

**Referees report to the State Water Resources Control Board, Sacramento, California for  
the review draft entitled:**

“Toxicological Summary and Suggested Action Levels to Reduce Potential Adverse Health  
Effects of Six Cyanotoxins.”

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This external scientific peer review has been structured based on attachment 2, and comments specifically on the scientific basis used to generate the action levels in the draft. The following sections contain my comments on what the “staff” have been identified as being particularly relevant to the review process. As you will read most of my comments deal with item 3 of attachment 2.

**General Approach**

**Point 1.** The objectives of the report are sound and necessary, namely to dissociate alert levels from cyanobacterial cell counts alone and to consider the actual toxin content of cyanobacterial blooms. It is well known that cell numbers do not equate to toxicity and that a few cyanobacteria are able to synthesize copious amounts of toxin while dense blooms can be non-toxic. A bloom is also able to increase or decrease its toxin production rate, and hence content, depending on prevailing environmental conditions, nutrient availability, and bloom species composition. Tools have been developed over the last decade that allow water monitoring bodies to assess the potential of a bloom to produce a toxin and also to identify the class of toxin. The application of these molecular detection techniques is addressed in point C in the final section of this referee’s report.

The review draft focuses on calculating the following 4 parameters that will be addressed individually in this report:

Toxicity assessments for humans, dogs and cows.

Recreational Exposure for humans.  
Animal exposure for dogs and cattle.  
Computation of action levels.

Section I (Introduction) is vague regarding the exact motivation for this report within the context of California and its water quality needs. While presenting some information about the overall occurrence of cyanobacterial blooms, it does not clearly state the current situation within California as opposed to the rest of the USA followed by brief correlations, where necessary, with the worldwide situation. It introduces the different toxins and then introduces some of their health effects. This last part of the introduction is duplicated in section II that contains the health-based criteria for cyanotoxins.

Overall the review draft of June 2009 is quite confusing. There is little adherence to the numbering listed in the contents page (page V) that makes the document hard to follow and refer to at times. The equations are haphazardly numbered, if at all (see example on page 11 which refers to equation 1 and also on page 30). The equations on page 45 are not numbered. There is no consistency as to the application for determining the RfD and other parameters for each toxin.

### **Toxicity Criteria for the Six Chemicals**

**Point 2.** The title of this section and the emphasis on 6 cyanobacterial toxins is misleading since essentially only three cyanobacterial toxin types were assessed, namely microcystin-LR, anatoxin-a and cylindrospermopsin. The exclusion of a carcinogenic exposure level is valid since there is not enough data available to establish and prove clear cause and effect of exposure to cyanobacterial toxins leading to carcinogenesis. This reflects the findings of the International Agency for Research on Cancer, as mentioned on page 13 of the review draft.

**Point 3.** It is the opinion of this peer reviewer that focusing on toxic extracts administered orally to animals to determine the toxicity of specifically microcystin, was very restrictive. An extract of toxin does not reflect the “true” situation as the cells are lysed before being administered and are often concentrated to higher levels than those occurring in a natural bloom. In addition, there

exist studies (Yoshida et al., 1997; Fawell et al., 1999) that indicate a 5-100 fold increase in oral LD<sub>50</sub> values when compared to intraperitoneal values for microcystins in mice and rats. The range being dependent on age, nutritional status, and species of animal. By excluding a large amount of this type of research data the authors have essentially restricted the science used to support their final guideline values.

Four microcystin variants were included in calculating the alert levels for toxicity assessments yet it is generally accepted that the variant microcystin-LR is the most toxic and hence all alert levels focus on using this variant as a worst-case scenario. The review draft has focused on the following 4 microcystin variants listed with their intraperitoneal mouse LD<sub>50</sub> values as published in Table 3.2 in the WHO supported book: “Toxic Cyanobacteria in Water” edited by Ingrid Chorus and Jamie Bartram:

- MCLA with an LD<sub>50</sub> listed as 50 µg/kg
- MCYR with an LD<sub>50</sub> listed as 70 µg/kg
- MCRR with an LD<sub>50</sub> listed as 600 µg/kg
- MCLR with an LD<sub>50</sub> listed as 50 µg/kg

As is evident from this table, there is great variability in the toxicity of different isoforms. The differences depend on numerous factors including binding to the uptake receptors, hydrophobicity/hydrophilicity, and their ability to bind protein phosphatases 1 and 2A. However, the draft review states on page 13 that these congeners “appear to have similar toxicological effects.”

Given that recent reports of nodularin being produced or found in freshwater systems it may be useful to establish a guideline value for this cyanotoxin to pre-empt any potential occurrence of associated poisonings in the near future.

The following section specifically addresses **toxicity criteria as it pertains to the health-based criteria for cyanotoxins** (section III of the draft report). In order to determine the toxin reference dose (RfD) the authors first identified “the best study” (page 11) that provided quantitative information. They do not however provide any criteria as to what constitutes a good

or the best study. They also limit themselves to a single study on which to base their analysis instead of determining a range of values and then calculate a dose that does not cause adverse health effects by extrapolating from existing work. This level is determined in the analysis of experimental values that are fed into a range of formulas with the end result falling outside any experimental study. The best result (again not clearly explained) is then used as the no adverse effect level.

The authors accept that a single study conducted by Heinze is the best study to use for determining the RfD for microcystin. They do not mention which toxin he used. They also state that rats are more sensitive to microcystin in the Heinze study and that a mouse study formed the basis of the WHO study, thereby implying their analysis is better. This is erroneous as mice and rats show different responses to microcystins based on time after eating, species of rat or mouse, as well as numerous other parameters. Hooser et al. (1989), in contrast to Heinze, demonstrated rats were more resilient to microcystin than mice. While the Heinze study is valid, as an impartial referee I cannot see why this study would be considered superior to other studies, including the Fawell (1999) study that was used to determine the WHO guidelines. The Heinze study has only been cited a total of 10 times whereas other studies, such as Solter et al. (1998), have been cited more than 36 times. It may be useful to tabulate the studies with both oral and intraperitoneal exposures over the various periods investigated to illustrate how the “best studies” were chosen. This tabulation of data would also allow for an average value to be obtained and used for further calculations of RfD values. This is most relevant to MCLR toxicology as this cyanotoxin is the most recognized and best studied.

It is not at all clear how the RfD values for 4 microcystin variants were determined. On page IV it is stated in the caption that Microcystins LA, LR, RR and YR all had the same RfD which seems highly unlikely given that MCRR has an LD<sub>50</sub> 12 times greater than that of MCLR. Notably, the Heinze study on which the RfD was based only studied the effects of MCLR.

The application of EPA benchmark dose response software to fit mathematical models to dose-response data for estimating the 10% response rate (BMD) (page 14) is not suitable for studies that have only two dose levels, or three if the control group is included. In the case of

microcystin the study had two doses, one at 50 µg/kg per day that resulted in liver lesions in 6/10 rats, while the 150 µg/kg per day dose resulted in liver lesions in 9/10 rats which led to the calculation of 6 µg/kg as the body mass dose limit (BMDL, page 15), well out of the range of the study used to calculate the value. This analysis forces an implied response based on a mathematical analysis of two data points on 10 rats each. The authors state that the log-probit fit of the data was determined to be the best fitting model without explaining the basis for this calculation.

Overall, the application of mathematical analysis to the dose levels obtained from the literature is not clearly presented. For example, in the calculation of the acute reference dose in domestic animals for microcystins (page 15) the authors refer to Appendix IV without stating which equation/or page to consult.

In calculating the values it would be advisable to clearly differentiate between the published results/literature, and what was calculated in this work.

One would expect each toxin to be analyzed similarly yet the reference dose for humans for cylindrospermopsin was calculated using a different mathematical model (page 17) to that for microcystin, excluding the highest dose group. The section on anatoxin-a starts with a paragraph describing the toxicology of anatoxin-a, a useful summary that was not provided for microcystin and cylindrospermopsin. I have concerns regarding the determination of values for anatoxin-a as there is simply not enough information to make sound calculations on the RfD. This is illustrated with the problem of calculating the sub-chronic reference dose in domestic animals that, if calculated according to their procedure for microcystin and cylindrospermopsin, would be above the RfD for short-term exposure (page 21). However, as there are only limited toxicological studies available for cylindrospermopsin and anatoxin-a, the authors have done the best they can to calculate the action levels needed for this project.

The following section of the peer review deals with the **health-based water concentrations for human recreational exposures** (section IV of the draft review). While I agree with the interpretation that ingestion is by far the most toxic route of exposure to microcystin and

cylindrospermopsin, I have trouble with the statement, “Based on their chemical properties, microcystin and cylindrospermopsin are not likely to penetrate the skin or vaporize from water” without citing the reference or providing the chemical analysis as support for this assertion. Merely stating “they are large zwitterions” (page 33) is not scientifically accurate. While having a formula, such as that used in equation 2 on page 30, is useful. The information related to  $K_p$  values, however, should have accompanied equation 2 on page 30 and illustrates a problem with the draft review, that is, proper cross-referencing to the supporting appendices and calculations. Detailed editing of the draft should address this problem.

The authors fail to include the potential of particulate matter or clumps of cyanobacteria being splashed into the eyes and inhaled into the upper respiratory tract of an exposed individual that would allow for localized higher exposure than pure aerosolized toxin.

#### **Exposure Assessment and Microcystin Ecotoxicology (points 4-6 of attachment 2)**

I do not have any comments related to the issues as I felt the author’s comments in this regard were justified and accurate. They have done a commendable job of interpreting the limited data available in these cases and proposing what appear to be sound and feasible guideline values. In respect to point 6 of attachment 2, there are a several more publications regarding the levels of microcystin found in fish tissue and a selection are provided at the end of this report. These studies, however, do not always reflect an environmental dose situation or time of exposure thereby making accumulation estimations difficult.

#### **Peer Reviewer’s Comments on the Broader Perspective and Recommendations**

a) I am not aware of any additional scientific issues not described in this report regarding toxicity assessments. I have, however, suggested alternative screening methods to reduce the costs of chemical detection methods in section C below. The table of action levels, as presented on page IV of the review draft, accurately reflect the current status of scientific knowledge. The calculation of the RfD value for microcystin is lower than that recommended the WHO (1  $\mu\text{g/L}$ ) for human consumption and may result in (unnecessary) additional costs during its

implementation for appropriate water management procedures to reduce toxin exposure. It is the feeling of this referee that the argument for reducing this particular level is not convincing as it is based on a single study to determine the RfD which was then directly applied to the formula for exposure for swimmers, considered to be the high risk users and most endangered target group for this study.

b) The actions levels determined in this study are based on the currently available scientific data. This peer assessment has noted the lack of information pertaining to cyanobacterial toxicity, especially for those toxins not included in the review, namely saxitoxin and lyngbyatoxin. The data available for anatoxin-a and cylindrospermopsin is also very sparse and more focused studies are needed to provide certainty as to the alert levels calculated in this draft review. The concluding alert levels determined by the analysts are as sound and reasonable as the limited published data to which they refer.

c) The present draft does not mention which methods shall be employed to detect and quantify cyanotoxin levels within the recreational waters of California. However, as toxin concentration is the key variable in the exposure equations listed, and different protocols deliver different toxin estimates, this is a point that requires clarification. As acknowledged in the draft, cell numbers do not always correlate well with toxin levels, and neither do physiological traits and morphological characteristics such as cell size and shape. In fact, toxin profiles vary widely across and within the five orders of cyanobacteria (Sivonen and Jones, 1999). The detection and quantification of cyanotoxins via animal bioassays has been extensively utilized in the past. However, low sensitivity, ethical issues, and high associated costs have driven the search for alternative testing methods. The elucidation of the biochemical structures of the cyanotoxins subsequently permitted accurate assessment via analytical methods such as high-pressure liquid chromatography (HPLC) and matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry (Lawton et al., 1999; Welker et al., 2002). These analytical methods deliver structural information as well as precise measurements of toxin concentration in a given sample, however, they necessitate expensive specialized equipment and purified toxin standards (some of which are difficult to obtain), and cannot be used to assess a blooms potential for toxin production.

Contemporary guidelines for water safety are frequently based upon a combination of animal bioassays and analytical techniques, thereby enabling assignment of LD<sub>50</sub> values to particular toxin isoforms or subclasses. Indeed the present draft review relies on data generated by animal bioassays (Appendix III). The main drawback of these methods is that they can only be applied to samples in which toxin is already present. As previously mentioned, they cannot be used to assess *potential* toxicity and hence prevent or reduce the impact of a given bloom event.

The recent characterization of the cyanotoxin synthetase gene clusters has resulted in an explosion of molecular detection methods for these organisms and their toxins (Tillett et al., 2000; Moffitt et al., 2004; Kellmann et al., 2007; Mihali et al., 2008; Mejean et al., 2009). Conventional polymerase chain reaction (PCR) tests targeting cyanotoxin biosynthesis genes provide a rapid and sensitive means for detecting potentially toxic populations of cyanobacteria in water supplies (for a review of these methods see Pearson and Neilan, 2008). The adaptation of these simple PCR tests into quantitative methods has additionally enabled the monitoring of dynamic bloom populations and the identification of particularly problematic species. More recently, DNA microarray technology has been applied to cyanobacterial diagnostics offering a high-throughput option for detecting and differentiating toxic genotypes in complex samples. Together, these molecular methods are proving increasingly important for monitoring water quality.

While numerous genetic loci have been targeted for the detection and differentiation of toxic cyanobacteria, the toxin biosynthesis genes themselves are unquestionably the most informative. Conventional and/or quantitative PCR tests have been described for the major cyanotoxins including, microcystin, cylindrospermopsin, and anatoxin-a (Pearson and Neilan, 2008; Al Tebrineh et al., 2011; Mejean et al., 2009). In general, the best PCR targets for detecting toxic cyanobacteria are those that are essential for toxin production, and are conserved within the target group of cyanobacteria, but divergent from the wider population of microorganisms. For example, in the case of the microcystin biosynthesis gene cluster, the *mcyE* gene is essential for toxin biosynthesis and will therefore be present in every microcystin-producing cyanobacterium. *mcyE*-based PCR will in theory identify toxigenic cyanobacteria producing all microcystin

isoforms including those listed in the present draft review, that is, microcystin-LR, -RR, -YR and -LA. On the other hand, these PCR tests will not provide information as to which isoform is being produced. This molecular approach has been adopted not only for the detection, differentiation and quantification of toxic cyanobacteria, but also for investigating the regulation of toxin biosynthesis.

Both conventional and qPCR techniques are highly sensitive and can be tailored according to desired specificity. However, qPCR has the added advantage of being able to quantify the genetic target. In practical terms, this means it is possible to determine the concentration of toxigenic cyanobacteria, and even maximum toxin levels, in a bloom, be it a complex or unialgal sample.

Most of the qPCR methods described to date are uniplex, that is, they utilize a single primer pair that targets an individual toxin gene. Primers can be designed to be highly specific (e.g. to target a toxin gene from a single species) or broad-range (e.g. to target multiple species producing the same toxin), however, uniplex reactions are limited to a single genetic target. Multiplex qPCRs on the other hand can be tailored to target multiple toxin genes from a number of toxigenic species in a single reaction. These quantitative PCR assays are usually very sensitive, with reliable detection limits of only a few cells per reaction and can be applied directly to water (or other environmental) samples. Quantitative real-time PCR may prove to be a powerful tool for deciphering the complexities of bloom dynamics. For example, by quantitatively monitoring species within natural bloom communities, it may be possible to identify particularly problematic strains and hence implement certain protocols that target their removal. Furthermore, quantitative PCR may provide insight into which environmental factors promote/inhibit the growth of toxigenic species and may thus bring us closer to understanding the physiological and ecological parameters that regulate cyanotoxin production.

Oligonucleotide microarrays are proving to be increasingly popular diagnostic tools for analyzing complex clinical and environmental samples. While only recently applied to the study of cyanobacterial diversity, microarray technology is beginning to show great promise for the high-throughput analysis of bloom samples (Rudi et al., 2000; Castiglioni et al., 2004; Rantala et

al., 2008). However, the initial onset costs and the need for specialized, expensive equipment have prevented the widespread use of microarrays in the field of cyanobacterial diagnostics.

While numerous tests have been described for the detection and quantification of toxigenic cyanobacteria in the scientific literature (Pearson and Neilan, 2008), for brevity, we shall only list a few of the most recent and most effective assays in this review. Neilan and co-workers recently developed a novel multiplex qPCR assay targeting four different cyanotoxin gene clusters: *mcy* (microcystin), *nda* (nodularin), *cyr* (cylindrospermopsin), and *sxt* (saxitoxin). This assay, which utilizes TaqMan technology, was designed to target all the major microcystin, nodularin, cylindrospermopsin and saxitoxin-producing cyanobacteria (Al Tebrineh et al., 2011). In addition, they incorporated an internal control based on a conserved region of the 16S rRNA gene present in toxic and non-toxic cyanobacterial species. While anatoxin-a genes were not targeted in this assay, the recent publication of the anatoxin-a biosynthesis gene cluster (Mejean et al., 2009) could enable this in the near future. Detection of anatoxin-a producing cyanobacteria via PCR would circumvent problems currently encountered testing for the toxin itself as mentioned in the draft “Dr. Carmichael explained that the analytical method he used to measure anatoxin-a in the biological samples can misidentify phenylalanine, a common amino acid, as anatoxin-a (Carmichael et al, 2004).”

In summary, molecular detection and quantification methods for cyanotoxins offer numerous advantages over conventional animal bioassays and analytical techniques. These methods would be applicable to water samples and sediments from Californian recreational water bodies and could thus constitute the basis for the exposure equations described in the present draft review. However, in situations where accurate diagnosis is paramount (e.g. when assessing the quality of drinking water supplies), supplementary toxicity tests (e.g. physicochemical or biochemical) are always advised. Furthermore, as PCR-based methods only detect the toxin genes and not the toxins themselves, they are not appropriate for measuring toxins that have accumulated in the tissues of animals that may be consumed by humans or for detecting new toxins.

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