PEER REVIEW COMMENTS ON:

TOXICOLOGICAL SUMMARY AND SUGGESTED ACTION LEVELS TO REDUCE POTENTIAL ADVERSE HEALTH EFFECTS OF SIX CYANOTOXINS

AND

THE OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT'S RESPONSES

May 2012

Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

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Office of Environmental Health Hazard Assessment
California Environmental Protection Agency
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Preface

Four individuals were selected to review this document: Dr. Adam Bownik of the John Paul II Catholic University of Lubin, Poland; Dr. Wayne Carmichael of Wright State University, United States; Dr. James Haney of the University of New Hampshire, United States; and Dr. Brett Neilan of University of New South Wales, Australia. Peer reviewer selection was facilitated through the University of California.

The reviewers were asked to comment on four specific areas related to the document: 1) General approach, 2) Toxicity criteria for the six chemicals, 3) Exposure assessment and 4) Microcystin ecotoxicology. Reviewers were also asked to contemplate the broader perspective by commenting on any additional scientific issues related to the scientific basis of the action levels. Finally, reviewers were asked whether the action levels are based upon sound scientific knowledge, methods, and practices. Instructions to peer reviewers and their final comments are available at: http://www.waterboards.ca.gov/water_issues/programs/peer_review/peer_review_cyanotoxins.shtml

The Office of Environmental Health Hazard Assessment (OEHHA) appreciates the thorough reviews provided by these referees. Their comments and insight have prompted us to clarify and improve the cyanotoxin report in several areas. In this document, we reproduce the comments from each reviewer and insert our responses using ***bold, blue italic text.***

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Wayne W. Carmichael, Ph.D.
Professor Emeritus
Department of Biological Sciences
Wright State University
Dayton, Ohio

Date: June 10, 2011

From: Wayne W. Carmichael, Professor Emeritus

Subject: Peer Review of Cyanotoxin Toxicity Criteria and Health Based Water Concentrations to Protect Human Swimmers, Dogs and Cattle.

Prepared for: State Water Resources Control Board-Division of Water Quality

Att: Dominic Gregorio Senior Environmental Scientist Chief, Ocean Unit

Summary: In organizing and presenting comments to the cyanotoxin toxicity criteria document I have focused on the major points requested -- namely toxicity criteria, exposure criteria and the general approach used by OEHHA in writing the document. To accomplish these goals I have added detail where needed, additional text or edited text, plus references to support the new or edited text. I believe my edits will contribute to a more accurate, usable and defensible document for setting reference doses (RfD) and the use of those RfDs to estimate maximum concentration levels. These in turn will help protect the public's health from Cyanobacteria Harmful Algae Blooms (CyanoHABs) and their toxins.

The Office of Environmental Health Hazard Assessment's (OEHHA) responses and notes are provided in bold, blue italic.

Review Contents:

Comments on the General Approach Used for Action Level Development:
"OEHHA has limited the scope of the cyanotoxin assessment to four forms of microcystins plus anatoxin-a and cylindrospermopsin. There at least 76 forms of microcystins (Mcysts) but the four under consideration (Mcyst-LA, YR, RR and LR) are the most commonly found ones in U.S. and California waters. Comments for cylindrospermopsin are generic even though there are two known variants, 7-epicylindrospermopsin, with equal toxicity to cylindrospermopsin, and deoxycylindrospermopsin with lower toxicity than other two (Meriluoto and Codd, 2005). Likewise for anatoxin-a, criteria was developed based on only anatoxin-a. In addition to anatoxin-a, Homoanatoxin-a and 4hydroxyhomoanatoxin-a have been described. Some photodegradation products of anatoxin-a, namely dihydroanatoxin and epoxyanatoxin have also been identified (Meriluoto and Codd, 2005).

"Two of these three cyanotoxin groups are currently the most common found in U.S. and California water supplies -- the microcystins and anatoxin-a. Of the microcystins, the four reviewed are also the most common found. These two groups plus cylindrospermopsin are the top three priorities for health risk and for detection methods development as listed by the U.S. EPA. It is therefore appropriate and prudent that these toxin groups be the ones reviewed by OEHHA. However some cautions involving occurrence, health risk and legal points should be considered as the document is developed and considered for adoption. These include:

> "1) Cylindrospermopsin was placed on the EPA priority list because it was perceived to be an emerging cyanotoxin with regards to occurrence and hazard. To date it has not been identified in California waters and in only a few U.S. water supplies (i.e. Florida, Indiana and Oregon)."

OEHHA agrees that cylindrospermopsin has not emerged as a focal hazard in the United States. Nevertheless, we feel the work we have completed on this cyanotoxin remains relevant and may be helpful in the future.

> "2) If these become the only cyanotoxins monitored for, it is very likely some will be missed (i.e. other microcystins, anatoxin-a(s) and saxitoxins), in any monitoring program based upon assessment of only these 6 cyanotoxins, and possible guidelines or regulations that may be adopted based upon an evaluation of these 6 cyanotoxins."

OEHHA acknowledges the importance of this concern. Although monitoring of cyanotoxins in California waters is important, this report is not intended to define monitoring goals or practices. The State Water Resources Control Board (SWRCB) asked OEHHA to assess the risk of the six cyanotoxins addressed in the report. Additional cyanotoxins were not addressed due to lack of toxicological information and/or funding limitations.

> "3) Because only 6 cyanotoxins are being reviewed and assessed there may be legal issues that arise from occurrences, exposures and/or toxicities due to Cyanobacteria Harmful Algae Blooms (CyanoHABs) that contain other cyanotoxins."

In response to this comment, OEHHA has added information in the document to further acknowledge that not all cyanotoxins are covered. In the first section of the Introduction, under the subsection "Not all cyanotoxins have toxicological criteria", the following text was added:

> ***"However, this report does not address all of the important cyanotoxins such as anatoxin-a(s), saxitoxins and other analogs of microcystins. Toxicological criteria are also needed for these cyanotoxins and should be developed in the future."***

Comments on Toxicity Criteria Used for the Six Chemicals.

"The reasoning and text for the Toxicity Criteria-Assessment (pages 10-21) is overall very good and complete. Acute and acute-lethal poisoning from microcystins are the only toxicities that have been confirmed. Liver carcinogenesis has not been demonstrated except in laboratory experiments and then only when initiation from a proven carcinogen such as aflatoxin is also used. There is however one statement that does need editing. On page 11 under "Existing Health-Based Criteria" – the sentence **"WHO (2) considered the ability of microcystins to promote liver tumors, but the international Agency for Research on Cancer found the evidence for microcystins to cause cancer in humans inadequate"**-is correct in that WHO did discuss the topic but did not consider it. And it is correct that IARC found the evidence inadequate—however the two are not linked in the sense that one might have influenced the other. WHO did their study in 1998 and published it in 1999 (ref 2). IARC did their evaluation in 2005 and published it in 2006 (ref 62). The real reason WHO did not act on any evidence for linking microcystins to cancer was that the Australian representatives to the WHO deliberations were explicitly asked not to consider the question of microcystins and cancer. It is therefore more correct to say that WHO simply did not address the issue at all, following the Australian request against it."

This has been corrected in the document.

"The use of cyanobacterial extracts (generally greater than 90% pure) is an acceptable criteria for assessing cyanotoxin action levels. There are no certified reference standards for any cyanotoxins. The best reference materials are 95% pure (or better)-however they have not been quality controlled (certified) by more than two methods-usually HPLC peak purity or by the use of extinction coefficients. In turn purity of these standards have been determined using reference materials not certified by NMR or LC/MS."

The reviewer agrees with OEHHA's approach.

Comments on Exposure Assessment Assumptions

"Addressing exposure assessment for humans (recreational waters), and domestic animals (livestock and pets), covering pages 21-29, is a good approach. This reviewer finds the calculations for water intake and related action levels determined to be the best ones possible given the available data on toxicity and exposure scenarios. Likewise the Exposure calculations in Appendix I through VI (pages 30-46) are also appropriate. The professional judgments used in estimating exposure to dogs is acceptable to this reviewer. The only caveat to this, from this reviewer, is that dogs do exhibit a fairly rapid acute toxicity from licking fur matted with bloom material that contains anatoxin-a or anatoxin-a(s). Dogs are also attracted to fermenting mats of cyanobacteria near shorelines of waterbodies. In other words-dogs may be unusually sensitive to cyanotoxin neurotoxins. This attraction and rapid toxicity was discussed in a paper by Codd et al. 1992:

> "Codd, G.A. , Edwards, C., Beattie, K.A. Barr, W.M., and Gunn, GJ. (1992) Fatal Attraction to Cyanobacteria? Nature. 359:110-111."

The reviewer agrees with OEHHA's approach with one caveat regarding exposure to dogs. OEHHA agrees with this and other reviewers that have suggested similar concerns. An uncertainty factor of 3 has been added to the acute and subchronic domestic animal exposure assessments for all of the cyanotoxins covered in the report. The added uncertainty factor represents the uncertainty of exposure due to preferential consumption by domestic animals. This approach assumes that animals may eat or drink up to three times their normal intake due to preferential consumption of cyanobacteria.

Comments on Microcystins, Anatoxin-a and Cylindrospermopsin Ecotoxicology

"An assessment of the ecotoxicology of cyantoxins is a very important topic. Indeed it may be even more important than the risk to humans and domestic animals. This is primarily because human activities leading to eutrophication and alteration of water supplies are the primary drivers for the increased incidence and duration of Cyanobacteria Harmful Algae Blooms (CyanoHABs). Aquatic and terrestrial systems are widely affected by CyanoHABs. However as the review points out on page 46-the

topic is “complex and evolving”. Equally important to setting action levels to reduce adverse health effects of cyanotoxins should be programs and actions to reduce human impacts on aquatic systems that are responsible for the increases in CyanoHABs and their significant impacts on natural populations of plants and animals. While there are many more examples and studies that show ecotoxicological effects from cyanotoxins the discussion and examples on pages 51-72 are good examples to have used for the document.”

The reviewer agrees with OEHHA’s approach.

Specific Editing Changes to the Document: “Some general edits are needed in the document. These are as follow:

1) “Page 2-line line 8. The number of papers reviewed by OEHHA (2025) represents about half of the scientific papers on the topic to 2004. It is estimated, that about another 2000 have been published since 2004, meaning the OEHHA review is based on about one-third of the available publications. The initial 2004 publication list is available for the USEPA at;

http://nlquery.epa.gov/epasearch/epasearch?typeofsearch=epa&filterclause=%28tssms:ogwdw000%29%20AND%20&max_results=100&referer=http%253A%252F%252Fwww.epa.gov%252Fsafewater%252Findex.html&result_template=epafiles_default.xsl&areaname=Ground%20Water%20%20%20Drinking%20Water&areapagehead=epafiles_pagehead&areapagefoot=epafiles_pagefoot&areasidebar=search_sidebar&stylesheet=http://www.epa.gov/epafiles/s/epa.css&sort=term_relevancy&faq=no&results_per_page=10&cluster=both&sessionid=FB8AEAD369DCEA87FAF295DEC7CBA1CB&querytext=cyanobacteria%20toxins&start=11&doctype=all ”

OEHHA’s literature search was limited to the topic of adverse health effects and exposure to cyanotoxins in humans, domestic animals, and wildlife. Many of the available scientific papers on cyanotoxins focus on other topics, such as cyanobacterial identification and production of specific toxins.

2) “Page 4. Table 1. The molecular weights reported for the four microcystins vary with instrument at the decimal point numbers. It is best to omit the mass fraction numbers (i.e. 910 not 910.06). Also the Molecular Weight reported is actually mass plus 1. Therefore the column heading should be changed to read: “Molecular Weight plus H”.”

This has been corrected in the document.

3) “Page 5. Line 4. It is now accepted that the species *Aphanizomenon flos-aquae* is not a known toxin producer. Previous references to it as a producer have proven to be other species in the genus or from mixed populations where another cyanobacteria actually was the toxin producer. For example see ref:

> Carmichael, W.W. 2001. Health Effects of Toxin Producing Cyanobacteria: "The CyanoHABS". Human and Ecological Risk Assessment. 7(5): 1393-1407.
>
> Li, R., Carmichael, W.W., Liu, Y. and Watanabe, M.M. 2000. Taxonomic re-evaluation of Aphanizomenon flos-aquae NH-5 based upon morphology and 16S rRNA gene sequences. Hydrobiologia, 438(1): 99-105.
>
> Li, R.H. and W.W. Carmichael. 2003. Morphological and 16S rRNA gene evidence for reclassification of the paralytic shellfish toxin producing Aphanizomenon flos-aquae LMECYA 31 as Aphanizomenon issatschenkoi. J. of Phycol. 39. 814-818.”

This has been corrected in the document.

4) “Page 6. Line 14. There is some published material on biodegradation of cylindrospermopsin. One paper by Wormer et al found no bacterial degradation over 40 days. See below references:

> Smith, M. J., Shaw, G. R., Eaglesham, G. K., Ho, L., and Brookes, J. D. (2008). Elucidating the factors influencing the biodegradation of cylindrospermopsin in drinking water sources. Environ. Toxicol. 23, 413-421.
>
> Wormer, L., Cires, S., Carrasco, D., and Quesada, A. (2008). Cylindrospermopsin is not degraded by co-occurring natural bacterial communities during a 40-day study. Harmful Algae 7, 206-213.”

This has been corrected in the document.

5) “Page 7. Line 8. It is my understanding that Clear Lake in Northern California has a history of microcystin occurrence. Also a just published paper details microcystin in Pinto lake and transfer to Monterey Bay –see:

> Miller MA, Kudela RM, Mekebri A, Crane D, Oates SC, et al. Evidence for a Novel Marine Harmful Algal Bloom: Cyanotoxin (Microcystin) Transfer from Land to Sea Otters. (2010) PLoS ONE 5(9): e12576. doi:10.1371/journal.pone.0012576”

We did add information on Pinto Lake and Clear Lake. However, we are not able to review and incorporate all of the relevant scientific information published after our original 2009 draft. We did add the following sentence to the report section: “Cyanotoxins have occurred elsewhere in California – the above citations are not intended as a comprehensive review of occurrences.”

6) “Page 7. Bottom paragraph. A more complete ref for the Brazil human deaths is Carmichael et al. 2001. In this outbreak report 100 patients developed acute liver failure (of 116/131 with symptoms) – 76 died and 52 were confirmed with cyanotoxin poisoning. See:

> Carmichael, W.W., Azevedo, M.F.O., An, J.S., Molica, R.J.R., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eagelsham, G.K. 2001 Human Fatalities from Cyanobacteria: Chemical and Biological Evidence for Cyanotoxins. Environmental Health Perspectives. 109 (7):663-668.”

This has been corrected in the document.

7) “page 9-dog deaths. Oregon has also reported dog deaths from anatoxin-a. see:

http://public.health.oregon.gov/HealthyEnvironments/Recreation/HarmfulAlgaeBlooms/Pages/new2009.aspx”

OEHHA has added information on the Oregon dog deaths.

8) “page 68-Food Web Transfer. The recent exposure of microcystins to Sea Otters in Monteray Bay should be included in this section. See: Miller MA, Kudela RM, Mekebri A, Crane D, Oates SC, et al. Evidence for a Novel Marine Harmful Algal Bloom: Cyanotoxin (Microcystin) Transfer from Land to Sea Otters. (2010) PLoS ONE 5(9): e12576. doi:10.1371/journal.pone.0012576”

In response to this comment, the following text was added to the Food Web Transfer subsection in Appendix VII:

> ***“A recent study provided clear evidence of the trophic transfer of microcystins from Microsystis spp. (and free microsystins) to marine bivalves and sea otters (cite Miller 2010). The deaths of 21 sea otters in the Monterey Bay National Marine Sanctuary were linked to microcystin poisoning. The source of Microcystis spp. was Pinto Lake and its downstream tributaries draining into the Sanctuary. In this case, the marine bivalves did biomagnify the microcystins.”***

A footnote was added to clarify that the inclusion of a recent report was an exception:

> ***“[1] Information on this study was added during the final edits in response to peer review comments. The literature review for this report extended through 2008.”***

Additionally, the following text was added to the first paragraph of Appendix VII:

> ***“Readers should be aware that the information presented in this appendix is based on a review of the literature published through 2008. In the meantime, more literature on cyanotoxins has been published. In general, literature published after 2008 was not integrated into this document. However one pertinent recent study that was highlighted by a peer reviewer was added to this appendix.”***

9) “Terminology-page 48-51. On page 48 the definition of purified toxin should be modified to indicate an important topic in developing methods of analysis and toxicology mechanisms. There is a distinction between “reference standards” and “certified reference material”. Purified toxin does not indicate degree of purity and this varies widely from different sources using different extraction methods and whether multiple methods for quality control have been used. Reference standards do not carry the same degree of purity testing and usual only have had one or two QC methods applied-i.e. HPLC purity as compared against another reference material whose purity might be no more than 90-95%. Certified Reference materials would have multiple QC methods

applied-i.e. HPLC, extinction coefficient. LC-MS or MS-MS and even NMR. These "standards" would be used as the ultimate comparison for purity of an extract and should be 99% or better pure. For some applications reference materials are suitable but for others only certified reference material should be used. Therefore it is suggested the terms "Reference Standard and "Certified Reference Standard" be put in this table."

This has been added to the document.

10) "Page 13-Microcystins and Cancer. It is true that the question of cancer has been addressed by IARC and OEHHA has handled the question appropriately. There is a new review published on the topic which might be good to consider and insert in this document. It is:

> Zegura. B., Straser, A., Filipic, M. 2011. Genotoxicity and potential carcinogenicity of cyanobacterial toxins – a review. Mutation Research/Reviews in Mutation Research. 727:1-2. 16-41."

In response to this comment, OEHHA discussed the final IARC document and called attention to the Zegura review on pages 13-14 of the document.

References Cited in this Editors Review:

Meriluoto, J. and Codd, G.A. 2005. Cyanobacterial Monitoring and Cyanotoxin Analysis. Abo Akademi University Press. ISSN 0001-5105; Vol. 65, no. 1. Pp. 148.

Miller MA, Kudela RM, Mekebri A, Crane D, Oates SC, et al. Evidence for a Novel Marine Harmful Algal Bloom: Cyanotoxin (Microcystin) Transfer from Land to Sea Otters. (2010) PLoS ONE 5(9): e12576. doi:10.1371/journal.pone.0012576

Carmichael, W.W. 2001. Health Effects of Toxin Producing Cyanobacteria: "TheCyanoHABS". Human and Ecological Risk Assessment. 7(5): 1393-1407.

Li, R., Carmichael, W.W., Liu, Y. and Watanabe, M.M. 2000. Taxonomic re-evaluation of Aphanizomenon flos-aquae NH-5 based upon morphology and 16S rRNA gene sequences. Hydrobiologia, 438(1): 99-105.

Li, R.H. and W.W. Carmichael. 2003. Morphological and 16S rRNA gene evidence for reclassification of the paralytic shellfish toxin producing Aphanizomenon flos-aquae LMECYA 31 as Aphanizomenon issatschenkoi. J. of Phycol. 39. 814-818.

Carmichael, W.W., Azevedo, M.F.O., An, J.S., Molica, R.J.R., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eagelsham, G.K. 2001 Human Fatalities from Cyanobacteria: Chemical and Biological Evidence for Cyanotoxins. Environmental Health Perspectives. 109 (7):663-668.

Smith, M. J., Shaw, G. R., Eaglesham, G. K., Ho, L., and Brookes, J. D. (2008). Elucidating the factors influencing the biodegradation of cylindrospermopsin in drinking water sources. Environ. Toxicol. 23, 413-421.

Wormer, L., Cires, S., Carrasco, D., and Quesada, A. (2008). Cylindrospermopsin is not degraded by co-occurring natural bacterial communities during a 40-day study. Harmful Algae 7, 206-213.

Zegura. B., Straser, A., Filipic, M. (2011). Genotoxicity and potential carcinogenicity of cyanobacterial toxins – a review. Mutation Research/Reviews in Mutation Research. 727:1-2. 16-41.

Codd, G.A. , Edwards, C., Beattie, K.A. Barr, W.M., and Gunn, GJ. (1992) Fatal Attraction to Cyanobacteria? Nature. 359:110-111.

Respectfully Submitted

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The peer review of cyanotoxin toxicity criteria and health-based water concentrations to protect human swimmers, dogs, and cattle

Blooms of cyanobacteria is an emerging ecotoxicological problem and also health risk for humans and domestic animals all over the world. The idea of creating health based criteria to protect people during recreational use of surface water bodies and to protect dogs and livestock should be appreciated. I hope that my comments regarding the development of action levels will be useful.

The Office of Environmental Health Hazard Assessment's (OEHHAs) responses and notes are provided in bold, blue italic.

General Approach:

1. "The scope of the assessment was to establish the action levels of four variants of hepatotoxic microcystin, neurotoxic anatoxin-a, and cytotoxic cylindrospermopsin to protect people, dogs and cattle. The cyanotoxins selected by OEHHA have different mechanisms of their toxic action in mammals and induce different toxic effects. **The selection of the most toxic forms of microcystin: LA, -LR, -RR, -YR, anatoxin-a and cylindrospermopsin to determine the action levels is, in my opinion, very relevant.** The four variants of microcystin are similar in

structure but they have different water solubility, cell membrane permeability and, as a consequence, their toxicity. However, much more adequate literature is available on the toxicity of microcystin-LR than three other variants of this cyanotoxin. Cytotoxic cylindrospermopsin and neurotoxic anatoxin-a are very commonly produced by many strains of cyanobacteria and their impact on health of many mammalian species is very evident, so action levels for these cyanotoxins should also be developed.

"If neurotoxic saxitoxin is found in waters of California, I suggest determination of action levels also for this cyanotoxin, because scientific data indicate that it could also be a serious threat to domestic animals and humans. Neurotoxic saxitoxin is produced by marine dinoflagellates *Alexandrium*, *Gymnodinium* and also by freshwater cyanobacteria such as *Anabaena sp.*, some strains of *Aphanizomenon*, *Cylindrospermopsis* and *Planktothrix* very commonly found in the freshwater environment."

OEHHA agrees that determining action levels for saxitoxin could benefit the State. However, the scope of this report was limited by available funds through the SWRCB contract. We were not able to include saxitoxin in our assessment.

"I agree that the correlation between cyanobacteria cell count and the cyanotoxin level is not consistent and cell count is not efficient basis for action level. The WHO developed guidelines for health protection on the basis of : 1) low probability of adverse health effects from water ≤ 20 000 cells/mL or 10 μg chlorophyll-a/L where cyanobacteria are the dominant species, 2) moderate probability of adverse health effects from waters with 100 000 cells/mL or 50 μg chlorophyll-a/L, in case of bloom formation on the water surface. These guidelines are rather based on cell concentrations but not on toxin concentrations. In a cyanobacterial bloom toxigenic (cyanotoxin-producing) and non-toxigenic strains of the same species of cyanobacteria that can exist together. By using light microscopy cyanobacteria cell count, it is not possible to determine the quantity of toxigenic or non-toxigenic strains in a sample. Even if a sample contains only toxigenic strain of cyanobacteria one cannot predict the amount of cyanotoxin produced because the same number of cyanobacteria can contain different amounts of cyanotoxins. The same strain produces various amounts of cyanotoxins depending on certain unknown conditions, and some strains can be more toxic by producing microcystins simultaneously with some other cyanotoxins, such as anatoxin-a (Sivonen 1996). The best basis for developing the action levels would be determination of concentration of cyanotoxin in the cyanobacterial extract by HPLC and LC/MC methods. Recently the ELISA techniques for some algal toxins have been developed. The ability to produce cyanotoxins can be determined by PCR-techniques.

"Some health-protective guidelines for recreational water levels in the USA exist. Vermont Department of Health (http://healthvermont.gov/enviro/bg_algae/bgalgae.aspx) suggests that in case of a visible cyanobacterial scum in a recreational water reservoir and microcystin-LR (equivalent) and anatoxin-a concentration in the water is above 6 and 10 μg/l respectively the beaches should be closed."

The reviewer agrees with OEHHA's approach.

Toxicity criteria

2. "One of the toxicity criteria is cancerogenesis. Microcystin-LR is considered as 'possibly cancerogenic to humans', a potent chemical stimulating proliferation of hepatic tumor at low doses but the mechanisms of this effect is still unclear. Some authors associated inhibitory activity of microcystin on protein phosphatases 1 and 2 with tumor promotion. Microcystin at moderate and high concentrations is not directly genotoxic (does not form DNA adducts), but causes production of reactive oxygen species inducing DNA damage and lipid peroxidation leading to formation of liver tumors. It is also suggested that the microcystin-induced oxidative stress is the cause of liver apoptosis. Many short-term studies revealed possible pro-cancerogenic influence of microcystin-LR on hepatocytes, however there is a need for more appropriate long-term studies which is difficult to perform because time-consuming lifetime bioassays should be used. Currently, there are no adequate dose-response results on carcenogenesis induced or promoted by microcystins to use them as the basis for action level development. Some bioassay studies were planned by the National Toxicology Program to expose rats and mice for 24 months to a mixture of microcystins LR and LA but the results are currently not available yet. **As there is a lack of adequate studies for computation of a criterion based on tumor promotion the reference dose for microcystin-LR should be based on liver toxicity."**

The reviewer agrees with OEHHA's approach of basing the microcystin-LR reference dose on liver toxicity.

3. "Data on the toxicity of purified or pure cyanotoxins is limited. Most of the toxicological studies on the acute toxicity of cyanotoxins were performed with the use of cyanobacterial extracts and the some results suggest that cyanobacterial extracts induce more severe toxic effects than purified or pure cyanotoxins. Therefore, reference doses based on the extract toxicity would be even more health-protective. A

cyanobacterial extract used in toxicological studies usually includes the cyanotoxin of known concentration, however it may also contain some other toxic compounds such as lipopolisacharides or substances of unknown identity even more active than the known toxin or potentiating its toxic effects. Cyanobacterial extracts used in toxicological studies may simulate more adequately natural conditions than a solution of purified or pure cyanotoxin. On the other hand use of extract may not reflect the toxicity of the single cyanotoxin.

"A lack of data on acute toxicity of purified toxins and more severe toxic effects induced by cyanobacterial extracts than purified cyanotoxins suggest that results from studies based on cyanobacteria extracts with known concentration of a toxin are an adequate basis to develop the acute reference doses for microcystin and cylindrospermopsin in domestic animals."

The reviewer agrees with OEHHA's approach.

Exposure assessment

4. "**The scientific data to predict air concentration of microcystins is too limited and more sufficient studies are needed.** Some approach was made recently by Backer et al. (2008) who determined the concentrations of microcystin in water, aerosol of bloom-free lake and blood of 97 people recreating near the lake. The cyanotoxin was found at low concentrations in water (2-5 µg/l) and the aerosol samples (0,1 ng/m^3). Blood levels of microcystins for all patients were below the limit of detection (0,147 µg/l). The study was performed when the water and aerosol concentrations of the toxin were very low, however it can be assumed that microcystin even when it is at low level in water it can be aerosol-borne and inhaled from during water skiing or from other water activities. Moreover, other scenarios of cyanotoxin inhalation for recreating people should be also considered. For example, dried cyanobacterial cell debris remaining on the shores and beaches of recreational lakes may contain high amounts of cyanotoxins that could be airborne and inhaled or digested when swallowed."

OEHHA agrees that additional studies are needed to characterize the risk associated with airborne concentrations of microcystins. We used the studies available to us at the time of the report. Further review is currently limited by lack of available studies and funding. According to data from Cheng [113], exposure to water skiers is much lower than to swimmers – so the swimmer action level

should be protective of the water skier. Information on inhalation of dried algal particulates was not available.

5. "**In my opinion, estimation of water amount ingested via gulping during swimming should be also included in the assessment of the canine exposure.** Some amounts of cyanotoxin-contaminated water can be ingested by dogs during gulping and also afterwards, via licking the coat. The amount of absorbed water and cyanobacterial scum seems to be dependent on the length of a dog's hair. The longer hair of a dog, the more water is retained and higher doses of toxic cyanobacteria could be absorbed and then ingested. Assumption that the water forms a 2 mm layer on the coat may not be applicable to all dog breeds. In case of a small dog with long hair such as Yorkshire terrier the surface of cyanotoxin absorbance would be larger and given that average body weight is smaller in comparison to other dog breeds, the suspected toxic effects would be more pronounced. However, it should be also taken into account that dogs have a natural ability to get rid of the water and cyanobacterial scum by rapid shaking the water off. As a result of this action, the total amount of water ingested during grooming would be smaller. For developing the action levels some other ways of dog exposure to cyanotoxins during exercises should also be considered: via skin, especially for some skin-penetrable cyanotoxins such as anatoxin-a and by inhalation of aerosols or dried cyanobacterial debris containing cyanotoxins when exercising at the edge of water."

OEHHA agrees with this and other reviewers that have suggested similar concerns. An uncertainty factor of 3 has been added to the acute and subchronic domestic animal exposure assessments for all of the cyanotoxins covered in the report. The added uncertainty factor represents the uncertainty of exposure due to preferential consumption by domestic animals. This approach assumes that animals may eat or drink up to three times their normal intake due to preferential consumption of cyanobacteria. No information was found on inhalation of aerosols or dried cyanobacterial debris in dogs. Dermal exposure was not estimated because OEHHA focused on the major pathways of exposure.

Microcystin Ecotoxicology

6. "Cyanobacterial toxin-positive blooms are very frequently found in in many water reservoirs abundant in many species of fish. Toxicological studies show that these aquatic animals are sensitive to cyanotoxins. Development of action level for those

animals seems to be very important issue since these organisms play an essential ecological role and they are also essential for human consumption. However, it is rather impossible to develop the action levels for cylindrospremopsin and anatoxin-a in fish, because there is too little data on the toxicity of these cyanotoxins. On the other hand, there are many toxicological results on the influence of frequently detected microcystin-LR on different endpoints of fish health, such as growth rate, osmoregulation, heart rate, behavior, liver, intestine, kidneys, heart, spleen and gills. Data on microcystin developmental toxicity and immune system also exist. Microcystin toxicity to fish depends on the exposure route. In most studies on acute toxicity in fish this cyanotoxin was administered intraperitoneally and this way of exposure is not natural. The cyanotoxin given into the body cavity usually is more toxic, it is absorbed faster and has different pathways of metabolism. In a number of studies fish were also administered orally freeze-dried cyanobacterial cells and results would be most sufficient for the determination of action levels. Other natural routes of intoxication should also be considered, such as uptake of microcystin directly from water by immersion. In natural conditions the transfer of algal toxins by the food web with zooplankton, crustaceans and smaller fish is also possible. In a such scenario the absorbed doses of cyanotoxins could be much higher in comparison to direct exposure from water. **I suggest consideration of developing action levels for fish for one cyanotoxin: microcystin-LR** for two reasons:

1. Fish are a very important taxonomical group of animals for human consumption and play an important role in aquatic water ecosystems.
2. It seems that there are enough data for developing the reference dose of microcystin-LR for fish. Consideration of some new results could also be useful for developing the reference dose: such as dietary threshold for microcystin-LR in quart medaka (Deng, 2010)."

OEHHA agrees that developing microcystin action levels for fish health would be beneficial. This is something that the State Water Resources Control Board (SWRCB) or OEHHA may be able to pursue in the future. OEHHA did develop action levels for human consumption of sportfish, described in Section V and Appendix II of the report.

A broader perspective of the scientific issues

a) "Analysis and the development of human and animal action levels is a complex scientific effort. Possible teratogenic and dermatotoxic effects induced by some

cyanotoxins were not considered in this report. These are essential endpoints of the toxicity criteria, however, there are too little adequate studies on possible teratogenic effects of cyanotoxins. Some teratogenic influence of anatoxin-a at sublethal dose was found by Astrachan et al (1980) on hamsters such as fetal stunting but not on rats and mice. No teratogenic effects were also found in mice and toads exposed to microcystin-LR however development of Aphrican clawed frog (*Xenopus laevis*) eggs was altered. Microcystins and cylindrospermopsin are assumed to be not able to penetrate the skin, however some authors suggest that these cyanotoxins could induce skin toxicity such as allergy or skin irritation. Microcystin-LR was documented to cause eye irritation, it is also an allergenic agent but only at very high concentrations (1,5 mg/ml). Experimental studies revealed that cylindrospermopsin induces delayed-contact hypersensitivity reactions in Balb/c mice. Some reports also suggest highly irritant potency of cylindrospermopsin."

OEHHA agrees that more studies of potential teratogenic effects of cyanotoxins are needed. Dermal exposure was considered in OEHHA's human health evaluations. In general, however, this route of exposure was not considered to be predominant. OEHHA focused on the major routes of exposure and toxicities of these cyanotoxins based on information available at the time.

"There is a great need for adequate studies to develop the action levels for poultry and currently no toxicological results are available. Possible drinking of cyanotoxin-contaminated water could be a great risk for this essential group of animals used for human consumption. Tissue accumulation and possible further transfer of the cyanotoxins with food to humans should be also considered. The results could be also useful for development of the action levels for birds living in the wild."

OEHHA agrees that more studies of the effects of cyanotoxins on birds are needed.

b) "Action levels are based on commonly used methods of toxicity and exposure assessment. However, I recommend considering some new results that have been published during recent 2 years."

Unfortunately, we are not able to review and incorporate all of the relevant scientific information published after our original 2009 draft. We have incorporated some recent information from publications specifically highlighted

by reviewers. The recent results listed by this reviewer involve fish health and thus would not change any action levels developed in our report.

c) “HPLC and LC/MS are very common techniques used for determination of water concentrations of various cyanotoxins. There are some new methods that have been developed recently. ELISA tests for the detection of cylindrospermopsin, microcystins, nodularin and saxitoxins in different media, including water samples and human serum. A good method for determination of microcystin toxicity is colorimetric test measuring protein phosphatases inhibition.

“There are methods used to monitor the cyanotoxin production. The antibody-based methods (CQ-ELISA) can be used for the detection of cyanobacterial strains producing cyanotoxins. The ability of a strain to produce cyanotoxins could be determined by the use of PCR-based techniques by which the presence of genes coding certain cyanotoxins can be detected and quantified. The Fluorescent in Situ Hybridization (FISH) allows to localize the cyanotoxin genes in mixed phytoplankton populations. These methods are early warning system that allows to obtain results very quickly, within 1-3 hours.”

The reviewer describes important information regarding cyanotoxin detection methods. However, OEHHA was not asked to review analytical methods. The SWRCB may review this topic when developing any future cyanotoxin monitoring programs.

Additional comments

1. “There is a sentence in the draft on page number 52 that there are 70 congeners of microcystin. Currently, over 80 variants of this chemical have been described.”

This has been updated.

2. “Anatoxin-a is an alkaloid and it has a similar chemical structure to cocaine. Considering the determination of swimmer exposure this cyanotoxin could be not only from the stomach and intestines but also sublingually and from mucous membranes in the mouth.”

OEHHA agrees that this exposure may occur. However, we focused on the major exposure pathway of swallowing water. The action level for recreational swimmers is based on children age 7 – 10, estimated to ingest 250 ml of water per swimming event (5 hrs). This is the group with the greatest exposure level. We expect absorption through the mucous membranes in the mouth or sublingually to be small relative to ingestion.

List of useful reference:

I added the list of reference that I used in my review.

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4. Fawell J.K., Mitchell R.E., Hill R.E., Everett D.J. 1999. The toxicity of cyanobacterial toxins in the mouse: II anatoxin-a. Hum Exp Toxicol 18, 168-73.
5. Chernoff N., Hunter E.S.Hall L.L, Rosen M.B, Brownie C.F, Malarkey D., Marr M.,Herkovits J. 2002. Lack of teratogenicity of microcystin-LR in the mouse and toad. J Appl Toxicol. 22, 13-17.
6. Deng DF. Zheng K., Teh F-C., Lehmann P.W., Teh S.J. 2010. Toxic threshold of dietary microcystin (-LR) for quart medaka. Toxicon 55, 787-794.
7. Backer L.C., Carmichael W., Kirkpatrick B., Williams C., Irvin M., Zhou Y., Johnson T.B., Nierenberg K., Hill V.R., Kieszak S.M., Cheng Y-S. 2008. Recreational exposure to low concentrations of microcystins during an algal bloom in a small lake. Mar Drugs , 6 389-406.

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Peer review of cyanotoxin toxicity criteria and health based water concentrations to protect human swimmers, dogs and cattle

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The Office of Environmental Health Hazard Assessment's (OEHHAs) responses and notes are provided in bold, blue italic.

1. General Approach and General Comments of the Reviewer:

a. "The OEHHA report addresses the need for informing the public of the potential public and animal health threats of cyanotoxins. As with any rapidly emerging environmental problem, the scientific data are often complex and, in some cases, contradictory.

b. "The six candidate cyanotoxins selected seem reasonable and appropriate considering the 1) widespread occurrence the toxins 2) high toxicities related to humans and wildlife and 3) scientific literature available on these cyanotoxins at this time.

"The OEHHA has reviewed and discussed the relevant literature on the six candidate cyanotoxins, up to the date of release of the draft proposal. Although the toxicology of cyanobacteria is a rapidly developing field with a growing literature, this reviewer is not aware of any findings published since 2009 that would significantly alter the findings of the OEHHA report.

c. "The report does not deal with the question of analytic methods needed to quantify the six cyanotoxins, an important but perhaps not critical to the immediate goals of the report. This point is covered later in more detail."

The reviewer agrees with OEHHA's approach.

2. Toxicity Criteria for the six chemicals

a. “Epidemiological studies in China suggest possible long-term effects of microcystins on the incidence of liver cancers (Falconer 2005, Yu et al. 2002, Yu & Yuan 2004). However, as noted by the authors, such studies cannot exclude the role of other toxic substances as well as other microbes associated with polluted waters. In the absence of controlled long-term experiments on carcinogenic effects of microcystins, the focus of the OEHHA report on liver toxicity is well justified.”

The reviewer agrees with OEHHA’s approach.

b. “The OEHHA use of studies using cyanobacteria extracts to set toxin action levels raises some important considerations. On the one hand, experiments using a single purified toxin allow for clear association between the toxin level and the response. In contrast, extracts of cyanobacteria contain a measurable concentration of the toxin of interest, but cyanobacteria extracts often contain additional toxic substances as well as a broad spectrum of other chemicals, with unknown effects on the test species. Thus, one might assume that tests conducted with purified toxins would be the ideal basis for setting toxicity criteria. However, an additional factor should be considered, i.e. the cyanotoxins under consideration are generally endotoxins, contained within the cyanobacteria cells, unless released through decomposition or cell breakage, such as through sonification and freeze-thaw treatments commonly employed in preparation of samples for testing. Thus, for example, organisms consuming cyanobacteria contaminated water would ingest a mixture of both dissolved and intracellular toxin. The free dissolved fraction might be expected to correspond to the effects seen with the purified toxin, whereas the toxin contained in the cells, often a large portion of the toxin present, is not available to become toxic until it is released in the digestive system. There is little known about the ability of humans or other mammals to digest cyanobacteria, although it is likely to be variable, depending on the type and condition of cyanobacteria cells. Based on studies of with crustacean zooplankton such as *Daphnia*, cyanobacteria with protective gelatinous sheaths may pass through the gut unharmed (Porter 1975). Thus, studies employing purified cyanotoxins might be expected to overestimate the effect of the single toxin, but do not allow for the combined or synergistic effects caused by other chemical and cyanobacteria metabolites present and thus may underestimate the toxicity that would occur in nature. Considering the paucity of studies with vertebrates using purified toxins, especially cylindrospermosin and anatoxin-a, the OEHHA decision to use the results from studies using cyanobacteria extracts seems reasonable as the best information available at this time.”

The reviewer agrees with OEHHA's approach.

c. ***Technical points***:

i. "In Table 13, it is not clear how the anatoxin-a subchronic level of 100 for dairy was derived and whether it is based on experimental data or assumptions. This section is somewhat confusing and difficult to follow."

In response to this comment we expanded the text in Section VI, Domestic Animal Exposure Assessment, and specifically addressed the final derivation of these action levels. We also provided text in Section VII, Summary, that described the summary tables and linked the data in each table to sections of the report describing that data.

ii. "Table 4 footnote #3 states that "apply action levels to the sum of all microcystins variants until subchronic toxicities of other variants are clarified". There is ambiguity as to whether "all microcystins variants" refers to the four microcystins variants considered in this document or the broader array of microcystins analogs that would be measured with an ELISA technique."

"The sum of all microcystin variants" was intended as all detected microcystins, or total microcystins. This has been corrected in the report.

iii. "Laboratory techniques used for analysis of cyanotoxins in tissues may involve the use of fresh tissue or freeze-dried material. To avoid confusion and potential errors, where tissue levels are concerned it is important to clearly state whether the units for the tissue are wet weight or dry weight (e.g. Table 4. This could be done as a footnote or as often is done in the units of measure as ng/g tissue dw or ww."

This has been added to Table 4 and elsewhere in the report.

iv. "Concerning the table on page iv, footnote 4 should probably read 'subchronic' rather than 'subacute'."

This has been corrected in the table on page iv of the report.

3. Exposure Assessment

a. ***MC in aerosols***: “Recent studies conducted along coastal beaches have confirmed the presence of red tide neurotoxins in ocean aerosols generated by bubbles. These studies also confirm a correlation between the concentration of the toxin in the water and in the aerosols. Less is known about the importance of aerosol-borne cyanotoxins on or near lakes.

“Numerous reports suggest a higher incidence of illnesses such as flu-like symptoms, rashes and respiratory irritation in persons living near lakes with cyanobacteria blooms (Stewart et al. 2006), although there are few quantitative data to support these claims. Intake of cyanotoxins in airborne aerosols represents a potentially important pathway, because of the broad range of dispersal potential exposure to large populations. The few studies that have been conducted on aerosols emitted from water bodies have found microcystins present, but in low concentrations. The OEHHA dismisses aerosols as a potential source of microcystins for lake recreation users based on the data from Cheng (2007) that had some field results, but focused largely on laboratory examination of aerosol formation. More relevant data were collected in a recent study by Backer et al. (2010) of two California lakes that found an average of 0.3 ng MC/m3 (<0.1 – 2.89 ng MC/m3) in the aerosols collected at these lakes during periods of cyanobacteria blooms. Assuming an adult inhalation rate of 25 liters per minute, Backer et al. estimated an inhalation of 0.8 ng MC for a 106 min exposure of swimming or boating. It also appeared from this study that most of the inhaled toxins were deposited on the upper respiratory tract, a potentially effective area for absorption of the toxins into the body. These calculations were based on average recreational periods of less than two hours, however, and do not consider that in addition to the period of active recreation many lake visitors are likely to spend a significant amount of time at or near the water body at other activities, thereby having considerably longer exposure times. Of course, exposures would be even longer for residents living at or working near the lake. Although this newer information on toxins in aerosols near lakes suggests inhalation could be a pathway for exposure to cyanobacteria toxins, it would appear that OEHHA action level for microcystins in lake water of 0.7 µg MC/L should provide an adequate level of protection against inhalation of harmful levels of MC in the air during recreation activities. Further studies are badly needed to evaluate long-distance dispersal and potential long-term effects of aerosolized hepatotoxins and neurotoxins on both lake users and lake residents.”

The recent study by Backer et al. (2010) provides valuable data and represents an important issue for further study. It will likely be difficult to relate air concentrations to water concentrations of cyanotoxins until this issue is better understood. Presently, OEHHA agrees with the reviewer regarding the overarching protection provided by the microcystin action level for recreation.

b. ***Exposure of dogs to cyanotoxins*:**

"Estimation of action levels of microcystins, anatoxin-a and cylindrospermopsin for dogs is especially difficult to determine. The OEHHA has accounted for the intake by dogs of water and cyanobacteria attached to the fur following swimming. The assumed ingestion of a 2 mm coating of water seems plausible, although this is likely to be variable and dependent on the size and breed of the dog. It is perhaps more difficult to account for the highly selective drinking habits of dogs, that generally drink close to the shore, where concentrations of cyanobacteria also tend to be the highest, especially when surface blooms are blown shoreward. Thus, dogs may be exposed to higher toxin levels than those sampled at sampling stations somewhat offshore. Also problematic is the possibility that some animals, including dogs, may be attracted to water containing cyanobacteria (Codd et al. 1992, Lopez et al. 1999) and thus may actively select to drink from areas of the lake with highest cyanobacteria concentrations and the highest levels toxicity. According to the above authors, this "fatal attraction" may be responsible for the frequent reports of acute deaths of dogs and cattle after exposure to lakes and streams. Selective near shore drinking is potentially a more important consideration that the gulping of water during swimming.

"The acute action canine drinking water exposure levels of 500, 400 and 500 µg/L for microcystins, anatoxin-a and cylindrospermopsin, respectively, would seem adequate, but dependent on a sampling protocol that actually collects water for testing from the immediate shoreline area that dogs would normally use for drinking. Based on the limited studies available, the subchronic action levels recommended are likely to provide safe levels of cyanotoxins where repeated exposures are expected. It is important to consider that action levels are only meaningful when tests are conducted on the near shore water that is likely to be consumed by dogs. Although it would be difficult to develop an accurate metric to account for selective drinking, it might be useful to consider the addition of an uncertainty factor to account for the tendency for near shore drinking and the possible attraction to higher than average levels of cyanotoxins."

OEHHA agrees with this and other reviewers that have suggested similar strategies. An uncertainty factor of 3 has been added to the acute and

subchronic domestic animal exposure assessments for all of the cyanotoxins covered in the report. The added uncertainty factor represents the uncertainty of exposure due to preferential consumption by domestic animals. This approach assumes that animals may eat or drink up to three times their normal intake due to preferential consumption of cyanobacteria.

c. Cyanobacteria crusts:

"Presumably, many of the crusts of cyanobacteria deposited on the shores of ponds, lakes and streams are the result of surface cyanobacteria blooms of planktonic cyanobacteria that have aggregated along the shore or blown on land and. Benthic cyanobacteria often form dense mats that periodically rise to the surface, buoyed by gas that has accumulated underneath the mat. It seems probable that some of the crusts that occur on shore could be derrived from floating benthic mats. There is evidence that attached forms of benthic cyanobacteria such as *Phormidium* and *Oscillatoria* produce microcystins and anatoxin-a and ingestion of dislodged mats have been linked to the death of cattle in Switzerland (Mez et al. 1997) and dogs in Scottland and France (Gugger et al. 2005, Edwards et al. 1992). Thus, OEHHA might consider including benthic mats in the category with "crusts", since it is likely that at times these are one and the same. This designation might also result in an awareness of the potential risks associated with submerged, floating and landed cyanobacteria mats and the determination of their toxicity."

In response to this comment, OEHHA has included surfaced or landed benthic mats with crusts as seen in Section VI, Domestic Animal Exposure Assessment, and in Tables 7, 8, 10, and 14.

4. ***Microcystin Ecotoxicology***:

"The OEHHA review of the research on ecotoxicology of cyanotoxins included a reasonable sampling of papers published in this field as well as an accurate assessment of the state of the understanding for the microcystins, cylindrospermosin and anatoxin-a. As noted in the OEHHA report, most of the research on aquatic food webs has examined the production and accumulation of microcystins in components of the web, including zooplankton, fish and, to a lesser extent, freshwater mussels. Although microcystins do not generally biomagnify as has been seen for some toxins, such as DDT and mercury, trophic levels do retain the relatively stable microcystins so that they are effectively transferred to the higher trophic levels (Kotak et al. 1996).

“Since most studies conducted on cyanotoxins in lake food webs have measured static quantities of toxins in the various trophic levels we have little understanding about the dynamics of the transfer of cyanotoxins in lake ecosystem. For example, bloom forming cyanobacteria such as *Anabaena* and *Microcystis* are relatively large forms and are highly inedible for many of the zooplankton grazers, such as *Daphnia*. Thus, the seemingly simple question of how toxins enter and move through the food web cannot be answered at this time. Also, it is not known whether some of the toxicity detected in lake water is actually produced by the smallest cyanobacteria or picocyanobacteria (< 2 µm). These abundant phytoplankton are not considered in most studies of cyanobacteria toxicity, although these potentially grazeable cells are capable of producing microcystins (Domingos et al. 1999).

“The OEHHA accurately concluded that the research to date is inadequate to allow for setting toxin limits to protect fish species. Among the many toxicological issues that are at present inadequately addressed are 1) differences in the tolerance levels of fish species to the biotoxins 2) ontogenetic changes in sensitivity to the toxins with age of the fish and 3) the ability of some fish and invertebrates (Williams et al. 1997, Smith et al. 2010) to covalently bind toxins such as microcystins to proteins where they are effectively stored in a non-toxic state and possibly slowly released through excretion (Smith and Haney 2006).”

The reviewer agrees with OEHHA’s approach.

5. **Broader perspective points and questions**:

a. ***Data availability***: “The report entitled “Toxicological summary and suggested action levels to reduce potential adverse health effects of six cyanotoxins” is comprehensive and clearly describes the rationale and scientific basis for the toxicity and exposure assessments as well as the proposed action levels for the six cyanobacteria toxins under consideration. The subject is complex and many areas in this field have had little research, such as the carcinogenic potential of cyanobacteria toxins. Also, there is little known about the potential effects of chronic exposure to the neurotoxins, such as anatoxin-a.”

The reviewer agrees with OEHHA’s approach.

b. “The OEHHA report makes an important and necessary step in updating the recommendations of the World Health Organization, still widely used although it was first proposed in 1998. The proposed action levels for human recreation, dogs and cattle

make useful distinctions between target types (humans, dogs and cattle) as well as between acute and subchronic effects, where possible. It is not clear how these categories will be eventually applied to specific situations, although it appears this information is designed to assist state and local agencies in setting appropriate limits."

The reviewer agrees with OEHHA's approach.

c. ***Testing methods***: "Although methodologies for measuring the candidate cyanotoxins was not in the OEHHA report, implementation of these findings will require a review and careful analysis of the most appropriate detection methods. Development of SOPs for the testing of cyanotoxins will not be simple. For example, the report clearly identifies the four microcystins analogs, LR, YR, RR and LA, selected in in part because they had comparable RfD levels. To determine the concentrations of each analog at this time one could use HPLC-MS. From a practical standpoint, however, state and local agencies may find it more efficient and less costly to measure the microcystins levels with an ELISA kit, as this is highly sensitive, can be carried out with minimal laboratory facilities and personnel. The results of the testing, however, will differ with the two methods, as ELISA antibody reactions generally have a wide range of cross reactivity, measuring more than the four selected MC analogs, and doing so with differing degrees of reactivity. Considering the importance of turn-around- time for getting samples tested when public health is involved, the ELISA method may be preferable, but it will not be possible to know which microcystins were present if that technique is used."

OEHHA agrees that implementation of our findings will require a review and careful analysis of the most appropriate detection methods. The reviewer describes the important variables that must be considered. However, OEHHA was not asked to review analytical methods. The State Water Resources Control Board (SWRCB) may review this topic when developing any future cyanotoxin monitoring programs.

Bound and free forms of MC: "Microcystins are generally extracted by exposure of tissues to aqueous methanol. This treatment does not extract microcystins that are covalently bound to proteins. Williams et al. (1997) raised the question of the importance of microcystins bound in cells to protein phosphatases when they determined that the majority of the total body load of MC in blue mussels and Dungeness crab was present in the protein bound form. This and other studies using Lemieux oxidation to release the bound MC have indicated that a large fraction of the

total MC pool in organisms is in the covalently bound form. However, the relevance of this finding is not clear, since the covalently bound MC is presumably non-toxic to the organism containing it, although and there is evidence that bound MC may contribute to the transfer of MC through the food web (Smith et al. 2010)."

OEHHA agrees that this is an important issue when evaluating the ecotoxicology of cyanotoxins. More studies are needed to determine the implications of protein-bound microcystins to wildlife.

d. ***Surrogate methods***: "It is difficult to ignore the difficulties and costs associated with measuring cyanobacteria toxins. As noted in the OEHHA report, many states have employed testing procedures that utilize counts of cyanobacteria cells as a proxy for toxicity testing. Although the OEHHA report deals solely with cyanobacteria toxins, it might be useful to examine other methods as surrogates for estimating the risk from toxic cyanobacteria. Despite many limitations, one of these methods that shows promise is the use of fluoroprobes that measure the fluorescence of phycobilin pigments found in cyanobacteria. When calibrated with standardized laboratory culture of a known strain of cyanobacteria such as *Microcystis aeroginosa*, rapid assessment can be made of the total population of cyanobacteria, Leboulanger et al (2002) has demonstrated phycocyanin fluorescence counts can be used to predict the levels of cyanobacteria and the probably levels of microcystins present. This relationship works best at high concentrations of cyanobacteria, when potentially interfering forms such as cryptophytes are not abundant (McQuaid et al. 2011). The advantages of this method, when properly calibrated, are that it is rapid, relatively inexpensive and the measurements can be conducted either at the *in situ* at the lake. Rapid assessment can be especially important when evaluating water condition in recreational waters, since the local conditions in a particular region of the lake can change rapidly, depending on weather conditions. As with any method there are potential problems that must be addressed including:

1) microscopic examination should also be conducted to assure that other phycocyanin/ phycoerythrin containing phytoplankton such as cryptomonads and dinoflagellates are not present

2) background fluorescence by colored dissolved organic matter may create errors in humic rich waters and

3) quenching of fluorescence signals at high turbidity levels may result in underestimates of cyanobacteria abundance. Because of the need for a rapid cyanobacteria assessment method, major producers of these fluorescence probes are currently developing methods that will improve the accuracy of this technique, such as built-in corrections for high turbidity and humic water color. As these methods are further tested and improved they may prove to be a valuable addition to the direct measurement of cyanotoxins. For example, on-lake fluorometry could provide the first level of indication of a water quality problem that could be followed up with more accurate analysis of the cyanotoxins present."

OEHHA recommends direct measurement of the toxin rather than an estimation of the cyanobacteria population. The complexity of the relationship between the presence and quantity of cyanobacteria and concentrations of cyanotoxins appears to preclude the estimation of toxin concentrations from cyanobacterial density. Cyanobacterial population estimates can overestimate the risk of cyanotoxin poisoning if cyanobacteria are present but not producing toxin. They can also underestimate the risk of cyanotoxin poisoning because cyanotoxins may persist in the water after a cyanobacterial bloom has subsided and is no longer visible. However, as the reviewer stated, this method may be successful in confirming the presence of cyanobacteria.

e. ***Sampling protocols***: "Bloom-forming cyanobacteria present a particularly challenging sampling problem, since their buoyancy and relatively large size give them in-lake mobility not generally seen with other toxins found in lakes. The most sophisticated water testing procedures mean little if the sampling is not carefully conducted. It would be especially useful if the OEHHA could develop recommended sampling protocols for the different water bodies likely to be involved in cyanobacteria testing. The objectives of the sampling must first be determined, e.g., is the intent of the sample to provide evidence of the average conditions for the water body, or to represent the condition of an isolated region such as a beach or recreation area. The sampling must also consider the vertical mobility of cyanobacteria blooms, especially if these are concentrated at the water surface. Since many of the commonly occurring cyanobacteria such as *Anabaena*, *Planktothrix* and *Microcystis* can adjust their buoyancy according to light conditions, coming to the surface under low light or at night and easily mixing deeper in the water column with bright light and with light wind action. Thus, for example, it is important to avoid using grab samples that may hit or miss the population, depending on the depth sampled. Collection with a form of integrated tube sampler would minimize spatial variability due to depth. The horizontal distribution across the lake is also transient and highly patchy, requiring an integrated horizontal

sampler (our lab uses a continuous peristaltic pump) or multiple sampling sites. Sampling designs may follow a general protocol, but specifics will most likely vary with each system, dependent on the lake or pond size and morphometry. I emphasize this last point, as it is not often considered as a fundamental part of water quality testing programs, despite its potential importance."

This is valuable information that will likely be of assistance to the SWRCB. OEHHA was contracted by SWRCB to provide a toxicological review and risk assessment of the six cyanotoxins. Sampling protocols are the purview of the SWRCB and state or local health agencies.

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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."

by B. A. Neilan PhD

11th June, 2011

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.

The Office of Environmental Health Hazard Assessment's (OEHHAs) responses and notes are provided in bold, blue italic.

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 reviewer agrees with OEHHA's general approach.

"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."

OEHHA agrees that a summary of the cyanobacteria problem at local, federal and global levels would be an excellent addition to this report. However, as stated in the Executive Summary and Preface, this document is designed solely to provide a toxicological review and risk assessment information on the designated cyanotoxins and is a deliverable item under a contract between the State Water Resources Control Board (SWRCB) and OEHHA. Characterizing the occurrences of cyanobacterial blooms in California did not fall within the scope of this project. SWRCB provides additional information on cyanobacterial blooms through other documents and website links.

"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."

We have reviewed these sections and cannot identify any significant duplication between them. The section "Health-Based Criteria for Cyanotoxins" (now Section III) begins by summarizing some key points from the previous section, "Cyanotoxins and Potential Health Effects" (now Section II), in order to establish the need for the information provided in this section (reference doses for the selected cyanotoxins in humans and domestic animals). This summary is brief and includes one paragraph. We may have misinterpreted this comment, but have provided due diligence to avoid duplication.

"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."

The report has been reformatted and made clearer. The format follows the Table of Contents and the sections are clearly marked throughout the report. All equations have been numbered, including those in the appendices. We have also included several new internal references within the report to connect information between the sections.

"There is no consistency as to the application for determining the RfD and other parameters for each toxin."

OEHHA acknowledges that different approaches were used to determine the various RfDs in this document. The most appropriate model was used to fit the available toxicity data for a particular RfD. The uncertainty factors used for human RfDs were consistent. For domestic animals, the uncertainty factor was tailored to the available data on toxicity and exposure for each cyanotoxin.

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."

Microcystin (MC)-LA, MC-RR and MC-YA were also assessed but no studies were found that could support an RfD. However, we found that all of the microcystins assessed here had similar modes of toxicities and apical endpoints. For this reason, we applied the MC-LR RfD to the other three microcystin variants.

"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."

The reviewer agrees with OEHHA's approach.

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_{50} 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."

Studies using intraperitoneal (i.p.) exposure were not considered because they do not reflect actual exposure routes to dogs or livestock. Oral and i.p. exposures lead to different pathways and rates of metabolism of the toxin, which leads to dramatically different effect levels. We are interested in effects associated with ingestion. Additionally, studies using fresh cyanobacteria, rather than extracts, were rare.

"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_{50} 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_{50} listed as 50 µg/kg

MCYR with an LD_{50} listed as 70 µg/kg

MCRR with an LD_{50} listed as 600 µg/kg

MCLR with an LD_{50} listed as 50 µg/kg"

Only one microcystin variant, MC-LR, was included in calculating the RfDs and action levels provided for the four microcystin variants. This was done because a) the variants have similar mechanisms of toxicities and apical endpoints and b) no data were available for oral toxicity levels in the other variants. From the i.p. toxicities provided above, we see that MC-LA, -YR and –LR are quite similar while MC-RR shows much lower toxicity through the i.p. route. OEHHA focused on oral toxicity levels because they represent the most realistic exposure. Toxicity levels for oral and i.p. exposures are significantly different for MC-LR.

"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."

This section was describing the mechanism of toxicities and apical endpoints of the microcystin variants, not the threshold of toxicity.

"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."

OEHHA agrees that RfDs and action levels for nodularin would be beneficial. However, the scope of this project was limited to the six cyanotoxins addressed in the report. This limitation is now clearly described in the preface of the report through the following text: "SWRCB asked OEHHA to provide toxicological assessments, exposure assessments and action levels for six cyanotoxins that had been prioritized by the USEPA: anatoxin-a, cylindrospermopsin, microcystin LR, microcystin RR, microcystin YR and microcystin LA. Several other cyanotoxins are present in California and require the attention of regulatory and resource agencies. Limited funds and availability of toxicological information narrowed the scope of this report to these particular cyanotoxins." The SWRCB may choose to address nodularin in the 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."

Developing toxicity criteria on the single best study is standard procedure in human health risk assessment. The basis of choosing the best study relies on professional judgment by the toxicologist as based on toxicological principles. The toxicity criteria generally fall outside of the experimental doses because high doses are used in these studies on few animals. There are numerous interspecies differences including those due to pharmacokinetics and body size. The effects seen at experimental doses are typically severe effects; however less severe effects that would occur at lower dose levels are also a concern. Mathematical models are used to estimate a safe dose between a dose that causes an effect and one that does not, or the control. If there is insufficient data to estimate a safe dose using mathematical models, then the lowest dose that results in effects is used as the Lowest Observed Adverse Effect Level (LOAEL) and the highest benign dose below that is the No Observable Adverse Effect Level (NOAEL).

"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."

Heinze used microcystin LR, as stated on page 15 of the report: "Heinze [83] exposed two groups of ten rats each to microcystin-LR-laced drinking water for 28 days."

"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."

In response to this comment, OEHHA has expanded our discussion of identifying the most appropriate study on which to base the microcystin-LR RfD. Both the Fawell (1999) and Heinze (1999) studies found liver toxicity and used overlapping doses. The study on mice by Fawell identified a NOAEL of 40 µg/kg-d and a LOAEL of 200 µg/kg-d, which was the next highest dose level. The study on rats by Heinze used lower doses and identified a LOAEL of 50 µg/kg-d. OEHHA chose the Heinze study as the basis of the RfD because it evaluated more endpoints, utilized a better experimental design, included lower toxin doses, showed greater target organ specificity (intrahepatic hemorrhage) in the histopathological analysis, and showed a clear dose-response trend. Additionally, the rats of the Heinze study showed a greater sensitivity to microcystin-LR than the mice of the Fawell study. The most sensitive model is generally used when extrapolating human toxicity from a rodent model. This is taken as a precautionary measure.

The problem with i.p. injection studies, such as Hooser et al. (1989) and Solter et al. (1998), is that the potency of the toxin is considerably different between oral exposure and i.p. administration. For example, Fawell et. al. (1999) found that "microcystin-LR is 30 - 100 times less toxic via oral ingestion than via intraperitoneal injection".

"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_{50} 12 times greater that of MCLR. Notably, the Heinze study on which the RfD was based only studied the effects of MCLR."

The derivation of the RfD for MC-LR is explained in detail on pages 14 - 16 of the report. The MC-LR RfD was also used for MC-LA, -RR and -YR, as explained on page 13 of the report: "the toxicity criteria computed for microcystin-LR will be used for microcystins LA, RR and YR." This is because "the LA, RR and YR congeners appear to have similar toxicological effects: these congeners induce histological changes in rodent liver similar to microcystin-LR and have been shown to inhibit the same phosphatases [75]." The i.p. LD_{50}'s provided by the reviewer above show that three of the microcystins have similar i.p. toxicity levels (MC-LR, -LA and -YR). However, MC-RR does appear less toxic by the i.p. route. But as noted above, the route of exposure can have a profound effect on the potency of these toxins.

"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."

In response to this comment, the following text was added to the benchmark dose discussion in the report:

"OEHHA's use of the BMD approach here does have limitations: only two dose levels were used in the study and the 95 percent lower confidence limit on the BMD (BMDL) is well outside of the dose range tested. It is helpful to point out here that an alternative standard protocol of dividing the LOAEL, 50 µg/kg-d in Heinze (1999), by 10 to estimate a NOAEL of 5 µg/kg-d provides a very similar point of departure as achieved using the BMD approach, 6.4 µg/kg-d."

"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."

The mistake in the example given was due to a typographical error. The information is found in Appendix III rather than IV. Appendix III is one page and only describes this conversion. To address the broader comment, we have added explanatory text to the calculation of RfDs and, to a greater extent, exposure assessments and action level derivations in domestic animals.

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

In response to this comment we have added explanatory text throughout the descriptions of our RfD determinations, exposure estimates and action level derivations.

"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 analyses utilized standard practices in applying the benchmark dose approach to establish RfDs. For both toxins, we used the model that best described the dose-response relationship.

"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."

A description of the toxicology of each toxin is provided in this section. Microcystins and cylindrospermopsin toxicology are described on pages 12 and 17, respectively.

"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 reviewer agrees with OEHHA's general approach.

"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 Kp 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."

We have updated these sections to add internal references. In Appendix I, under the subsection Volatility and Skin Permeability of Cyanotoxins (page AI-6), we clarify that "No information on dermal absorption could be obtained [for cylindrospermopsin]. But due to its large size and charged nature, like microcystins, it was assumed not to penetrate the dermis." When skin permeability is touched upon in discussion of the equations in Appendix I, the reader is directed to the detailed discussion in the subsection Volatility and Skin Permeability of Cyanotoxins (page AI-6). We also pointed out that the variables for equations A.I-1 through A.1-4 at the beginning of Appendix I are shown in the tables on the following pages.

"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."

Information on exposure to cyanobacteria through these mechanisms was not available. OEHHA focused on characterizing the most prominent exposure pathways.

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."

The reviewer agrees with OEHHA's approach.

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 µg/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."

Developing toxicity criteria on the single best study is standard procedure in human health risk assessment. Similarly, applying the human health risk assessment to the most sensitive population is an appropriate conservative approach and is needed to prevent human illness.

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.”

The reviewer agrees with OEHHA’s approach.

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.”

Review of analytical methods or monitoring strategies fell outside of the scope of this project. However the information provided by the reviewer here will likely be of assistance to SWRCB.

“Contemporary guidelines for water safety are frequently based upon a combination of animal bioassays and analytical techniques, thereby enabling assignment of LD_{50} 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."

OEHHA appreciates the above review of molecular detection and quantification methods for cyanotoxins. Some exciting developments have occurred on this front. As mentioned above, the review of analytical methods fell outside of the scope of this project. However the information provided by the reviewer here will likely be of assistance to SWRCB.

Literature Cited in this Peer Review

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