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Cyanobacterial toxins: cylindrospermopsins

Background document for development of WHO *Guidelines for drinking-water quality* and *Guidelines for safe recreational water environments*

World Health Organization

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Preface

Access to safe drinking-water is essential to health, a basic human right and a component of effective policy for health protection. A major World Health Organization (WHO) function to support access to safe drinking-water is the responsibility "to propose ... regulations, and to make recommendations with respect to international health matters ...", including those related to the safety and management of drinking-water.

The first WHO document dealing specifically with public drinking-water quality was published in 1958 as *International standards for drinking-water*. It was revised in 1963 and 1971 under the same title. In 1984–1985, the first edition of the WHO *Guidelines for drinking-water quality* (GDWQ) was published in three volumes: Volume 1, Recommendations; Volume 2, Health criteria and other supporting information; and Volume 3, Surveillance and control of community supplies. Second editions of these volumes were published in 1993, 1996 and 1997, respectively. Addenda to Volumes 1 and 2 of the second edition were published in 1998, addressing selected chemicals. An addendum on microbiological aspects, reviewing selected microorganisms, was published in 2002. The third edition of the GDWQ was published in 2004, the first addendum to the third edition was published in 2006, and the second addendum to the third edition was published in 2008. The fourth edition was published in 2011, and the first addendum to the fourth edition was published in 2017.

The GDWQ are subject to a rolling revision process. Through this process, microbial, chemical and radiological aspects of drinking-water are subject to periodic review, and documentation relating to aspects of protection and control of drinking-water quality is accordingly prepared and updated.

Since the first edition of the GDWQ, WHO has published information on health criteria and other information to support the GDWQ, describing the approaches used in deriving guideline values, and presenting critical reviews and evaluations of the effects on human health of the substances or contaminants of potential health concern in drinking-water. In the first and second editions, these constituted Volume 2 of the GDWQ. Since publication of the third edition, they comprise a series of free-standing monographs, including this one.

For each chemical contaminant or substance considered, a background document evaluating the risks to human health from exposure to that chemical in drinking-water was prepared. The draft health criteria document was submitted to a number of scientific institutions and selected experts for peer review. The draft document was also released to the public domain for comment. Comments were carefully considered and addressed, as appropriate, taking into consideration the processes outlined in the *Policies and procedures used in updating the WHO guidelines for drinking-water quality* and the WHO *Handbook for guideline development*. The revised draft was submitted for final evaluation at expert consultations.

During preparation of background documents and at expert consultations, careful consideration was given to information available in previous risk assessments carried out by the International Programme on Chemical Safety, in its Environmental Health Criteria monographs and Concise International Chemical Assessment Documents; the International Agency for Research on Cancer; the Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Meeting on Pesticide Residues; and the Joint FAO/WHO Expert Committee on Food Additives (which evaluates contaminants such as lead, cadmium, nitrate and nitrite, in addition to food additives).

Further up-to-date information on the GDWQ and the process of their development is available on the WHO website and in the current edition of the GDWQ.

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The work of the following experts was crucial in the development of this document and others in the second addendum to the fourth edition:

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The draft text was discussed at expert consultations held on 14–16 July 2016, 28–30 March 2017 and 13–14 July 2018 on the second addendum to the fourth edition of the GDWQ or *Toxic cyanobacteria in water*. Input from all those who participated in these meetings is gratefully acknowledged. The final version of the document also takes into consideration comments from both peer reviewers and the public, as well as other experts who contributed to the text. These include N Chernoff, Environmental Protection Agency, United States of America; I Chorus, formerly Federal Environment Agency of Germany; I Falconer, Univesity of Adelaide, Australia; J Fastner, Federal Environment Agency of Germany; AM Cameán Fernández, University of Sevilla, Spain; A Foss, Greenwater Labs, United States of America; B Lampe, NSF International, United States of America; L Lawton, Robert Gordon University, Scotland; and M Welker, independent consultant, Germany.

The coordinator was Ms J De France, WHO, with support from Dr V Bhat, formerly of NSF International, United States of America. Strategic direction was provided by Mr B Gordon, WHO. Dr A Tritscher, formerly of WHO, and Dr P Verger, WHO, provided liaisons with the Joint FAO/WHO Expert Committee on Food Additives and the Joint FAO/WHO Meeting on Pesticide Residues. Dr R Brown and Ms C Vickers, WHO, provided liaisons with the International Programme on Chemical Safety. Dr M Perez contributed on behalf of the WHO Radiation Programme. Dr Andina Faragher, Biotext, Australia, was responsible for the scientific editing of the document.

Many individuals from various countries contributed to the development of the GDWQ. The efforts of all who contributed to the preparation of this document are greatly appreciated.

Acronyms and abbreviations

ALF	alert level framework
bw	body weight
CYN	cylindrospermopsin
CYP450	cytochrome P450
GD	gestational day
GSH	glutathione
GV	guideline value
i.p.	intraperitoneal
LC	liquid chromatography
LD_{50}	median lethal dose
LOAEL	lowest-observed-adverse-affect level
MS	mass spectrometry
NOAEL	no-observed-adverse-affect level
TCiW	*Toxic cyanobacteria in water* (WHO guidebook)
UF	uncertainty factor
WHO	World Health Organization

Contents

Information on cyanobacterial toxins, including cylindrospermopsins, is comprehensively reviewed in a volume to be published by the World Health Organization, *Toxic cyanobacteria in water* (TCiW; Chorus & Welker, in press). TCiW covers chemical properties of the toxins and information on the cyanobacteria producing them, as well as guidance on assessing the risks of toxin occurrence, monitoring and management. In contrast, this background document focuses on reviewing the toxicological information and other considerations for deriving guideline values for cylindrospermopsins in water. Sections 1, 2 and 7 are largely summaries of chapters in TCiW, and readers are referred to corresponding chapters in TCiW for further information, including references to original publications.

Executive summary

Cylindrospermopsin (CYN) and its variants (CYNs) are naturally occurring alkaloids produced by strains of various species of cyanobacteria, primarily in freshwater environments. Drinking-water is the most likely exposure route. Limited data suggest that CYNs may also accumulate in some food items. Country- or region-specific assessments should therefore take into account whether food may significantly contribute to exposure – particularly molluscs, fish and shellfish from bloom-ridden water bodies. Recreational activities in lakes with cyanobacterial blooms may also expose individuals intermittently to high concentrations of CYNs.

The main driver of high amounts of cyanobacterial biomass is nutrients from anthropogenic sources such as agricultural runoff and wastewater. Hence, control of these sources is the primary long-term management option. Drinking-water can usually be treated to acceptable levels by a well-run conventional treatment plant implementing coagulation, flocculation, filtration and chlorination. If this is not sufficient, ozonation and activated carbon filtration or addition of powdered activated carbon can be effective, and such steps are more likely to be necessary for removing CYNs than for other cyanotoxins because of the high proportion of CYN that is released from cells into the water and so not removed during filtration.

CYN was first identified in a cyanobacterial strain isolated from a drinking-water reservoir that was the likely cause of a mass human poisoning incident. CYN is a potent inhibitor of protein synthesis, and also has cytochrome P450 (CYP450)-dependent effects on other processes – for example, DNA damage and induction of cellular stress responses.

The provisional guideline values (GVs) for CYN (lifetime drinking-water GV: 0.7 µg/L; short-term drinking-water GV: 3 µg/L; recreational exposure: 6 µg/L) are based on studies in mice that demonstrated adverse effects in a range of organs (including liver, kidneys and testes), as well as perturbations of protein and cholesterol metabolism. The provisional GVs are based on effects in the kidneys, identified as the most sensitive organ. Limited evidence suggests that the CYN analogues 7-deoxy-CYN and 7-epi-CYN may be of similar potency to CYN. It is therefore recommended that they be included in calculations of total CYN when comparing against the GV.

The provisional short-term drinking-water GV is intended to indicate the extent to which the lifetime value can be exceeded for periods of up to 2 weeks until water treatment can be augmented to bring the concentration of CYN back under control. It is not intended to allow for repeated seasonal exceedences of the lifetime value. The short-term value is derived for adults. As a result of their higher water consumption per unit body weight, it is recommended, as a precautionary measure, that bottle-fed infants and small children be provided with an alternative water source if the lifetime GV is exceeded.

1 General description

1.1 Identity

Cylindrospermopsins (CYNs) are alkaloids produced by a number of species of cyanobacteria. Naturally occurring structural variants are 7-epi-CYN, 7-deoxy-CYN, 7-deoxy-desulfo-CYN and 7-deoxy-desulfo-12-acetyl-CYN.

All CYN variants consist of a tricyclic guanidino moiety linked via a hydroxylated bridging carbon (C7) to uracil (Fig. 1.1). The uracil moiety is required for toxicity.

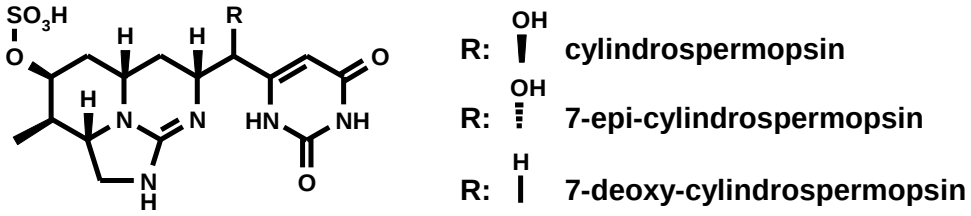

Fig. 1.1. Molecular structure of common cylindrospermopsins

1.2 Physicochemical properties

CYN molecules are zwitterionic – that is, dipolar ions with localized positive and negative charges. As a result, they are very hydrophilic and highly soluble in water, with saturation concentrations several orders of magnitude higher than the World Health Organization (WHO) guideline values (GVs) (see section 8.1). Known physicochemical properties are summarized in Table 1.1. A pKa of 8.8 for CYN was estimated using a computer program that estimates physical properties based on structure–activity relationships (Onstad et al., 2007).

The molecular extinction coefficient (ε) of CYN has been inconsistently reported, ranging from 5800/M/cm (Ohtani, Moore & Runnegar, 1992) to 9800/M/cm (Sano et al., 2008) at maximum absorbance of 262 nm. The most recent ε (9800/M/cm) was confirmed using certified reference material (Sano et al., 2008) and has been applied to more recent investigations (Chernoff et al., 2018). Therefore, concentrations of CYN published before 2008 are likely to have been lower than reported, if they were determined using spectrophotometry.

Table 1.1 Properties of common cylindrospermopsins

Property	Cylindrospermopsin	7-deoxy-cylindrospermopsin
CASRN	143545-90-8	NA
Chemical formula	$C_{15}H_{21}N_5O_7S$	$C_{15}H_{21}N_5O_6S$
Average molecular weight[a] (g/mol)	415.42	399.42
Monoisotopic mass (Da)	415.116	399.121
Colour/physical state	White powder	White powder
K_{ow}[b]	–2.6	–1.4
Solubility in water	High	High

CASRN: Chemical Abstracts Service Registry Number; NA: not applicable

[a] Average molecular weight for natural isotopic composition calculated based on conventional atomic weights as given in Table 3 of Meija et al. (2016)

[b] K_{ow} computation with XLOGP3 (Cheng et al., 2007)

1.3 Organoleptic properties

None of the known cyanobacterial toxins (microcystins, CYNs, saxitoxins, anatoxins) have been shown to affect the taste or odour of water. However, some cyanobacterial species produce other compounds, such as geosmin and methyl-isoborneol, that do affect taste or odour, indicating the presence of cyanobacteria in raw water. As this applies only to some strains of some cyanobacterial species, the absence of these typical tastes or odours is not a reliable indicator of the absence of cyanotoxins. For an overview of the relationship between organoleptic properties and toxins, see *Toxic cyanobacteria in water* (TCiW), Kaloudis (in press).

1.4 Major uses and sources

CYN and its variants (CYNs) occur naturally, although high concentrations are typical for fresh waters influenced by human activity – for example, by wastewater or runoff from agricultural land that introduces nutrients that fertilize the growth of phototrophic organisms, including cyanobacteria. There are no known commercial applications of CYNs. CYNs are produced by strains of various species within a number of cyanobacterial genera, primarily in the order Nostocales. They have most frequently been reported from the genera *Raphidiopsis* (formerly *Cylindrospermopsis*), *Aphanizomenon* (some species of which are now classified as *Cuspidothrix* and some as *Chrysosporum*), *Anabaena* (some species of which are now classified as *Dolichospermum*) and *Umezakia*. Known CYN producers within the order Oscillatoriales include *Microseira* (formerly *Lyngbya*) and *Oscillatoria*, many of which are primarily benthic (i.e. grow on sediments or other submerged surfaces). For more informaton on the new classification of genera, see TCiW, Vidal et al. (in press). Not all strains within potentially toxigenic species produce CYNs. Interestingly, the distribution of CYN-producing strains of some species follows a geographic pattern, which has changed in recent decades: whereas *Raphidiopsis raciborskii* is a major producer in Australia, New Zealand and Asia, strains of this species from Europe, the Americas and Africa have not been found to synthesize CYNs. In Europe, CYN production is largely confined to *Aphanizomenon* spp. and *Dolichospermum* spp.

Among CYN-producing strains, the relative share of the CYN structural variants produced varies. Most strains produce CYN as the highest share, but in rare cases more deoxy-CYN is

produced. Where CYNs occur, CYN is dominant in most field samples, but occasionally deoxy-CYN is dominant, probably reflecting the clonal composition of the bloom. The CYN content is highly variable between individual strains, ranging from trace amounts up to 10 mg/g dry weight across all producing species and geographical regions. Amounts of CYN reported in planktonic species range from <10 to 190 fg/cell, and from 0.3 to 3.5 µg/mm^3 of biovolume.

The biosynthesis of CYN has been largely elucidated. It starts with an amidinotransferase and is completed by nonribosomal peptide synthetases, polyketide synthases and tailoring enzymes. The genes for biosynthesis are organized in a large cluster (*cyrA-O*, approximately 44 kbp) that is known from several species (*Raphidiopsis raciborskii*, *Chrysosporum ovalisporum*, *Aphanizomenon* spp., *Oscillatoria* spp., *Raphidiopsis curvata*). The regulation of gene transcription and hence CYN production is thought to be coupled to the cells' nitrogen metabolism. The presence of the biosynthesis cluster in the genome of a particular strain is a strong indicator of CYN production – that is, CYN is synthesized constitutively by toxigenic strains.

An intermediate of CYN biosynthesis, guanidinoacetate, is known to be toxic and accumulates in individual CYN-producing strains. It may contribute to the total toxicity.

For more details on CYN-producing organisms and biosynthesis, see TCiW, Humpage & Fastner (in press).

2 Environmental levels and human exposure

2.1 Air

CYNs are not volatile, and so exposure via inhalation is possible only through spray carrying cyanobacterial cells or toxins – for example, via overhead irrigation, during storms or in the wake of a power boat. No data on inhalation exposure or concentrations in sprays were found.

2.2 Food

Data on food items potentially contaminated with CYNs – including fish, shellfish and edible crops – are scarce, and systematic monitoring is not undertaken. In addition, the reliability of some of the methods used to study CYN in food is under question (Testai et al., 2016).

After transfer to water free from CYNs, fish readily eliminate the toxins. CYNs are not degraded by boiling, and so cooking is not likely to reduce their concentration in food. No field data are available on CYN occurrence in crops; however, in experimental settings, transfer of CYN from roots to leaves was observed. CYNs have not been reported from algal dietary supplements.

For more information on CYNs in food, see TCiW, Ibelings, Foss & Chorus (in press).

2.3 Water

Since CYN-producing cyanobacteria are found primarily in freshwater environments, in many settings the primary waterborne route of human exposure to CYN is the consumption of drinking-water, if it is produced from surface waters that are untreated or insufficiently treated. Another exposure route – important in some settings – is the recreational use of lakes and rivers. Depending on the seasonal patterns of cyanobacterial blooms and water body use, patterns of exposure may be episodic. Blooms of most CYN-producing cyanobacteria show an intermediate tendency to form surface scums.

CYNs have been detected in surface waters around the globe. The in situ CYN concentration is largely coupled to the abundance of producing organisms. Highest CYN concentrations occur during peak cyanobacterial blooms in water. However, in contrast to other cyanobacterial toxins, particularly microcystins, up to 90% of the total CYN in surface water is in the dissolved fraction, rather than intracellular, through release of CYN from viable cells. In laboratory experiments, the release of CYN and the proportion of dissolved CYN relative to the total CYN pool exceeded 50%, but the mechanisms and the regulation of this process are not understood. CYN concentrations in a water body may remain high long after the producing species has disappeared – for example when the water temperature has been low for weeks. Degradation experiments with bacterial consortia showed CYN to resist microbial degradation for several weeks. Hence, the concentration of dissolved CYN can be high relative to the cell-bound CYN concentration, exceeding the GV. This is highly relevant for drinking-water treatment (see below).

In most surveys, CYN concentrations in surface waters, particularly open waters, are in the low µg/L range (usually well below 10 µg/L and often below the limit of quantification) and only occasionally up to 800 µg/L. CYNs probably do not often reach the high concentrations observed for microcystins because the main producing organisms (*Raphidiopsis*, *Aphanizomenon*, *Dolichospermum*, *Chrysosporum*) rarely form the massive scums caused by *Microcystis* blooms that lead to an increase in concentrations by orders of magnitude. Globally, CYNs are detected less frequently than microcystins, presumably due to the less frequent occurrence of CYN-producing strains in surface waters. However, the number of extensive surveys including all known cyanotoxins is low; CYNs may well occur more frequently on a regional or local scale. This seems to be the case in tropical and subtropical regions and has been reported from other regions (e.g. northern Germany).

CYN concentrations in finished drinking-water up to 97 µg/L have been reported, although concentrations detected are usually at trace levels (usually well below 0.7 µg/L). However, the number of investigations of CYNs in drinking-water is quite limited. CYN in raw water could potentially break through treatment into finished drinking-water, especially because dissolved CYN can pass filtration or flocculation steps in the treatment process.

Recreational activity in surface waters with cyanobacterial blooms can cause exposure to CYN (and other toxins in blooms and scums), mainly through unintentional swallowing of water, particularly where surface blooms or scums accumulate. Although high levels of CYN in scum have not been reported, some CYN-producing cyanobacteria (e.g. *Aphanizomenon*, *Dolichospermum*) can form scums. Recreational activity typically takes place at near-shore sites where surface blooms or scums accumulate.

Inhalational exposure may be a relevant pathway for specific recreational activities, such as waterskiing or jet-skiing, and for specific occupational situations involving spraying with water containing bloom material, such as spray irrigation or dust suppression. Therefore, recreational activity and in some cases occupational activity may be a potentially substantial exposure route, although in most cases for a limited time.

For more information on CYN occurrence in the environment and drinking-water, see TCiW, Humpage & Fastner (in press) and Humpage & Cunliffe (in press).

2.4 Estimated total exposure and relative contribution of drinking-water

Where surface water is used as the source for drinking-water, this is the most likely source of exposure to CYNs. However, this assumption is a starting point, and country- or region-specific assessments should take into account whether food may significantly contribute to exposure – particularly molluscs, fish and shellfish from bloom-ridden water bodies. Recreational activities in lakes with cyanobacterial blooms may also expose individuals to high concentrations of CYNs as described in section 2.3. For most situations for the general population, the oral route is the main route of concern.

Patterns and duration of exposure are strongly influenced by region and lifestyle. Estimating total exposure or the relative contribution of particular exposure routes (e.g. food, drinking-water) requires specific analyses of CYN concentrations in samples from the respective media in a given setting. Exposure may be higher in tropical regions where blooms of CYN-producing cyanobacteria may persist for extended periods and where there is greater consumption of local freshwater fish and/or shellfish. Chapter 5 of TCiW gives further guidance and background information on assessing routes of exposure.

For specific population groups, exposure may occur via the parenteral route – for example, associated with use of contaminated water for haemodialysis or infusions. Risks are potentially high if water from contaminated surface waters is used for haemodialysis, which was the major source of exposure to microcystin and possibly CYN (including some lethal exposures) documented in the cases discussed in TCiW, Azevedo (in press).

3 Kinetics and metabolism in humans and laboratory animals

3.1 Absorption

Information on absorption of CYNs in humans was not identified. Following oral dosing, gastrointestinal absorption of CYN is inferred by effects on the liver and other tissues in mice (Shaw et al., 1999, 2000; Shaw, Seawright & Moore, 2001; Humpage & Falconer, 2003) and in fish (Guzman-Guillén et al., 2014). Since CYNs are small hydrophilic molecules, intestinal absorption may occur through a combination of mediated transport and passive diffusion mechanisms. In vitro investigations showed that the integrity of monolayers of Caco-2 (human tissue culture) cells was not compromised by CYN at high concentrations (20 µg/mL; 8.3 M), but CYN permeation across the Caco-2 cell monolayer was limited (Fernández et al., 2014; Pichardo, Cameán & Jos, 2017). Information about the activity of putative transporters in the Caco-2 cells was not reported. Since evidence also supports paracellular passage of CYN (via diffusion) rather than transcellular transport, Caco-2 studies may underestimate intestinal CYN permeation – this is because the cell junctions in these monolayers are tighter than in the human intestine (Pichardo et al., 2017). Additional observations with Caco-2 cells indicate the possible involvement of active transport in the secretory direction (not absorptive), and a minor transcellular-facilitated (but not active) absorptive transport, which could be glutathione (GSH) and H^+ dependent (Pichardo et al., 2017).

Absorption through skin has not been quantitatively investigated for CYN. If CYN was aerosolized, in vitro evidence suggests that it may directly damage and pass through upper respiratory tract cells (Kubickova et al., 2019). After intratracheal administration of semi-pure CYN (70 µg/kg body weight [bw]) to BALB/c mice, CYN was detected in lung cytosol after 24 hours and in liver cytosol at 96 hours, indicating that in vivo absorption had occurred, but the mechanisms involved are unknown (Oliveira et al., 2012).

3.2 Distribution

Following oral exposure of laboratory animals to purified CYN, a range of tissues is affected, including liver, kidneys, heart, thymus and intestines (Terao et al., 1994; Humpage & Falconer, 2003; Bazin et al., 2010). This implies systemic distribution of CYN.

Tissue distribution of CYN was assessed via intraperitoneal (i.p.) injection of [^{14}C]-labelled CYN (0.2 mg/kg bw) in male Quakenbush mice (Norris et al., 2001). Six hours after i.p. injection, radiolabelled CYN was detected in all examined specimens (liver, kidney, heart, lung, spleen and blood), except bile. Radiolabelled CYN was mainly recovered in the liver (21%) and kidney (4.3%). By 5–6 days, these recoveries were 3% and 0.2%, respectively, with high variation between animals. A week after dosing, about 2% of the label was still detectable in the liver (Norris et al., 2001).

Once CYN is absorbed into the circulatory system, mechanisms for uptake into the different tissues are poorly understood. Two bile acids (cholate and taurocholate) added to culture media of hepatocytes with CYN (800 ng/mL) provided some protection from cytotoxicity at 48 hours, but no protection at 72 hours (Chong et al., 2002). Furthermore, cytotoxic effects were observed in a cell line (KB) not expressing a bile acid transport system (Chong et al., 2002). Energy-independent uptake into renal (Vero) cells has been shown to be slow and progressive (Froscio et al., 2009).

Distribution of CYN in fish was investigated using immunochemistry techniques following administration (i.p. or gavage) of 200 µg/kg bw to tilapia (*Oreochromis niloticus*) (Guzman-Guillén et al., 2014). Five days after exposure, immunostaining was highest in the liver, followed by kidney, intestines and gills, for both routes of exposure.

3.3 Metabolism

After i.p. administration of [^{14}C]-labelled CYN (0.2 mg/kg bw) to mice, most of the urinary radiolabelled CYN (about 76%, corresponding to around 50% of the administered dose) was associated with the parent compound (Norris et al., 2001). This indirectly indicates that about half of the parent compound undergoes biotransformation. A radiolabelled compound that was more hydrophilic than CYN was recovered from both urine and liver, although high variability between individuals was observed (Norris et al., 2001).

Laboratory studies provide some indirect evidence that cytochrome P450 (CYP450) enzymes are involved in the metabolic activation of CYN to a more cytotoxic compound, both in vitro (Froscio et al., 2003) and in vivo (Shaw, Seawright & Moore, 2001; Norris et al., 2002). The hypothesis is supported by the localization of hepatic toxicity mainly in the periacinar region, which is also where substantial CYP450-mediated xenobiotic metabolism occurs, and by the in vivo and in vitro effects of inducers and inhibitors of CYP450 on CYN toxicity (Norris et al., 2002; Humpage et al., 2005; Bazin et al., 2012). In Caco-2 cells, the presence of ketoconazole (a CYP450 inhibitor) reduced cytotoxicity and genotoxicity to 50%; in HepaRG cells (a metabolically competent human hepatic cell line), the presence of ketoconazole prevented micronucleus induction and led to significant protection from CYN cytotoxicity at all tested concentrations (Bazin et al., 2010).

However, specific CYN metabolites have not yet been identified. An investigation using HepaRG cells, as well as rat and human liver tissue fractions, failed to identify any phase I metabolites using untargeted liquid chromatography – high resolution mass spectrometry (LC-HRMS) and tandem mass spectrometry (MS/MS) analyses (Kittler et al., 2016). Targeted LC-

MS/MS analysis resulted in the near complete recovery of unmetabolized CYN (only 9% was lost). Co-treatment of HepaRG cells with CYN and ketoconazole (a CYP3A4 inhibitor) resulted in a slight, but statistically significant, decrease in cytotoxicity. The authors hypothesized that ketoconazole could inhibit the cellular uptake of CYN, but inhibition of formation of low (undetectable) levels of CYP450-mediated metabolites, more toxic than the parent compound, cannot be excluded (Funari & Testai, 2008; Buratti et al., 2017). When male Quakenbush mice were pre-treated with piperonyl butoxide (an inhibitor of CYP450) before i.p. administration of CYN (0.2 mg/kg bw), a significantly higher 7-day survival rate was observed, further implicating CYP450 as playing a role in CYN toxicity (Norris et al., 2002). Additional work is needed to confirm CYN bioactivation and to identify phase I metabolites.

Phase II metabolism has not been directly investigated. The observed reduction in GSH levels following CYN administration in vivo and in vitro has been hypothesized to be due to inhibition of GSH synthesis or conjugate formation (Runnegar et al., 1995, Norris et al., 2002), but neither mechanism has been confirmed.

3.4 Elimination

Limited studies have examined elimination routes for CYN. Information on the elimination or excretion of CYN in exposed humans was not identified. A single laboratory animal study quantified the excretion of CYN following i.p. dosing in rodents. When male Quackenbush mice were treated i.p. with either a sublethal dose (0.1 mg/kg bw) or a median lethal dose (0.2 mg/kg bw) of [^{14}C]-CYN, the majority (about 73%) of radiolabelled CYN was excreted in the first 12 hours, primarily through the urine (about 65% of the administered dose) (Norris et al., 2001). However, variability between individuals was seen in excretion patterns; faecal [^{14}C]-CYN excretion ranged from 0.7% to 55.8% (average 8.1%).

4 Effects on humans

4.1 Acute exposure

In the USA and Australia, several different cyanobacterial toxins have been implicated as the cause of human illness from certain municipal water supplies, often after algal blooms had been treated with copper sulfate (Bourke et al., 1983; Falconer, Beresford & Runnegar, 1983; Ressom et al., 1994). In most cases, the cyanobacteria involved were identified (and sometimes the toxins), but the levels of toxin associated with illness have not been established in any outbreak. The Palm Island mystery disease in Australia, affecting about 140 people (mostly children), occurred after a dense cyanobacterial bloom in a water supply reservoir was treated with copper sulfate. Within a week, severe illness was seen in consumers of the treated drinking-water, characterized by vomiting, hepatomegaly and kidney dysfunction, with loss of electrolytes, glucose and plasma protein; recovery took 1–3 weeks (Byth, 1980). The cause of illness was not initially identified. Where blooms were treated with copper sulfate, high copper concentrations may explain symptoms such as diarrhoea, vomiting, stomach cramps and nausea; however, this would require concentrations above the 1–2 mg/L at which it is used as an algicide (see WHO, 2017, for a discussion of copper toxicity and derivation of GVs). A subsequent cyanobacterial bloom in the same water supply reservoir was shown to be highly toxic to mice (Hawkins et al., 1985). The causative organism was *Cylindrospermopsis raciborskii*, from which CYN was later isolated and characterized (Ohtani, Moore & Runnegar, 1992). An illness called Barcoo fever afflicting early settlers in Australia may also have been caused by consumption of *C. raciborskii*-contaminated water (Hayman, 1992).

CYN, as well as microcystin, was detected in water treatment filters from a dialysis clinic where 100 people developed liver failure following intravenous exposure to poorly treated water containing high levels of cyanobacteria (Jochimsen et al., 1998; Pouria et al., 1998; Carmichael et al., 2001). Some elements of the pathology, such as the presence of multinucleated hepatocytes and an unexplained coagulinopathy, were consistent with CYN-induced effects (Jochimsen et al., 1998; Pouria et al., 1998). However, the role of CYN in the etiology of the illness cannot be properly assessed because analysis of microcystins in tissue samples was prioritized, and the extraction method may not have been optimal for analysis of CYN (Carmichael et al., 2001).

Effects following dermal exposure to CYN-containing cyanobacterial cells were evaluated using skin-patch testing in humans (Pilotto et al., 2004; Stewart et al., 2006a). Exposed individuals showed mild skin irritation, but no statistically significant dose–response relationship was found between skin reactions and increasing cell concentrations for either whole or lysed cells; there was also no time–response relationship (Pilotto et al., 2004). No detectable skin reactions were seen in individuals exposed to lyophilized *C. raciborskii* (Stewart, Schluter & Shaw, 2006).

CYN was detected in water in two of 11 disease outbreaks associated with algal blooms that were described by the United States Centers for Disease Control among users of freshwater lakes in 2009 and 2010; however, the contribution of CYN to the symptoms (nausea, vomiting, diarrhoea, abdominal cramps and anorexia) could not be determined because other cyanotoxins (microcystin and anatoxin-a) were also present (Hilborn et al., 2014).

4.2 Long-term exposure

There have been no studies of long-term human exposure to CYN.

5 Effects on experimental animals and in vitro systems

Most animal studies on CYN have used extracts of the producing cyanobacteria – usually *Raphidiopsis raciborskii* (formerly *Cylindrospermopsis raciborskii*) or *Chrysosporum ovalisporum* (formerly *Aphanizomenon ovalisporum*). This is problematic for understanding CYN toxicity because many studies have suggested that some effects in animals caused by extracts of *C. raciborskii* cannot be explained by the known CYN content of the extracts (Falconer et al., 1999; de la Cruz et al., 2013).

5.1 Acute exposure

Cattle deaths have been attributed to consumption of *Cylindrospermopsis raciborskii*-contaminated water, although toxin analyses were not conducted (Thomas et al., 1998).

A number of studies have investigated the acute oral toxicity of extracts of *C. raciborskii* (Falconer et al., 1999; Shaw et al., 2000; Falconer & Humpage, 2001; Shaw, Seawright & Moore, 2001), but none of these studies have used purified CYN; this limits the ability to estimate a median lethal dose (LD_{50}). In male MF1 mice, the lowest lethal gavage dose of extracts of *C. raciborskii* strains PHAWT/M or PHAWT/1 was 4.4 mg/kg bw (as CYN-equivalent); the highest nonlethal 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 histopathology showed effects on the liver, spleen, thymus, heart, oesophagus, gastric mucosa and ocular orbits (Seawright et al., 1999). When Quackenbush mice were given oral doses of *C. raciborskii* strain AWT 205 extract, mortality occurred in 2/4 mice at 6 mg/kg bw (CYN-equivalent) (in

5 days) and 4/4 mice at 8 mg/kg bw (in 24–48 hours) (Shaw et al., 2000; Shaw, Seawright & Moore, 2001).

Single i.p. doses of purified toxin (Ohtani, Moore & Runnegar, 1992), or toxic extracts from *C. raciborskii* (Falconer et al., 1999; Shaw et al., 2000; Shaw, Seawright & Moore, 2001) or *Umezakia natans* (Terao et al., 1994) induced a progressive toxicity over at least 7 days. Pathological lesions were observed in the liver, kidney, adrenal gland, lung, intestine, thymus and heart. The reported estimates of the i.p. LD_{50} derive from the only study using purified CYN (Ohtani, Moore & Runnegar, 1992) and varied with observation time: 2.1 mg/kg bw after 24 hours, and 0.2 mg/kg bw at 120–144 hours (5–6 days). However, the primary focus of this study was structural characterization of CYN, and the LD_{50} value was stated in a footnote without any further experimental detail. The data are in line with results obtained with freeze-dried cyanobacterial extracts: an i.p. LD_{50} at 7 days of 0.18 mg/kg bw (CYN-equivalent). When the same extract was administered via the oral route, the LD_{50} was 4.4–6.9 mg/kg bw after 2–6 days, indicating a lower toxicity (Seawright et al., 1999). The difference between the LD_{50} values obtained following oral and i.p. administration are likely due to kinetic differences. For example, absorption might differ because passage through the gastrointestinal mucosa is bypassed by i.p. injection, meaning that CYN will be directly available for internalization into liver and other organs (Buratti et al., 2017).

The CYN variant 7-epi-CYN was shown to be as potent as CYN by mouse bioassay (methodology not reported; Banker et al., 2001). When three Quackenbush mice were dosed i.p. with deoxy-CYN at 0.8 mg/kg bw, no histological signs of hepatotoxicity were seen after 5 days of observation, suggesting that deoxy-CYN is at least 4-fold less toxic than CYN (Norris et al., 1999). However, results from in vitro studies indicated similar potency to CYN (Looper, Runnegar & Williams, 2005; Neumann, Bain & Shaw, 2007). Further in vivo studies are required to elucidate the toxicity of these CYN analogues.

Information on acute toxicity is also available from intratracheal injection of semipurified extracts in mice at a dose of 70 µg/kg bw. Effects observed were impaired lung mechanics, associated with parenchymal inflammation and production of inflammatory mediators (e.g. interleukins IL-1b and IL-6, and neutrophil chemokines), induced alveolar collapse, polymorphonuclear cells, fibre deposition and oxidative stress (Oliveira et al., 2012, 2015a, b).

Lyophilized extracts from CYN-producing cyanobacteria have been associated with moderate skin irritation and skin sensitization potential (Törökné, Palovics & Bankine, 2001). However, cell components other than CYN could be the major causative agent since, in a mouse ear swelling test, purified CYN produced a response in only 22% of animals, whereas a *C. raciborskii* suspension produced a response in 80% of animals (Stewart et al., 2006b) .

5.2 Short-term exposure

In mice given daily gavage doses of purified CYN for 14 days, the no-observed-adverse-affect level (NOAEL), based on fatty infiltration of the liver, was 50 µg/kg bw/day and the lowest-observed-adverse-affect level (LOAEL) was 150 µg/kg bw/day (Shaw, Seawright & Moore, 2001). Mice that were given 600 µg/L of purified CYN in drinking-water for 3 weeks (estimated daily CYN dose of 66 µg/kg) showed increased liver and testes weights, whereas the urine excretion rate decreased. Liver cholesterol was significantly reduced by 29% compared with the control, whereas cholesterol in the plasma and erythrocyte membranes was significantly increased, by 23% and 14%, respectively (Reisner et al., 2004).

5.3 Long-term exposure

No chronic studies of CYN were identified. Subchronic exposure studies with CYN extracts have been conducted in mice via drinking-water exposure for 10 or 42 weeks, or gavage exposure for 11 weeks (Humpage & Falconer, 2002, 2003). In male Swiss albino mice given daily gavage doses of purified CYN for 11 weeks, the NOAEL was 30 µg/kg/day, and the LOAEL was 60 µg/kg bw/day, based on statistically significant increases in relative kidney weight of 12%, 23% and 20%, respectively, in the 60, 120 and 240 µg/kg bw/day dose groups. Other statistically significant effects seen included decreased urinary protein/creatinine (approximately 50% and 60% at 120 and 240 µg/kg bw/day, respectively) and urine specific gravity (20% at 240 µg/kg bw/day), and increased relative liver weight (13% at 240 µg/kg bw/day). "Minor" liver histopathology was reported: necrotic or inflammatory foci were observed in the livers of 60% of mice at 120 µg/kg bw/day and 90% of mice at 240 µg/kg bw/day, compared with 10% of mice in the control group; unquantified renal proximal tubule damage (at 240 µg/kg bw/day) was also seen (Humpage & Falconer, 2002, 2003).

When *C. raciborski* AWT 205 extract was given to male Swiss albino mice in drinking-water, at CYN-equivalent doses of 216, 432 or 657 µg/kg bw/day for 10 weeks, the top two doses caused reduced body weights; all doses increased relative liver and kidney weights, increased serum total bilirubin, and decreased serum total bile acids; and the top two doses decreased urine protein concentration (g/mmol creatinine) (Humpage & Falconer, 2002, 2003).

Male and female ICR mice were given culture medium from toxic *Chrysosporum* (*Aphanizomenon*) *ovalisporum* as their drinking-water for up to 42 weeks (Sukenik et al., 2006). The CYN concentration in the drinking-water was increased every 8 weeks, ranging from 100 to 550 µg/L over the dosing period, so that the estimated daily CYN doses increased from 10 µg/kg to 55 µg/kg over the 42 weeks. Relative kidney weights were significantly increased in males and females at 20 weeks (when estimated daily dose was 30 µg/kg) and at 42 weeks. Relative liver weights were increased at 42 weeks, and relative testes weights were increased in males at 42 weeks. Perturbations of cholesterol distribution and erythrocyte morphology were also observed. Results qualitatively support the findings of studies described above, confirming the liver and kidney as the major targets for CYN-induced toxicity, but cannot be used to derive a NOAEL for guideline formulation.

A detailed study of CYN toxicity in mice was reported by Chernoff et al. (2018). Male and female CD-1 mice were treated with purified CYN (>95% purity) by gavage (0, 75, 150 and 300 µg/kg bw/day in water) for 90 days. Clear differences between the sexes were seen in the pattern of toxic effects: although both sexes showed histopathological changes in the liver (including death and hypertrophy of hepatocytes, and inflammation), females showed more marked adverse effects than males. The reverse was true for renal effects: males showed significant histopathology in the cortical tubules (including dilation and basophilia, intraluminal protein accumulation, nuclear crowding, and epithelial cytoplasmic alteration) at all doses tested, whereas the females showed less marked effects and only at the two highest doses. Gene expression studies showed an up-regulation of the pro-apoptotic gene *BAX* and 60S ribosomal protein L6, which is involved in liver regeneration and expressed after liver injury. The fatty acid binding protein gene (*FABP4*), which is associated with hepatic dysfunction, was down-regulated. Genes produced in the liver and associated with inhibition of coagulation were significantly down-regulated, whereas others involved in platelet function were not altered in this 90-day oral study, although they had been in previous i.p. studies (Chernoff et al., 2014). No bleeding was seen in the 90-day study, whereas it had been in the

i.p. study, suggesting that a relatively high dose may be required to produce this effect. Significant effects were seen at 75 µg/kg bw/day in gene expression, liver and kidney body weight ratios, histopathology, and blood urea nitrogen, so a NOAEL could not be identified.

In summary, available subchronic studies indicate that the liver and kidneys are major target organs for CYN-induced toxicity in mice; structural changes in erythrocytes and a haemorrhage/coagulinopathy syndrome have also been observed. The lowest NOAEL for increased relative kidney weight was identified as 30 µg/kg bw/day.

5.3.1 Reproductive and developmental toxicity

In pregnant Wistar rats given daily gavage doses of purified CYN at 0, 0.03, 0.3 or 3 µg/kg during gestation days (GD) 1–20, no maternal toxic effects were observed (Sibaldo de Almeida et al., 2013). Furthermore, no statistically significant effects were observed on numbers of corpora lutea, implantations, resorptions, live fetuses, or visceral or skeletal malformations in the offspring,

In pregnant CD-1 mice receiving purified CYN via i.p. injections on GD 8–17, three daily doses of 64 µg/kg or greater were lethal to the majority of dams. Four of 20 dams given five daily doses of 32 µg/kg died. Relative liver weight was significantly increased in surviving dams, but there was no effect on kidney weight. No effects were seen on fetus weight, mortality, skeleton or soft tissues (Rogers et al., 2007).

In experiments in which pregnant CD-1 mice were given i.p. injections of 50 µg/kg bw/day, the incidence of mortality was much greater in dams treated during GD 8–12 (62%) than in dams treated during GD 13–17 (3%). However, the surviving pups from the GD 13–17 group had reduced birth weight and postnatal growth compared with controls or pups from the GD 8–12 group (Rogers et al., 2007; Chernoff et al., 2011, 2014).

Human chorionic gonadotropin (hCG)–stimulated progesterone production was inhibited in primary cultures of human granulosa cells exposed to a noncytotoxic concentration of CYN (1 µg/mL) for 24 hours (Young, Micklem & Humpage, 2008). However, in a second experiment, CYN up to 3 µM (1.25 µg/mL for 6-hour treatment) was not cytotoxic and did not alter production of progesterone or estrogen, with or without hCG stimulation. Protein synthesis was significantly inhibited by 3 µM CYN alone and by 0.3–3.0 µM CYN when cells were stimulated with hCG (Young et al., 2012).

5.3.2 Genotoxicity and carcinogenicity

Bacterial mutagenicity studies using *Salmonella* Typhimurium strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strains WP2 uvrA and WP2 [pKM101] did not demonstrate mutagenic activity of CYN at doses up to 10 µg/mL (Sieroslawska, 2013). However, various mammalian cell genotoxicity assays have shown effects. Human hepatocytic (HepaRG, HepG2), enterocytic (Caco-2) and lymphoblastoid (Wil2-NS) cell lines have shown increased multinucleated and binucleated cells at doses (0.5–6.0 µg/mL, depending on the study) that did not induce significant cytotoxicity (Humpage et al., 2000; Bazin et al., 2010; Štraser, Filipič & Žegura, 2011). In Wil2-NS cells, clastogenicity, indicated by fluorescent staining of centromeres in micronuclei, was statistically significant at 1.0 µg/mL (Humpage et al., 2000). DNA strand breaks were observed in primary mouse hepatocytes by the comet assay at a subcytotoxic concentration of 0.05 µM (0.02 µg/mL). Furthermore, the CYP450 inhibitors SKF525A (50 µM) and omeprazole (100 µM) prevented this effect when tested using a CYN

concentration of 0.8 µg/mL (Humpage et al., 2005). In contrast, genotoxicity was not seen in Chinese hamster ovary (CHO-K1) cells exposed to CYN at 0.5 or 1 µg/mL, despite the induction of noncytotoxic effects on cell morphology and microtubule structure (Fessard & Bernard, 2003). This negative result may possibly be due to an inability of these cells to metabolically bioactivate the toxin (Lankoff et al., 2007).

DNA strand breakage was induced in the livers of BALB/c mice in a time-dependent manner over 72 hours after a single i.p. dose of CYN at 0.2 mg/kg bw (Shen et al., 2002). Covalent binding between DNA and an adduct presumed to be CYN or a metabolite was detected in the livers of Quackenbush mouse administered a single i.p. dose (unspecified amount) of a cell-free extract of *Cylindrospermopsis raciborskii* (Shaw et al., 2000). Bazin et al. (2012) exposed male Swiss albino mice to purified CYN either by i.p. injection (50, 100 or 200 µg/kg) or by gavage (1, 2 or 4 mg/kg) and examined the occurrence of DNA damage by the comet and micronucleus assays in various organs 24 hours after treatment. DNA damage was detected in the colon after i.p. injection of CYN at 100 and 200 µg/kg, and in the colon and bone marrow after gavage exposure to 4 mg/kg, or 1 and 2 mg/kg, respectively.

Maire et al. (2010) investigated the in vitro carcinogenic potential of CYN using the cell transformation assay on Syrian hamster embryo cells. A significant ($P < 0.01$) increase in morphological cell transformation was seen at extremely low concentrations of CYN, ranging from 1×10^{-10} to 1×10^{-5} µg/mL. Transformation was not seen at concentrations above 1×10^{-5} µg/mL, and cytotoxicity was not seen until 0.01 µg/mL. It was hypothesized that this pattern may have been caused by a CYN-mediated CYP450 inhibition occurring at higher concentrations, as has been observed in vivo (Terao et al., 1994) and in vitro (Humpage et al., 2005).

Swiss albino mice were given up to three doses of extract of freeze-dried *Cylindrospermopsis raciborskii* AWT 205 over 6 weeks (one dose every 2 weeks; each dose estimated to contain CYN at 2.75 or 8.25 mg/kg bw), and then fed liquid food with or without the tumour promoter tetradecanoylphorbol acetate (TPA) twice a week for 30 weeks. Neoplastic changes were found in five CYN-treated mice, but not in any of the 27 control mice; however, this difference was not statistically significant. There was no pattern to the neoplastic changes – they occurred in different animals, target organs and treatment groups, thus providing equivocal evidence for carcinogenicity (Falconer & Humpage, 2001). This result was further confounded by the unusual study design and the limited number of animals tested.

5.3.3 Immunological effects

Although there have not been any specific studies on the potential immunotoxicity of CYN, a number of acute toxicity studies have reported necrotic lesions in the thymus and spleen following lethal i.p. or oral doses of CYN extract or purified toxin (Terao et al., 1994; Seawright et al., 1999; Shaw et al., 2000; Shaw, Seawright & Moore, 2001).

In addition, in vitro studies reported that inhibition of lymphocyte proliferation in human peripheral blood samples resulted from exposure to CYN at 1 µg/mL (Poniedziałek, Rzymski & Wiktorowicz, 2012). This was possibly attributable to induction of oxidative stress and resulted in a reduced capacity to fight pathogenic microorganisms (Poniedziałek, Rzymski & Karczewsk, 2014, 2015).

5.3.4 Haematological effects

Morphological changes in erythrocytes have been reported in ICR mice after 3 weeks of ad libitum exposure to CYN in drinking-water (0.6 mg/L; estimated dose of 66 µg/kg bw/day). This effect may be linked to observed changes in cholesterol levels in the liver, plasma and erythrocyte cell membrane (Reisner et al., 2004; Sukenik et al., 2006). Many studies have reported haemorrhagic lesions in a range of tissues, including the gastrointestinal tract, eye orbit, tail and vagina, following exposure to *Cylindrospermopsis raciborskii* extracts or purified CYN (e.g. Shaw et al., 2000; Chernoff et al., 2011, 2014, 2018). The cause of these lesions has not been further investigated.

5.4 Mode of action

Based on available studies, the liver, kidneys and erythrocytes may be important targets of CYN toxicity; however, the mode of action for CYN-mediated toxicity has not been fully elucidated. Although not clearly understood, more than one mode of action may be involved in liver toxicity (US EPA, 2015a). The mode of action may depend on the magnitude and frequency of dose, exposure duration, life stage, age or sex of animals, and duration of observation of animals following dosing (Buratti et al., 2017; Pichardo, Cameán & Jos, 2017).

Inhibition of protein synthesis is involved in liver toxicity (Terao et al., 1994; Froscio et al., 2003). This effect is not decreased by broad-spectrum CYP450 inhibitors, suggesting that it is mediated by the parent compound (Froscio et al., 2003). Potent inhibition of the isolated protein synthesis apparatus by purified CYN in vitro confirms this (Froscio et al., 2008). Studies specifically investigating inhibition of protein synthesis in the kidneys are not available, although the results of the 11-week oral toxicity study in mice (Humpage & Falconer, 2003) are consistent with inhibition of protein synthesis in these organs.

Hepatocytotoxicity appears to be also partially dependent on metabolite formation, likely mediated by CYP450 and other mechanisms (Norris et al., 2002; Froscio et al., 2003; Humpage et al., 2005; Kittler et al., 2016), based on the following considerations:

- Pretreatment with the CYP450 inhibitor α-naphthoflavone partially protected against CYN-induced cytotoxicity and cellular GSH depletion (Runnegar et al., 1995); ketoconazole inhibited CYN-induced cytotoxicity and genotoxicity in HepaRG and Caco-2 cells (Bazin et al., 2010; Kittler et al., 2016); and piperonyl butoxide protected mice against CYN toxicity (Norris et al., 2002).
- Liver histopathology in response to CYN exposure is mainly induced in the periacinar region, where CYP450-catalysed xenobiotic metabolism occurs (Shaw et al., 2000; Shaw, Seawright & Moore, 2001).
- CYN-induced up-regulation of genes coding for phase I enzymes (CYP1A1, CYP1B1, ALDH1A2 and CES2) and phase II enzymes (UGT1A6, UGT1A1, NAT1 and GSTM3) occurs in CYN-exposed HepG2 cells (Štraser, Filipič & Žegura, 2013).

Although these findings support the hypothesis that toxicity is metabolite mediated, all phase I enzymes examined, apart from CYP2A4, were down-regulated in CYN-exposed mice in another study (Chernoff et al., 2011), and no metabolites were detected in vitro (Kittler et al., 2016). The specific enzymes involved have not yet been identified.

The role of GSH in CYN toxicity has not yet been clearly defined. CYN decreased GSH levels, and synthesis of GSH and protein in cultured rat hepatocytes (Runnegar et al., 1994, 1995,

2002). Inhibition of GSH synthesis was considered the predominant mechanism for the reduction in GSH; other mechanisms were effectively ruled out – for example, 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 (Runnegar et al., 1995). GSH depletion occurred at nontoxic CYN concentrations and preceded the onset of observable toxicity at higher concentrations (Runnegar et al., 1994). However, in male Quackenbush mice, a single i.p. dose of CYN at 0.2 mg/kg bw induced only a small reduction in hepatic GSH that rebounded within hours. The magnitude of the decrease could not be explained by GSH conjugate formation. In contrast, in Caco-2 cells, γ-glutamylcysteine synthetase was induced, and GSH levels were higher than in controls at higher CYN concentrations (Gutiérrez-Praena et al., 2012a).

In vitro evidence from cultured human dermal fibroblasts and HepG2 cells suggested that induction of stress responses may be involved in the mode of action. Activation of the p53 transcription factor was observed, as well as concentration-dependent increases in mRNA levels of p53 target genes *CDKN1A, GADD45a, BAX* and *MDM2* (Bain, Shaw & Patel, 2007). Involvement of oxidative stress and formation of reactive oxygen species were observed in various studies: in a human umbilical vein endothelial cell line (HUVEC) (Gutiérrez-Preana et al., 2012a); in HepG2 cells (Ŝtraser, Filipič & Žegura, 2013); in primary rat hepatocytes, with induction of transcription of the antioxidant response element (ARE)–binding factor Nrf2 (López-Alonso et al., 2013); and in human intestinal Caco-2 cells (Gutiérrez-Praena et al., 2012b). However, a toxicogenomic study on HepG2 cells showed that, among the oxidative response genes, only catalase (*CAT*) and thioredoxin reductase (*TXNRD1*) were up-regulated (Ŝtraser et al., 2013). An in vitro study of CYN genotoxicity demonstrated DNA damage in the comet assay without significant increases in reactive oxygen species (Humpage et al., 2005). Hence, the involvement of oxidative stress in the induction of stress responses such as p53 remains uncertain.

6 Overall database and quality of evidence

6.1 Summary of health effects

Human data on the toxicity of CYNs are limited by a lack of quantitative information, and by potential co-exposures to other cyanobacterial toxins and microorganisms (US EPA, 2015a). No chronic exposure study in laboratory animals was identified. Available subchronic studies indicate that the liver and kidneys are major target organs for CYN-induced toxicity in mice; structural changes in erythrocytes have also been shown to be an end-point 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/day.

The most appropriate study for derivation of GVs for CYN is Humpage & Falconer (2003). In this study, the most sensitive effects observed were related to kidney damage (increased relative kidney weight at ≥60 μg/kg bw/day and decreased urinary protein at ≥120 μg/kg bw/day). The study identified a NOAEL and LOAEL of 30 and 60 μg/kg bw/day, respectively. The findings of Humpage & Falconer (2003) have recently been corroborated by Chernoff et al. (2018), who identified a LOAEL of 75 μg/kg bw/day for changes in gene expression, liver and kidney to body weight ratios and histopathology, and blood urea nitrogen.

6.2 Quality of evidence

The toxicological database for CYN has a number of major deficiencies that limit confidence in the conclusions that can be drawn regarding the toxic potency CYN and its effects in humans.

The majority of studies have used extracts of *Raphidiopsis* (formerly *Cylindrospermopsis*) *raciborskii* or poorly characterized toxin preparations, and the i.p. route of exposure. Other deficiencies include the lack of studies using high-purity and well-characterized toxin, acute oral studies identifying a NOAEL, pharmacokinetic studies that include identification of metabolites and their role in toxicity, oral studies corroborating effects on reproductive end-points, chronic oral studies on toxicity and carcinogenicity, and oral studies on congener comparisons.

Nevertheless, some main themes have emerged. These include:

- identification of protein synthesis inhibition, mediated by the parent compound, as a key molecular initiating event; and
- the observation that metabolic activation appears to be important in mediating some other adverse outcomes, including DNA and genomic damage in eukaryotic cells in vitro and in vivo.

7 Practical considerations

CYN and its variants are some of the cyanotoxins that are found most frequently, although, as discussed above, usually at low concentrations. Where blooms occur, concentrations of CYNs can fluctuate as a result of uneven distribution of blooms in a water body, heterogeneity of clones within blooms and variation in the amount of toxin produced by individual clones.

Chapters 7–10 of TCiW give guidance on multiple barriers to reduce cyanotoxin levels in water, including controlling nutrient loads from the catchment, managing water bodies, optimizing sites for drinking-water off-takes or recreation, applying drinking-water treatment to remove cyanobacteria and cyanotoxins, and providing information or warnings for recreational use of water bodies with blooms. TCiW includes guidance on planning, managing and documenting the measures used to mitigate cyanotoxin risks by developing a water safety plan (Bartram et al., 2009; TCiW, Chorus & McKeown, in press).

Unlike the other cyanotoxins, a variable – but often high – fraction of CYN is found outside the producing cells in the water, and it may persist after the producing cyanobacteria are no longer present. Also, some producing species do not form scums that would provide warning of their presence. These considerations necessitate including this aspect in the risk assessment and risk management approach for CYNs (see section 8).

7.1 Monitoring

Depending on a range of conditions, including climate, cyanobacteria can be present in surface waters throughout the year or as short-lived seasonal blooms; in both cases, they may produce significant concentrations of toxins. Monitoring of source waters should include assessing factors that can affect the growth of cyanobacteria, including total phosphorus, temperature, water residence time and pH (for details, see TCiW, Padisák et al., in press). On-site visual assessment for turbidity with greenish discolouration or scums – for example, using a Secchi disc to measure water transparency – and microscopy are effective, low-cost, direct methods that can trigger increased vigilance if CYN-producing cyanobacteria are observed. Monitoring over several seasons can often establish the likely occurrence and timing of favourable conditions for cyanobacterial growth, as well as the taxonomic composition and magnitude of blooms. For example, a lake with regular seasonal blooms of *Aphanizomenon* in late summer

is unlikely to shift to perennial blooms of *Raphidiopsis* from one year to the next (TCiW, Ibelings, Foss & Chorus, in press).

Monitoring programmes should be adaptive, so that sampling and analyses are increased when there is evidence of increasing amounts of cyanobacteria. For early warning and to trigger short-term management responses, alert level frameworks (ALFs) are useful both for drinking-water and for recreational water use. To trigger alerts, these frameworks primarily use levels of cyanobacterial biomass (measured as biovolume or chlorophyll *a*; Table 7.1) below which hazardous concentrations exceeding the health-based values of cyanotoxins for drinking-water (acute, short-term or lifetime) or recreational water are unlikely.

Table 7.1 Alert levels for cyanobacterial biomass indicators that trigger management responses

Alert level	Indicators of cyanobacterial biomass	
	Biovolume (mm^3/L)	Chlorophyll *a* (with cyanobacteria dominant) (µg/L)
Alert Level 1 threshold for drinking-water	0.3	1
Alert Level 2 threshold for drinking-water	4	12
Alert level threshold for recreational water use	8	24

As described in the ALF, monitoring of source waters can start with simple site inspections for appearance of visible blooms, assessing transparency using a Secchi disc. However, not all CYN producers form surface scums or strong discolouration; those that do not may be overlooked. Therefore, if the presence of cyanobacteria is suspected, microscopic examination for the presence of cyanobacteria that could potentially produce CYNs is important. As blooms develop, monitoring can be expanded to include quantitative measures of cyanobacterial biomass that could indicate potential toxin concentrations, such as cyanobacterial biovolumes or chlorophyll *a*, or direct analyses of CYN concentrations.

CYN dissolved in water may persist after the CYN-producing cyanobacteria have disappeared. Therefore, and also because concentrations associated with cyanobacterial blooms can vary substantially, toxin analyses should be performed, if possible, when CYN is suspected. The data from toxin analyses may allow restrictions of site use to be avoided or lifted where these were based on biovolume or chlorophyll *a* concentrations.

Template alert level decision trees for monitoring cyanobacteria and responding to exceedances are given in TCiW, Humpage & Cunliffe (in press) for drinking-water and in TCiW, Chorus & Testai (in press) for recreational water exposure. Further considerations for CYN monitoring in the context of ALFs are included in TCiW, Chapter 5 (in press).

7.2 Analytical methods and achievability

Analytical techniques are available for the range of parameters associated with cyanobacterial blooms and associated CYNs. The complexity, expertise requirements and costs of monitoring vary. Techniques range from relatively simple visual inspections; to testing for phosphorus, pH, Secchi disc transparency, cell numbers, species identification, biovolumes, chlorophyll *a*; to toxin analysis. Limits of quantification below 0.7 µg/L can be achieved with all established methods outlined below. For less sensitive detection methods, appropriate sample

concentration is required. More sensitive methods generally require less sample preparation, but costs per analysis tend to be higher.

For cell-bound and total (cell-bound and extracellular) CYNs, extraction (e.g. by freeze–thaw cycles) is performed before analysis. Filtration can be used to separate cells so that intracellular and extracellular fractions can be tested separately. The high hydrophilicity of CYNs means that polar extraction solvents such as water or methanol/water are effective. Solid-phase extraction using graphitized carbon can be used to concentrate CYNs to achieve lower detection limits or for sample clean-up.

Analytical methods available for CYNs include liquid chromatography (LC) coupled with absorbance detection (ultraviolet [UV], photodiode array [PDA]) and/or mass spectrometry (MS). The method with the highest specificity and sensitivity is liquid chromatography – tandem mass spectrometry (LC-MS/MS). US EPA Method 545 based on LC-MS/MS has a reported detection limit of 0.065 µg/L (Shoemaker & Dietrich, 2017). Although reference standards are available for CYN, deoxy-CYN and epi-CYN, only that for CYN is certified. ELISA (enzyme-linked immunosorbent assay) kits are commercially available, with reported limits of detection of 0.05 µg/L.

These methods were developed for analysis of water samples. Their application to more complex matrices (e.g. food, stomach/tissue contents) requires identification of matrix effects, prior clean-up, and determination of recovery rates by spiking samples with known amounts of CYNs.

Molecular tests have been developed to identify the presence of one gene fragment involved in the production of CYNs for various cyanobacterial taxa. These methods do not provide information about actual toxin production or concentrations, but can provide early warning of potential occurrence (see TCiW, Padisák et al, in press, for further information).

The choice of methods depends on local or regional accessibility, costs and, in particular, the purpose of the analyses. Purposes may include screening for risk assessment, assessing compliance with GVs and research.

For more informaton on methods for chemical analyses of CYN, see TCiW, Lawton et al. (in press).

7.3 Source control

For planktonic toxic cyanobacteria, preventing blooms in source waters is the key to long-term control of the risks they present. The most sustainable approach is to keep concentrations of plant nutrients low. Most cyanobacteria proliferate under eutrophic conditions – that is, elevated concentrations of nutrients, especially phosphorus – and total phosphorus concentrations below 20–50 µg/L (with the threshold depending on water body characteristics) will often limit the development of cyanobacterial blooms (TCiW, Chorus & McKeown, in press; Zessner & Chorus, in press).

Other measures can be applied to water bodies to mitigate cyanotoxin occurrence, including artificial water column mixing, nutrient reduction through sediment removal or treatment, and biomanipulation. Success of these measures is highly dependent on the specific conditions in the water body, as discussed in TCiW, Burch, Brookes & Chorus (in press).

Many reservoir off-take structures (towers) can take water from multiple depths to account for vertical heterogeneity. Variable off-takes enable water layers containing the highest concentrations of cyanobacteria to be avoided. If multiple off-takes are not available (e.g. in small systems), it may be possible, as a temporary measure, to siphon water from a specific depth. However, this strategy may be less effective for CYNs than for other cyanotoxins because of the large extracellular fraction of toxin.

Where conditions allow, the use of bank filtration between source waters and treatment plant inlets can be effective in removing cyanobacteria and in biodegrading dissolved CYNs (TCiW, Brookes et al., in press).

Where possible, sites for recreational activities are best located upwind of bays where scums tend to accumulate.

7.4 Treatment methods and performance

Treatment processes to reduce CYNs in drinking-water are based on two approaches: reducing cell-bound CYN by physical removal of the cells and reducing dissolved CYN. Cell-bound CYN is removable by coagulation followed by flocculation, clarification and rapid media filtration, as well as slow sand filtration or membrane filtration. Even where cell removal does not sufficiently control concentrations of dissolved CYN, it is important to reduce the high organic load caused by a substantial amount of cyanobacteria in raw water.

Filtration processes require care to avoid shear stress that may rupture cells. As cells may lyse in more acidic water, the pH should be kept above 6. Care also needs to be taken to ensure that cyanobacterial and CYN concentrates (e.g. filter backwash, sludges, sludge supernatants) are not allowed to return to the head of the filtration plant during a bloom.

To enhance flocculation, pre-oxidation is sometimes used. Depending on the amounts applied, oxidants can lyse the cells, causing toxin release; at sufficiently high concentrations, they can also degrade the released toxins (see below). However, elevated cyanotoxin concentrations (including CYN) typically occur during blooms, which cause a high organic load at the treatment plant. Oxidizing this material without prior filtration is likely to cause high concentrations of disinfection by-products, so filtration before oxidation is recommended.

Because a high proportion of the CYN is likely to be dissolved in water, advanced treatment – ozonation, powdered activated carbon (PAC) or granular activated carbon (GAC) – are more likely to be required. Dissolved CYN can be removed by adsorption onto PAC or GAC. Efficacy of removal can be influenced by the type of activated carbon, doses and points of application (PAC), contact times (PAC), flow rates (GAC) and water quality. Biological degradation of CYN during slow sand filtration and on GAC filters can be very effective, although it may require a lag phase for the degrading bacteria to establish.

Oxidation by chlorine or ozone can be effective in degrading dissolved CYN under conditions normally applied for optimal disinfection of drinking-water. However, the type and concentration of organic substance, as well as pH, strongly affect the amount of disinfectant needed. Elevated organic carbon in bloom situations will substantially increase the disinfectant demand. It is therefore important to validate the disinfectant dose and contact time under the specific conditions of the treatment train and at the point of disinfection. Other oxidants such as chloramine and chlorine dioxide are ineffective against CYN.

The treatment methods discussed above can reduce CYN concentrations to below 0.7 µg/L. However, validation of efficacy under specific local conditions is important: efficacy is highly dependent on water quality and other conditions in the treatment system. Validation may include field trials and laboratory investigations such as jar testing. Removal of CYN during blooms should be verified by monitoring of CYN in the finished drinking-water.

After effective treatment, it is important to ensure that drinking-water remains free from cyanobacterial regrowth. This can be accomplished by ensuring that any channels and storages are covered and dark, so that cyanobacteria lack the light necessary for growth. Maintaining chlorine residuals throughout the distribution system will also suppress cyanobacterial regrowth.

For further information, see TCiW, Newcombe, Ho & Capelo-Neto (in press).

8 Conclusions

8.1 Derivation of the provisional guideline values

The point of departure has been identified as the NOAEL of 30 µg/kg bw/day from the Humpage & Falconer (2003) study. By applying an uncertainty factor (UF) of 1000 (10 for interspecies variability, 10 for intraspecies variability, and 10 for the lack of chronic toxicity studies and deficiencies in the overall toxicological database), a provisional tolerable daily intake (NOAEL/UF) value of 0.03 µg/kg bw/day can be derived. The value is provisional because of deficiencies in the CYN toxicological database, essentially related to the limited availability of studies with purified toxins, lack of in vivo data on reproductive end-points and the unclear role of metabolites, especially related to potential genotoxicity. The Sukenik et al. (2006) 42-week drinking-water study provides supporting qualitative evidence for CYN toxicity, but the experimental design does not allow derivation of a robust reference value (Funari & Testai, 2008). The study by Chernoff et al. (2018) observed many of the same effects as seen previously and demonstrates that the NOAEL is below 75 µg/kg bw/day.

The toxicological database is more limited for CYN than for microcystin-LR – for example, data on reproductive effects following oral dosing are lacking. Critically, there is evidence for potential in vivo genotoxicity of CYN. However, the lack of chronic dosing studies does not affect derivation of the short-term GV. Therefore, an uncertainty factor of 3 was used to allow for these uncertainties in the derivation of the provisional short-term drinking-water GV and recreational water GV.

For deriving the provisional lifetime drinking-water GV, the fraction of exposure allocated to drinking-water was 80% (rather than the 20% default) because drinking-water is expected to be the most likely long-term source of exposure. All other default assumptions were applied as described in WHO (2009) for deriving the lifetime as well as the short-term drinking-water GVs, and WHO (2003) for deriving the recreational water GV. The GVs for CYN are considered provisional due to inadequacies in the database, as reflected in section 6.2, and the database UF of 1000 for the lifetime GV.

Calculation of provisional lifetime drinking-water GV for CYN:

$$\mathrm{GV_{chronic}} = \frac{\mathrm{NOAEL} * \mathrm{bw} * \mathrm{P}}{\mathrm{UF} * \mathrm{C}} = \frac{30 * 60 * 0.8}{1000 * 2}\,\mu\mathrm{g/L} = 0.72\ \mu\mathrm{g/L} \approx 0.7\ \mu\mathrm{g/L}$$

where

$GV_{chronic}$ = GV for chronic (lifetime) exposure

NOAEL = no-observed-adverse-effect level (30 µg/kg bw/day, based on Humpage & Falconer, 2003)

bw = body weight (default = 60 kg for an adult)

P = fraction of exposure allocated to drinking-water (80%, because other sources of exposure, such as air, food and soil, are considered minor)

UF = uncertainty factor (1000 = 10 for interspecies variation × 10 for intraspecies variation × 10 for database deficiencies, including use of a subchronic study)

C = daily drinking-water consumption (default = 2 L for an adult).

Calculation of provisional short-term drinking-water GV for CYN:

To develop a short-term GV, the same logic was applied, except that a UF of 3 was used for database limitations:

$$GV_{short-term} = \frac{NOAEL * bw * P}{UF * C} = \frac{30 * 60 * 1.0}{300 * 2} \mu g/L = 3\ \mu g/L$$

where

$GV_{short-term}$ = GV for short-term exposure

NOAEL = no-observed-adverse-effect level (30 µg/kg bw/day, based on Humpage & Falconer, 2003)

bw = body weight (default = 60 kg for an adult)

P = fraction of exposure allocated to drinking-water (default for short-term exposure = 100%, as drinking-water is expected to be the most likely source of exposure)

UF = uncertainty factor (300 = 10 for interspecies variation × 10 for intraspecies variation × 3 for database deficiencies)

C = daily drinking-water consumption (default = 2 L for an adult).

Calculation of provisional recreational water GV for CYN:

$$GV_{recreation} = \frac{NOAEL * bw}{UF * C} = \frac{30 * 15}{300 * 0.25} \mu g/L = 6\ \mu g/L$$

where

$GV_{recreation}$ = GV for recreational water exposure

NOAEL = no-observed-adverse-effect level (30 µg/kg bw/day, based on Humpage & Falconer, 2003)

bw = body weight (default = 15 kg for a child)

UF = uncertainty factor (300 = 10 for interspecies variation × 10 for intraspecies variation × 3 for database deficiencies)

C = daily incidental water consumption (default = 250 mL for a child).

8.2 Considerations in applying the provisional guideline values

The public should be informed about cyanobacterial blooms in source waters when the water is used for recreation or for producing drinking-water. This is particularly important if toxin concentrations in finished drinking-water exceed the GVs. As well, cyanobacterial blooms tend to impair the taste and odour of drinking-water, even when cyanotoxins are absent, and informing the public that the water remains safe is important to avoid people turning to other, less safe sources of water.

For recreational sites with blooms, information and warnings are particularly important. The most common situation is that monitoring cannot occur at sufficiently short time intervals (i.e. daily rather than weekly) to ensure that it captures situations with heavy scums or pronounced greenish turbidity (to the extent that one can only barely see one's feet when knee-deep in the water). Site users therefore need information about avoiding scum contact and ingestion of water under such situations. Temporary closure of sites is an option if blooms contain high toxin concentrations, exceeding the recreational water GV (for further detail, see TCiW, D'Anglada, in press). In determining toxin concentrations that trigger such responses, it is important to consider the actual site of water use (e.g. for raw water abstraction, bathing), since averaged CYN concentrations may underestimate the risk at a particular site.

The provisional GVs are based on toxicological data for CYN. The limited evidence on the relative potency of other CYN congeners suggests that they are probably similar in potency to CYN. Therefore, for assessing risk, as a conservative approach, it is suggested that the sum of of CYNs (on a molar basis) be evaluated against the GV.

In some regions, other sources of exposure besides drinking-water can be significant (see section 2.4). This includes food from locations where blooms have a long duration and there is high consumption of locally affected food items. In such situations, it may be appropriate to consider reducing the allocation factor for the lifetime and short-term drinking-water GVs based on relative exposure data for the population.

The short-term drinking-water GV is intended to provide guidance on how much the lifetime GV can be exceeded for short periods of about 2 weeks until enhanced water treatment or other measures can be implemented. It is not intended to allow repeated seasonal exceedances of the lifetime GV.

The short-term drinking-water GV is based on exposure of adults. Since infants and children can ingest a significantly larger volume of water per body weight (e.g. up to 5 times more drinking-water/kg bw for bottle-fed infants than for adults), it is recommended that alternative water sources, such as bottled water, are provided for bottle-fed infants and small children when CYN concentrations are greater than 0.7 µg/L even for short periods, as a precautionary measure.

As described in section 7.1, GVs can be used within the context of ALFs for early warning and to trigger short-term management responses. For further information on CYN monitoring relative to GVs in the context of ALFs, see TCiW, Chapter 5 (in press).

References

Azevedo SMFO (in press). Understanding exposure: renal dialysis. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Bain P, Shaw G, Patel B (2007). Induction of p53-regulated gene expression in human cell lines exposed to the cyanobacterial toxin cylindrospermopsin. J Toxicol Environ Health A. 70:1687–93.

Banker R, Teltsch B, Sukenik A, Carmeli S (2001). 7-Epicylindrospermopsin, a toxic minor metabolite of the cyanobacterium *Aphanizomenon ovalisporum* from Lake Kinneret, Israel. J Nat Prod. 63:387–9.

Bartram J, Correales L, Davison A, Deere D, Drury D, Gordon B, et al. (2009). Water safety plan manual: step-by-step risk management for drinking-water suppliers. Geneva: World Health Organization.

Bazin E, Mourot A, Humpage AR, Fessard V (2010). Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines: Caco-2 and HepaRG. Environ Mol Mutagen. 51:251–9.

Bazin E, Huet S, Jarry G, Hégarat LL, Munday JS, Humpage AR, et al. (2012). Cytotoxic and genotoxic effects of cylindrospermopsin in mice treated by gavage or intraperitoneal injection. Environ Toxicol. 27:277–84.

Bourke A, Hawes R, Neilson A, Stallman N (1983). An outbreak of hepato-enteritis (the Palm Island mystery disease) possibly caused by algal intoxication. Toxicon. 21:45–8.

Brookes JD, Burch MD, Grützmacher G, Klitzke S (in press). Managing cyanotoxin risks at the drinking water offtake. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Buratti FM, Manganelli M, Vichi S, Stefanelli M, Scardala S, Testai E, et al. (2017). Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. Arch Toxicol. 91:1049–130.

Burch MD, Brookes JD, Chorus I (in press). Assessing and controlling the risk of cyanobacterial blooms: water-body conditions. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Byth S (1980). Palm Island mystery disease. Med J Aust. 2:40–2.

Carmichael WW, Azevedo SMFO, An JS, Molica RJR, Jochimsen EM, Lau S, et al. (2001). Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environ Health Persp. 109:663–8.

Cheng T, Zhao Y, Li X, Lin F, Xu Y, Zhang X, et al. (2007). Computation of octanol−water partition coefficients by guiding an additive model with knowledge. J Chem Inform Model. 47:2140–8.

Chernoff N, Rogers EH, Zehr RD, Gage MI, Malarkey DE, Bradfield CA, et al. (2011). Toxicity and recovery in the pregnant mouse after gestational exposure to the cyanobacterial toxin, cylindrospermopsin. J Appl Toxicol. 31:242–54.

Chernoff N, Rogers E, Zehr R, Gage M, Travlos G, Malarkey D, et al. (2014). The course of toxicity in the pregnant mouse after exposure to the cyanobacterial toxin cylindrospermopsin: clinical effects, serum chemistries, hematology, and histopathology. J Toxicol Environ Health A. 77:1040–60.

Chernoff N, Hill D, Chorus I, Diggs D, Huang H, King D, et al. (2018). Cylindrospermopsin toxicity in mice following a 90-d oral exposure. J Toxicol Environ Health A. 81:549–66.

Chong M, Wong B, Lam P, Shaw G, Seawright A (2002). Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. Toxicon. 40:205–11.

Chorus I, McKeown RM (in press). Introduction to understanding and managing water use systems. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Chorus I, Testai E (in press). Understanding exposure: recreation and occupational activities. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Chorus I, Welker M, editors (in press). Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

D'Anglada L (in press). Public health surveillance, public communication and participation. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

de la Cruz AA, Hiskia A, Kaloudis T, Chernoff N, Hill D, Antoniou MG, et al. (2013). A review on cylindrospermopsin: the global occurrence, detection, toxicity and degradation of a potent cyanotoxin. Environ Sci Process Impacts. 15:1979–2003.

Falconer IR, Humpage AR (2001). Preliminary evidence for in vivo tumour initiation by oral administration of extracts of the blue-green alga *Cylindrospermopsis raciborskii* containing the toxin cylindrospermopsin. Environ Toxicol. 16:192–5.

Falconer IR, Beresford AM, Runnegar MT (1983). Evidence for liver damage by toxin from a bloom of the blue-green alge, *Microcystis aeruginosa*. Med J Aust. 1:511–14.

Falconer IR, Hardy SJ, Humpage AR, Froscio SM, Tozer GJ, Hawkins PR (1999). Hepatic and renal toxicity of the blue-green alga (cyanobacterium) *Cylindrospermopsis raciborskii* in male Swiss albino mice. Environ Toxicol. 14:143–50.

Fernández DA, Louzao MC, Vilariño N, Fraga M, Espiña B, Vieytes MR, et al. (2014). Evaluation of the intestinal permeability and cytotoxic effects of cylindrospermopsin. Toxicon. 91:23–34.

Fessard V, Bernard C (2003). Cell alterations but no DNA strand breaks induced in vitro by cylindrospermopsin in CHOK1 cells. Environ Toxicol. 18:353–9.

Froscio SM, Humpage AR, Burcham PC, Falconer IR (2003). Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environ Toxicol. 18:243–51.

Froscio SM, Humpage AR, Wickramasinghe W, Shaw G, Falconer IR (2008). Interaction of the cyanobacterial toxin cylindrospermopsin with the eukaryotic protein synthesis system. Toxicon. 51:191–8.

Froscio SM, Canon E, Lau HM, Humpage AR (2009). Limited uptake of the cyanobacterial toxin cylindrospermopsin by Vero cells. Toxicon. 54:862–8.

Funari E, Testai E (2008). Human health risk assessment related to cyanotoxins exposure. Crit Rev Toxicol. 38:97–125.

Gutiérrez-Praena D, Pichardo S, Jos Á, Moreno FJ, Cameán AM (2012a). Alterations observed in the endothelial HUVEC cell line exposed to pure cylindrospermopsin. Chemosphere. 89:1151–60.

Gutiérrez-Praena D, Pichardo S, Jos Á, Moreno FJ, Cameán AM (2012b). Biochemical and pathological toxic effects induced by the cyanotoxin cylindrospermopsin on the human cell line Caco-2. Water Res. 46:1566–75.

Guzmán-Guillén R, Gutiérrez-Praena D, de los Ángeles Risalde M, Moyano R, Prieto AI, Pichardo S, et al. (2014). Immunohistochemical approach to study cylindrospermopsin distribution in Tilapia (*Oreochromis niloticus*) under different exposure conditions. Toxins. 6:283–303.

Hawkins PR, Runnegar MTC, Jackson ARB, Falconer IR (1985). Severe hepatotoxicity caused by the tropical cyanobacterium (blue–green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju isolated from a domestic supply reservoir. Appl Environ Microbiol. 50:1292–5.

Hayman J (1992). Beyond the Barcoo: probable human tropical cyanobacterial poisoning in outback Australia. Med J Aust. 157:794–6.

Health Canada (2017). Guidelines for Canadian drinking water quality: guideline technical document – cyanobacterial toxins. Ottawa: Health Canada.

Hilborn ED, Roberts VA, Backer L, Deconno E, Egan JS, Hyde JB, et al. (2014). Algal bloom-associated disease outbreaks among users of freshwater lakes: United States, 2009–2010. MMWR Morb Mortal Wkly Rep. 63:11–15.

Humpage AR, Cunliffe DA (in press). Understanding exposure: drinking water. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Humpage AR, Falconer IR (2002). Oral toxicity of cylindrospermopsin: no observed adverse effect level determination in male Swiss albino mice. Adelaide: Cooperative Research Centre for Water Quality and Treatment.

Humpage AR, Falconer IR (2003). Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environ Toxicol. 18:94–103.

Humpage AR, Fastner J (in press). Cylindrospermopsins. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Humpage AR, Fenech M, Thomas P, Falconer IR (2000). Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mutat Res. 472:155–61.

Humpage AR, Fontaine F, Froscio S, Burcham P, Falconer IR (2005). Cylindrospermopsin genotoxicity and cytotoxicity: role of cytochrome P-450 and oxidative stress. J Toxicol Environ Health A. 68:739–53.

Ibelings BW, Foss A, Chorus I (in press). Understanding exposure: food. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Jochimsen EM, Carmichael WW, An JS, Cardo DM, Cookson ST, Holmes CEM, et al. (1998). Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. N Engl J Med. 338:873–8.

Kittler K, Hurtaud-Pessel D, Maul R, Kolrep F, Fessard V (2016). In vitro metabolism of the cyanotoxin cylindrospermopsin in HepaRG cells and liver tissue fractions. Toxicon. 110:47–50.

Kubickova B, Laboha P, Hildebrandt JP, Hilscherová K, Babica P (2019). Effects of cylindrospermopsin on cultured immortalized human airway epithelial cells. Chemosphere. 220:620–8.

Lankoff A, Wojcik A, Lisowska H, Bialczyk J, Dziga D, Carmichael WW (2007). No induction of structural chromosomal aberrations in cylindrospermopsin-treated CHO-K1 cells without and with metabolic activation. Toxicon. 50:1105–15.

Lawton L, Metcalf JS, Žegura B, Junek R, Welker M, Törökné AK, et al. (in press). Analysing cyanotoxins. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Looper RE, Runnegar MTC, Williams RM (2005). Synthesis of the putative structure of 7-deoxycylindrospermopsin: C7 oxygenation is not required for the inhibition of protein synthesis. Angew Chem Int Ed. 44:3879–81.

López-Alonso H, Rubiolo JA, Vega F, Vieytes MR, Botana LM (2013). Protein synthesis inhibition and oxidative stress induced by cylindrospermopsin elicit apoptosis in primary rat hepatocytes. Chem Res Toxicol. 26:203–12.

Maire M-A, Bazin E, Fessard V, Rast C, Humpage A, Vasseur P (2010). Morphological cell transformation of Syrian hamster embryo (SHE) cells by the cyanotoxin, cylindrospermopsin. Toxicon. 55:1317–22.

Meija J, Coplen TB, Berglund M, Brand WA, De Bièvre P, Gröning M, et al. (2016). Atomic weights of the elements 2013 (IUPAC Technical Report). Pure Appl Chem. 88:265–91.

Neumann C, Bain P, Shaw G (2007). Studies of the comparative in vitro toxicology of the cyanobacterial metabolite deoxycylindrospermopsin. J Toxicol Environ Health A. 70:1679–86.

Newcombe G, Ho L, Capelo-Neto J (in press). Controlling cyanotoxin occurrence: drinking-water treatment. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Norris RL, Eaglesham GK, Pierens G, Shaw GR, Smith MJ, Chiswell RK, et al. (1999). Deoxycylindrospermopsin, an analogue of cylindrospermopsin from C*ylindrospermopsis raciborskii*. Environ Toxicol. 14:163–5.

Norris RL, Seawright AA, Shaw GR, Smith MJ, Chiswell RK, Moore MR (2001). Distribution of 14C cylindrospermopsin in vivo in the mouse. Environ Toxicol. 16:498–505.

Norris RLG, Seawright AA, Shaw GR, Senogles P, Eaglesham GK, Smith MJ, et al. (2002). Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. Toxicon. 40:471–6.

Ohtani I, Moore RE, Runnegar MTC (1992). Cylindrospermopsin, a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. J Am Chem Soc. 114:7941–2.

Oliveira V, Carvalho G, Avila M, Soares R, Azevedo S, Ferreira T, et al. (2012). Time-dependence of lung injury in mice acutely exposed to cylindrospermopsin. Toxicon. 60:764–72.

Oliveira VR, Avila MB, Carvalho GMC, Azevedo SMF, Lima LM, Barreiro EJ, et al. (2015a). Investigating the therapeutic effects of LASSBio-596 in an in vivo model of cylindrospermopsin-induced lung injury. Toxicon. 94:29–35.

Oliveira VR, Mancin VG, Pinto EF, Soares RM, Azevedo SM, Macchione M, et al. (2015b). Repeated intranasal exposure to microcystin-LR affects lungs but not nasal epithelium in mice. Toxicon. 104:14–18.

Onstad GD, Strauch S, Meriluoto J, Codd GA, Von Gunten U (2007). Selective oxidation of key functional groups in cyanotoxins during drinking water ozonation. Environ Sci Technol. 41:4397–404.

Padisák J, Chorus I, Welker M, Maršálek B, Kurmayer R (in press). Laboratory analyses of cyanobacteria and water chemistry. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

Pichardo S, Cameán AM, Jos A (2017). In vitro toxicological assessment of cylindrospermopsin: a review. Toxins. 9:402.

Pichardo S, Devesa V, Puerto M, Vélez D, Cameán AM (2017). Intestinal transport of cylindrospermopsin using the Caco-2 cell line. Toxicol In Vitro. 38:142–9.

Pilotto L, Hobson P, Burch MD, Ranmuthugala G, Attewell R, Weightman W (2004). Acute skin irritant effects of cyanobacteria (blue-green algae) in healthy volunteers. Aust N Z J Public Health. 28:220–4.

Poniedziałek B, Rzymski P, Wiktorowicz K (2012). First report of cylindrospermopsin effect on human peripheral blood lymphocytes proliferation in vitro. Centr Eur J Immunol. 37:314–17.

Poniedziałek B, Rzymski P, Karczewski J (2014). Cylindrospermopsin decreases the oxidative burst capacity of human neutrophils. Toxicon. 87:113–19.

Poniedziałek B, Rzymski P, Karczewski J (2015). The role of the enzymatic antioxidant system in cylindrospermopsin-induced toxicity in human lymphocytes. Toxicol In Vitro. 29:926–32.

Pouria S, de Andrade A, Barbosa J, Cavalcanti RL, Barreto VTS, Ward CJ, et al. (1998). Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet. 352:21–6.

Reisner M, Carmeli S, Werman M, Sukenik A (2004). The cyanobacterial toxin cylindrospermopsin inhibits pyrimidine nucleotide synthesis and alters cholesterol distribution in mice. Toxicol Sci. 82:620–7.

Ressom R, Soong FS, Fitzgerald J, Turczynowicz L, El Saadi O, Roder D, et al. (1994). Health effects of toxic cyanobacteria (blue–green algae). Canberra: National Health and Medical Research Council.

Rogers EH, Zehr RD, Gage MI, Humpage AR, Falconer IR, Marr M, et al. (2007). The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation. Toxicon. 49:855–64.

Runnegar MT, Kong SM, Zhong YZ, Ge JL, Lu SC (1994). The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem Biophys Res Commun. 201:235–41.

Runnegar MT, Kong SM, Zhong YZ, Lu SC (1995). Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochem Pharmacol. 49:219–25.

Runnegar MT, Xie CY, Snider BB, Wallace GA, Weinreb SM, Kuhlenkamp J (2002). In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. Toxicol Sci. 67:81–7.

Sano T, Kikuchi S, Kubo T, Takagi H, Hosoya K, Kaya K (2008). New values of molecular extinction coefficient and specific rotation for cyanobacterial toxin cylindrospermopsin. Toxicon. 51:717–19.

Seawright AA, Nolan CC, Shaw GR, Chiswell RK, Norris RL, Moore MR, et al. (1999). The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska). Environ Toxicol. 14:135–42.

Shaw GR, Seawright AA, Moore MR (2001). Toxicology and human health implications of the cyanobacterial toxin cylindrospermopsin. In: de Koe WJ, Samson RA, van Egmond HP, et al., editors. Mycotoxins and phycotoxins in perspective at the turn of the millennium: proceedings of the Xth International IUPAC Symposium on Mycotoxins and Phycotoxins. Guaruja, Brazil: IUPAC and AOAC International, 435–43.

Shaw GR, Sukenik A, Livne A, Chiswell RK, Smith MJ, Seawright AA, et al. (1999). Blooms of the cylindrospermopsin containing cyanobacterium, *Aphanizomenon ovalisporum* (Forti), in newly constructed lakes, Queensland, Australia. Environ Toxicol. 14:167–77.

Shaw GR, Seawright AA, Moore MR, Lam PKS (2000). Cylindrospermopsin, a cyanobacterial alkaloid: evaluation of its toxicologic activity. Ther Drug Monit. 22:89–92.

Shen XY, Lam PKS, Shaw GR, Wickramasinghe W (2002). Genotoxicity investigation of a cyanobacterial toxin, cylindrospermopsin. Toxicon. 40:1499–501.

Shoemaker J, Dietrich W (2017). Single laboratory validated method for determination of cylindrospermopsin and anatoxin-a in ambient water by liquid chromatography/tandem mass spectrometry (LC/MS/MS). Washington, DC: United States Environmental Protection Agency.

Sibaldo de Almeida C, Costa de Arruda AC, Caldas de Queiroz E, Matias de Lima Costa HT, Fernandes Barbosa P, Araujo Moura Lemos TM, et al. (2013). Oral exposure to cylindrospermopsin in pregnant rats: reproduction and foetal toxicity studies. Toxicon. 74:127–9.

Sieroslawska A (2013). Assessment of the mutagenic potential of cyanobacterial extracts and pure cyanotoxins. Toxicon. 74:76–82.

Stewart I, Schluter PJ, Shaw GR (2006). Cyanobacterial lipopolysaccharides and human health: a review. BMC Environ Health. 5:1–23.

Stewart I, Robertson IM, Webb PM, Schluter PJ, Shaw GR (2006a). Cutaneous hypersensitivity reactions to freshwater cyanobacteria: human volunteer studies. BMC Dermatol. 6:6.

Stewart I, Seawright AA, Schluter PJ, Shaw GR (2006b). Primary irritant and delayed-contact hypersensitivity reactions to the freshwater cyanobacterium *Cylindrospermopsis raciborskii* and its associated toxin cylindrospermopsin. BMC Dermatol. 6:5.

Štraser A, Filipič M, Žegura B (2011). Genotoxic effects of the cyanobacterial hepatotoxin cylindrospermopsin in the HepG2 cell line. Arch Toxicol. 85:1617–26.

Štraser A, Filipič M, Žegura B (2013). Cylindrospermopsin induced transcriptional responses in human hepatoma HepG2 cells. Toxicol In Vitro. 27:1809–19.

Sukenik A, Reisner M, Carmeli S, Werman M (2006). Oral toxicity of the cyanobacterial toxin cylindrospermopsin in mice: long-term exposure to low doses. Environ Toxicol. 21:575–82.

Terao K, Ohmori S, Igarashi K, Ohtani I, Watanabe MF, Harada K-I, et al. (1994). Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from the blue-green alga *Umezakia natans*. Toxicon. 32:833–43.

Testai E, Buratti FM, Funari E, Manganelli M, Vichi S, Arnich A, et al. (2016). Review and analysis of occurrence, exposure and toxicity of cyanobacteria toxins in food. EFSA Supporting Publications. 13(2):998E.

Thomas A, Saker ML, Norton JH, Olsen RD (1998). Cyanobacterium *Cylindrospermopsis raciborskii* as a probable cause of death in cattle in northern Queensland. Aust Vet J. 76:592–4.

Törökné A, Palovics A, Bankine M (2001). Allergenic (sensitization, skin and eye irritation) effects of freshwater cyanobacteria: experimental evidence. Environ Toxicol. 16:512–16.

US EPA (United States Environmental Protection Agency) (2015a). Health effects support document for the cyanobacterial toxin cylindrospermopsin. Washington, DC: US EPA (EPA 820R15101).

Vidal L, Ballot A, Azevedo SMFO, Padisák J, Welker M (in press). Introduction to cyanobacteria. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.

WHO (World Health Organization) (2003). Guidelines for safe recreational water environments. Volume 1: Coastal and fresh waters. Geneva: WHO.

WHO (World Health Organization) (2009). Policies and procedures used in updating the WHO guidelines for drinking-water quality. Geneva: WHO.

WHO (World Health Organization) (2017). Factsheet on copper. In: Guidelines for drinking-water quality, fourth edition incorporating the first addendum. Geneva: WHO, 340.

Young FM, Micklem J, Humpage AR (2008). Effects of blue-green algal toxin cylindrospermopsin (CYN) on human granulosa cells in vitro. Reprod Toxicol. 25:374–80.

Young FM, Zebian D, Froscio S, Humpage A (2012). Cylindrospermopsin, a blue-green algal toxin, inhibited human luteinised granulosa cell protein synthesis in vitro. Toxicol In Vitro. 26:656–62.

Zessner M, Chorus I (in press). Catchment management. In: Chorus I, Welker M, editors. Toxic cyanobacteria in water, second edition. Boca Raton, Florida: CRC Press, on behalf of the World Health Organization, Geneva.