammalian embryo culture studies revealed no adverse effects. Yavasoglu et al. (2008) intraperitoneally administered 50, 100 or 150 µg/kg bw per day anatoxin-a (commercially available anatoxin-a fumarate diluted in physiological saline [0.9%]) to groups of 10 male mice for 7 consecutive days. Effects included significantly reduced absolute and relative weights of cauda epididymis in the 100 and 150 µg/kg bw per day groups; significantly reduced (in a dose-dependent manner) sperm count in the cauda epididymis in all treatment groups; dose-dependent degeneration in seminiferous tubules, intercellular disassociation of spermatogenic cell lines, sloughing of germ cells into tubular lumen, vacuolization in Sertoli cells and loss of germ cells; and significantly decreased (in a dose-dependent manner) epithelial thickness of seminiferous tubules in all treatment groups. The LOAEL was 50 µg/kg bw per day, based on reduced sperm count in cauda epididymis.

In summary, limited evidence shows that anatoxin-a can affect mouse pup weight following *in utero* exposure (via intraperitoneal injection) to as low as 125 µg/kg bw; however, this was shown to be dependent on the gestational period during which the dose was

administered. Exposure of male mice to as low as 50 µg/kg bw anatoxin-a (via intraperitoneal injection) has also been shown to affect male reproduction by impacting the testes and sperm. More research is required in order to better understand the impacts of anatoxin-a on development and reproduction in animals following oral exposure.

*9.2.5.3 Cylindrospermopsin*

The only studies located investigating the reproductive and developmental toxicity of cylindrospermopsin implemented intraperitoneal injection as the means of exposure. Although injection is not considered to be a relevant route of exposure for assessing the risks from chemicals in drinking water, results from these studies are briefly summarized for the purpose of hazard identification.

Rogers et al. (2007) conducted a teratology study comprising three experiments exposing CD-1 mice by intraperitoneal injection to purified cylindrospermopsin (> 98% in distilled water). Experiment 1 exposed pregnant females on GDs 8–12 to 0, 8, 16, 32, 64, 96 or 128 µg/kg bw ($n$ = 20–25 per treatment). Experiments 2 and 3 investigated the effects of *in utero* exposure to 50 µg/kg bw on GDs 8–12 and on GDs 13–17 (two groups of 23–51 mice per group) on prenatal and postnatal development. Experiment 1 results showed increased mortality in dams exposed to 32 µg/kg bw or higher and a significant dose-related increase in relative liver weight in the 8, 16 and 32 µg/kg bw groups. Results from experiments 2 and 3 included significant reductions in litter size, pup size, pup body weight, pup body weight gain and pup viability; however, these effects occurred at doses higher than those observed to elicit toxicity in dams.

Chernoff et al. (2011) evaluated the effects of cylindrospermopsin in pregnant CD-1 mice during different gestational periods. The first study exposed pregnant mice by daily intraperitoneal injections to 0.05 mg/kg bw cylindrospermopsin (purified from bulk cultures of *Cylindrospermopsis raciborskii*; > 98% pure) for 5 days on GDs 8–12 ($n$ = 42); controls received distilled water (intraperitoneally) ($n$ = 26). Animals (2–3 controls and 3–5 exposed dams) were sacrificed on GD 13 and post-treatment days 7, 14, 28 and 42. Fifty-five percent of treated animals died by GD 13. Reported effects included vaginal bleeding ($n$ = 13) and occasional orbital and tail bleeding; reduced maternal body weight gain; elevated enzymes associated with liver injury in mice sacrificed 1 day post-exposure ($n$ = 2–5 per measurement), which returned to control levels by day 7 post-exposure; significantly increased creatinine and blood urea nitrogen 1 day post-exposure, which returned to control levels 7 days post-exposure; increased incidence of liver necrosis, nephrosis and/or renal inflammation in mice treated on GDs 8–12, with histopathology returning to normal within 7 days of exposure; and altered expression of genes involved in ribosomal biogenesis, xenobiotic and lipid metabolism, inflammatory response and oxidative stress up to 2 weeks post-treatment. The second study used the same exposure scenario, but for 5 days on GDs 13–17 ($n$ = 42 per method). No deaths or vaginal bleeding occurred; however, gastrointestinal bleeding occurred ($n$ = 3) on either day 1 or day 7 post-exposure. Other effects included elevated enzymes associated with liver injury (in mice sacrificed 1 day post-exposure), which returned to near control levels within 7 days; significantly increased blood urea nitrogen 1 day post-exposure, which returned to control levels 7 days post-exposure; significantly increased relative liver weight 7 days after dosing; increased incidence of liver necrosis, with histopathology returning to normal within 7 days of exposure; and altered expression of genes involved in ribosomal biogenesis, xenobiotic and lipid metabolism, inflammatory response and oxidative stress for 2 weeks post-treatment.

In summary, limited evidence indicates that cylindrospermopsin may act as an *in utero* developmental toxicant; however, the effects in pups were observed at a dose higher than that

eliciting toxicity in the dams (50 µg/kg bw); maternal toxicity was observed to occur at doses below 50 µg/kg bw cylindrospermopsin. Further research is needed in order to better understand the impacts of cylindrospermopsin on development and reproduction in animals following oral exposure.

### *9.2.6 Chronic toxicity*

#### *9.2.6.1 Microcystins*

Ueno et al. (1999) evaluated the toxicity of MC-LR in mice chronically exposed via drinking water. Two hundred 6-week-old female BALB/c mice were randomly assigned to receive either no treatment or drinking water (*ad libitum*) containing 20 µg MC-LR/L for 7 days/week for a period of up to 18 months; mean cumulative MC-LR intake for the 18 months was estimated at 35.5 mg per mouse. The MC-LR had been isolated from lyophilized cyanobacterial bloom materials from Lake Suwa in Nagano, Japan, and had been characterized as 95% pure by HPLC. Twenty animals from each group were sacrificed at 3, 6 and 12 months, whereas the remaining 40 animals were retained for chronic toxicity evaluation and sacrificed at 18 months. Based on weekly estimates of water consumption, the cumulative intake of MC-LR over 18 months was calculated to be 35.5 µg/mouse. No clinical signs of toxicity or impacts on survival, body weight, food or water consumption or hematology were observed; however, hematology data from the 3-month sacrifice were lost due to sampling errors. Treated mice were reported to have a statistically significant decrease in ALP at month 12 (13%) and a significant increase in cholesterol at month 18 (22%). Neither effect was considered by the authors to be toxicologically significant in the absence of other treatment-related effects; however, the increase in cholesterol could be related to the interaction of MC-LR with bile acid transport in the liver. The authors observed no difference in the incidence of liver histopathology between treated and control mice. Immunohistochemistry of the liver revealed no accumulation of MC-LR.

Ito et al. (1997b) evaluated the carcinogenicity and liver toxicity of chronic gavage doses of MC-LR. A water bloom from Lake Suwa, Japan, served as the source of the MC-LR, which was isolated and dissolved in ethanol and saline for dosing. The purity of the isolated MC-LR was not specified. Twenty-two ICR mice (13 weeks old) were given either 80 or 100 gavage doses of 80 µg MC-LR/kg bw over the course of 28 weeks. No change in mean liver weight was observed in the MC-LR-treated animals compared with controls. The authors reported “light” injuries to hepatocytes in the vicinity of the central vein in 8/15 mice sacrificed immediately after treatment and in 5/7 mice that were withdrawn from treatment for 2 months after exposure. No fibrous changes or neoplastic nodules were observed. Analysis for MC-LR and its metabolites by immunohistochemistry failed to detect either the parent compound or any metabolites in the livers of mice sacrificed immediately after treatment.

Falconer et al. (1988) conducted a chronic exposure experiment using an extract of a *Microcystis aeruginosa* water bloom in Swiss albino mice. A concentration-dependent increase in mortality, reduced body weight and a concentration-dependent increase in ALT levels were observed among groups of mice receiving serial dilutions of the extract as their drinking water for 1 year. There was some evidence that bronchopneumonia incidence was related to the concentration of the extract. No significant differences in liver histopathology were observed, although the observed liver changes were slightly more prevalent in treated animals. The data showed some indication of sex differences in susceptibility; male mice showed effects (including mortality and enzyme level increases) at lower concentrations than for females.

X.X. Zhang et al. (2010) administered MC-LR (commercial product; ≥ 95% purity) to 8-week-old male C57Bl/6 mice (10 per treatment group) via drinking water at a concentration of 0,

1, 40 or 80 µg/L (reported as 0, 0.2, 8.0 and 16.0 µg/kg bw per day) for 180 days. A significant ($p < 0.01$) decrease in body weight, accompanied by an increase in relative liver weight, was reported at 8.0 and 16.0 µg/kg bw per day (data not given). Histopathology revealed infiltrating lymphocytes and fatty degeneration in the liver of mice treated with 8.0 and 16.0 µg/kg bw per day, but incidence and severity data were not provided. There was a significant increase in the area stained positive for matrix metalloproteinase 9 (MMP9) in all treatment groups and for MMP2 at 8.0 and 16.0 µg/kg bw per day. The concentrations of MMP9 protein were also increased at all doses, but the MMP2 protein concentration was significantly increased only in the high-dose group. mRNA expression for both MMPs was significantly increased in the mid- and high-dose groups. MC-LR also increased the phosphorylation of the mitogen-activated protein kinase (MAPK)s, extracellular signal-regulated protein kinase (ERK) 1/2 and p38.

Zhang et al. (2013) administered MC-LR (commercial product; ≥ 95% purity) to 8-week-old male C57Bl/6 mice (10 per treatment group) via drinking water at a concentration of 0, 1, 40 or 80 µg/L for 270 days. Histopathology revealed infiltrating lymphocytes and fatty degeneration in the livers of mice (doses not specified). MMP expression and protein levels for both MMP2 and MMP9 were significantly increased in all dose groups. mRNA levels were increased in all dose groups for MMP2 and in the mid- and high-dose groups for MMP9. The changes in MMP expression and protein levels were not considered adverse.

In summary, existing chronic toxicity studies provide varying results following different MC-LR exposure scenarios. Oral gavage exposure to 80 µg/kg bw per day over 28 weeks resulted in mild hepatocytic injury in mice; however, no liver or other toxicity was reported in female mice administered a lower dose (20 µg/kg bw per day) over a longer period (18 months). Male mice administered 8.0 or 16.0 µg MC-LR/kg bw per day via the drinking water for 180 days showed mild hepatocytic injury and increased relative liver weight (accompanied by decreased body weight); however, the relevance of these effects cannot be verified owing to the absence of reported incidence and severity data.

### *9.2.7 Carcinogenicity*

#### *9.2.7.1 Microcystins*

Falconer and Buckley (1989) and Falconer (1991) reported evidence of skin tumour promotion by extracts of *Microcystis*. The extract was administered via drinking water at a concentration of 40 µg microcystin/mL to mice pretreated topically with an initiating dose of dimethylbenzanthracene (DMBA). After 52 days, the total skin tumour weight in mice exposed to *Microcystis* extract was significantly higher than that of mice receiving only water after initiation. The number of tumours per mouse was only slightly increased in mice receiving the extract; the weight difference was largely due to the weight of individual tumours (Falconer and Buckley, 1989). The total weight of tumours in this group also exceeded that of mice pretreated with DMBA and subsequently treated with topical croton oil, with or without concurrent consumption of *Microcystis* extract. Details of the tumour incidence in the mice were not provided by the authors.

When *Microcystis* extract was provided in the drinking water (0, 10 or 40 µg/mL) of mice pretreated with two oral doses of *N*-methyl-*N*-nitrosourea, no evidence of promotion of lymphoid or duodenal adenomas and adenocarcinomas was observed, and no primary liver tumours were observed (Falconer and Humpage, 1996).

Humpage et al. (2000) administered *M. aeruginosa* extract in drinking water to mice pretreated with azoxymethane. The content of microcystins in the drinking water was determined by mouse bioassay, HPLC, capillary eletrophoresis and protein phosphatase inhibition. The

estimated doses of total microcystins were 0, 382 and 693 μg/kg bw per day at the midpoint of the trial. Mice were sacrificed at intervals up to 31 weeks after commencement of extract exposure. Enzyme analysis showed a concentration-dependent increase in ALP and decrease in albumin in mice treated with extract. A concentration-dependent increase in the mean area of aberrant crypt foci of the colon was observed, although the number of foci per colon and the number of crypts per focus were not different among the groups. The authors proposed that increased cell proliferation caused the increase in size of foci. The livers of mice treated with extract showed more leukocyte infiltration in animals treated with the highest concentration of extract compared with those receiving a low concentration.

IARC (2010) has classified MC-LR as possibly carcinogenic to humans based on inadequate evidence of carcinogenicity in both animals and humans but sufficient evidence for tumour promotion in animals. IARC (2010) summarized studies in rats and mice in which MC-LR or microcystins, administered by intraperitoneal injection, promoted preneoplastic lesions in the liver. In one limited study in male mice (Ito et al., 1997b), repeated intraperitoneal injections of MC-LR induced liver foci, which were probably benign tumours. In three experiments in male rats that were initiated with *N*-nitrosodiethylamine (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994; Sekijima et al., 1999) and one experiment in male rats that were initiated with aflatoxin B1 (Sekijima et al., 1999), multiple intraperitoneal injections of MC-LR increased the incidence of liver GST placental form–positive foci, which are considered to be preneoplastic lesions.

In summary, microcystins administered to mice and rats following treatment with known tumour initiators have been shown to act as tumour promoters; when administered in the absence of initiator pretreatment, direct tumour induction activity was not observed. Further research is required to fully determine the carcinogenic potential of microcystins.

#### *9.2.7.2 Anatoxin-a*

No studies investigating the carcinogenicity of anatoxin-a were identified.

#### *9.2.7.3 Cylindrospermopsin*

The tumour initiating activity of cylindrospermopsin was tested *in vivo* in male Swiss mice using *O*-tetradecanoylphorbol 13-acetate (TPA) as the promoter. Mice were administered a gavage dose of saline (27 mice) or 500 mg/kg bw of a saline extract of freeze-dried *Cylindrospermopsis raciborskii* (strain AWT 205) cells (34 mice) every other week for three doses. Other groups received a single dose of 1500 mg extract/kg bw (14 mice) or two doses of 1500 mg extract/kg bw separated by 2 weeks (17 mice). Most (70%) of the mice in the group receiving two doses of 1500 mg extract/kg bw died within 1 week of the second dose, leaving five survivors for use in the rest of the study. Based on a reported cylindrospermopsin content of 5.5 mg/g extract, the cylindrospermopsin-equivalent doses in the 500 and 1500 mg extract/kg bw groups were 2.75 and 8.25 mg/kg bw, respectively. Two weeks after the final dose, the saline and 500 mg extract/kg bw groups were divided into subgroups of 13–18 mice that were fed liquid food containing TPA dissolved in dimethyl sulphoxide (DMSO), or food containing DMSO alone, for 24 hours twice weekly for 30 weeks. All of the mice in both 1500 mg extract/kg bw groups were similarly exposed to TPA-containing liquid food (no 1500 mg/kg bw mice were exposed to food containing DMSO alone). Neoplastic changes were found in five cylindrospermopsin-treated mice, but not in any of the 27 control mice, a difference that was not statistically significant. There was no pattern to the neoplastic changes, as they occurred in different animals, target organs and treatment groups, thus providing equivocal evidence for carcinogenicity (Falconer and Humpage, 2001).Marie et al. (2010) investigated the *in vitro*

carcinogenic potential of cylindrospermopsin using the cell transformation assay on Syrian hamster embryo cells. Purified cylindrospermopsin, supplied by the Australian Water Quality Centre (> 98% purity; Adelaide, Australia) was dissolved in water and applied to Syrian hamster embryo cells at concentrations of $1 \times 10^{-8}$ to $1 \times 10^{-3}$ µg/mL (first experiment) and $1 \times 10^{-10}$ to $1 \times 10^{-6}$ µg/mL (second experiment) for 7 days. Pooled results from both experiments indicate a significant ($p < 0.01$) increase in morphological cell transformation at concentrations ranging from $1 \times 10^{-10}$ to $1 \times 10^{-5}$ µg/mL. In this study, the transforming potential of cylindrospermopsin was observed at concentrations lower than the ones shown to be genotoxic *in vitro*. Morphological cell transformation occurred at $1 \times 10^{-10}$ µg/mL, which indicates a carcinogenicity hazard at very low doses. It is noted that in this study, cylindrospermopsin concentrations above $1 \times 10^{-5}$ µg/mL failed to induce cell transformation, but cylindrospermopsin was not cytotoxic until 0.01 µg/mL (one of several concentrations used in a preliminary test to determine cytotoxicity). This may be due to the biotransformation pattern of cylindrospermopsin being different at low and high doses.

In another study, a significant decrease in hepatic CYP of mice treated (via intraperitoneal injection) with 0.2 mg/kg bw cylindrospermopsin *in vivo* was observed compared with controls. At high cylindrospermopsin concentrations, the decrease of CYP enzymes may reduce the production of active metabolites involved in cell transformation. It was concluded by the authors that the cell-transforming activity indicates carcinogenic potential (Terao et al., 1994).

Lankoff et al. (2007) studied the carcinogenic potential of cylindrospermopsin *in vitro* via Chinese hamster ovary (CHO-K1) cells in a chromosome aberration assay to assess DNA damage. Cylindrospermopsin isolated from two cultures of *C. raciborskii*, AWT 205 (Australian Water Technology Center) and Thai (fish pond in Thailand), was prepared in solution, and CHO-K1 cells were exposed to 0, 0.05, 0.1, 0.2, 0.5, 1 or 2 µg/mL with and without metabolic activation (9000 × *g* supernatant fraction from rat liver homogenate, or S9) for 3, 16 and 21 hours. Results indicated no significant influence on the frequency of chromosome aberrations in cells treated with cylindrospermopsin with or without S9 compared with control groups. Neither cylindrospermopsin nor the S9 fraction–induced metabolites were clastogenic in CHO-K1 cells. However, significant ($p < 0.05$) decreases in frequencies of mitotic indices after various exposure durations were observed at exposure concentrations of 0.1 µg/mL and above. Furthermore, significant ($p < 0.05$) increases in frequencies of apoptotic cells (1 µg/mL and above) and necrotic cells (0.5 µg/mL and above) after 21 hours were observed compared with the controls in a dose- and time-dependent manner. The presence of metabolic activation influences the susceptibility to necrotic cell death, but not to apoptotic cell death.

In summary, the data are inadequate to determine the carcinogenic potential of cylindrospermopsin. It is unclear whether cylindrospermopsin acts as an initiator; however, evidence of morphological cell transformation has been observed following exposure to low levels of cylindrospermopsin, indicating the potential for carcinogenicity. More research is required to further elucidate the carcinogenic potential of cylindrospermopsin.

### *9.2.8 Immunotoxicity*

#### *9.2.8.1 Microcystins*

The only studies available investigating the immunotoxicity of microcystins are intraperitoneal injection studies. Although intraperitoneal injection is not considered to be a relevant route of exposure for assessing the risks from chemicals in drinking water, a brief summary of the results from several intraperitoneal studies is provided for the purpose of hazard identification.

Limited animal evidence indicates that exposure to microcystins (isolated from extracts of water blooms) by single or multiple intraperitoneal injections at doses ranging from 4.97 to 50 µg MC-$LR_{equiv}$/kg bw may alter immune function via effects on phagocytic capacity, B-lymphocyte proliferation, humoral immune response, plasma white blood cell levels, and cytokine and interferon levels (Shen et al., 2003; Shi et al., 2004; Yuan et al., 2012). T. Chen et al. (2004, 2005) reported a dose-dependent inhibition of nitric oxide production in activated macrophages, as well as repression of cytokine formation at the mRNA level, following exposure to 1–1000 nmol MC-LR/L. Further research is required in order to further elucidate the immunotoxicity of microcystins following oral exposure.

#### *9.2.8.2 Anatoxin-a*

No studies investigating the immunotoxicity of anatoxin-a were identified.

#### *9.2.8.3 Cylindrospermopsin*

No information was located regarding effects of cylindrospermopsin on immune function, although immune system tissues appear to be a target of short-term, high-level exposures.

Massive necrosis of lymphocytes in the thymus of male ICR mice was reported following a single 0.2 mg/kg bw intraperitoneal dose of cylindrospermopsin purified (purity not reported) from cultured *Umezakia natans* cells (Terao et al., 1994).

Effects observed in MF1 mice administered a single gavage dose of a suspension of freeze-dried *Cylindrospermopsis raciborskii* cells, in the lethal dose range of 4.4–8.3 mg/kg bw, included atrophy in lymphoid tissue of the spleen and thymus (Seawright et al., 1999).

Lymphophagocytosis was observed in the spleen of Quackenbush mice exposed to a cell-free extract of freeze-dried and sonicated *C. raciborskii* cells by gavage at a non-lethal dose level of 0.05 mg/kg bw per day for 14 days. These effects were considered to be normal responses of the immune system to the stress of severe intoxication (Shaw et al., 2000, 2001).

### *9.2.9 Hematological effects*

#### *9.2.9.1 Microcystins*

Several studies have noted thrombocytopenia in laboratory animals treated with microcystins or bloom extracts reportedly containing microcystins (Slatkin et al., 1983; Adams et al., 1985, 1988; Takahashi et al., 1995). Early investigations explored whether microcystins had a direct effect on platelets and whether platelets might be responsible for pulmonary thrombi (Slatkin et al., 1983; Jones, 1984). However, *in vitro* studies have shown that MC-LR neither induces nor impedes the aggregation of platelets (Adams et al., 1985). Pulmonary thrombi apparently consist of necrotic hepatocytes circulating in the blood. More recent information supports the hypothesis that hematological effects observed in animals acutely exposed to microcystins are secondary effects of liver hemorrhage (Takahashi et al., 1995).

#### *9.2.9.2 Cylindrospermopsin*

Reisner et al. (2004) reported significant increases in hematocrit and acanthocytes (abnormal red blood cells characterized by a spiked external membrane) in 4-week-old male ICR mice following exposure to purified cylindrospermopsin from *Aphanizomenon ovalisporum* (isolated from Lake Kinneret, Israel, during a 1994 bloom) (see Section 9.2.3.3 for a description of the study). At the end of 3 weeks, hematocrit levels were significantly altered ($p < 0.05$) in the treated animals compared with the controls. Acanthocyte-like red blood cells were observed in blood samples collected at the end of each exposure week, leading to an exposure duration–

related increase in hematocrit values. The cholesterol content of the red blood cell membranes and plasma was significantly ($p < 0.05$) greater in treated animals compared with controls after 3 weeks of exposure; liver cholesterol levels were significantly lower ($p < 0.05$) in treated animals compared with controls.

In investigating the potential effects of cylindrospermopsin in mice following 42 weeks of exposure via drinking water, Sukenik et al. (2006) (see Section 9.2.3.3 for a description of the study) observed significantly ($p < 0.05$) increased hematocrit levels in both male and female mice after 16 weeks of exposure compared with controls. The observed changes in the hematocrit level were accompanied by increased numbers of acanthocytes in the blood. At 20 weeks, many red blood cells were present as acanthocytes, and at 42 weeks, very few normal cells were present in the collected blood samples. The conversion of red blood cells to acanthocytes appears to be related to increased cholesterol in the red blood cell membrane; thus, the authors measured the amount of cholesterol in the red blood cell membrane, plasma and liver for eight males and eight females at 20 and 42 weeks. Cholesterol was significantly increased in the red blood cell membrane and decreased in the liver for both males and females at 42 weeks. At 20 weeks, there was a significant decrease in cholesterol in the livers of males, but not females. Plasma cholesterol was increased only at 42 weeks, and the difference was significant only for the females. The authors proposed a LOAEL of 20 µg/kg bw per day for structural changes in red blood cells at 20 weeks; the identified LOAEL also applies to observed increases in relative kidney weight (see Section 9.2.3.3). It should be noted, however, that the confidence in the proposed LOAEL is weak due to the design of the experiment (i.e., changing dose over the duration of the experiment).

In summary, both short- and long-term exposure to cylindrospermopsin resulted in structural changes in red blood cells (acanthocytosis), which was found to be associated with increased hematocrit. It is postulated that an increase in the ratio of red blood cell membrane cholesterol to phospholipids may be a factor responsible for acanthocyte formation. Further studies of the role of cylindrospermopsin in acanthocyte formation are needed to better understand how cylindrospermopsin can affect the cholesterol content of the red blood cell membrane.

### *9.2.10 Mutagenicity and genotoxicity*

#### *9.2.10.1 Microcystins*

The evidence for the mutagenicity and genotoxicity of microcystins is conflicting. As cancer is not considered to be a key endpoint of concern for the derivation of an HBV for microcystins in drinking water, only a brief qualitative summary of the available evidence is provided below.

Microcystin-containing extracts gave positive results in the Ames assay (Ding et al., 1999; Huang et al., 2007b; Sieroslawska, 2013), whereas negative results were observed using *Microcystis aeruginosa* extracts, as well as purified microcystin (Grabow et al., 1982; Repavich et al., 1990; Wu et al., 2006). Positive genotoxicity results were observed in mammalian cell lines (Suzuki et al., 1998; Zhan et al., 2004; Žegura et al., 2006, 2008a, 2008b, 2011; Nong et al., 2007; D. Li et al., 2011), but *in vivo* animal studies have shown conflicting results (Dong et al., 2008; Gaudin et al., 2008, 2009; Abramsson-Zetterberg et al., 2010; X. Zhang et al., 2011). Evidence for MC-LR-induced DNA damage as measured by the comet assay has been called into question by the finding that apoptosis can lead to false-positive findings in this assay (Lankoff et al., 2004). Some evidence exists for a clastogenic effect of MC-LR (Repavich et al., 1990; Ding et al., 1999; Zhan et al., 2004; Lankoff et al., 2006). Metabolic activation has been found to decrease MC-LR

mutagenicity. The inconsistent outcomes from the mutagenicity studies may be related to differences in the cell uptake of MC-LR or the metabolism of MC-LR in the test system.

DNA fragmentation was significantly increased with MC-LA and MC-YR in rat neutrophils, but not in human neutrophils (Kujbida et al., 2008). MC-YR has also been found to induce DNA damage in the blood (lymphocytes), liver, kidney, lung, spleen and brain of mice administered 10 µg MC-YR/kg bw via intraperitoneal injection every other day for 30 days (Filipič et al., 2007). Lankoff et al. (2003) showed that MC-LR, through its effect on microtubules, damages the mitotic spindle, leading to the formation of polyploid cells.

In terms of tumour promotion, mechanistic data indicate that at low doses, MC-LR may increase cell proliferation. MC-LR has been shown to increase the expression of the Bcl-2 protein that inhibits apoptosis and decrease the expression of the Bax protein that induces apoptosis (Hu et al., 2002; Lei et al., 2006; Weng et al., 2007; G. Li et al., 2011); upregulate the transcription factors c-fos and c-jun, leading to abnormal proliferation (Zhao and Zhu, 2003); inhibit PP2A, which regulates several MAPKs (Gehringer, 2004); and increase MAPK phosphorylation in livers (X.X. Zhang et al., 2010). The MAPK cascade regulates transcription of genes required for cell proliferation, including c-jun and c-fos, and the activation of the MAPK cascade has been postulated to inhibit apoptosis and thus increase cell proliferation. Finally, MC-LR has been reported to increase phosphorylation of p53 (Gehringer et al., 2004; Fu et al., 2005; Hu et al., 2008; Xing et al., 2008; Žegura et al., 2008a; G. Li et al., 2011), which is involved in the regulation of the cell cycle and apoptosis.

#### *9.2.10.2 Cylindrospermopsin*

As cancer is not considered to be a key health endpoint for the derivation of an HBV for cylindrospermopsin in drinking water, only a brief summary of the available data on the mutagenicity and genotoxicity is provided in this section.

Studies investigating the *in vitro* and *in vivo* genotoxicity of cylindrospermopsin are relatively few in number. *In vitro* mutagenic and genotoxic cell assays with cylindrospermopsin have shown varied results, with some indications of potential damage to DNA (Fessard and Bernard, 2003; Humpage et al., 2005; Lankoff et al., 2007; Bazin et al., 2010; Straser et al., 2011). The human hepatocytic and enterocytic models consisting of HepaRG and Caco-2 cells showed increased multinucleated binucleate cells, and micronucleated cells were observed in a study with human lymphoblastoid WIL2-NS cells (Bazin et al., 2010). DNA breaks have been observed in primary hepatocytes by the comet assay, indicating that DNA strand breakage could be a mechanism for cylindrospermopsin-induced cytogenetic damage (Humpage et al., 2005). Following intraperitoneal exposure, DNA strand breakage was observed in the liver of BALB/c mice (Shen et al., 2002), and covalent binding between DNA and cylindrospermopsin, or a metabolite, occurred in Quackenbush mouse liver (Shaw et al., 2000). However, these data are limited, and there has been no long-term bioassay of purified cylindrospermopsin. The limited study by Falconer and Humpage (2001) on initiation with TPA promotion did not support classification of cylindrospermopsin as a tumour initiator.

## 9.3 Mode of action

### *9.3.1 Microcystins*

Although the mode of action for microcystins (MC-LR) has not been fully elucidated, some mechanistic studies (including *in vivo* investigations in laboratory animals, *in situ* studies in isolated perfused organ systems and *in vitro* assays in isolated cell preparations) have evaluated the following key events: the reason for target organ and cell type specificity of microcystins;

interaction with Ser/Thr protein phosphatases (i.e., PP1 and PP2A) as the molecular target for microcystins; the role of cytoskeletal effects; and the importance of oxidative stress and apoptosis as a mode of toxic action.

*9.3.1.1 Key event 1: Uptake of microcystins by organic anion–transporting polypeptides (OATPs)*

Microcystins do not passively permeate cells and are hepatotoxic because hepatocytes express OATPs, which facilitate their uptake. Evidence of the importance of OATPs (also known as bile acid transporters) in microcystin-mediated liver toxicity has been provided by *in vivo* and *in vitro* studies using bile acids and bile acid transport inhibitors (Runnegar et al., 1981, 1993, 1995a; Runnegar and Falconer, 1982; Eriksson et al., 1990a; Hermansky et al., 1990a, 1990b, 1991). Fischer et al. (2005) showed that rat OATP1B2 and human OATP1B1 and OATP1B3 are responsible for MC-LR transport into hepatocytes, and human OATP1A2 mediated the transport of MC-LR transport across the blood–brain barrier.

The testes have also been reported as a target organ for microcystin toxicity in *in vivo* studies after intraperitoneal injection in male mice or rats (Kirpenko et al., 1981; Ding et al., 2006; Li et al., 2008; Liu et al., 2009; Y. Chen et al., 2011; D. Li et al., 2011; X. Wang et al., 2012); however, the way in which MC-LR enters the testes is unclear. Augustine et al. (2005) demonstrated a lack of OATP1 mRNA expression (the family of OATPs confirmed to facilitate microcystin uptake by hepatocytes and across the blood–brain barrier) in both isolated Sertoli cells and whole testis. OATP2 and OATP3 mRNA expression was measurable in both isolated Sertoli cells and whole testis; however, no evidence was located to indicate whether these transporters actively transport MC-LR across cell membranes. Later publications by Klaassen and Aleksunes (2010) and Svoboda et al. (2011) reported that several OATPs (1A4, 1A5, 2A1, 2B1, 3A1, 6A1, 6B1, 6C1 and 6D1) are active in the testes, although no studies determining their specific contribution to the testicular toxicity of microcystins have been identified. Zhou et al. (2012) demonstrated the presence of five OATPs (1A5, 3A1, 6B1, 6C1 and 6D1) in the spermatogonia of rats and that the expression of all the identified OATPs, especially OATP3A1, was affected by MC-LR exposure. In addition, through gel electrophoreses with targeted western blot analysis along with observed cytotoxicity, the authors were able to provide evidence of MC-LR entry into spermatogonia. Wang et al. (2012b) observed that MC-LR was not able to enter Leydig cells and thus did not induce cytotoxicity in these cells. Given the lack of clear evidence on how MC-LR is taken up into the testis, further studies are needed in order to elucidate how effects on sperm are induced by MC-LR and if these effects are induced by oral doses lower than the NOAEL for liver effects.

As the OATPs responsible for transporting microcystins across cell membranes have been detected in human hepatocytes, microcystin-mediated liver effects are possible in humans. Evidence of such OATPs in human testes has not been located; thus, it is unclear whether the uptake of microcystins by the testes in humans is biologically plausible. Further research is required in order to better understand how MC-LR impacts male reproductive function in animals and whether these effects are plausible in humans.

*9.3.1.2 Key event 2: Inhibition of serine/threonine (Ser/Thr) protein phosphatase*

The toxic action of microcystins is a consequence of their inhibition of cellular Ser/Thr protein phosphatases leading to altered phosphorylation homeostasis, thus affecting cell functions and structures that are controlled by changes in phosphorylation. Microcystins have been shown to potently inhibit the activities of Ser/Thr protein phosphatases types 1 and 2A (PP1, PP2A) in the hepatocytes (Eriksson et al., 1990b; Matsushima et al., 1990; Yoshizawa et al., 1990;

Runnegar et al., 1991, 1999). The mechanism of action in the toxicity of microcystins is the specific inhibition of PP1 and PP2A in the cytoplasm and the nucleus, as observed in both *in vivo* and *in vitro* studies (Honkanen et al., 1990; MacKintosh et al., 1990; Matsushima et al., 1990; Yoshizawa et al., 1990; Runnegar et al., 1993, 1995b; Sim and Mudge, 1993; Leiers et al., 2000; Xu et al., 2000b; Becchetti et al., 2002; Billam et al., 2006, 2008; Jayaraj and Rao, 2006). This inhibition, along with a loss of coordination between kinase phosphorylation and phosphatase dephosphorylation, results in a destabilization of the cytoskeleton initiating cellular apoptosis and necrosis followed by altered cell function (Barford et al., 1998). Together, cellular kinases and phosphatases maintain the balance between phosphorylation and dephosphorylation of key cellular proteins that control metabolic processes, gene regulation, cell cycle control, transport and secretory processes, organization of the cytoskeleton and cell adhesion.

The concerted action of protein phosphatases and kinases regulating the phosphorylation of the cytoskeleton is also known to be important to sperm physiology. In a study of human normozoospermic (normal motile sperm) and asthenozoospermic (sperm with reduced motility) samples, Fardilha et al. (2013) identified a significant decrease in the cellular distribution of the PP1 and PP2 subfamilies that correlated with the low motility for the asthenozoospermic samples. The progressive motility of sperm in the asthenozoospermic samples was about 10% of that for the normal sperm, and the number of immotile sperm was about twice that for the normal samples. This study indicates that the inhibition of protein phosphatases can adversely impact sperm motility, providing that microcystins can reach this target after oral administration. More research is required in order to determine whether microcystins can impact sperm motility via this pathway in humans.

#### *9.3.1.3 Key event 3: Cytoskeletal disruption*

Protein phosphatase inhibition by microcystins relates to changes in cytoskeletal structure and cell morphology (Eriksson and Golman, 1993). Ser/Thr proteases are of critical importance in maintaining cytoskeletal integrity (Eriksson et al., 1992a, 1992b) because of their dephosphorylating impact on phosphoprotein cytoskeletal precursors. The cytoskeletal effects of microcystins in the liver have been visually demonstrated in several studies using light, electron and fluorescent microscopy (Runnegar and Falconer, 1986; Eriksson et al., 1989; Hooser et al., 1989, 1991b; Falconer and Yeung, 1992). Ultrastructural changes in rats given a lethal dose of microcystin include a widening of intracellular spaces; progressive cell–cell disassociation followed by rounding, blebbing and invagination of hepatocytes; a loss of microvilli in the space between the hepatocytes and sinusoids; a breakdown of the endothelium; hemorrhage; and loss of lobular architecture (Hooser et al., 1989).

Similar histopathological changes in the rat testes have been described by Chen et al. (2013). Repeated intraperitoneal dosing resulted in increased space between the seminiferous tubules, cytoplasmic shrinkage, cell membrane blebbing, swollen mitochondria and deformed nuclei. Transcriptional levels of β-actin and β-tubulin were significantly decreased.

In considering the similarities in basic cell structure and function between experimental animals and humans, it is possible that microcystin-mediated cytoskeletal disruption as reported in animal liver and testes could also occur in humans; however, more research is required in order to determine any biological plausibility in humans.

#### *9.3.1.4 Key event 4: Apoptosis and oxidative stress*

The ultrastructural changes observed in hepatocytes following exposure to microcystins suggest that cell death is related to apoptosis and not necrosis. These changes include cell

shrinkage (decreased volume and increased density), condensation of chromatin and segregation of organelles separated by apoptotic microbodies presumably related to the cytoskeletal damage discussed in the previous section (Boe et al., 1991; Fladmark et al., 1998; McDermott et al., 1998; Ding et al., 2000b; Mankiewicz et al., 2001). The effects of microcystins on the signalling pathways involved in rapid apoptosis are supported by several studies (Ding et al., 1998a, 1998b, 2000b, 2001, 2002; Ding and Ong, 2003; Feng et al., 2011; Huang et al., 2011; Ji et al., 2011).

Botha et al. (2004) demonstrated that apoptosis and oxidative stress can be induced in non-hepatic cells by microcystins. MC-RR changed the concentration of several proteins associated with apoptosis in FL human amniotic epithelial cells (Fu et al., 2009). LDH leakage and increased apoptotic indices were observed in the human colon carcinoma cell line (Caco-2) and MCF-7 cells (deficient in pro-caspase-3). These changes were accompanied by increased hydrogen peroxide formation and increased calpain activity.

Y. Chen et al. (2011) found an increase in apoptosis in testes cells in male mice orally administered low doses of MC-LR. Wang et al. (2013) showed apoptosis in testes of mice given 7.5 µg MC-LR/kg bw and higher by intraperitoneal injection; mRNA expression for Bax, caspase-3 and caspase-8 was upregulated, and increased phosphorylation of p53 and Bcl-2 was measured. H. Zhang et al. (2011) also observed apoptosis in isolated rat Sertoli cells incubated with 10 µg/mL of MC-LR for 24 hours. Accompanying this were increases in p53, Bax and caspase-3 and a decrease in Bcl-2.

Oxidative stress may play a role in the onset of apoptosis. Ding et al. (2001) illustrated generation of superoxide and hydrogen peroxide radicals preceding microfilament disorganization and cytotoxicity. Hepatocellular GSH levels were affected by microcystins, and administration of *N*-acetylcysteine was shown to protect against cytoskeletal alterations (Ding et al., 2000a). Lipid peroxidation was observed 2 hours after exposure in the livers of male mice administered a single intraperitoneal injection of 55 µg MC-LR/kg bw (Wei et al., 2008). The effects of MC-LR on ROS and enzyme activities indicated that MC-LR-induced liver injury in mice begins with the production of ROS, which stimulated the sustained activation of c-Jun N-terminal protein kinase (JNK) as well as transcription factor AP-1 and the Bcl-2 family member Bid; these changes led to mitochondrial dysfunction, followed by apoptosis and oxidative liver injury.

Lipid peroxidation was induced in the testes of immature male rabbits with a single intraperitoneal injection of 12.5 µg MC-$LR_{equiv}$/kg bw of a crude extract (Liu et al., 2010). Other indicators of oxidative stress included increased hydrogen peroxide, catalase, superoxide dismutase, GSH peroxidase, GST and GSH.

In considering the similarities in basic cell structure and function between animals and humans, it is possible that microcystin-mediated apoptosis and oxidative stress as reported in animal liver and testes could also occur in humans; however, more research is required in order to determine any biological plausibility in humans.

### *9.3.2 Anatoxin-a*

The mode of action for anatoxin-a has not been fully elucidated. *In vitro* studies have demonstrated that (+)-anatoxin-a mimics the action of acetylcholine at neuromuscular nicotinic receptors (Carmichael et al., 1975, 1979; Biggs and Dryden, 1977; Aronstam and Witkop, 1981; Swanson et al., 1986) and is significantly more potent than acetylcholine and nicotine as an agonist (see Section 9.2.4). Anatoxin-a has become a very useful agent for investigating nicotinic acetylcholine receptors because it is resistant to enzymatic hydrolysis by acetylcholinesterase and because it is 100-fold more selective for nicotinic acetylcholine receptors than for muscarinic acetylcholine receptors (Aronstam and Witkop, 1981). When acetylcholine is released at the

neuromuscular junction of motor neurons, it binds to muscle cell receptor molecules consisting of a neuromuscular binding site and an ion channel, which triggers ionic currents that induce muscle cell contraction. Extracellular acetylcholinesterase acts on acetylcholine by degrading the neurotransmitter to prevent overstimulation of the muscle cells. Because anatoxin-a is not degraded by cholinesterase or any other known cellular enzymes, muscle cells continue to be stimulated, causing muscular twitching, fatigue and paralysis. Severe overstimulation of respiratory muscles may result in respiratory arrest and rapid death, as observed in acute lethality studies in animals (Carmichael et al., 1975, 1977; Devlin et al., 1977; Stevens and Krieger, 1991b).

Anatoxin-a also acts as a nicotinic cholinergic agonist at receptors in the cardiovascular system of rats, resulting in increased blood pressure and heart rate (Sirén and Feuerstein, 1990; Adeyemo and Sirén, 1992; Dube et al., 1996), as well as in rat and human brain neurons (Zhang et al., 1987; Thomas et al., 1993; Durany et al., 1999). Anatoxin-a is a potent agonist of the secretory response of bovine adrenal chromaffin cells, presumably via neuronal-type nicotinic receptor activation (Molloy et al., 1995).

Anatoxin-a is capable of eliciting the release of neurotransmitters from presynaptic neuromuscular and brain cell terminals. Several studies (Rowell and Wonnacott, 1990; Gordon et al., 1992; Soliakov et al., 1995; Clarke and Reuben, 1996; Wonnacott et al., 2000) indicate that anatoxin-a can bind to presynaptic nicotinic receptors to trigger neurotransmitter release. Increased neurotransmitter release could contribute to increased stimulation of postsynaptic receptors.

### *9.3.3 Cylindrospermopsin*

Based on available studies, the liver, kidneys and red blood cells appear to be important targets of cylindrospermopsin toxicity; however, the mode of action for cylindrospermopsin-mediated toxicity has not been fully elucidated.

The mechanism for liver toxicity is not completely characterized, but involves inhibition of protein synthesis (Terao et al., 1994; Froscio et al., 2003). Evidence indicates that the protein synthesis inhibition is not decreased by broad-spectrum CYP inhibitors, suggesting that it is mediated by the parent compound (Froscio et al., 2003). Hepatocytotoxicity occurring at higher levels of exposure to cylindrospermopsin, however, appears to be CYP dependent, indicating the involvement of metabolites and other mechanisms (Norris et al., 2002; Froscio et al., 2003; Humpage et al., 2005). Pretreatment with the CYP inhibitor α-naphthoflavone partially protected against cytotoxicity and cellular GSH depletion, indicating involvement of the CYP enzyme system in cylindrospermopsin metabolism and that one or more metabolites might be more active than the parent compound in inhibiting GSH synthesis (Runnegar et al., 1995c). Additional support for the involvement of CYP in the hepatotoxicity of cylindrospermopsin is the finding that liver histopathology is mainly induced in the periacinar region where CYP-catalyzed xenobiotic metabolism occurs (Shaw et al., 2000, 2001). Cylindrospermopsin has also been shown to deplete mouse hepatic GSH *in vivo* (Norris et al., 2002) as well as decrease GSH levels and synthesis of GSH and protein in cultured rat hepatocytes (Runnegar et al., 1994, 1995c, 2002). Inhibition of GSH synthesis was the predominant mechanism for the reduction in GSH; other mechanisms, including increased consumption of GSH, increased formation of oxidized glutathione, increased GSH efflux, hidden forms of GSH, decreased GSH precursor availability and decreased cellular adenosine triphosphate, were effectively ruled out (Runnegar et al., 1995c). GSH depletion occurred at non-toxic cylindrospermopsin concentrations and preceded the onset of observable toxicity at higher concentrations (Runnegar et al., 1994).

Studies specifically investigating the inhibition of protein synthesis in the kidneys are not available, although the results of the 11-week oral toxicity study in mice (Humpage and Falconer, 2003) are consistent with an inhibition of protein synthesis. Effects in this study included decreased urinary protein and, at a higher dose, proximal renal tubular lesions; however, the biological relevance of these effects requires further investigation. Potential mechanisms for a decrease in urinary protein include a decrease in glomerular filtration (i.e., filtered load) of protein, an increase in resorption of filtered protein and a decrease in secretion of nephrogenic protein. A decrease in glomerular filtration of protein (e.g., µg protein/day) could result from a decrease in serum protein concentration or a decrease in glomerular filtration rate. The predominant serum protein in the urine of healthy animals (e.g., mice, rats and humans) is albumin (approximately 50% of serum proteins in urine). In the Humpage and Falconer (2003) study, serum albumin concentration increased in mice exposed to cylindrospermopsin, and serum creatinine (a marker of glomerular filtration rate), which was measured but not discussed in the results, was indicated as unchanged. Therefore, it is unlikely that glomerular filtration of serum proteins decreased in response to cylindrospermopsin (if a change occurred, it is likely to have been an increase in the rate of filtration of albumin). Furthermore, serum proteins normally account for approximately 15% of total urinary protein (Pesce and First, 1979), whereas the decrease in urinary excretion of protein observed in Humpage and Falconer (2003) was substantially larger (approximately 50%), indicating that the decrease in urinary protein cannot result solely from a decrease in excretion (i.e., glomerular filtration) of serum proteins.

No information is reported in Humpage and Falconer (2003) that would allow an assessment of tubular resorption of filtered protein (e.g., plasma-to-urine clearance of protein, excretion of low-molecular-weight proteins such as $\beta_2$-microglobulin or retinal binding protein).

In the absence of a decrease in filtration or increased resorption of filtered serum protein, the substantial decrease in urinary protein (i.e., approximately 50%) may involve decreased excretion of Tamm-Horsfal protein (THP), the predominant mammalian protein in urine. THP is synthesized exclusively in the thick ascending limb of the loop of Henle. A sustained change in THP excretion would likely reflect a functional change in this region of the nephron. Increases and decreases in THP have been observed in various kidney diseases and in association with experimental treatments that induce hypertrophy of the thick ascending limb of the loop of Henle, including increased dietary protein (Bachmann et al., 1991). The decrease in urinary specific gravity in animals exposed to cylindrospermopsin in the Humpage and Falconer (2003) study may be indicative of impaired urine concentrating ability and possibly related to impaired function of the thick ascending limb of the loop of Henle (i.e., impairment of transport activity in this region of the nephron impairs urine concentrating ability) and/or decreased synthesis of THP. Proximal renal tubular damage (type and severity of lesions not reported) was also reported by Humpage and Falconer (2003) at the high dose and, when considered with decreased protein excretion at lower doses, suggests a dose-severity progression.

Cylindrospermopsin exposure has also been reported to significantly affect (i.e., increase) hematocrit levels in mice via the formation of acanthocytes (abnormal red blood cells) (Reisner et al., 2004; Sukenik et al., 2006). Studies in humans and rats (McBride and Jacob, 1970; Ulibarrena et al., 1994; Gallagher and Forget, 2001) indicate that acanthocyte formation results from alterations in the lipid content of the red blood cell membrane, resulting in an increased ratio of membrane cholesterol to phospholipid content (Williams et al., 1990). Acanthocyte formation appears to be secondary to changes in plasma lipoproteins as well as decreased activity of lecithin–cholesterol acyltransferase (LCAT), the enzyme responsible for regulating the transfer of cholesterol between the red blood cell membrane and plasma. Plasma LCAT catalyzes the

depletion of cholesterol from the red blood cell membrane; therefore, in the absence of LCAT, a net accumulation of free cholesterol in the red blood cell membrane occurs (Hochgraf et al., 1997). Although direct evidence for the involvement of LCAT in the physiological response to cylindrospermopsin is lacking, it has been reported that severe liver or renal failures inactivate LCAT (Cooper and Jandl, 1968; Gillet et al., 2001). Further studies on the role of cylindrospermopsin and acanthocyte formation are needed in order to further deduce the mode of action for cylindrospermopsin-mediated effects on red blood cells.

## 9.4 Toxic equivalency

### *9.4.1 Microcystins*

MC-LR has been shown to be one of the most potent microcystin variants, with the lowest reported intraperitoneal $LD_{50}$ values, ranging between 50 and 60 µg/kg bw (Chorus and Bartram, 1999). Limited comparative testing of *in vitro* protein phosphatase inhibition ($IC_{50}$) for MC-LR, MC-RR and MC-YR resulted in $IC_{50}$ values of 1.6, 3.4 and 1.4 nmol/L, respectively (Yoshizawa et al., 1990); comparable results from other studies (Hoeger et al., 2007; Monks et al., 2007; Fischer et al., 2010; Vesterkvist et al., 2012) indicate that microcystin variants may be relatively similar with respect to protein inhibition potency, despite differences in their variable amino acids. Pharmacokinetic differences among the various variants may be at least partially responsible for observed variations in lethal potency (Ito et al., 2002; Fischer et al., 2010; Buratti and Testai, 2015). Microcystin variants of varying hydrophobicities were shown to interact differently with lipid monolayers (Vesterkvist and Meriluoto, 2003), and effects on membrane fluidity may also alter the cellular uptake of these toxins.

Toxicity equivalency factors (TEFs) have been proposed by Wolf and Frank (2002) for MC-LR, MC-LA, MC-YR and MC-RR based on $LD_{50}$ values obtained following intraperitoneal administration. Using MC-LR as the index compound (TEF = 1.0), the authors proposed TEFs of 1.0 for both MC-LA and MC-YR and 0.1 for MC-RR. TEFs derived from intraperitoneal $LD_{50}$ values may serve as indicators of relative toxicity; however, they are not appropriate for deriving HBVs, given that differences in lipophilicity and polarity of the variants may lead to variable absorption through the oral route of exposure. Further, the differences between the $LD_{50}$ information and the $IC_{50}$ data (as reported above) reduce confidence in the $LD_{50}$-derived TEFs.

Gupta et al. (2003) evaluated the comparative toxicity of the three most predominant microcystin variants, MC-LR, MC-RR and MC-YR, in mice. Mice were administered a one-time $LD_{50}$ dose of 43, 235.4 and 110.6 mg/kg bw for MC-LR, MC-RR and MC-YR, respectively, and biochemical and histological variables were determined at 30 minutes post-treatment and at mean time to death (time to death expressed as a mean due to variability in time to death of experimental animals). A significant increase in liver body weight index was induced by all of the variants. Serum levels of AST, ALT and GGT increased significantly, compared with controls, as early as 30 minutes post-exposure for all variants and was further enhanced (by 3- to 4-fold) at the mean time to death. Enhanced LDH leakage, DNA fragmentation and depletion of hepatic GSH were also observed at 30 minutes post-treatment for all variants. No changes in levels of serum protein, albumin or albumin/globulin ratio were observed. Liver histology showed time-dependent severe pathological lesions, such as congestion, haemorrhage, portal mononuclear cell infiltration and obliteration of chromatin material. Lung lesions were predominantly in the bronchi and parenchyma. Although lesions were qualitatively identical for all three microcystin variants, the degree of liver and lung lesions varied quantitatively among the variants. A uniform breathing pattern and respiratory frequency were observed in mice up to 90 minutes following intraperitoneal administration of the toxin; however, after 90 minutes, an abrupt change in the

respiratory pattern was observed, followed by instantaneous death. Based on biochemical and histological observations, the authors concluded that MC-LR was the most potent variant, followed by MC-YR and MC-RR.

## 10.0 Classification and assessment

### 10.1 Microcystins

The majority of toxicological data on the effects of microcystins pertain to the MC-LR variant. A single poorly described study, reported only in a secondary source, is available for the MC-LA variant, and data on the MC-YR and MC-RR variants are limited to $LD_{50}$ values and measures of relative inhibition of protein phosphatases following intraperitoneal administration. TEFs have been proposed for MC-LR, MC-LA, MC-YR and MC-RR based on $LD_{50}$ values obtained following intraperitoneal administration; however, these values serve as indicators of relative toxicity only. As such, there are inadequate data to determine HBVs for any microcystin variants aside from MC-LR.

Human epidemiological data on the oral toxicity of microcystins are limited by the lack of quantitative information on exposure and by potential co-exposure to other types of cyanobacterial toxins and microorganisms. Anecdotal reports involving exposure to cyanobacterial blooms via recreational contact or drinking water cite numerous symptoms, including headache, muscle weakness, eye, ear and throat irritation, nausea, stomach pain and diarrhea. Other epidemiological studies have reported an association between consumption of drinking water containing cyanobacteria and microcystins and liver or colon cancer in certain areas of China; however, individual exposures to microcystins were not estimated, and it is unclear whether these studies adequately controlled for confounding factors, such as hepatitis infection or aflatoxin exposure. Thus, there are no adequate epidemiological studies to serve as the basis for the derivation of a proposed maximum acceptable concentration (MAC) for total microcystins in drinking water.

The evidence for genotoxicity and mutagenicity is conflicting, with suggestive evidence of impacts on DNA through ROS and oxidative stress; the mechanisms by which MC-LR induces DNA damage and cancer development, however, are not well understood and require further elucidation. MC-LR has also been shown to act as a tumour promoter in animals in the presence of known tumour initiators.

Limited information is available on potentially susceptible populations; however, the role of GST in the metabolism (detoxification) of microcystins suggests possible susceptibilities for GST-null genotypes. In laboratory animals, liver effects following acute exposure to high levels of MC-LR ($\geq$ 500 µg/kg, determined to be lethal for adults) have been reported to be absent or greatly reduced in juvenile animals (aged 5-6 weeks) compared to adults; age-dependent differences in toxicity were observed after both oral and intraperitoneal exposure, suggesting that differences in gastrointestinal uptake were not entirely responsible for the effect of age. The available data on developmental toxicity in animals indicates that repeated oral exposure of pregnant mice to as high as 600 µg/kg MC-LR resulted in no evidence of effects on developmental toxicity (fetal body weight, skeletal ossification, resorptions, litter size, external, visceral or skeletal abnormalities); no difference in number of litters, number of pups per litter, sex ratio or litter weight was also observed in pups born from male and female mice orally exposed to 14 µg/ml (approximately 2700 µg/kg bw) of toxin from an extract from *M. aeruginosa* from weaning (for 17 weeks prior to mating) through mating. The relevance of these age-related differences to acute toxicity in humans, however, is unclear.

The liver is a major target organ for microcystins, as hepatocytes are among only a few cell types in the body that actively take up these toxins. Severe liver damage was reported in dialysis patients exposed to untreated water containing an estimated 19.5 µg/L of cyanobacterial toxins (both microcystins and cylindrospermopsin) in the dialysate. Heinze (1999) showed that oral exposure of rats to 50 µg/kg bw per day of MC-LR over 28 days resulted in increased liver weight as well as slight to moderate liver lesions with hemorrhages. Fawell et al. (1999a) reported mild liver lesions as well as increased ALT and AST in male mice exposed orally to 200 µg/kg bw per day of MC-LR over a 13-week period; a NOAEL of 40 µg/kg bw per day for liver effects was identified by the study authors. No liver toxicity was reported in female mice exposed to approximately 3 µg/kg bw per day of MC-LR in drinking water for 18 months (Ueno et al., 1999). Male mice exposed to 8 or 16 µg/kg bw per day of MC-LR in drinking water for up to 270 days showed mild hepatocytic injury, increased relative liver weight and decreased body weight (X.X. Zhang et al., 2010; Zhang et al., 2012); these studies, however, provide insufficient data for dose–response analyses.

Although the liver has traditionally been considered to be the most important target of microcystin toxicity, more recently, evidence of impacts of MC-LR on the male reproductive system and sperm development has been reported following both *in vivo* and *in vitro* exposures. Several intraperitoneal studies and a recent drinking water study by Y. Chen et al. (2011) (many of which originate from the same research laboratory) have reported various effects on the male reproductive system, including decreased absolute and relative testes and epididymis weights, decreased sperm concentration, viability and motility, as well as increased sperm abnormalities, following exposure to MC-LR. None of these studies provide an adequate basis for the development of an HBV for total microcystins due to the mode of administration (intraperitoneal injection) or weaknesses in experimental design and reporting. These results require validation through additional research in other animal species implementing good laboratory practices before male reproductive effects can be confidently considered as the basis for developing a guideline for total microcystins.

In Canada, available data indicate that exposure to microcystins from municipally treated drinking water supplies generally occurs only over a short duration (< 30 days), as blooms are seasonal and protocols are in place to address any exceedances, hence mitigating the potential for longer-term exposure. In addition, the reported occurrence of liver toxicity following short-term exposure to MC-LR provides convincing evidence that adverse liver effects may occur from shorter-term exposures, thus warranting a short-term guideline for total microcystins. Heinze (1999) represents the best available study for the basis of a short-term limit for total microcystins given the duration of the study (28 days), the mode of administration (oral exposure via drinking water), the use of an adequate number of animals and the analysis of organ weights (liver, kidneys, adrenals, thymus and spleen), hematology, serum biochemistry and histopathology of the liver and kidneys. A tolerable daily intake (TDI[2]) from this study can be derived as follows:

$$\text{TDI} = \frac{\text{LOAEL}}{\text{UF}}$$

$$= \frac{50\ \mu\text{g/kg bw per day}}{900}$$

[2] Although a TDI refers to the amount of the substance in air, food, soil, drinking water and consumer products that can be taken in daily over a lifetime without appreciable health risk, the terminology is also being used in the derivation of a short-term HBV for total microcystins.

≈ 0.056 µg/kg bw per day

where:

- 50 µg/kg bw per day is the LOAEL for increased liver weight and slight to moderate liver lesions with hemorrhages in rats as reported by Heinze (1999); and
- 900 is the uncertainty factor: ×10 for intraspecies variability, ×10 for interspecies variability, ×3 for database deficiencies and ×3 for the use of a LOAEL instead of a NOAEL.

Using this TDI, the HBV for total microcystins can be derived as follows:

$$\text{HBV} = \frac{0.056\ \mu g/kg\ bw\ per\ day \times 70 \times 0.80}{1.5\ L/day}$$

= 2.1 µg/L

≈ 2 µg/L (rounded)

where:

- 0.056 µg/kg bw per day is the TDI as derived above;
- 70 kg is the average body weight of a Canadian adult;
- 0.80 is the "ceiling value" allocation factor, as the majority of exposure to microcystins is expected to be through drinking water; the remaining 0.20 allows for allocation to other non-negligible exposures from other media (Krishnan and Carrier, 2013); and
- 1.5 L/day is the average daily volume of drinking water consumed by a Canadian adult.

### 10.2 Anatoxin-a

A limited amount of information is available on the health effects of anatoxin-a in humans. Cases of non-lethal poisonings, manifested mainly as acute gastrointestinal disturbances, have been attributed to ingestion of lake or pond water containing anatoxin-a-producing *Anabaena* sp. None of these case reports provides dose information or unequivocally establishes anatoxin-a as the causal agent.

In animals, the majority of experimental studies for anatoxin-a are *in vitro* studies. Information on the *in vivo* effects of anatoxin-a in orally exposed laboratory animals includes only a limited number of dose–response data on systemic toxicity and developmental toxicity owing to limitations in experimental design and reporting, including insufficient numbers of dose levels as well as study endpoints. In particular, the available oral toxicity database is limited by a few NOAELs and no LOAELs. As a result, the data for anatoxin-a toxicity are insufficient for deriving a proposed MAC in drinking water.

### 10.3 Cylindrospermopsin

The main source of information on the toxicity of cylindrospermopsin in humans is from qualitative reports of a hepatoenteritis-like illness that is attributed to the acute or short-term consumption of drinking water containing *Cylindrospermopsis raciborskii*. The clinical signs reported include fever, headache, vomiting, bloody diarrhea, hepatomegaly and kidney damage

with loss of water, electrolytes and protein; however, no data were provided on the exposure levels of cylindrospermopsin that induced these effects.

The main targets of cylindrospermopsin toxicity include the liver and kidneys, and possible modes of action include inhibition of protein synthesis, bioactivation to a reactive intermediate and covalent binding of the parent compound or a metabolite to DNA. Exposure to cylindrospermopsin has also been shown to induce morphological changes in red blood cells that may be linked to cylindrospermopsin-mediated effects on the liver and kidney.

The database on the oral toxicity of purified cylindrospermopsin in animals is limited by a small number of studies with appropriate exposure durations; most of the studies, however, suffer from insufficient reporting of effects considered to be key toxicity endpoints. No information was located regarding the neurotoxicity or developmental/reproductive toxicity of cylindrospermopsin following oral exposure. Studies on genotoxicity indicate the potential for cylindrospermopsin to affect liver DNA in mouse bioassays; however, the data are limited and inadequate to support a determination of the carcinogenic potential of cylindrospermopsin. Limited chronic and subchronic studies of cylindrospermopsin show multiple similarities in terms of the effects observed, which include increased relative liver and kidney weights as well as morphological changes in red blood cells.

## 10.4 International considerations

This section presents the various drinking water guidelines and standards from other national and international organizations. Variations in these limits can be attributed to the age of the assessments or to differing policies and approaches, including the choice of key study and the use of different consumption rates, body weights and allocation factors.

WHO (2003) established a provisional guideline value of 0.001 mg/L (1 µg/L) for total MC-LR (free plus cell bound). This is based on a TDI of 0.04 µg/kg bw per day derived from a NOAEL of 40 µg/kg bw per day for liver pathology as reported by Fawell et al. (1999a), divided by an uncertainty factor of 1000 to account for intraspecies and interspecies variation and for limitations in the database (in particular, lack of data on chronic toxicity and carcinogenicity). An adult consumption rate of 2 L/day was applied to the TDI, as well as an adult body weight of 60 kg and an allocation factor of 0.80 for the proportion of daily exposure arising from drinking water.

The Australian National Health and Medical Research Council established a guideline in 2001 for total microcystins in drinking water at 0.0013 mg/L (1.3 µg/L) expressed as MC-LR toxicity equivalents (NHMRC, 2004). The guideline is based on the same TDI and adult consumption rate used by WHO (2003); however, an allocation factor of 0.9 was used to account for the proportion of daily exposure from drinking water. Non-regulatory health advisory levels of 1 µg/L and 3 µg/L were developed for cylindrospermopsin and saxitoxins, respectively.

The U.S. EPA has not established regulations or guidelines for cyanobacteria or cyanotoxins. Cyanobacteria and cyanobacterial toxins are currently on the Drinking Water Contaminant Candidate List 3—a list of contaminants that are currently not subject to any proposed or promulgated national primary drinking water regulations, that are known or anticipated to occur in public water systems and that may require regulation under the *Safe Drinking Water Act*. Many states/jurisdictions have developed health advisories or action levels as part of their management strategies for water bodies in which harmful cyanobacterial blooms occur (Chorus, 2012), with only Ohio and Oregon implementing drinking water regulations or guidelines. The state of Ohio has established a maximum contaminant level of 1 µg/L for microcystins, and the state of Oregon has established limits of 3 µg/L for anatoxin-a, 1 µg/L for

cylindrospermopsin, 1–12 µg/L (indicated as "to be determined") for microcystins and 3 µg/L for saxitoxin (Chorus, 2012).

Drinking water standards for MC-LR similar to that of WHO (2003) have been set by other international jurisdictions, including the Czech Republic, Singapore and Uruguay; Brazil, France and Spain, have also established drinking water standards of 1 µg/L, however, for all microcystins (Chorus, 2012). New Zealand has established provisional maximum acceptable values of 1 µg/L for microcystins (as MC-$LR_{equiv}$), 1 µg/L for cylindrospermopsin, 3 µg/L for saxitoxin (as equivalents), 6 µg/L for anatoxin-a, 1 µg/L for anatoxin-a(s), 2 µg/L for homoanatoxin-a and 1 µg/L for nodularin (Chorus, 2012).

## 11.0 Rationale

Cyanobacteria form naturally in surface waters under the right environmental conditions which include adequate temperature, sunlight and nutrients. Cyanobacterial blooms form in surface water bodies in Canadian provinces, but have not been observed to date in the territories. Although most scientific studies on cyanobacterial toxins focus on microcystins, there are many types of cyanobacteria, which produce an even greater number of toxins. A bloom may contain more than one type of cyanobacteria; these do not necessarily produce any toxin but could produce more than one type of toxin. This assessment considered scientific information regarding microcystins, anatoxin-a and cylindrospermopsin. The data available for anatoxin-a are insufficient for deriving a proposed MAC in drinking water. A MAC for cylindrospermopsin in drinking water is not proposed, as the toxin has not been detected in Canadian drinking water sources.

Microcystins are generally considered to be the most important of the freshwater cyanotoxins. The main source of exposure to microcystins is drinking water, followed by accidental ingestion of surface water during recreational activities. Some exposure may also occur through foods affected by contaminated water (primarily fish and shellfish, some crops) and natural health products (including algal supplements) that have been contaminated. Studies on the health effects of cyanobacterial toxins focus primarily on microcystin-LR.

The liver continues to be considered the most important target organ of microcystin toxicity for both animals and humans. Long-term epidemiological studies indicate elevated liver enzyme levels in humans exposed to high levels of microcystins. While the evidence for its genotoxicity and mutagenicity in humans is conflicting, microcystin-LR has been shown to act as a tumour promoter in animals in the presence of known tumour initiators. There have been recent reports of effects of microcystin-LR on the male reproductive system in animals, but these still require validation through additional research.

A short-term MAC is proposed for total microcystins as studies have found liver toxicity in rats following short-term exposure to microcystin-LR. The HBV of 2.0 µg/L for total microcystins applies to both dissolved and intracellular toxins and is deemed to be protective of human health against exposure to all microcystin variants. It is measurable by current analytical methods and achievable by current treatment technologies

A seasonal MAC of 0.0015 mg/L (1.5 µg/L) is proposed for total microcystins in drinking water based on the following considerations:

- An HBV of 0.002 mg/L (2 µg/L) can be calculated using Heinze (1999). However, Canadian jurisdictions have shown that the lower proposed MAC is already being achieved.

- Most analytical methods available are capable of accurately measuring microcystins at concentrations well below the proposed MAC.
- Well operated and optimized drinking water treatment plants can remove total microcystins to concentrations well below the proposed MAC.

Although the current scientific evidence for MC-LR mediated liver effects in young animals, and developmental toxicity in fetuses (exposed in utero) indicates that younger animals are less sensitive to MC-LR compared to adults, it is unknown whether infant animals are more or less sensitive to MC-LR following direct oral exposure. In humans, an infant can consume up to 5 times more drinking water per kilogram of body weight than an adult thus increasing their exposure to any microcystins present in drinking water. Using the TDI derived above and applying an infant (0-6 months) body weight of 7 kg and a consumption rate of 0.75 L/day, a reference value of 0.4 µg/L can be derived for the basis of recommending alternative sources of drinking water for bottle-fed infants as a precautionary measure during a bloom event. Consequently, as a precautionary approach during a cyanobacterial bloom, drinking water authorities should inform the public in the affected area that an alternate suitable source of drinking water (such as bottled water) should be used to reconstitute infant formula.

As part of its ongoing guideline review process, Health Canada will continue to monitor new research in this area and recommend any change to this guideline technical document that it deems necessary.

## 12.0 References

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## Appendix A: List of acronyms

| | |
|---|---|
| ALP | alkaline phosphatase |
| ALS-PDC | amyotrophic lateral sclerosis/parkinsonism–dementia complex |
| ALT | alanine aminotransferase |
| ANSI | American National Standards Institute |
| AOP | advanced oxidation process |
| APCI | atmospheric pressure chemical ionization |
| AST | aspartate aminotransferase |
| BMAA | β-methylamino-L-alanine |
| bw | body weight |
| CI | confidence interval |
| CT | concentration × time |
| CYP | cytochrome P450 |
| DAF | dissolved air flotation |
| DBP | disinfection by-product |
| DMBA | dimethylbenzanthracene |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DOC | dissolved organic carbon |
| EBCT | empty bed contact time |
| ELISA | enzyme-linked immunosorbent assay |
| EPA | Environmental Protection Agency (U.S.) |
| equiv | equivalents |
| ERK | extracellular signal-regulated protein kinase |
| ESI | electrospray ionization |
| FSH | follicle stimulating hormone |
| GAC | granular activated carbon |
| GC | gas chromatography |
| GD | gestation day |
| GGT | gamma-glutamyltransferase |
| GnRH | gonadotropin-releasing hormone |
| GSH | glutathione |
| GST | glutathione *S*-transferase |
| HAA | haloacetic acid |
| HBV | health-based value |
| HPLC | high-performance liquid chromatography |
| HU | Hazen unit |
| $IC_{50}$ | median inhibitory concentration |
| ISO | International Organization for Standardization |
| LC | liquid chromatography |
| LCAT | lecithin–cholesterol acyltransferase |
| $LD_{50}$ | median lethal dose |
| LDH | lactate dehydrogenase |
| LDTD | laser diode thermal desorption |
| LH | luteinizing hormone |
| LOAEL | lowest-observed-adverse-effect level |

| | |
|---|---|
| LOQ | limit of quantification |
| M | moles per litre (molar) |
| MAC | maximum acceptable concentration |
| MALDI | matrix-assisted laser desorption/ionization |
| MAPK | mitogen-activated protein kinase |
| MC | microcystin variant (e.g., MC-LR, MC-LA, MC-YA, MC-RR, MC-YR) |
| MDL | method detection limit |
| MF | microfiltration |
| MMP | matrix metalloproteinase |
| MMPB | 2-methyl-3-methoxy-4-phenylbutyric acid |
| MRL | minimum reporting level |
| mRNA | messenger ribonucleic acid |
| MS/MS | tandem mass spectrometry |
| MTD | maximum tolerated dose |
| MWCO | molecular weight cut-off |
| NF | nanofiltration |
| NOAEL | no-observed-adverse-effect level |
| NOM | natural organic matter |
| NSF | NSF International |
| NTU | nephelometric turbidity unit |
| OATP | organic anion-transporter polypeptide |
| OR | odds ratio |
| PAC | powdered activated carbon |
| PCR | polymerase chain reaction |
| PND | postnatal day |
| POE | point of entry |
| POU | point of use |
| PP1 | protein phosphatase 1 |
| PP1c | protein phosphatase 1c |
| PP2A | protein phosphatase 2A |
| PPIA | protein phosphatase inhibition assay |
| ppm | part per million |
| QA | quality assurance |
| QC | quality control |
| PDA | photodiode array |
| PQL | practical quantitation level |
| RNA | ribonucleic acid |
| RO | reverse osmosis |
| ROS | reactive oxygen species |
| S9 | 9000 × *g* supernatant fraction from rat liver homogenate |
| SCC | Standards Council of Canada |
| SPE | solid-phase extraction |
| SRR | standardized rate ratio |
| TDI | tolerable daily intake |
| TEF | toxicity equivalency factor |
| THM | trihalomethane |
| THP | Tamm-Horsfal protein |

| | |
|---|---|
| TOC | total organic carbon |
| TOF | time of flight |
| TPA | *O*-tetradecanoylphorbol 13-acetate |
| UF | ultrafiltration |
| UPLC | ultra-high-performance liquid chromatography |
| UV | ultraviolet |
| Vis | visible |

# Appendix B: Considerations for implementing the guideline for total microcystins in treated drinking water supplies

## B.1 Have an up-to-date action plan in place before a cyanobacterial bloom forms

A quick and effective response to the detection of a cyanobacterial bloom is critical to the provision of safe drinking water. Preparation is key, as many decisions that have to be made during a cyanobacterial bloom event rely on information that is best prepared in advance. Similarly, the impact of a bloom can be reduced by taking actions early in its development, such as collecting and analysing samples of raw water (in addition to the treated water samples) to allow the operator to assess the efficiency of treatment processes and adjust them as required. Consequently, an action plan should be established or updated prior to bloom season for systems using a surface water source that is or may be vulnerable to cyanobacterial blooms.

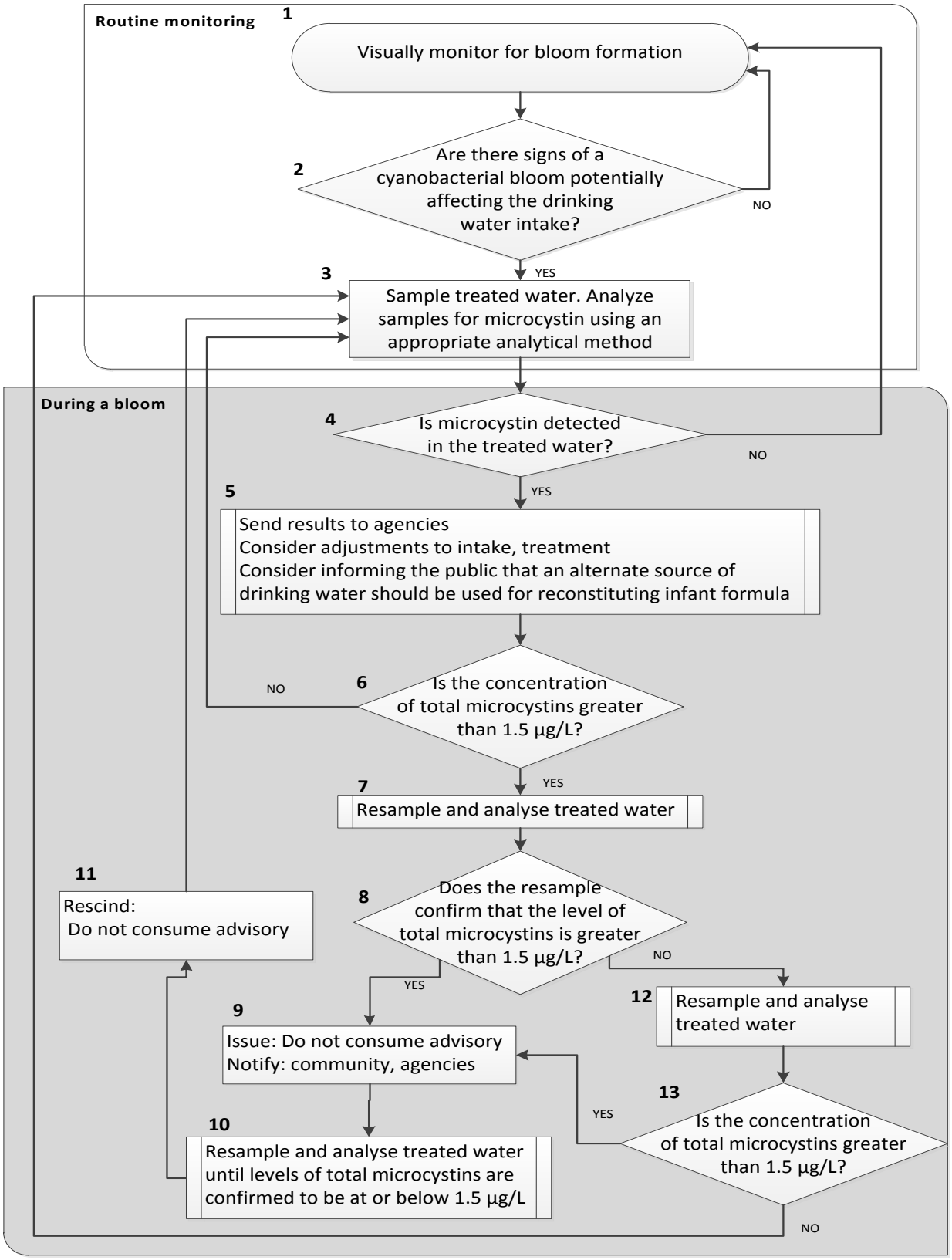
### Elements of the action plan

The action plan should lay out what to do in case a cyanobacterial bloom is visually detected in the water source. It should:

- describe the sampling strategy (parameters, frequency, timing, locations) to be followed for the duration of the bloom with respect to both routine sampling and resampling when microcystins are detected;
- identify the analytical laboratory or laboratories that can do microcystin analysis;
- ensure that agreement(s) and protocol(s) are in place with lab(s) for receiving and processing samples in a timely manner, and for communicating results from the lab to the appropriate contact people;
- outline individual responsibilities for how samples will be collected and delivered to the laboratory;
- specify the method(s) of microcystin detection/analysis that can be used;
- identify the appropriate contact people to receive the results from the lab and who they must notify if microcystins are detected;
- identify which authority or authorities are responsible to decide further notifications and actions;
- identify which authority will take the lead role in notifying communities and other appropriate agencies or authorities;
- set out a communications plan describing the circumstances and target groups for notifications, including when an advisory is issued or rescinded;
- include sample communications products, such as messages, Qs &As and press releases, to deal with different situations (e.g., microcystins level above guideline, microcystins detected below guideline level but still of concern for infants) and to provide clear guidance to the public; and
- identify any corrective actions (e.g., treatment adjustments) and the triggers for such actions.

Information that may aid the risk assessment process can include the history of blooms and microcystins (if previously tested) at the site, the size and location of the bloom, the location and position of the raw water intake, the treatment technologies in place, existing uses of the source water (recreational versus domestic uses) and environmental, climate or weather factors conditions that might affect the bloom (e.g., currents/flows, temperature, winds, rainfall).

## B.2 Flow-chart



## B.3 Interpreting the flow-chart

### 1. Visually monitor for bloom formation

Visual monitoring of surface water sources should be conducted throughout the peak bloom season. As accumulations of algae and pollen can be mistaken for cyanobacterial blooms, monitors should receive appropriate training to correctly identify blooms. Drinking water authorities should develop a site-specific approach for visually monitoring water bodies that are considered susceptible to cyanobacterial blooms, particularly near the intake.

The frequency of visual inspections will vary depending on the time of year and weather conditions (including wind direction and speed). The approach would require visual monitoring for evidence of bloom formation at regular intervals (one or two weeks). It would establish increased monitoring frequencies (e.g., weekly, daily) once a bloom is suspected or confirmed, or when microcystins are detected in the treated water. It would also identify criteria for reducing visual monitoring frequency once microcystin levels decrease and the bloom shows signs of dissipation or breakdown.

It may be advisable to also conduct visual monitoring inside treatment facilities (e.g., clarifier, filter) as cyanobacteria may accumulate there even in the absence of a bloom in the source water.

### 2. Are there signs of a cyanobacterial bloom potentially affecting the drinking water intake?

Early signs of cyanobacterial growth may be the presence of small green particles in the water. These may be more easily seen by collecting a jar of water and inspecting the sample under good lighting. As cell densities increase, the water may appear soupy. Cyanobacterial blooms can have the appearance of small clumps, filaments, fine grass clippings, spilled paint or a film on the surface of the water. Most blooms have a bright or olive green to blue-green colour, although some may appear red or brown. Measuring water transparency (using a Secchi disc, if available) can also provide useful information. Other evidence of a developing bloom may include high levels of nutrients, increases in cell counts or biovolume, and chlorophyll-a levels.

The position of the intake pipe will also influence whether a bloom can affect the drinking water. An intake located at least 6 m below the water surface is less likely to be affected by cyanobacteria present in the water body.

If no signs of a bloom are found, continue to visually monitor source water near the intake (Box 1). If signs of a bloom are detected, proceed to Box 3.

Note: Authorities may wish to issue a precautionary do not consume advisory during a toxic bloom event for communities that lack the necessary treatment capabilities to ensure the safety of the water supply.

### 3. Collect and analyse water samples using an appropriate method

The water provider or authorities should collect samples of finished water in the treatment plant, after the water has undergone the final treatment step and prior to its entry into the distribution system. Samples should be collected, stored and transported following instructions from the analytical laboratory. Section 6.3 provides additional information on sample collection and relevant references. In general, samples should be clearly labelled and kept cool (not frozen) and in the dark (e.g., in coolers) after collection and during transport.

It is recommended that water providers establish early contact with the analytical laboratory as part of the action plan in order to minimize the time required for sample analysis and management response. The samples collected should be analysed using laboratory ELISA,

PPIA or a physico-chemical method such as LC-MS/MS. If microcystins are detected using ELISA or PPIA, it is recommended that a portion of the samples also be analyzed using LC-MS/MS to identify and measure the individual microcystin variants which may be present.

Field test kits cannot be used to determine whether drinking water concentrations meet the guideline value. They can be used as qualitative tools to determine whether a bloom is toxic or if treatment plant operations need to be adjusted during a bloom event.

Water providers or utilities should consider also collecting raw water samples at this time to measure microcystins and cell counts and identify cyanobacteria species to help determine the efficiency of the treatment process.

**4. Are microcystins detected in the treated water?**

If no microcystins are detected in the treated water, return to Box 1 and continue to visually monitor for intensification of the existing bloom, or bloom recurrence if still in the season where cyanobacterial blooms could develop.

If microcystins are detected in the treated water, proceed to Box 5.

**5. Send results to agencies, consider adjustments to intake and/or treatment, consider informing the public that an alternate source of drinking water should be used to reconstitute infant formula**

When microcystins are detected in the treated water, it is important to ensure that appropriate agencies and contact people (identified in the action plan) are informed in a timely manner. Where feasible, appropriate actions should be taken to reduce microcystin levels in the treated water. These may include adjustments to the intake to reduce the amounts of microcystins entering the treatment plants, as well as adjustments to the treatment process to increase its efficiency for microcystins removal. The results of raw water analysis (recommended in 3 above) would be useful to monitor the effectiveness of the treatment process and determine appropriate treatment adjustments.

In addition, as a precautionary approach during a cyanobacterial bloom, or when microcystins are detected in finished water, drinking water authorities should consider informing the public in the affected area that an alternate suitable source of drinking water (such as bottled water) should be used to reconstitute infant formula.

**6. Is the concentration of total microcystins in the treated water greater than 1.5 µg/L?**

If total microcystins are detected at concentrations up to 1.5 µg/L, the treated water meets the guideline but monitoring needs to continue as exceedances of the MAC may develop. Return to Box 3.

If the concentration of total microcystins is above 1.5 µg/L, the treated water does not meet the guideline and further action is required. Proceed to Box 7.

**7. Resample and analyse treated water**

Additional sampling and analysis of the treated water should be conducted to confirm the concentration of total microcystins and identify follow up actions. Refer to Box 3 for guidance on collection and analysis.

**8. Does the resample confirm that the total microcystins level exceeds 1.5 µg/L?**

If the resample confirms a concentration of total microcystins above the guideline of 1.5 µg/L, proceed to Box 9. If the concentration of total microcystins in the resample is at or below 1.5 µg/L, proceed to Box 12.

**9. Issue a "Do not consume" advisory, notify community and agencies**

If total microcystin levels are confirmed to be above the guideline of 1.5 µg/L, responsible authorities should refer to the facility-specific action plan developed as per section B.1. The lead authority should issue a "Do not consume" advisory and notify communities and other appropriate agencies or authorities. If possible, an alternate source of drinking water (such as bottled water) should be identified. Dialysis treatment providers or units in the community should also be notified.

**10. Resample and analyse treated water until levels of microcystins are confirmed to be at or below 1.5 µg/L**

Authorities and water providers should continue to resample and analyse the treated water until the concentration of total microcystins is confirmed to be at or below 1.5 µg/L. Proceed to Box 11.

**11. Rescind the "Do not consume" advisory**

Once the concentration of total microcystins meets the guideline of 1.5 µg/L, the advisory can be rescinded. However, authorities should continue to advise parents of infants of the approach to take when reconstituting infant formula, until microcystins are no longer detected in the treated water. Return to Box 3.

**12. Resample and analyse treated water**

As the results previous analyses (Boxes 6 and 8) are not consistent, another sample and analysis is needed to determine whether levels of total microcystins are a concern for the general population.

**13. Is the concentration of total microcystins greater that 1.5 µg/L?**

If the results show a concentration of total microcystins above 1.5 µg/L, then this confirms the results from Box 6: Proceed to Box 9. If the results show a concentration of total microcystins at or below 1.5 µg/L, then this confirms the results from Box 8: Return to Box 3 but continue to advise parents of infants of the approach to take when reconstituting infant formula until microcystins are no longer detected in the treated water.

## Appendix C: Provincial and territorial anticipated impacts

Note: The information below has been provided by the individual province or territory and is included in the language in which it was received.

**Prince Edward Island**

Because all of our drinking water supply comes from groundwater sources and is not affected by the types of influences that would result in the presence of cyanobacterial toxins, there are no anticipated impacts in Prince Edward Island.

**Newfoundland and Labrador**

Newfoundland and Labrador has no recorded incidents of cyanobacterial blooms in drinking water supplies. There have been limited observed incidents in water bodies not utilized as drinking water supplies. The province has an excellent source water protection program. Of the 298 surface water public water supplies in the province, 254 (85%) are protected under the Water Resources Act. This provides for an extensive source water protection program that reduces the risk of contamination for drinking water sources.

There is limited agriculture in Newfoundland and Labrador due to soil conditions. The risk of cyanobacterial blooms is limited as nutrient levels are typically low in drinking water supplies throughout the province. The province will continue to be observant regarding algae blooms. NL will ensure that any observed or reported blooms are evaluated to ensure that they are not cyanobacterial blooms.

**Nova Scotia**

Although Nova Scotia Environment (NSE) does not have data for total microcystins, the data reviewed for microcystin -LR (although likely an underestimate of exposure to total microcystins) suggests that the proposed revision to the guideline will have a minimal impact. Nova Scotia has an existing protocol for responding to algal blooms in drinking water sources that includes requirements for: surveillance; notification; sampling; laboratory analysis; and risk communication including the issuance of drinking water advisories, if deemed necessary.

In light of the proposed revisions to the guideline for cyanobacterial toxins, NSE will update its response protocol to include requirements for enhanced surveillance, precautions for bottle-fed infants, and a current list of laboratories that are capable of performing the required testing. There are currently no accredited laboratories in Nova Scotia that perform testing for microcystins.

**New Brunswick**

This guideline is not anticipated to have any significant impact on compliance related matters with regard to the regulation of drinking water systems. However, from a public health perspective the addition of the cautionary statement may create additional alarm and confusion for the public and could result in regulated systems being required to conduct additional sampling and monitoring beyond what would be required for compliance purposes.

**Quebec**

Au Québec, depuis quelques années, des fleurs d'eau de cyanobactéries ont été observées dans certains plans d'eau servant de source d'approvisionnement en eau potable. Les installations de production d'eau potable alimentées par ces plans d'eau réalisent un suivi selon une procédure

établie par le ministère du Développement durable, de l'Environnement et de la Lutte contre les changements climatiques, en collaboration avec le ministère de la Santé et des Services sociaux. Une moyenne annuelle de 15 installations de production d'eau potable réalisent un suivi conformément à cette procédure.

Depuis le mois de mars 2013, le Règlement sur la qualité de l'eau potable fixe une norme au regard des microcystines, soit 1,5 µg/L (en équivalent toxique de microcystine-LR). À ce jour, aucun échantillon prélevé à l'eau traitée de l'ensemble des installations de production d'eau potable ayant réalisé un suivi n'a présenté une concentration en microcystines dépassant la norme. Considérant ces informations, les impacts attendus de la révision de la recommandation pour la qualité de l'eau potable sur les microcystines sont négligeables.

**Ontario**

The information presented in this document will have minimal impact to Ontario as the current provincial drinking water quality standard for microcystin-LR remains at the same value of 1.5 µg/L.

Ontario has developed a 12-point response plan for algal blooms that combines surveillance, laboratory analyses of finished drinking water, and public health unit notification of potentially toxic blooms to the public to ensure the delivery of safe, high-quality drinking water.

**Manitoba**

In Manitoba, the majority of regulated public and semi-public water systems draw their water from groundwater sources. Approximately 20 surface water sourced public water systems have been identified as potentially impacted by cyanobacteria toxins. These systems range in size from small campgrounds to relatively large municipal systems serving more than 5,000 people. However, those large systems have adequate treatment capabilities in place to deal with cyanobacterial toxins. A study was conducted by the Office of Drinking Water during the summer of 2015, with most of these systems participating. No cyanobacteria toxins were detected in the treated water supplies. These results are consistent with previous, though limited, event based monitoring results. The impact of this guideline is therefore expected to be minimal, with most of the compliance costs attributable to monitoring. Private (residential and cottage) water systems are not regulated in Manitoba, and may or may not be monitored. Fact sheets are being developed to assist private water system owners in assessing potential risks from cyanobacterial toxins.

**Saskatchewan**

The Water Security Agency (WSA) has reviewed the proposed guideline document on cyanobacterial toxins in drinking water and agrees with the proposed seasonal MAC of 1.5 microgram/L for total microcystins in drinking water. Recently, regulations governing drinking water in Saskatchewan have been revised with the new Waterworks and Sewage Works Regulations (effective June 1, 2015) adopting a drinking water standard of 1.5 microgram/L for microcystin-LR (frequently occurring and most researched form of cyanobacterial toxins) in treated water. The water treatment plants (WTPs) in Saskatchewan that are regulated by the WSA are required under the new regulations to meet the new drinking water standard for Microcystin-LR by July 1, 2020.

Before adopting the standard, the WSA conducted a study during 2013 to determine the levels of microcystin-LR in treated water at selective locations in the province. The results showed that the levels are well below 1.5 microgram/L and/or were not detected in drinking water samples. The WSA also believes this work to be generally representative of total microcystin

concentrations in treated drinking water supplies. The WSA is also working on revising the "Municipal Drinking Water Quality Monitoring Guidelines - EPB 202" incorporating the microcystin-LR monitoring guidelines for the WTPs in the province. Owners of regulated WTPs in Saskatchewan will soon be advised to monitor microcystin-LR and total microcystins in raw water, and where detected, then treated water. The WSA will also request WTP owners/operators in the province to monitor algal blooms in source water supplies. Since studies showed that many of the water treatment systems including conventional water treatment filtration systems, GAC filtration systems, RO systems, are effective in removing microcystins, and since these type of treatment systems are in existence and/or are being increasingly adopted for use in WTPs in the province, the WSA believes and expects that the new standard for total microcystins will have minimal or no impact including cost to municipal and other regulated waterworks in the province.

**Alberta**

Systems that do not see algae blooms will not have to test; systems that do see algae would likely have more testing required than under the current approvals. Most of the systems that do see algae blooms are doing the testing anyway as a precaution or based on the recommendation of Alberta Health Services. The biggest cost will be for the smallest systems who will now have another complex decision making process of when to sample and what to sample for. There may be significant costs associated with driving in the samples to the labs in order to reduce the time between taking the sample and getting the results.

**British Columbia**

The proposed guideline for cyanobacterial toxins reaffirms a MAC of 0.0015 mg/L (1.5 µg/L) for total microcystins in drinking water and adds new guidance for bottle-fed infants. This advice is consistent with, and supports the advice given by the Decision Protocols for Cyanobacterial Toxins in B.C. drinking water and recreational water, and minimal impact is expected.

**Yukon**

It is not anticipated that the Guideline Technical Document for cyanobacterial toxins will have a significant impact on Yukon large public drinking water systems at this point in time.

**Northwest Territories**

There is little impact from cyanobacterial toxins in NWT due to our water sources generally being from fast flowing water sources.

**Nunavut**

No impact is expected for Nunavut.