

## Cyanobacterial Toxins — Microcystin-LR

### Guideline

The maximum acceptable concentration (MAC) for the cyanobacterial toxin microcystin-LR in drinking water is 0.0015 mg/L (1.5 µg/L). This guideline is believed to be protective of human health against exposure to other microcystins (total microcystins) that may also be present.

### Identity of Cyanobacterial Toxins

Cyanobacterial toxins are toxins produced by cyanobacteria, or blue-green algae. They include neurotoxins (e.g., anatoxins), hepatotoxins (e.g., microcystins), skin irritants and other toxins. Both hepatotoxins and neurotoxins are produced by cyanobacteria commonly found in surface water supplies and therefore appear to be of most relevance to water supplies at present.<sup>1-3</sup> However, the neurotoxins are relatively unstable and, as such, are not considered to be as widespread as hepatotoxins in water supplies; in addition, they do not appear to pose the same degree of risk from chronic toxicity.<sup>3</sup> It should be noted, however, that, due to limited analytical capabilities, there are only limited quantitative data available on the levels of neurotoxins in water supplies. Cyanobacterial toxins were detected during a survey in the summer (July/August) of 2000 in Onondaga Lake and Oneida Lake in upstate New York, USA.<sup>4</sup> Microcystins were detected in only one of 13 samples from Onondaga Lake, as was anatoxin-a. However, 50% of the 22 samples from Oneida Lake tested positive for microcystins (seven or eight tested >1.0 µg/L), and two samples were positive for anatoxin-a. Anatoxin-a was less common than microcystins, with levels ≤0.85 µg/L. It may be possible that neurotoxins are more widespread than is currently believed, particularly since many of the neurotoxin-producing algae have been linked to deaths of both livestock and domestic animals.

Most of the hepatotoxins are collectively referred to as microcystins, because the first hepatotoxin was isolated from *Microcystis aeruginosa*. About 50 different microcystins have been isolated, and several of these

may be produced during a bloom. Structurally, the microcystins are monocyclic heptapeptides that contain two variable L-amino acids and two novel D-amino acids. Microcystins are named according to their variable L-amino acids — for example, microcystin-LR contains leucine (L) and arginine (R), whereas microcystin-YA contains tyrosine (Y) and alanine (A).<sup>5</sup> The two novel D-amino acids in microcystins are N-methyldehydroalanine (Mdha), which hydrolyses to methylamine, and a unique non-polar-linked amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, also known as ADDA.<sup>6</sup> The key component for biological activity appears to be linked with the ADDA side chain, as cleavage of the ADDA side chain from the cyclic peptide renders both components non-toxic.<sup>1,5,6</sup> Nodularins, hepatotoxins that were first isolated from the blue-green alga *Nodularia*, are cyclic pentapeptides<sup>2</sup> found mostly in saltwater and hence have not been a focus in drinking water.

Microcystin-LR, produced as a secondary metabolite by *Microcystis aeruginosa* and other blue-green algal species, appears to be the most commonly occurring microcystin.<sup>1</sup> To date, most of the work on microcystins has been conducted using this variant because of its presence in most countries reporting toxic episodes.<sup>3</sup> Microcystin-LR has a molecular weight of about 1000 daltons. Its toxicity does not appear to vary greatly from that of the other microcystins.<sup>5</sup> There have been over 65 variant microcystins reported, the LD<sub>50</sub>s of which range from 50 to 300 µg/kg bw. Microcystin-LR is one of the most toxic, with an LD<sub>50</sub> of 50 µg/kg bw.<sup>7</sup>

Calculated octanol-water partition coefficients (which measure solubility and interaction with water molecules) for the majority of microcystin variants suggest that their adsorption by activated carbon would be similar to, or greater than, that of microcystin-LR.<sup>7</sup>

### Occurrence of Cyanobacteria

The cyanobacteria, or blue-green algae, owe their name to the presence of photosynthetic pigments. Freshwater cyanobacteria are known to occur throughout the

world. Their classification and identification have been reviewed,<sup>8</sup> and some 40 toxigenic species have been identified. Freshwater cyanobacteria may accumulate in surface water supplies as “blooms” and may concentrate on the surface as blue-green “scum.”

Cyanobacterial genera known to occur in Canada and most often associated with poisonings are *Anabaena*, *Aphanizomenon*, *Microcystis*, *Oscillatoria* and *Nodularia*.<sup>5</sup> Cultures of toxic *Microcystis* were first isolated from the Rideau River in Ottawa in the 1950s by P.R. Gorham and his associates at the National Research Council. A high degree of polymorphism of *Microcystis aeruginosa* has been reported, with variability between species in the northern and southern hemispheres.<sup>9</sup>

It has generally been found that 50–75% of bloom isolates are capable of producing toxins, with often more than one toxin being present. More than 70% of over 380 bloom biomass samples from 19 lakes in Alberta between 1990 and 1992 showed detectable levels of toxin (>1 µg of microcystin-LR per gram of dry biomass).<sup>10</sup> The overall toxicity of a bloom can be uncertain because of variations in toxin concentration temporally and spatially within a water body experiencing a bloom.<sup>10</sup>

The growth of cyanobacteria and the formation of blooms are influenced by a variety of physical, chemical and biological factors; these were reviewed by the NRA Toxic Algae Task Group<sup>11</sup> and Ransom *et al.*<sup>6</sup> and are discussed below. As a result of the interplay of these factors, there may be large year-to-year fluctuations in the levels of cyanobacteria and their toxins.<sup>12</sup> There is also seasonal variation with regard to which species predominate. Timing and duration of the cyanobacterial bloom season are dependent upon climatic conditions; in temperate zones such as Canada, cyanobacterial occurrences are most prominent during the late summer and early autumn and may last 2–4 months. However, blooms of some species of cyanobacteria have been found in winter under ice in Scandinavian and German lakes<sup>7</sup> and early spring and summer in the Midwest, which can lead to a year-round problem.

### Physical Factors

As water temperatures increase in the spring, there is a natural succession of algal groups from diatoms and green algae to cyanobacteria. Different genera of cyanobacteria have different minimum temperature tolerances. For example, *Microcystis* is less tolerant of cooler temperatures than *Oscillatoria*. The length of daylight required to optimize growth is species dependent. For example, *Microcystis* is more adapted to shorter days than *Anabaena*. This is perhaps one of the reasons why *Microcystis* species are the dominant species in North America in late summer, when day length is shorter. Some cyanobacteria, such as

*Cylindrospermopsis*, can tolerate low light levels and can therefore compete more efficiently with other planktonic algae for available light, owing primarily to the presence of photosynthetic pigments. These pigments also allow photosynthesis in coloured waters.

In addition, some cyanobacteria, such as *Microcystis aeruginosa*, can optimize their position in the water column in response to available light by actively regulating their buoyancy. This characteristic also allows cyanobacteria to migrate through thermal gradients and utilize nutrients confined to the cooler water below. The main control operates through photosynthesis (through the production of carbohydrates) and breaks down if there is too little carbon dioxide. Buoyancy cannot be adjusted during the night.

Increased turbidity favours cyanobacteria over other algae. As noted above, cyanobacteria can use a wide spectrum of light for photosynthesis and are able to migrate to the surface to maximize light intensity. However, very high turbidity can reduce the availability of phosphate and thus limit their growth. Turbulence and high water flows, on the other hand, are unfavourable to the growth of cyanobacteria, as these interfere with their ability to maintain a position in the water column.

Heavy rain storms can increase runoff and nutrient levels in the water and thus encourage the formation of blooms.

### Chemical Factors

As is the case for other photosynthetic organisms, the availability of the macronutrients phosphorus and, to a lesser degree, nitrogen controls the growth of cyanobacteria. In general, cyanobacteria do not have as high a demand for phosphorus as do other phytoplankton but are efficient at phosphorus storage. As phosphorus readily adheres to reactive surfaces (organic and inorganic), much of the phosphorus in a water body will be associated with sediments. Unnaturally high levels of phosphorus in waters are indicative of disturbances in the watershed. Important point and non-point sources of phosphorus include raw and treated sewage, detergents and urban and agricultural runoff. Mass occurrences of toxic cyanobacterial blooms are not always associated with human activities causing pollution. For example, toxic blooms have been reported in Australian reservoirs with pristine or near-pristine watersheds.

Iron and molybdenum are particularly important micronutrients for cyanobacteria because of their direct involvement in nitrogen fixation and photosynthesis (iron) as well as carbon fixation and nitrogen uptake (molybdenum). *Microcystis* is not a nitrogen-fixing cyanobacterial genus.

Alkalinity and pH determine the chemical speciation of inorganic carbon, such as carbonate, bicarbonate and carbon dioxide. Low carbon dioxide concentrations favour the growth of several cyanobacterial

species. Hence, water conditions such as low alkalinity and hardness and the consumption of carbon dioxide during photosynthesis by algae, increasing the pH, give cyanobacteria a competitive advantage.

### Biological Factors

The role of cyanobacteria in aquatic food webs is very complex. In general, phytoplankton are grazed upon by zooplankton, which in turn are consumed by fish. Cyanobacteria are not easily digested by zooplankton; therefore, their populations may increase in relation to other, more easily digestible algae. Macrophytes compete with cyanobacteria and other phytoplankton for nutrients and light and may also suppress phytoplankton by releasing inhibitory compounds. Other aquatic bacteria can also compete with cyanobacteria for nutrients.

### Formation of Surface Scum

The formation of surface scum requires a sudden change in weather conditions. Initially, there may be high barometric pressure and light to moderate winds, accompanied by constant circulation in a water body in which a substantial population of cyanobacteria may have optimally positioned itself in the water column to take advantage of those conditions. If the wind stops and circulation also stops, the cyanobacteria may suddenly become “overbuoyant.” If they cannot adjust their buoyancy fast enough or at all (if it is night), then they will float to the surface and form a surface scum. Thus, scums may often be formed overnight. The scum may drift downwind and may settle at lee shores and quiet bays, where the cyanobacteria may eventually die, releasing their toxins.

### Toxin Production and Persistence

The factors inducing the production of toxins by cyanobacteria are not well known. Laboratory studies demonstrate that some of the same environmental factors as above, such as temperature, light, nitrogen concentrations, carbon availability (in the form of bicarbonate, carbonate and carbon dioxide), phosphate concentrations and pH, could be important. As toxin production varies greatly among different strains of the same species, genetic differences and metabolic processes may also be important in the production of these secondary metabolites. Studies have shown that the ability to produce toxins can vary temporally and spatially at a particular site.<sup>6</sup>

Cyanobacterial toxins tend to be associated with cyanobacterial cells and may be membrane bound or occur free within the cells. In laboratory studies, most of the toxin release occurs as cells age and die and passively leak their cellular contents; some active release of toxins can also occur from young, growing cells.<sup>5,11</sup>

Toxin levels do not necessarily coincide with maximum algal biomass; there can be significant variation in

the amount of toxin per unit biomass of cyanobacteria over time, which is independent of changes in the blue-green algal population. Kotak *et al.*<sup>13</sup> found higher concentrations of microcystins in blooms taken during the day than at night, whereas a study in Australia found no significant difference in toxin concentrations from cyanobacteria incubated for 24 hours at different depths in a reservoir.<sup>14</sup>

Microcystins are relatively persistent in the aquatic environment. Studies in Australia have shown that microcystin-LR was present up to 21 days following treatment of a *Microcystin aeruginosa* bloom with an algicide.<sup>15</sup> Studies conducted in natural waters in the United Kingdom indicated that five days were required for the destruction of 50% of the toxin.<sup>16</sup> Biodegradation and photolysis are means by which released microcystin-LR can naturally decrease in concentration.<sup>17,18</sup> Cousins *et al.*<sup>19</sup> demonstrated that the primary biodegradation of microcystin-LR in reservoir water had a first half-life of approximately four days. It was noted, however, that the half-life of this toxin in natural waters would likely vary considerably with changes in water temperature and the size of the microbial population.

Levels of microcystin-LR in Alberta lakes and dug-out ponds, measured using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (according to the method of Harada *et al.*,<sup>20</sup> as modified slightly by Kenefick *et al.*<sup>21</sup>), ranged from 4 to 605 µg/g dry weight (dw) of biomass<sup>22</sup> or up to 1500 µg/g.<sup>10</sup> Similarly, microcystin-LR levels in natural blooms of *Microcystis* in Japan between 1989 and 1991 ranged from 27 to 622 µg/g dw of biomass.<sup>12</sup> In the same blooms, the levels of microcystin-RR and microcystin-YR ranged from 11 to 979 and from 9 to 356 µg/g dw of biomass, respectively, with a total maximum microcystin level of 1732 µg/g dw of biomass.<sup>12</sup>

### Exposure to Microcystins

The major route of human exposure to cyanobacterial toxins is the consumption of drinking water. Minor exposure routes are the recreational use of lakes and rivers (oral and dermal routes) and, for some people, the consumption of certain algal food supplements (oral route); products containing non-*Spirulina* blue-green algae harvested from natural lakes have been found to contain microcystin toxins.<sup>23</sup> Another minor route of exposure would be from the use of showers (inhalation). The extent to which cyanobacterial toxins move up the food chain (freshwater mussels and fish) has not been widely investigated, although covalently bound microcystins have been detected in the tissues of both freshwater and saltwater mussels<sup>24,25</sup> and in the liver of salmon<sup>26</sup> in recent studies. There is also some preliminary experimental evidence that the toxins, or their transformation products, can move up the aquatic food

chain. Fish consuming *Daphnia magna* exposed to radiolabelled microcystin-LR accumulated the radiolabel in their tissues.<sup>27</sup> The duration of exposure to microcystins by these various routes would generally be shorter in countries such as Canada than in those with milder climates, such as Australia.

Absorption of microcystin-LR through skin contact is unlikely, as microcystin-LR does not readily cross cell membranes.<sup>28</sup> Microcystin-LR is water soluble and non-volatile; therefore, inhalation and absorption through the lungs are unlikely, unless microcystin-LR is present as an aqueous aerosol in air.<sup>29</sup> Although for some people intake may occur through the ingestion of blue-green algal products and possibly fish or seafood, for most people the major route of exposure to microcystin-LR is drinking water consumption.

The levels of microcystin in the raw intake water for two Alberta drinking water supplies (communities not specified), as measured by the sensitive phosphatase inhibition bioassay, ranged from 0.15 to 4.3 µg/L, with a large coefficient of variation (59%) for hourly fluctuations over an 11.5-hour period; in treated water, levels ranged from 0.09 to 0.64 µg/L, with a small coefficient of variation (10%). Over a five-week period, similar coefficients of variation were obtained in the two types of samples.<sup>10</sup>

In the summer of 1993, microcystin-LR was detected (detection limit 0.05 µg/L) in raw water samples (maximum 0.45 µg/L) collected from Shoal Lake, Ontario, and from within the treated tap water distribution system (maximum 0.55 µg/L) following the identification of *Microcystis aeruginosa* blooms in Shoal Lake.<sup>30,31</sup> No toxin-producing algae were detected in Deacon Reservoir, Winnipeg's main storage facility for water from Shoal Lake. In 1995, 160 surface water supplies, located mainly in southwestern Manitoba, were chosen for algal study. Microcystin-LR was detected (detection limit 0.1 µg/L) in 70% of the raw water supplies, 93% of the municipal water supplies, 57% of the dugouts used for domestic and shared domestic plus livestock water consumption, 84% of the dugouts used exclusively for livestock and 44% of the recreational use sites. Treated water samples were analysed only if the raw water supplies were found to have detectable levels of toxins. Toxin was present in 68% of the treated water samples collected from both the municipal sites and the dugout sites. Thus, it appears that conventional water treatment methods may be only partially successful in removing the toxins. Toxin concentrations ranged from <0.1 to 1.0 µg/L in raw water samples and from <0.1 to 0.6 µg/L in treated water samples. Dugouts used extensively for livestock consumption had the highest levels of toxin. Two samples taken directly from an algal bloom on a livestock dugout had toxin levels of 1.5 and 8.0 µg/L. A much higher microcystin-LR level has also been reported. On September 9, 1996, a sample

collected at Victoria Beach on Lake Winnipeg (used for domestic and recreational purposes) had a microcystin-LR concentration of 300 µg/L, whereas microcystin-LR levels had declined to 0.2 µg/L in a follow-up sample taken later in the month.<sup>32</sup> At that time, a large algal mass had been concentrated by the wind into the shore area; *Aphanizomenon flos-aqua*, *Microcystis flos-aqua* and *Microcystis aeruginosa* were the dominant algal species.

In a follow-up study, Jones *et al.*<sup>31</sup> investigated microcystin-LR in a smaller number of water supplies (n = 7) over a longer period of time (June–December). The data indicated that microcystin-LR was present throughout the entire sampling period and that it could persist for long periods (more than two months) following the decline of the algal population. No relationship was found between levels of microcystin-LR and blue-green algal densities or environmental variables, such as physico-chemical properties or nutrient loads. Microcystin-LR concentrations were ≤ 1 µg/L in all treated samples.

In a biweekly survey of dugouts in the Peace River area of northern Alberta in the summer of 1997, 11 of 12 dugouts (all used for domestic purposes, usually without treatment) had concentrations of microcystin-LR above 0.5 µg/L at least once during the sampling period (July 23 – August 20), with six having levels above 1.5 µg/L on at least one occasion.<sup>33</sup> In a follow-up study during the summer of 1998, 18 dugouts and nine municipal reservoirs were sampled on a biweekly basis between July 7 and September 24.<sup>34</sup> The study was designed to examine the effects of dugout aeration and nutrient loading on toxin levels. In contrast to the previous year's study, 97% of the samples had microcystin-LR levels below the detection limit (0.3 µg/L); the maximum reported concentration was 0.5 µg/L. The authors were unable to assess the effects of aeration or nutrient loading on toxin formation.

It is apparent that levels of microcystin-LR exceeding 0.5 µg/L occur from time to time in those provinces where measurements have been made so far. It is likely that microcystins also occur in the surface water supplies in other Canadian provinces, but no monitoring data are available. With appropriate treatment, the maximum concentration of total microcystins in drinking water in Canada is probably less than 1 µg/L, based on the above data. Average concentrations would probably be well below this.

A two-year American Water Works Association Research Foundation (AWWARF) survey on 45 utility waters in the United States and Canada was carried out between 1996 and January 1998.<sup>35</sup> Eighty percent of the 677 samples received contained microcystins (using enzyme-linked immunosorbent assay [ELISA]), with 4.3% of these having microcystin concentrations above 1 µg/L. Only two finished water samples had microcystin

levels of  $>1 \mu\text{g/L}$ , which indicated that most water treatment plants involved with the survey had adequate treatment regimes at the time to reduce toxin levels.

### Analytical Methods

The analysis of microcystins in drinking water is an emerging area of research, and there are few “standard methods” available (for a brief review, see Resson *et al.*<sup>6</sup>). For several methods, there is a need for inter-laboratory collaborative trials.

In comparing the various analytical methods being used for microcystin-LR and other microcystins and their detection limits, it may be useful to distinguish *screening* methods — such as the mouse bioassay, ELISA and the phosphatase bioassay, which are conducted prior to cleanup and which indicate the presence of toxins in samples — from methods that are conducted for the *identification* and *quantification* of the various individual microcystins.<sup>36</sup> Often more than one toxin may be present in a sample. The consensus among those using analytical methods is that a single method will not suffice. The best approach for monitoring is to use a combination of screening and more sophisticated and costly specific chemical methods.

Although the guideline is specific to microcystin-LR, it is important that one measures *total* microcystins. “Total microcystins” includes all microcystin variants, not just microcystin-LR, that are occurring free in the water and are bound to or inside cyanobacterial cells. Thus, sample preparation may need to include sonification (to break up cells) and a variety of extraction procedures in order to isolate the different (i.e., more lipophilic or polar) microcystins. So far, published studies on the levels of microcystins in water supplies have generally not clearly indicated whether total microcystins or free microcystins were measured. It is also important that samples be taken at a post-treatment site.

Purified standards are commercially available for microcystin-LR, -RR, -YR, -LW and -LF toxins.

### Screening

The mouse bioassay still plays an important role as a screening method, as it gives the total toxic potential of the sample within a few hours, and it is possible to distinguish hepatotoxins from neurotoxins.<sup>1</sup>

For screening, many scientists prefer the protein phosphatase bioassay. This method is sensitive to sub-nanogram levels of microcystins<sup>29,37,38</sup> and can be conducted on 30  $\mu\text{L}$  of sample. With this method, 100 samples can be analysed in one day.<sup>39</sup> The method, however, is not specific to microcystins and will indicate the presence of other substances inhibiting protein phosphatases. This should not be a problem, however, when monitoring a particular area where the potentially occurring

species and their possible toxins are known. In conjunction with HPLC, the method is useful in identifying toxic fractions. A colorimetric adaptation is also in use.<sup>40</sup>

A published ELISA method, using polyclonal antibodies, is available,<sup>41</sup> with a detection limit of 0.2 ng/mL; this method is commercially available. It is likely that methods using monoclonal or polyclonal antibodies raised against a single toxin (e.g., microcystin-LR) will have problems of cross-reactivity with other microcystins. Using the method of Chu *et al.*,<sup>41</sup> Carmichael<sup>42</sup> found that most of the important microcystins reacted with the antibody, but about 10% did not. A method using polyclonal antibodies is also under development in Canada (Prairie Biological Research Laboratories, Edmonton) and is awaiting validation.<sup>43</sup> A method using monoclonal antibodies is under development in Japan.<sup>44</sup>

### Identification

For identification, Harada<sup>39</sup> has used improved frit micro liquid chromatography/mass spectrometry (LC/MS) with fast atom bombardment (FAB), with a detection limit of 1 ng.<sup>45</sup> After isolation and purification by HPLC and thin-layer chromatography (TLC), Sivonen<sup>46</sup> assigned structures to many microcystins and their variants by FABMS, FABMS/MS and <sup>1</sup>H nuclear magnetic resonance (NMR).

### Separation and Quantification

There are several HPLC methods, many of which are variations on methods developed by Siegelman *et al.*<sup>47</sup> and Harada *et al.*<sup>20</sup>

In the United Kingdom, an official HPLC method with UV detection and a detection limit of 0.5  $\mu\text{g/L}$  has been developed by the Water Research Centre (WRC) in Medmenham. This method has been tested in a limited collaborative trial with five laboratories for drinking water and reservoir water.<sup>3</sup> It is designed to measure only free microcystin-LR; it does not measure total microcystin-LR (i.e., free plus cell bound) or microcystins other than microcystin-LR. It could be modified, however, to measure total microcystins.

For separation and quantification, HPLC with fluorescence or chemoluminescence detection (sub-nanogram detection limit) and micro LC/MS with FAB (single ion monitoring, or SIM) (detection limit  $\sim 1$  ng) are the preferred methods, partly because of their lower detection limits compared with the UK HPLC method.<sup>39</sup>

An HPLC method with photodiode array detection is being used by G. Codd (University of Dundee) but has not been published (see Edwards *et al.*<sup>48</sup>).

## Treatment Technology and Management

Appropriate technology to control cyanobacteria needs to be based on an understanding of their ecology (see above). Good control technology must reflect proper management of the watershed and reservoir to prevent algal growth, an appropriate monitoring program and correct treatment technology for both the cyanobacteria and their toxins.

### Prevention of Algal Growth

It is important that a proper strategy to control blue-green algal populations be developed for a particular water body. Management options are similar to common techniques used to control algal populations in reservoirs, but with a few exceptions. Nutrient deprivation can be achieved through good watershed management limiting input of nutrients (e.g., wastewater effluent, agricultural runoff) and the addition of chemicals to source waters to reduce nutrient availability (e.g., ferric sulphate to precipitate phosphorus).<sup>11</sup> These measures may take a number of years to become effective. Algal growth may also be controlled through physical means, such as light exclusion or artificial destratification of the reservoir.<sup>11</sup> One potential solution for the short-term control of algal blooms for small communities is the use of alum and gypsum as algistats.<sup>49</sup>

An inappropriate method of control is the use of an algicide, such as copper sulphate. The addition of copper sulphate (or chlorine) to a mature bloom will destroy the cyanobacteria but will cause the release of toxins into the water. As well, use of algicides would kill all algae, giving cyanobacteria less competition in a subsequent growth phase.<sup>11</sup>

### Monitoring for Cyanobacteria and their Toxins

An appropriate monitoring program is essential to the overall control of cyanobacteria and their toxins. Drinking water supplies suspected or known to be susceptible to blooms should be routinely monitored for the presence of cyanobacteria. Weather should also be closely monitored for conditions known to be conducive to bloom formation. The use of remote sensing to detect contributory conditions may be helpful. Monitoring sites for cyanobacteria (species identification and cell counts) should include the raw water intake, reservoir and various stages in the water treatment process. These sites could also serve as sampling points for toxins (identification and quantification) during cyanobacterial blooms. Timing, frequency and depth of sampling should take the ecology of cyanobacteria into consideration (e.g., their ability to float within the water column). Sampling and analysis of cyanobacteria are also required to determine the effectiveness of cyanobacterial management programs in watersheds or reservoirs.

In some countries, a system of alert levels, combining information on the number of cells, cyanobacterial species identification and toxin levels, is being used in guiding the appropriate response to be taken.<sup>11,50</sup> In Australia, a task force has also recommended that water suppliers be alerted when the level of cyanobacteria known to produce taste and odour (geosmin or 2-methylisoborneol) exceeds 2000 cells/mL.<sup>49</sup> This value has been determined to be the typical threshold value for consumer complaints. However, with *Microcystis*, the absence of taste and odour should not be equated with the absence of toxins. Studies conducted by Hruddy *et al.*<sup>51</sup> have demonstrated that the presence of microcystin-LR was not related to the presence of geosmin or 2-methylisoborneol. A more recent study of Canadian and U.S. utility waters by the AWWARF found that 82% of 181 samples that tested positive for taste and odour problems also tested positive for the presence of microcystins.<sup>35</sup>

When blooms occur, water suppliers must decide on an appropriate course of corrective action. The most common responses include one or all of the following: resample or test for toxicity, find an alternative supply or treat to remove toxins.

A flow chart illustrating those factors that should be considered during bloom events and recommendations on actions that may be taken to address the issue are given in Annex A.

### Treatment Technology

The final step in controlling cyanobacteria and their toxins is the drinking water treatment process.

Conventional surface water treatment plants, using coagulation (aluminum sulphate, ferric sulphate), clarification and filtration, are effective in removing cyanobacterial cells.<sup>52</sup> The use of chemicals or conditions that would lead to lysis of cyanobacterial cells must be avoided to prevent the release of their toxins. To remove the cyanobacterial cells from the treatment train, the frequency of sludge removal and filter backwash (to waste) should be increased. Recent studies have shown that toxin release from sludge depends upon the length of time that the sludge is retained in sedimentation tanks. A pilot plant study by Drikas *et al.*<sup>53</sup> showed that no additional toxin was released from cells during treatment; however, extracellular toxins already present in the water were not removed by the treatment process. In the study, total numbers of cells in the sludge were reduced by 50% after two days, but toxin release began immediately, reaching 100% after the two days. Toxin concentrations were reduced by approximately 80% after eight days, and toxins were completely removed after 13 days. In general, the final disposal of the water treatment plant's waste must be evaluated to guarantee that the waste is not recycled and that cyanobacterial cells are not reintroduced into the source water.

Conventional surface water treatment processes are only partially successful in removing or destroying cyanobacterial toxins.<sup>54</sup> However, certain oxidation procedures as well as activated charcoal have been found to be effective. Lambert *et al.*<sup>55</sup> examined the removal of microcystins from drinking water at two full-scale treatment plants in Alberta that employed coagulation-sedimentation, dual-media filtration and chlorination combined with either granular activated carbon (GAC) or powdered activated carbon (PAC) filtration. The two processes generally removed more than 80% of the microcystins from raw water, particularly when the raw water concentrations were high; however, a residual concentration of 0.05–0.2 µg microcystin-LR equivalents/L was observed at both treatment facilities. More recent studies by Chow *et al.*<sup>56</sup> of the effects of treatment chemicals, mechanical stirring and flocculation on *Microcystis aeruginosa* cells using jar test and a full pilot plant (coagulation/flocculation-sedimentation-filtration) resulted in no damage to the cells or additional release of microcystins from the cells into the finished water. Results from slow sand filtration experiments (over several hours) show some removal of cyanobacterial toxins through biodegradation.<sup>57</sup> Because the toxins are non-volatile, neither aeration nor air stripping would be effective in removing the soluble toxins.<sup>7</sup> Further investigation is required on large slow sand filters and on biological filtration processes.

With regard to oxidation, the residual oxidant level is important. Below pH 8, aqueous chlorine (largely present in the form of hypochlorous acid) at a concentration of 15 mg/L will destroy microcystins; at neutral pH values, chlorination is effective provided a chlorine residual concentration of at least 0.5 mg/L is present after a 30-minute contact time. Destruction is significantly reduced above pH 8 due to the rapid decrease in the concentration of hypochlorous acid with increasing pH. Ozone pretreatment at 1 mg/L can remove microcystins as long as a residual ozone level of 0.05–0.1 mg/L is maintained; the residual ozone level is significant because the effectiveness of ozone is affected by total organic carbon concentration. No new acute toxins are formed within 24 hours, based on the mouse bioassay for acute toxicity. With regard to other oxidation treatments, potassium permanganate at 1 mg/L was found to be effective, but further work is required; hydrogen peroxide, chloramine and chlorine dioxide were not effective; and UV radiation as a point-of-use treatment was not potent enough.<sup>52,58</sup> The effectiveness of a variety of oxidation techniques on raw or clarified waters was recently studied in the United Kingdom.<sup>52</sup> This study showed that certain oxidation processes were more effective when the oxidant was applied to treated water, presumably because raw water has higher levels of organic/inorganic materials, which will react with the oxidant and reduce the available dose for effective toxin

removal. Potassium permanganate and ozone, at doses of 2 mg/L, were highly effective in removing microcystin-LR from treated water. Under conditions similar to those for drinking water disinfection, the study found that chlorination was effective below pH 7; at higher pHs, however, longer contact time was required, which may be of relevance in long distribution systems that hold a chlorine residual. There was also evidence that the oxidants were causing cell lysis, resulting in toxin release; however, the authors concluded that, except for chloramine, intra- and extracellular toxins could be removed if sufficient oxidant was applied.

Different sources of activated carbon have been investigated for their ability to adsorb microcystin-LR. Wood-based products were found to be most effective because of their high mesopore volume. It was found that treatment with 25 mg/L of wood-based PAC, with a contact time of 30 minutes, could reduce the concentration of microcystin-LR from 50 to <1 µg/L.<sup>58</sup> The presence in the water of other substances (e.g., natural organics) that could be adsorbed by the PAC needs to be considered in studies of efficacy. Studies by Jones *et al.*<sup>54</sup> found that alum coagulation in conjunction with PAC adversely affected toxin removal. PAC may be capable of high toxin removal efficiencies; however, very high doses of PAC are required, and contact time is very important. Various GAC filters also appear to be effective in removing microcystin-LR. Although some studies have shown that the life of the GAC is limited,<sup>7</sup> others have found that the GAC filters, even when already exhausted by removal of dissolved organic carbon, were effective in reducing microcystin-LR levels from 20 to 1 µg/L.<sup>54,58</sup> Laboratory studies have indicated that a biologically active GAC would be able to completely remove toxins via adsorption and biodegradation, providing there is sufficient contact time to allow biological activity.<sup>52</sup>

Membrane processes such as microfiltration and nanofiltration may also be effective in the removal of both the cyanobacterial cells and the intracellular toxins.<sup>7</sup> Studies by Hart and Stott<sup>59</sup> and Muntisov and Trimboli<sup>60</sup> using microcystin-spiked natural water at concentrations between 5 and 30 µg/L found that toxin levels were reduced to less than 1 µg/L by nanofiltration. Testing of reverse osmosis membranes (2500–3500 kPa) for the elimination of microcystin-LR and -RR from tap water resulted in average retention levels of 96.7–99.6%; initial concentrations in the retentate were 70–130 µg/L.<sup>61</sup>

Toxin removal treatment for households and small community systems is of concern in rural areas that are subject to repeated growth of these organisms. Lawton *et al.*<sup>62</sup> tested three different domestic jug filtration units for the removal of toxin and algal cells; treatment in all units was based on activated carbon and ion exchange resin. Cell removal was found to be dependent upon the

morphological characteristics of the cells, with approximately 60% of the filamentous cells removed and only 10% of the single cells of *Microcystis* removed. Toxin removal (variants tested included LR, LY, LW and LF) ranged from 32 to 57% when using new cartridges, increasing to 88% with three repeated passages of the same water through the same filter. There is also the possibility of lysis of cells retained on the filters. Testing of filters that had reached the manufacturers' half-life showed a 15% reduction in toxin (LR) removal on one of the brands tested. More research and development are needed if these filters are to be suitable for household microcystin removal.

In summary, where feasible, the use of an oxidant such as ozone, potassium permanganate or chlorine and biologically activated GAC, after the removal of algal cells, is the preferred treatment. The specific concentrations of the various agents in the treatment process depend on the physical, chemical and biological quality of the water to be treated.<sup>46,52,58</sup> More research is required on approaches for household treatment.

### Health Effects

Blue-green algae have been known to cause animal and human poisoning in lakes, ponds and dugouts in various parts of the world for over 100 years.

### Effects in Humans

#### Recreational Water

Incidences of human illness have been linked to the recreational use of water contaminated by cyanobacterial blooms, including *Anabaena* and *Microcystis*, in North America, the United Kingdom, the Netherlands and Australia. No associated fatalities have been reported. In Canada, illnesses have been reported in Saskatchewan, and symptoms have included stomach cramps, vomiting, diarrhoea, fever, headache, pains in muscles and joints and weakness.<sup>63</sup> Similar symptoms as well as dermal irritation, sore red eyes, sore throat and allergic responses have also been reported elsewhere.<sup>6</sup> The reported instances of illnesses are few; however, because they are difficult to diagnose, such illnesses may be more common than it appears.<sup>64</sup>

Exposure has been mainly through skin contact and some inadvertent ingestion of the water containing dispersed cyanobacteria. Despite the high toxicity of cyanobacterial toxins in animals, serious acute human illness due to these toxins has been reported only rarely. This is probably because humans have an aversion to ingesting algal scum.

In one recent incident in the United Kingdom, 10 of 18 military recruits on an exercise in a reservoir with a bloom of *Microcystis aeruginosa* suffered abdominal pain, nausea, vomiting, diarrhoea, sore throat, dry cough, blistering at the mouth and headache. Two were

hospitalized and developed an atypical pneumonia, although it is possible that the pneumonia was caused by inspiration of algal material that may have also contained lipopolysaccharides. Serum enzymes indicating liver damage were elevated. Microcystin-LR was identified in the bloom material.<sup>65</sup>

#### Drinking Water

In the United States and Australia, several different cyanobacterial toxins have been implicated in human illness, often after algal blooms in certain municipal water supplies had been treated with copper sulphate.<sup>6,66,67</sup> Although a direct cause-and-effect relationship was not established in most of the outbreaks, there has been strong circumstantial evidence that cyanobacterial blooms were present at the water intake area or in open reservoirs. Although in most cases the cyanobacteria involved and sometimes the toxins involved have been identified, the levels of toxin associated with illness have not been established in any of the outbreaks. In at least one outbreak in Palm Island, Australia, in 1979,<sup>68</sup> acute copper poisoning has been suggested as an alternative cause,<sup>69</sup> although further study has shown that a toxin from a species of blue-green algae (*Cylindrospermopsis raciborskii*) may have been the causative agent.<sup>7</sup> In this case, complaints of bad taste and odour in a water supply, which were attributed to a cyanobacterial bloom, resulted in authorities treating the reservoir with copper sulphate. Within one week, numerous children developed severe hepatoenteritis, and 140 children and 10 adults required hospital treatment. No deaths were reported.

Possible liver damage, as evidenced by significant increases in  $\gamma$ -glutamyl transferase, was seen in persons drinking water from supplies containing blooms of *Microcystis* after treatment with copper sulphate (Malpas Dam, Armidale, Australia) compared with persons drinking uncontaminated water.<sup>70</sup>

To date, the most lethal outbreak attributed to exposure to cyanobacterial toxins in drinking water occurred in Brazil in 1988.<sup>71</sup> An immense cyanobacterial bloom developed in a newly flooded dam, resulting in more than 2000 cases of gastroenteritis, with 88 deaths reported (mostly children) over a 42-day period. It appears that the cyanobacterial proliferation resulted from the decomposing biomass and other conditions that prevailed in the newly flooded reservoir area.

More recently, in February 1996, liver failure and death were reported in haemolysis patients of a Brazilian dialysis clinic where the dialysate was found to be contaminated with fragmented microalgal and cyanobacterial cells and probably the microcystin-LR toxin.<sup>72,73</sup> Death was reported in approximately 50% of the dialysis patients exposed to the contaminated dialysate; however, no information was available on the type, abundance or toxicity of the cyanobacteria in the

reservoir that was the source of the water during the period in question. Liver histology confirmed the presence of acute toxic hepatitis similar to that observed in animals exposed to microcystins. Analytical examination of liver tissue and serum samples of patients and the carbon filter from the dialysis unit confirmed the presence of three microcystin derivatives (YR, LR and AR). It was concluded that inadequate additional treatment of the water used in the dialysis process at the clinic was most likely responsible for the toxins in the dialysate and that intravenous exposure to these microcystins was a major contributing factor in the deaths of these patients.<sup>74</sup> From further analysis of the phytoplankton from the dialysis centre and tissue and serum samples from the 76 victims and other affected patients, it was estimated that the water used in the dialysis treatment contained 19.5 µg microcystin/L.<sup>74</sup> Because of the susceptibility of dialysis patients to contaminated dialysate, dialysis centres should be informed if the source water from their local treatment plant is prone to blue-green algal blooms, so that they may provide additional treatment of the water, if necessary. Continuous monitoring of treatment plant performance and equipment is also necessary to ensure an adequate water supply.

Zilberg<sup>75</sup> postulated that seasonal acute childhood gastroenteritis observed during 1960–1965 in Salisbury, Rhodesia (now known as Harare, Zimbabwe), might be linked to annual algal blooms in the lake serving as the water supply. An adjacent water supply was not similarly affected and was not associated with this disease.

El Saadi and Cameron<sup>76</sup> reported on 26 cases (aged 1–64 years) with a variety of symptoms associated with exposure, during 1991–1992, to river water or rainwater (River Murray, Australia) that was stored in open tanks and contained *Anabaena* blooms. Symptoms following oral intake (drinking water) included diarrhoea, vomiting, nausea, muscle weakness, sore throat, respiratory difficulties and headaches. Complaints following skin contact (recreational activities) or oral mucosal contact included rash, itching, mouth blistering and eye irritation. Further case–control studies in the same area are ongoing.

There have been reports of cyanobacterial blooms in surface water used for drinking purposes in China, where there is a high incidence of primary liver cancer; however, data are lacking.<sup>6</sup> In an epidemiological study by Yu<sup>77</sup> of human primary liver cancer in Qidong county in China, the incidence of liver cancer was about eight times higher in people who drank pond and ditch water than in people who drank well water (no levels of algal toxins were determined). Further analytical epidemiological studies are required to elucidate a possible (additional) role of microcystins in this disease of multifactorial aetiology. Hepatitis B infection and dietary exposure to aflatoxin B<sub>1</sub> are two known risk factors for liver cancer and are present in the same area of China.

A similar relationship was not observed in a larger epidemiological study of primary liver cancer in 65 Chinese counties reported by Junshi.<sup>78</sup> In this study, the use of deep well water was directly associated with liver cancer, which is contrary to the findings of Yu.<sup>77</sup>

In a more recent epidemiological survey in Haimen City (Jian-Su province) and Fusui county (Guangxi province) in China, Ueno *et al.*<sup>79</sup> found a close relationship between the incidence of primary liver cancer and the use of drinking water from ponds and ditches. A combination of ELISA and affinity column chromatography was used to detect (detection limit 0.05 µg/L) very low levels of microcystins in the water samples without cleanup and concentration procedures (for method, see Nagata *et al.*<sup>80</sup>). In September 1993, three of 14 ditch water samples contained microcystins, in a concentration range of 0.09–0.46 µg/L. Following this, samples were collected from five ponds/ditches, two rivers, two shallow wells and two deep wells monthly throughout 1994. The data showed that the highest concentrations of microcystins occurred from June to September, with a range of 0.058–0.296 µg/L. A third trial on the 989 water samples collected from the different water sources in July 1994 revealed that 17% of the pond/ditch water, 32% of the river water and 4% of the shallow well-water contained microcystins, with average concentrations of 0.1, 0.16 and 0.068 µg/L, respectively. Microcystins were not detected in deep well-water. A similar survey on 26 drinking water samples in the Guangxi province showed a high frequency of microcystins in the water of ponds/ditches and rivers, but no microcystins were found in shallow or deep wells.

Pilotto *et al.*<sup>81</sup> examined the relationship between potential exposure to cyanobacterial toxins in drinking water during pregnancy and birth outcomes. The study examined >32 000 singleton live births between 1992 and 1994 in 156 Australian communities. Although significant differences were observed between exposure to cyanobacteria (estimated as cyanobacterial occurrence and cell density in the source drinking water) during the first trimester and the incidences of low and very low birth weights, the results do not suggest a causal link to cyanobacteria; no dose–response relationships were observed. The authors concluded that the study did not provide clear evidence of an association between cyanobacterial contamination of drinking water supplies and adverse pregnancy outcomes.

Since cyanobacterial blooms tend to occur repeatedly in the same water supply, some human populations are at risk of repeated ingestion of cyanobacterial toxins.

## Effects on Animals

### *Kinetics and Metabolism*

Although the most likely route of exposure to cyanobacterial toxins is via ingestion, there have been

few pharmacokinetic studies with orally administered microcystins. After intravenous or intraperitoneal injection of sublethal doses of variously radiolabelled microcystins in mice and rats, about 70% of the toxin is rapidly localized in the liver,<sup>82–87</sup> whereas oral administration resulted in less than 1% uptake into the liver of mice.<sup>87</sup> Although microcystin-LR does not readily cross cell membranes and does not enter most tissues, microcystins appear to be transported into hepatocytes and into the cells of the intestinal lining via the bile acid transport system<sup>88,89</sup>; microcystin-LR has also been found to cross the ileum through the multispecific organic ion transport system.<sup>90</sup> In hepatocytes, microcystin-LR is covalently bound to a 40 000-dalton protein (protein phosphatase 2A and possibly protein phosphatase 1) in the cytosol<sup>91</sup> (for a review, see Fujiki and Suganuma<sup>92</sup>). Microcystin congeners that are more hydrophobic than microcystin-LR may cross cell membranes by other mechanisms, such as diffusion.<sup>7,13</sup>

Plasma half-lives of microcystin-LR, after intravenous administration, were 0.8 and 6.9 minutes for the alpha and beta phases of elimination, but the concentration of radioactive (<sup>3</sup>H-microcystin-LR) label in the liver did not change through the six-day study period; about 9% of the dose was excreted early via the urinary route, with the remainder being excreted slowly (~1% per day) via the faecal route.<sup>85</sup> Based on the protective effect of microsomal enzyme inducers, it is evident that the liver plays a large role in the detoxification of microcystins.<sup>83</sup> Time-dependent appearance and disappearance of additional peaks, thought to represent detoxification products, were seen in urine, faeces and liver cytosol fractions,<sup>85</sup> but these products have not been structurally identified. Three metabolic products have been identified in rats and mice following intraperitoneal injection of microcystin-LR, including glutathione and cysteine conjugates and a conjugate with the oxidized ADDA diene.<sup>93</sup>

#### Acute Toxicity

Microcystin-LR is extremely toxic after acute exposure. Fatalities have been observed after animals consumed water containing large numbers (>10<sup>6</sup>/mL) of cyanobacterial cells.<sup>1</sup>

The LD<sub>50</sub> by the intraperitoneal route is approximately 25–150 µg/kg bw in mice; the oral (by gavage) LD<sub>50</sub> is 5000 µg/kg bw in mice and higher in rats.<sup>94,95</sup> This indicates that, even by the oral route, microcystin-LR is extremely toxic in mice following acute exposure; intraperitoneal injection is 30–100 times more toxic. Thus, a significant amount of microcystin-LR escapes the effects of peptidases in the stomach and is absorbed. The oral LD<sub>50</sub> of a toxic *Anabaena* extract in mice was also reported to be at least 170 times higher than the intraperitoneal LD<sub>50</sub> of the same extract.<sup>96</sup>

Yoshida *et al.*<sup>97</sup> reported that the LD<sub>50</sub> for orally (gavage) administered (10.0 mg/kg bw) microcystin-LR in six-week-old mice was 167 times higher than the intraperitoneal value (65.4 µg/kg bw). Histologically, both routes of administration resulted in similar types of injuries to hepatocytes, including haemorrhage and necrosis.

The intraperitoneal LD<sub>50</sub>s of several of the commonly occurring microcystins (microcystin-LA, -YR and -YM) are similar to that of microcystin-LR, but the intraperitoneal LD<sub>50</sub> for microcystin-RR is about 10-fold higher.<sup>98,99</sup> However, because of differences in lipophilicity and polarity between the different microcystins, it cannot be presumed that the intraperitoneal LD<sub>50</sub> will predict toxicity after oral administration.

The microcystins are primarily hepatotoxins. After acute exposure by intravenous or intraperitoneal injection of microcystins, severe liver damage is characterized by a disruption of liver cell structure (due to damage to the cytoskeleton), a loss of sinusoidal structure, increases in liver weight due to intrahepatic haemorrhage, haemodynamic shock, heart failure and death. Other organs affected are the kidney and lungs.<sup>100</sup> Intestinal damage is a consequence of the transport of microcystins through the lining cells, which are damaged in a similar manner to hepatocytes.<sup>89</sup>

#### Subchronic and Chronic Toxicity

In a study conducted at Quintiles by WRc in the United Kingdom, microcystin-LR was administered orally by gavage to groups of 15 male and 15 female mice at 0, 40, 200 or 1000 µg/kg bw per day for 13 weeks. The no-observed-adverse-effect level (NOAEL) for liver toxicity was 40 µg/kg bw per day. At the next highest dose level, there was slight liver pathology in one male and two female mice. At the highest dose level, all mice showed liver changes, which included chronic inflammation, degeneration of hepatocytes and haemosiderin deposits. In male mice at the two highest dose levels, alanine and aspartate aminotransferases were significantly elevated, serum γ-glutamyl transferase was slightly reduced and there were small but significant reductions in total serum protein and serum albumin; alkaline phosphatase was also significantly increased at the highest dose. In female mice at the highest dose level, only increases in alkaline phosphatase and alanine aminotransferase were observed. Male mice exhibited reduced body weight gain in all treatment groups, but there was no dose–response relationship, and the final body weight was depressed by only 7%.<sup>94</sup>

Another oral repeated-dose study was conducted with *Microcystis aeruginosa* extract supplied to mice (410 in total) at six concentrations (control, one-sixteenth dilution, one-eighth dilution, one-fourth dilution, one-half dilution and undiluted toxic extract;

undiluted extract had a toxin concentration of 56.6 µg/mL, estimated by LD<sub>50</sub> value, which can be calculated to be approximately equivalent to a dose of 11 300 µg/kg bw per day) in their drinking water for up to one year. The mortality rate increased with dose at the two highest doses. At the highest dose, body weight was reduced in both sexes at nine weeks, and liver weight was increased in females at five weeks; males showed significantly increased liver weight as a percentage of body weight at the second highest dose but not at the highest dose, owing to high mortality and loss of body weight. At the two highest concentrations, alanine aminotransferase levels were elevated at five and nine weeks, and chronic active liver injury was apparent following exposure for up to 13 weeks; after longer periods of exposure to lower doses, no pathological changes in the liver were found that were directly related to the effect of toxin on hepatocytes, and no hepatic neoplasms were noted. There was also some evidence for increased bronchopneumonia with increased concentrations of extract.<sup>101</sup>

Ito *et al.*<sup>102</sup> studied the effects of age on the liver of young and aged mice orally administered microcystin-LR. Twenty-nine 32-week-old (aged) and 12 five-week-old (young) male ICR mice received 500 µg/kg bw via gastric intubation; each group had three unexposed controls (aged and young). Animals from each group were sacrificed after two, five and 19 hours, and their livers and small intestines were examined. Sixty-two percent of the aged mice showed hepatic injury that could not be distinguished pathologically from hepatic injuries caused by intraperitoneal administration, indicating that microcystin-LR was incorporated into the liver following oral administration. The most severe damage in the small intestine of the aged mice was observed in the duodenum. In contrast, no effects, in either the liver or intestine, were observed in the young (five-week-old) mice. No significant differences were observed in either biochemical tests (glutamate–oxaloacetate transaminase and glutamate–pyruvate transaminase) or morphological examination of the livers of non-treated aged mice and young mice, indicating that the livers of the aged mice were healthy. Further testing in aged and young mice suggests that the uptake of the toxin via the oral route is related to the condition of the surface epithelial cells and the permeability of the capillaries in the villi of the small intestine and is strongly related to aging.

Heinze<sup>103</sup> studied the toxicity of pure microcystin-LR toxin in the drinking water of rats (10 animals/group) exposed to approximately 50 or 150 µg/kg bw per day for 28 days. Dose-dependent increases in relative liver weight and serum enzymes (lactate dehydrogenase and alkaline phosphatase) were observed. Liver damage, defined as “toxic hepatitis,” was clearly indicated by histological examination of the tissues; damage was more severe at the higher dose.

In a poorly described study, Fitzgeorge *et al.*<sup>104</sup> reported that intranasal instillation of microcystin-LR in mice resulted in extensive necrosis of the nasal mucosa epithelium in both the olfactory and respiratory zones. The necrosis progressed to destruction of large areas of mucosa down to the level of deep blood vessels. The reported LD<sub>50</sub> for this route was the same as the LD<sub>50</sub> for intraperitoneal administration (250 µg/kg bw), and dose-dependent liver lesions were observed. The authors also reported cumulative liver damage following repeated intranasal dosing. Although no increase in liver weight was observed following a single dose of 31.3 µg/kg bw, repeated daily administration of this same dose for seven days resulted in a 75% increase in liver weight, which was very near the effect observed from a single intranasal dose of 500 µg/kg bw (87% increase in liver weight).

In a subchronic study, *Microcystis aeruginosa* extract was given to groups of five pigs in their drinking water for 44 days at microcystin dose levels calculated to be equivalent to 0, 280, 800 and 1310 µg/kg bw per day (the potency of the extract used was based on its intraperitoneal LD<sub>50</sub> in mice). The extract contained at least seven microcystin variants, with microcystin-YR tentatively identified as the major peak. Liver function (as evidenced by changes in  $\gamma$ -glutamyl transpeptidase, alkaline phosphatase, total bilirubin and plasma albumin) was affected at the two highest doses, whereas visible liver injury was observed at all three doses; only one pig was affected at the lowest dose level. Thus, it may be appropriate to consider the 280 µg/kg bw per day dose level as a lowest-observed-adverse-effect level (LOAEL). LOAELs of a similar order of magnitude (ranging from 90 to 270 µg/kg bw per day) can be calculated using toxin contents of the dried cyanobacterial scum as determined by other laboratories.<sup>105</sup>

In a chronic toxicity study, three vervet monkeys were dosed intragastrically (three times per week) with microcystin-LA for 46 weeks; the dose levels were gradually increased from 20 to 80 µg/kg bw over the duration of the experiment. No statistically significant alterations in clinical or haematological parameters or serum enzyme levels were observed in treated animals compared with controls, and there were no histopathological changes in the liver or other organs of treated animals.<sup>106</sup> The results, although preliminary, suggest that the NOAEL of microcystin-LA in the vervet monkey is no less than the NOAEL of microcystin-LR observed in mice and may be higher.<sup>94</sup>

Ueno *et al.*<sup>107</sup> exposed BALB-c female mice to 20 µg microcystin-LR/L in their drinking water for seven days per week *ad libitum* for 18 months (567 days); control mice received water alone. Animals were sacrificed at three, six, 12 and 18 months. Mean cumulative microcystin-LR intake after 18 months of exposure was estimated at 35.5 µg per mouse. No chronic

toxicity or accumulation of the toxin in the liver was observed, nor was there any absorption from the intestines in the study. Neither water nor food consumption was affected by the treatment, and there were no treatment-related changes in a wide range of test parameters.

#### *Reproductive and Developmental Toxicity*

To investigate the effects of microcystin-LR on the embryonic and foetal development of the mouse, four groups of 26 time-mated female mice of the Cr1:CD-1(ICR)BR strain were dosed once daily by oral gavage with aqueous solutions of microcystin-LR from days 6 to 15 of pregnancy, inclusive. The dose levels were 0, 200, 600 and 2000 µg/kg bw per day. On day 18 of pregnancy, the females were killed and a necropsy was performed. The foetuses were weighed, sexed and subjected to detailed external, visceral and skeletal examinations for abnormalities. Only treatment at 2000 µg/kg bw per day was associated with maternal toxicity and mortality; seven of the 26 females died, and two were sacrificed prematurely during the dosing period because they showed signs of distress. There was no apparent effect of treatment at any dose level on litter size, incidence of resorption or the sex distribution of the live foetuses. Mean foetal weight was significantly lower in the high-dose group, and there was an increased incidence of foetuses with delayed skeletal ossification; both are common findings associated with maternal toxicity. Otherwise, there was no increased incidence of foetal abnormalities at any dose. The no-effect level for any aspect of developmental toxicity was 600 µg/kg bw per day.<sup>94,95</sup>

To examine the effect of toxic *Microcystis aeruginosa* extract on reproduction in mice, Falconer *et al.*<sup>101</sup> exposed male and female parents to a one-fourth dilution of extract (approximately 2800 µg/kg bw per day) in drinking water for 17 weeks prior to mating and through pregnancy and early lactation. No effects on fertility, embryonic mortality or teratogenicity were observed, other than reduced brain size in about 10% of the neonatal mice, compared with controls.

#### *Tumour Promotion*

There has been some evidence of tumour promotion in animal studies. In a modified two-stage carcinogenesis mouse skin bioassay, dimethylbenzanthracene (DMBA) (500 µg) in acetone was applied to the skin of four of six groups of 20 three-month-old Swiss female mice. After one week, the DMBA-treated mice received 1) drinking water, 2) *Microcystis* extract in drinking water (actual microcystin-YM dose not provided), 3) croton oil (as a positive control) applied to the skin (0.5% in 0.1 mL acetone twice a week) plus drinking water or 4) croton oil plus *Microcystis* extract; the control mice received drinking water or *Microcystis* extract in drinking water. After 52 days from initiation,

substantial skin tumours and ulcers were visible on the DMBA-treated mice consuming *Microcystis* extract. Tumour growth was less substantial in the other three groups of DMBA-treated mice. The mean weight of skin tumours per mouse was significantly higher in DMBA-treated mice given the *Microcystis* extract than in the DMBA-treated mice given water. The actual number of tumours per mouse and the weights of the tumours in relation to the weights of the animals were not provided. It was concluded by the authors that *Microcystis* extract consumed in drinking water may act as a promoter.<sup>96</sup> However, the mechanism of action is not clear, as microcystins have difficulty penetrating epidermal cells.<sup>108</sup> The tumour weight per mouse in DMBA-treated mice given both croton oil and the algal extract was slightly lower than in those given croton oil and drinking water. These latter findings could not be explained by the author.<sup>96</sup>

In a two-stage carcinogenicity bioassay, groups of 9–15 seven-week-old male Fischer 344 rats were initiated by intraperitoneal injection with diethylnitrosamine (200 mg/kg bw), followed by partial hepatectomy at the end of the third week. Tumour promotion was assessed by intraperitoneal injection of microcystin-LR at 1 or 10 µg/kg bw twice per week from the third week of the experiment. Tumour promotion, as indicated by an increase in glutathione S-transferase placental form (GST-P) positive liver foci, was seen after eight weeks in animals dosed with microcystin-LR at 10 µg/kg bw.<sup>109</sup> Microcystin-LR had no effect when given to non-initiated rats; as well, treatment with 1 µg/kg bw did not show any significant increase of foci. To confirm the tumour-promoting activity of microcystin-LR, the same authors administered microcystin-LR dose levels of 10 µg/kg bw before partial hepatectomy and 10, 25 or 50 µg/kg bw twice a week after partial hepatectomy to groups of 14–19 male rats. It was found that the increase in GST-P-positive foci following repeated intraperitoneal injections of microcystin-LR was dose related. According to the authors, the results suggest that microcystin is the strongest of the liver tumour promoters found to date. Even though the study involved intraperitoneal dosings, the authors suggested that tumour promotion by microcystin should be considered possible in humans as well.

Ito *et al.*<sup>110</sup> compared the formation of hepatic neoplastic nodules in mice exposed to microcystin-LR by intraperitoneal and oral routes without pretreatment with initiators. Multiple neoplastic nodules (up to 5 mm in diameter) were observed in all mice (13/13) receiving intraperitoneal injections of 20 µg microcystin-LR/kg (five times per week) for a total of 100 injections over 28 weeks. Five mice were sacrificed immediately following the last injection, and the remaining eight were allowed to recover for two months prior to sacrifice.

Liver weights in these two groups were 9.0% and 6.8% of total body weights, compared with 4.7% in controls. The same researchers exposed 22 mice to repeated intragastric intubation of 80 µg microcystin-LR/kg for 80 or 100 treatments over 28 weeks; seven mice were allowed a two-month withdrawal prior to sacrifice. Although there were injuries to the hepatocytes of some of the animals, there were no characteristic chronic injuries to the liver, such as fibrous changes and nodule formation, as were observed in the intraperitoneal study; mean liver weights were not significantly different from the controls.

In another tumour initiation and promotion assay aimed at evaluating possible tumour-promoting effects in the upper small intestine, Falconer and Humpage<sup>111</sup> orally administered two doses (40 µg/kg bw each) of the initiator N-methyl-N-nitrosourea to C57 black mice, one week apart, followed by drinking water containing various levels of *Microcystis* extracts (0, 10 or 40 mg *Microcystis* toxins per litre), estimated to be equivalent to 0, 1.2 or 4.2 mg microcystins/kg bw per day, for up to 154 days. No primary liver tumours were seen in any group, and there was no evidence of microcystin-induced promotion of lymphoid or duodenal tumours.

Microcystin-LR was found to be a potent inhibitor of eukaryotic protein serine/threonine phosphatases 1 and 2A both *in vitro*<sup>112,113</sup> and *in vivo*,<sup>114</sup> and this effect has become the basis of one of the bioassays to detect its presence. Such substances are considered to be 12-O-tetradecanoylphorbol-13-acetate (TPA) type tumour promoters. For the tumour promoter TPA, the mechanism of action is attributed to its activation of protein kinase C. Other substances that act similarly to microcystins are okadaic acid, nodularin, tautomycin and calyculin A (for a review, see Fujiki and Suganuma<sup>92</sup>). The protein phosphatases serve an important regulatory role in maintaining homeostasis in the cell. They slow down cell division by counteracting the effects of various kinases through dephosphorylation of proteins. Protein phosphatase inhibition results in a shift in the balance towards higher phosphorylation of target proteins. This is a major post-translational modification. It can result in excessive signalling and may lead towards cell proliferation, cell transformation and tumour promotion. In liver cells, cytoskeletal components (intermediate filaments followed by microfilaments) are affected, which may result in reduced contact with other cells.<sup>100,115</sup> The inhibition of protein phosphatase 2A by microcystin-LR can be effectively reversed in the presence of polyclonal antibodies against microcystin-LR.<sup>116</sup> The implications for low-level chronic exposure to microcystins in humans are not known.

#### Genotoxicity

No mutagenic response was observed for purified toxins derived from *Microcystis* in the Ames *Salmonella*

assay with or without S9 activation. The *Bacillus subtilis* multigene sporulation test was also negative with regard to mutagenicity using both the 168 and hcr-9 strains.<sup>117</sup> In contrast, results of a study in which the purified toxins were tested against human lymphocytes suggested that the toxins may be clastogenic, as indicated by increased and dose-related chromosomal breakage.<sup>117</sup> More recently, Ding *et al.*<sup>118</sup> reported that a microcystic cyanobacterial extract (extract prepared was derived from >90% *Microcystis aeruginosa*) showed a strong mutagenic response in the Ames test (strains TA97, TA98, TA100 and TA102; with or without S9 activation), induced significant DNA damage in primary cultured rat hepatocytes (comet assay) and produced bone marrow micronucleated polychromatic erythrocytes in mice.

#### Classification and Assessment

It is difficult to assess the risk to human health from microcystins in drinking water. Most of the relevant data have come either from reported human injury related to the consumption of drinking water from a source containing cyanobacteria or from limited work in experimental animals. There is evidence of liver injury in people who drank water contaminated by toxic cyanobacteria and evidence for tumour promotion by *Microcystis* or microcystins in animals. With microcystins, the underlying mechanism is thought to be protein phosphatase inhibition. Taking the information on genotoxicity into consideration, microcystin-LR could possibly be carcinogenic to humans, and for this reason it has been placed in Group IIIB (inadequate data in humans, limited evidence in experimental animals). It is therefore considered appropriate to use a LOAEL or NOAEL from the most suitable chronic or subchronic study, divided by appropriate uncertainty factors, to derive a tolerable daily intake (TDI) for microcystin-LR, the only microcystin for which there is sufficient information available with which to derive a guideline value.

For microcystin-LR, the TDI is derived as follows:

$$\text{TDI} = \frac{40 \text{ } \mu\text{g/kg bw per day}}{1000} = 0.04 \text{ } \mu\text{g/kg bw per day}$$

where:

- 40 µg/kg bw per day is the NOAEL for liver changes derived from the 13-week mouse study conducted at the WRc in the United Kingdom<sup>94</sup>
- 1000 is the uncertainty factor (×10 for intraspecies variation, ×10 for interspecies variation and ×10 for the less-than-lifetime study).

An additional uncertainty factor for limited evidence of carcinogenicity in animals was not considered necessary.

## Rationale

The maximum acceptable concentration (MAC) for microcystin-LR is calculated from the TDI, as follows:

$$\text{MAC} = \frac{0.04 \mu\text{g/kg bw per day} \times 70 \text{ kg bw} \times 0.80}{1.5 \text{ L/d}} \approx 1.5 \mu\text{g/L}$$

where:

- 0.04  $\mu\text{g/kg bw per day}$  is the TDI, as derived above
- 70 kg bw is the average body weight of an adult
- 0.80 is the proportion of total intake considered to be ingested in drinking water
- 1.5 L/d is the average daily consumption of drinking water for an adult.

The MAC of 1.5  $\mu\text{g/L}$  for microcystin-LR is believed to be protective against exposure to other microcystins (total microcystins, i.e., free plus cell bound) that may also be present. It is a conservative value, as it is derived on the basis of daily consumption of microcystin-LR over a full year. However, as there are spatial and temporal variations in the levels of microcystins within supplies and as there are also likely to be other microcystins present that could go undetected, this value is considered appropriate.

Also, as a precautionary note, dialysis centres should be aware or informed if the source water from their local treatment plant is prone to blue-green algal blooms so that they may undertake to provide additional treatment of the water, if necessary. This treatment can range from GAC filtration followed by reverse osmosis to much more complex membrane filtration systems (e.g., ultrafiltration). The extent of additional treatment will depend entirely on the quality of the municipal water supply. Continuous monitoring of performance and equipment will be required to ensure adequate quality of the water. As well, it is important that all manufacturers' specifications be evaluated for the local conditions.

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# Annex A: Stepwise Protocol for Microcystin-LR in Water Supplies Used for Human Consumption

## Preamble

Summer conditions can lead to the growth of blue-green algae in bodies of water (generally smaller or shallow lakes, reservoirs, sloughs or dugouts) throughout Canada. In many cases, blooms tend to recur within the same bodies of water year after year. While most species of blue-green algae are capable of producing nerve and liver toxins, not all blue-green algal blooms produce toxins, and, when present, the amount of toxins varies dramatically within the body of water and over time.

Analytical studies over the past few years in dugouts and other water supplies in Manitoba, Saskatchewan and the Peace River region of Alberta indicate that blue-green algal toxins are much more common in rural water supplies than originally thought. Although there are few quantitative data available, there are indications that these toxins may also be occurring in various water supplies in other provinces (e.g., Ontario, British Columbia, Quebec and Prince Edward Island).

The factors inducing the production of toxins by cyanobacteria are not well known. Laboratory studies demonstrate that some environmental factors, such as temperature, light, nitrogen concentrations, carbon availability (in the form of bicarbonate, carbonate and carbon dioxide), phosphate concentrations and pH, could be important. As toxin production varies greatly among different strains of the same species, genetic differences and metabolic processes may also be important in the production of these secondary metabolites. Studies have shown that the ability to produce toxins can vary temporally and spatially at a particular site.

Cyanobacterial toxins tend to be associated with cyanobacterial cells and may be membrane bound or occur free within the cells. In laboratory studies, most of the toxin release occurs as cells age and die and passively leak their cellular contents; some active release of toxins can also occur from young, growing cells.

Toxin levels do not necessarily coincide with maximum algal biomass; there can be significant variation in the amount of toxin per unit biomass of cyanobacteria over time, which is independent of changes in the blue-green algal population. Concentrations of

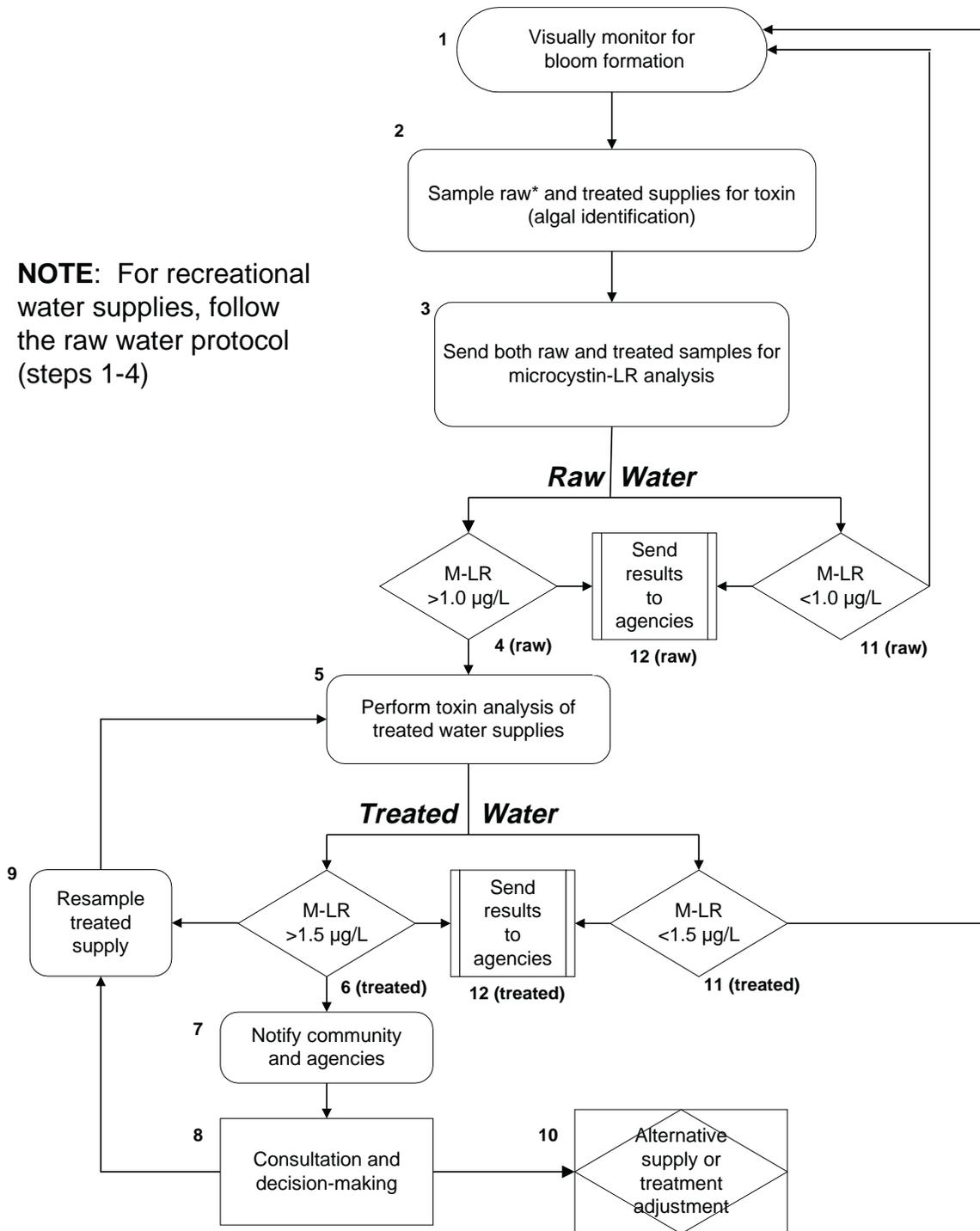
microcystins were higher in blooms taken during the day than at night in one study, for example, and no significant difference in toxin concentrations from cyanobacteria incubated for 24 hours at different depths in a reservoir was observed in another study. Microcystins are relatively persistent in the aquatic environment. Studies in Australia have shown that microcystin-LR was present up to 21 days following treatment of a *Microcystin aeruginosa* bloom with an algicide.

Recent studies have led to increasing concern by government agencies and the public regarding the safety of water supplies that may be potential sources of these toxins.

The purpose of this annex and the accompanying flow chart (see next page) is to provide water purveyors and health and environment authorities with a summary of the important factors that should be considered during bloom events and recommendations on actions that may be taken to address the issue.

**ANNEX A**  
**Cyanobacterial Toxins -- Microcystin-LR**  
**Flow Chart**  
**- Water Supplies for Human Consumption -**

**NOTE:** For recreational water supplies, follow the raw water protocol (steps 1-4)



\* A field kit could be used for screening. A validation sample should be sent to a laboratory for confirmation of actual levels following a positive field test.

**Step 1: Visually monitor for bloom formation**

As blooms tend to recur in the same water supplies, water bodies that have historically exhibited algal blooms should be visually monitored for bloom formation and hence toxin production during the peak season (usually late May to early October). Authorities should visually monitor supplies for algal growth and conduct sampling during and after collapse of the bloom. As well, bodies of water that, due to variables such as size, water depth and nutrient content, may be susceptible to algal blooms should be considered for monitoring programs. Toxins may persist following the collapse of blooms, particularly in the late summer and early fall, when the onset of colder temperatures and decrease in light intensity result in decreased rates of toxin degradation.

A visual bloom is identified by the appearance of “soupy” water. Colours can range from grey or tan to blue-green or bright blue or reddish. The appearance of blooms may also be described as fine grass clippings or small clumps.

**Step 2: Sample raw<sup>1</sup> and treated supplies for toxin (algal identification)**

Samples of raw and treated supplies (if applicable) should be taken at the same time in order to save time and money. For laboratory analysis, raw water samples should be collected prior to any treatment, including filtration; samples from the raw water tap at the treatment plant are acceptable if no pretreatment is applied. Sampling from a reservoir should be done as close to the inlet/shore and/or the bloom formation as possible. However, when possible, it is suggested that samples from several sites be pooled for the determination of toxicity, as cyanobacterial species/cell abundance and biomass vary spatially within a water body (e.g., cells may be transported by wind currents). Treated samples should be taken at the treated water tap of the treatment plant or within the distribution system.

Samples, in amounts required by the analytical laboratory, should be collected in glass containers, as studies indicate that the toxin, if present, can be adsorbed to the plastic.

Agencies may also wish to sample for algal identification. Species identification, especially from sites positive for toxin, can provide additional information regarding the source, conditions and type of other toxins that might be present.

1. A field test kit could be used as a screening tool to determine the presence or absence of toxin in a water supply. If the presence of toxin is detected in a sample using the field test kit, the sampling agency will need to submit a sample to a recognized laboratory for confirmatory analytical analysis (see Step 3).

**Step 3: Send both raw and treated samples for microcystin-LR analysis**

Both raw and treated samples should be sent (in coolers) to the laboratory for analysis as soon as possible (preferably within 24 hours). Sampling agencies should contact their local or regional departments of health or environment for information on laboratories capable of conducting toxin analyses.

Upon receipt of samples from sampling agencies and in order to avoid unnecessary costs, the laboratory should store the treated samples until the results of the microcystin-LR analysis of the raw samples are available. A microcystin-LR result of  $>1.0 \mu\text{g/L}$  in the raw sample will cause further action, as outlined in Steps 4–10.

**Step 4: Microcystin-LR  $>1.0 \mu\text{g/L}$  (raw)**

Results will be reported to the sampling agency, as per Step 12 (raw). A result of  $>1.0 \mu\text{g/L}$  should cause further action, as outlined in Steps 5–10.

**Step 5: Perform toxin analysis of treated water samples**

Testing will be performed on treated samples when a level of  $>1.0 \mu\text{g}$  microcystin-LR/L is found in a raw sample from the same site.

**Step 6: Microcystin-LR  $>1.5 \mu\text{g/L}$  (treated)**

A result of  $>1.5 \mu\text{g}$  microcystin-LR/L for the treated sample will cause the sampling agency to take appropriate action, as outlined in Steps 7–10. Microcystin-LR results of  $>1.5 \mu\text{g/L}$  in a treated supply will result in a resampling of the treated supply as soon as possible (Step 9) and notification of the community and appropriate agencies (Step 7).

**Step 7: Notify community and agencies**

Upon receiving the results, the sampling agency will follow standard protocol for notifying communities and appropriate agencies. Consideration will be given as to which agency should be taking the lead role in this regard. Additionally, the investigation should take into account the history of the source/supply and the type of treatment at the plant. Dialysis treatment units in the community should also be notified, especially if it is a first-time occurrence for blooms on this supply. A Health Canada fact sheet on microcystin-LR (*It's Your Health: Blue-Green Algae (Cyanobacteria) and their Toxins*) is available to help convey information to communities.

**Step 8: Consultation and decision-making**

In the case of community (municipal) supplies, risk assessment discussions should take place regarding additional action to be taken. Health agencies, municipal councils and the lead regulatory agency responsible for

municipal systems should be included in these discussions. The risk assessment discussion process should include, for example, such items as the history of the site, the size and location of the bloom, available treatment technology (if a treated site), uses of the source water (recreational versus domestic uses) and monitoring of the environmental conditions that might affect the bloom (e.g., wind). Where possible, lysing of the bloom by the addition of copper sulphate or Blue Stone should be avoided, as this action will immediately release toxin from the cells into the water supply. If the bloom is a common occurrence, nutrient monitoring may be considered. Weekly monitoring should be continued.

In the case of non-community (non-municipal) supplies, the sampling agency will consult with health agencies and agencies having treatment expertise.

**Note:** See Step 10.

#### **Step 9: Resample treated supply**

Following receipt of results indicating that the treated water contains  $>1.5 \mu\text{g}$  microcystin-LR/L, the treated supply will be resampled as soon as possible or as determined by the sampling agency.

#### **Step 10: Alternative supply or treatment adjustment**

During the Step 8 decision-making process, discussions regarding treatment adjustments or alternative supplies should be reviewed (Step 10). The lead agency should advise the owner of the supply of any treatment options, such as additional granular activated carbon filtration or ozonation. It should be pointed out that boiling is not effective in reducing or removing these toxins, although some point-of-use devices may be effective.

**Note:** As blooms may be of short duration (ranging from days to weeks), health and environment agencies may recommend, after consultations with the community (see Step 8), that consumers seek alternative supplies of safe drinking water until there is no longer a visible bloom and the toxin level has dropped below  $1.5 \mu\text{g/L}$ .

#### **Step 11 (raw): Microcystin-LR $<1.0 \mu\text{g/L}$**

A result of  $<1.0 \mu\text{g}$  microcystin-LR/L in the raw water will cause the sampling agency to continue to visually monitor the raw water for the recurrence of blooms and resample if necessary.

#### **Step 11 (treated): Microcystin-LR $<1.5 \mu\text{g/L}$**

A result of  $<1.5 \mu\text{g}$  microcystin-LR/L in the treated water will cause the sampling agency to continue to monitor the raw water as per Step 11 (raw).

#### **Step 12 (raw): Send results to agencies**

Microcystin-LR results from laboratories (and field test kit results when available) will be reported to the sampling agency as soon as possible from time of receipt of the sample. Sampling agencies are responsible for reporting results to the water supply owner.

#### **Step 12 (treated): Send results to agencies**

Treated water microcystin-LR results will be reported to the sampling agency as soon as possible from the time of the positive raw water results for the same sampling site. Sampling agencies are responsible for reporting results to the water supply owner and/or responsible agencies.