A.F.A., Inc
(Aphanizomenon Flos Aquae)
24308 Modoc Point Rd.
Chiloquin, Or 97624
Phone- 1-541-783-2903
Fax- 1-541-783-3360

5073 '97 MAY 12 P1:30

Dockets Management Branch
Food and Drug Administration
12420 Parklawn Dr. Room 1-23
Rockville, MD 20857

5-7-97

Dear Dockets Manager:

Regarding: Docket # 96N-0417) Federal Register Vol. 62, No 25- Part IV Department of Health and Human Services- Food and Drug Administration 21 CFR Ch 1-

A.F.A. Inc. is a harvester of Blue Green Algae on the Upper Klamath Lake in Oregon. We are a small business with per annum sales of over 1.5 million dollars, in the food supplement industry. A.F.A. has both Current Good Manufacturing Practices, and Good Harvesting Practices which provide for assurances of a safe and healthy food supplement.

A.F.A. supports the Industry Draft of the Current Good Manufacturing Practice in Manufacturing, Packing, or Holding Dietary Supplement: Proposed Rule. The contents of the rule are well designed. A.F.A. has modelled our revised Current Good Manufacturing Practices under the proposed standards and integrated these practices in the A.F.A. Policy Manual and the Health and Safety Plan.

There are refinements in this law that need to be worked on. In theory these are good ideas. A.F.A. Inc. wants to address the F.D.A.'s concerns as expressed in points 1-9 (pages 5707 & 5708) of the proposed points for consideration. A.F.A. is concerned that the law will integrate the points 1-9 (pages 5707 & 5708) of this law without the necessary inclusion of essential and key points outlined below that will protect industry from potential misinteruptation. Regardless of how this proposed law is applied- each industry must be consulted in developing the application to this standard in processing and quality control.

A.F.A. Inc, has comments to the following issues and concerns brought by the F.D.A. Points #1-9:

1. As a responsible company we believe that a reasonable standard be met for the development of defect action levels(DAL). This standard should be set in consultation with industry. A.F.A. Inc. has incorporated series of procedures for harvesting of a high quality product. Along with the harvest of the product can come unwanted cyanobacterias that have toxins in them. We aggressively test for these toxins in our product and have incorporated methodologies for the quality assurance in our product. We have been working with the Oregon Dept of Health toxicologist who is considering implementing the Canadian drinking water standard to be applied to the food

96N-0417

C45

supplement. The scientific studies he uses to base this level are from cyanobacteria with significantly differing strains from those our company harvests on the Upper Klamath Lake. The letter and intent of the food supplement law is to extend existing food industry practices into the food supplements. We request that drinking water standards not be used in application DAL. It is further requested the DAL action levels not be applied without specific quantified analysis of the problem with a standardised analytical methodology, *on the specific strain of the botanical in question*. (Attachment #1)

2. A.F.A. believes that there must be a generally available *standardised* analytical methodology for testing, that does not register false positives (Attachment #1). This testing methodology must be standardised by the AOAC. It is important to provide quality control of the product, a reasonable interim standard agreed upon by industry and health officials, using current methodologies might serve as an acceptable alternative.

3. A.F.A. believes that self regulating industries and associations of food supplement manufacturers or handlers, establishing internally high standards for their product, be allowed to submit their CGMP to the FDA, for acceptance. Industries that have incorporated safeguards and precautions known to the industrial community must be responsible for following through with the Good Manufacturing Practice, on a daily basis. The terminology on a day to day basis should not mean that a batch is indicative of a day. In the blue green algae industry, a batch may the harvest from the 10:00 pm through two days of harvest until 5000 gallons is harvested. This product is stored, chilled and homogeneously mixed. The monitoring of this product through the production and handling should be tracked and tested as established in the CGMPs on a day to day basis. A.F.A. Inc is concerned about placing into law a redefinition of a batch, as a daily figure.

4. If there is no counterpart in part 110 in the section 402(g), specific studies to determine these levels should be conducted by the National Institute of Health to arrive at the acceptable standard. It is our concern that as pointed out in item one- the water standard might be applied. It appears to be the responsibility of the Office of Dietary Supplement within the National Institutes of Health:

> (2). TO PROMOTE SCIENTIFIC STUDY OF THE BENEFITS OF DIETARY SUPPLEMENTS IN MAINTAINING HEALTH AND PREVENTING CHRONIC DISEASE AND OTHER HEALTH RELATED CONDITIONS.

5. A.F.A. Inc. promoted and assisted in sponsoring an event on April 26, 1997, at the Shilo Inn in Klamath Falls where we heard two of the leading national experts in Toxicology: Dr. Wayne Carmichael and Dr. Donald Anderson state that we should conduct very costly daily tests for toxins that have never seen in our product. Different strains of cyanobacteria have differing levels of toxins in differing locations. Item #6 on page 5709 Vol 62, No 25 requests comments on whether GMP should identify evaluate and respond to potential safety concerns with dietary ingredients. If we were to listen to the recommendations of these experts we would be conducting over $80,000.00 of testing per annum for toxins never found in our product to date after 14 years of testing. Testing for proven safety concerns is the responsibility of each company.

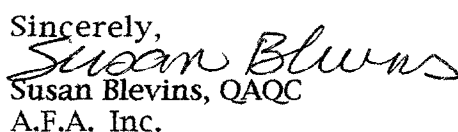
8. We need to be able to work with the FDA in developing the CGMP, for our industry. A.F.A. wants to work with the F.D.A. in developing acceptable standards. The Oregon Dept. of Agriculture and the Oregon Dept. of Health embargoed our entire inventory of product based upon a felonious report by a former disgruntled employee that have essentially the same product, same harvest, same lake same area The product tested out as clear of the contaminant- Microcystin- See attachments #3 (copies of the release of embargo and the testing results)- however due to the action of the Oregon Food Safety Division in applying a rule very similar to that proposed in item #8, we have suffered a financial burden. A.F.A. Inc., because of our first hand experience, believes that whenever the FDA declares any product an action item or consider applying the Hazard Analysis and Critial Control Points (HACCP), they must work with manufacturers of these companies. From our own experience, it was very difficult to hear the FDA was asking distributors questions, when we were not contacted to provide the information.

- Both the FDA- Mr. Nichols Duy(Special Nutritionals FDA) and Mr. Duncan Gilroy(Oregon Dept. of Health) have taken the liberty to call many of our large active accounts asking them directly if they sell our blue green algae product to children. These questions were not levelled to any distributor of competitors products. A.F.A. Inc. has wanted to see uniform treatment. With the official titles and positions these men have, it is important for them not to represent themselves to our marketplace in this manner. If a market survey is called for, it should be conducted by a third party marketing research firm in a professional manner. The design of the market survey should be developed so the information is gathered in a way that the industry is not hurt. If a retailer is placing product on the shelf, he may not know who is purchasing this product. These major retailers and resellers who are purchasing our algae are becoming alerted and have an extra level of concern because of the manner in which they were contacted. (Attachment 4)
- We have lost a major account because of the statements made by the Oregon Food Safety Division to account representatives, prior to the low clean test results in our product.

The FDA requests comment on how costly it would be to conform to the industry submission. To comply with the Industry draft of the proposed ruling should be considered the cost of doing business, for all food supplement businesses. Needless testing, as identified in item #5 of the above, and the cost of the FDA conducting market surveys to individually identified corporate accounts however, is very costly, to targeted businesses.

In conclusion, we support the Industry draft of the Proposed rules for Good Manufacturing Procedures. Thank-you for your time and thoughtful consideration. A.F.A. is requesting to be on the mailing list to recieve copies of any proposed regulations issued through this process.

Sincerely,



Susan Blevins, QAQC
A.F.A. Inc.

Attachment 3

FORM 1115 REV 6-75

STATE OF OREGON
DEPARTMENT OF AGRICULTURE

NOTICE OF EMBARGO

№ 2412

AGRICULTURE BUILDING
SALEM, OREGON 97310

TO ______________________________ ☐ OWNER

ADDRESS ______________________________ ☐ PERSON IN POSSESSION

PLANT NO. ______________ LICENSE NO. ______________ OTHER ID ______________

YOU ARE HEREBY NOTIFIED, PURSUANT TO ORS 561.605, THE FOLLOWING FOOD, ARTICLES OR PRODUCTS ARE HEREBY EMBARGOED.

	QUANTITY	SIZE	PRODUCT
1	[illegible]	[illegible]	[illegible]
2	[illegible]	[illegible]	
3	[illegible]	[illegible]	[illegible]
4	[illegible]	70 lbs [illegible]	[illegible]
5			
6			
7			
8			

REASON FOR EMBARGO—VIOLATION OF ORS [illegible]

LOCATION OF EMBARGOED ITEMS Freezer at 24[illegible] [illegible] Point and freezer at 151 Chocktoot Chiloquin [illegible]

ADDRESS
24508 Modoc Point Rd and [illegible] Chocktoot St Chiloquin OR.

NOTICE IS ALSO HEREBY GIVEN THAT:

1. IT IS UNLAWFUL FOR ANY PERSON TO REMOVE THE EMBARGOED ITEMS FROM THE ABOVE DESCRIBED PREMISES OR LOCATION WITHOU PRIOR WRITTEN APPROVAL OF THE STATE DEPARTMENT OF AGRICULTURE.
2. A WRITTEN REQUEST FOR A HEARING ON THE PROPRIETY OF THE EMBARGO MUST BE FILED WITH THE DEPARTMENT WITHIN 10 DAY FROM THE DATE THE OWNER OR PERSON IN POSSESSION HAS RECEIVED A NOTICE OF EMBARGO.

DATE ______________

OREGON DEPARTMENT OF AGRICULTURE

BY ______________ DIVISION ______________

THIS NOTICE SERVED:

☐ BY REGISTERED OR CERTIFIED MAIL ON ______________

☐ IN PERSON BY [illegible] DATE [illegible], 19__

COPY RECEIVED BY [illegible] [illegible] [illegible]
SIGNATURE POSITION/TITLE FIRM NAME

COPIES TO ______________

WHITE—OFFICE COPY
PINK—PERSON IN POSSESSION
BLUE—OWNER OR COPIES TO
GOLDENROD—COPIES TO
CANARY—INSPECTOR

FORM 1114 6/86

ORDER, AGREEMENT AND/OR RELEASE FROM EMBARGO PURSUANT TO ORS 561.605

OREGON DEPT. OF AGRICULTURE
635 CAPITOL STREET NE
SALEM, OR 97310-0110

TO: AFA Inc.

ADDRESS: 24308 Modoc Point Rd Chiloquin, OR. 97624

WHO IS ALLEGED TO BE: ☒ OWNER ☐ PERSON IN POSSESSION

THIS ORDER ☐ DOES NOT RELEASE ITEMS FROM EMBARGO ☐ RELEASES EMBARGOED ITEMS ☐ AUTHORIZES TRANSFER BUT CONTINUES EMBARGO ☒ SPECIFIES TERMS FOR RELEASE

NOTICE OF EMBARGO NUMBER: 2412 NOTICE OF EMBARGO DATE: Feb 7, 97

	QUANTITY	SIZE	PRODUCT
1	6257 Bucket @ aprox. 3.2 gal per bucket of Frozen		
2	blue-green algae from the following dates;		
3	Oct. 10, 21, 22, 23, 24, 25, 29, & 30 (No untested/		
4	unfiltered product as the same dates are to be released		
5	No other dates to be released until tested)		
6			
7			
8			

LOCATION OF EMBARGOED ITEMS: Freezer at Modoc Point and Chiloquin OR.

ADDRESS: Same as above

ORDER AND/OR AGREEMENT FOR RELEASE OF EMBARGOED ITEMS: Only above tested dates are to be released. Untested dates are still under embargo of Feb 7, 97

COMPLIANCE DATE:

OREGON DEPARTMENT OF AGRICULTURE

I (WE) AGREE TO THE ABOVE

DATE OF THIS ORDER: 5/5/97

FIRM: AFA Inc.

BY: Gail P. Flory R.S.

BY: Susan Blurns

DIVISION: Food Safety

THIS ORDER SERVED

☐ BY REGISTERED OR CERTIFIED MAIL ON ______, 19__

☐ IN PERSON BY Gail P. Flory R.S. DATE 5/5, 1997

COPY RECEIVED BY ______ SIGNATURE ______ POSITION/TITLE ______ FIRM NAME

COPY TO ______

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GOLDENROD—INSPECTOR

Food
Research
Institute

University
of Wisconsin-
Madison

College of Agricultural
& Life Sciences

Department of Food
Microbiology & Toxicology
1925 Willow Drive
Madison, WI 53706-1187
Telephone: 608/263-7777
Fax: 608/263-1114

FAX COVER SHEET

TO: Ms. Susan Blevins/A.F.A. Inc.

FROM: Prof. F. S. Chu

DATE: April 29, 1997 TIME: 4:00 p.m.

RE: Microcystin level in algal sample

NO. OF PAGES FOLLOWING THIS PAGE: 0

FAX NUMBER SENDING TO: 8-1-541-783-3360

MESSAGE OR COMMENTS: The MCYST-LR level, as determined by ELISA method, for the samples that you sent to us on March 24, 1997 is shown as follow. Please call me at (608) 263-6932 if you have additional questions.

Sample No.	MCYST (μg/g)
970424 - 1- 1	0.068
970424 - 1- 2	0.091
970424 - 1- 3	0.102
970424 - 1- 4	0.18
970424 - 2- 1	0.121
970424 - 2- 2	0.031
970424 - 2- 3	0.027
970424 - 2- 4	0.154
970424 - 3- 1	0.082
970424 - 3- 2	0.086
970424 - 3- 3	0.07
970424 - 3- 4	0.075
970424 - 3- 5	0.058

MicroForest, Inc.

145 Vallecitos de Oro, Suite E
San Marcos, CA 92069 USA
Phone: 619-736-3065
FAX: 619-736-3193

Attachment 1

Results from Evaluation of Klamath Lake *Microcystis aeruginosa*

Sampling Methods:

Two samples were taken at Klamath Lake June 1, 1996 by Jason Frank. One was taken near Modoc Point and the other near downtown Klamath Falls. The samples were approximately 750ml each and were place in sealed 1 liter containers and taken back to the laboratory two days later. The samples were transferred into incubator bottles, 0.25ml of concentrated MF/27.4 was added and the samples were placed in a Sherer GroLab growth chamber.

The samples were subcultured February 14, 1997 to select for actively growing cells and to concentrate *Microcystis aeruginosa* and *Aphanizomenon flos-aqua*. The samples were sent by overnight mail on March 19 for testing through the Southern Regional Research Center located in New Orleans, LA. The sample identies were verfified by Dr. Paul Zimba as consisting of *Microcystis aeruginosa* and *Aphanizomenon flos-aqua.* Included with the samples sent for testing was *Microcystis aeruginosa* UTEX LB2385 (University of Texas Culture Collection of Algae strain number LB2385). This is generally acknowledged as being a toxic microcystin-containing strain of *Microcystis aeruginosa*. This was included as an unknown to check the analytical accuracy of the testing methods of the laboratory.

Results:

Results of Testing March 20, 1997
Strains derived from sample MF5961

Method: Presence/Absence NMR Verification/Characterization of Positives by HPLC

Strain	Saxitoxin	Neo-saxitoxin	Microcystins
Klamath Lake *Microcystis aeruginosa*	absent	absent	absent
Microcystis aeruginosa UTEX LB2385	absent	absent	absent
Klamath Lake *Aphanizomenon flos-aqua*	absent	absent	absent

Attachment 2

HOARE and KOSHLAND (1967) reported that linkage of two protein molecules using the water-soluble carbodimide method could be enhanced by lowering the pH of the reaction buffer. Therefore in this study, the chemical reactions were carried out at pH = 6 using 10 mM phosphate buffer.

Purification and preparation of microcystin-LR standards for ELISA. MCYST-LR was purified from toxic blooms of *Microcystis aeruginosa* using a modified method of KRISHNAMURTHY *et al.* (1986). Freeze-dried *Microcystis aeruginosa* cells were extracted with water:methanol:butanol (75:20:5) overnight at room temperature (22°C) with stirring. The cell extract was either centrifuged (20,000 × *g*) for 30 to 60 min or filtered over glass wool and then centrifuged. The supernatant was air dried at 25–30°C overnight to remove methanol and butanol and the remaining aqueous solution was applied to Bond Elut (Analytichem) C-18 reverse phase columns. The columns were washed with water, 20% methanol, and then 80% methanol which eluted the MCYST-LR. The 80% methanol wash fraction was dried overnight at 20–25°C. Preparative HPLC using a Waters Prep Pak C-18 cartridge (47 × 300 mm, 55–105 μm particle size, 125 Å pore size) was used to purify MCYST-LR further. Mobile phase solvents used were 28% acetonitrile and 72% 20 mM NH_4OAc, pH 5.0. The eluent was monitored at 238 nm at a flow rate of 30 ml/min. Fractions containing the toxin were air-dried overnight to remove the acetonitrile. MCYST-LR was then desalted on another Waters Prep Pak C-18 column. This was followed by Gel chromatography on Toyo Pearl HW-40 F using 100% methanol for separation of MCYST-LR from the remaining pigments. The purity of MCYST-LR was estimated at 95–98% by analytical HPLC (55% methanol/45% 55 mM Na_2SO_4 pH 6.8) and Pico Tag amino acid analysis.

MCYST-LR (0.5 mg) was dissolved in 1 ml methanol and diluted to working concentrations of 0.5, 1, 5, 10 and 50 ng/ml with 0.01 M phosphate buffer saline (PBS), pH 7.4. The final methanol concentration in the standards was less than 1%.

Procedures for the direct competitive ELISA to detect microcystin and nodularin. The procedure for the direct competitive ELISA used in this study is as described by CHU *et al.* (1990).

Source of the microcystin and nodularin variants. Microcystin analogs isolated from natural water bloom samples were kindly provided by Dr M. NAMIKOSHI and Prof. K. L. RINEHART (Department of Chemistry, University of Illinois, U.S.A.). The microcystin variants used are listed in Table 1.

TABLE 1. AFFINITY OF DIFFERENT MICROCYSTINS AND NODULARINS WITH ANTIBODIES AGAINST MICROCYSTIN-LR

Toxin	LD_{50} (μg/kg, i.p. mouse)	ELISA reaction (IC_{50} ng/ml)
[DMAdda³]NODLN	150*	—§
[6(Z)Adda³]NODLN	nontoxic*	—§
[D-Asp¹]NODLN	75*	35
MCYST-FR	250*	10
MCYST-AR	250*	30
MCYST-WR	150–200*	10
[D-Asp³]MCYST-LR	200–500†	8
[DMAdda⁵]MCYST-LR	90–100*	—§
[D-Glu-OCH₃]MYCST-LR	nontoxic*	3.1
[6(Z)Adda⁵]MCYST-LR	nontoxic*	60
[Dha⁷]MCYST-LR	200–250*	30
MCYST-(H₄)YR	300–400*	8
[L-MeSer⁷]MCYST-LR	150*	5
MCYST-HilR	100*	3.4
Dimethylester NODLN (Methylester at Glu and MeAsp)	nontoxic‡	15
MCYST-YR	70*	10
MCYST-LR	50*	3.1
NODLN	50*	12

*RINEHART *et al.* (1994); †HARADA *et al.* (1991); ‡NAMIKOSHI *et al.* (unpublished data).

§No inhibition at a concentration up to 1 μg/ml.

Dha, Dehydroalanine; Hil, homoisoleucine; $(H_4)Y$, 1,2,3,4tetrahydro-tyrosine; MCYST, microcystin; NODLN, nodularin.

ATTN: SHARON

SENT BY: 5- 5-97 : 9:39AM : LPSL FAIRBANKS→ 5418847651:#18/19

Attachment 4

FILE COP

CLIENT COPY

Klamath Algae Harvesters
ASSOCIATION
P.O. Box 1070 • Keno, Oregon 97627-1070
(541) 885-8975 • Fax (541) 885-9586

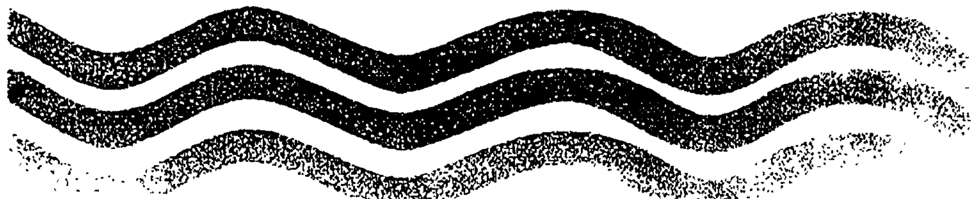
April 29, 1997

Mr. Nicholas Duy
Special Nutritionals, FDA
HFS 456 200 C Street, S.W.
Washington, D.C. 20204

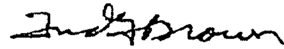
Dear Mr. Duy:

This association represents three of the largest blue green algae harvest companies on Upper Klamath Lake at Klamath Falls, Oregon. We have become concerned that Dr. Duncan Gilroy, with the State of Oregon, Division of Health has been giving you some misinformation with respect to our product. I am enclosing our letter to Laurna Youngs of the Oregon Department of Agriculture for your consideration. As you can see, the State of Oregon is considering establishing an interim standard of acceptable microcystis levels in lyophilized blue green algae product. It is doubtful that the strain of microcystis found in Upper Klamath Lake is toxic for human consumption. Some question does exist, however, as to whether **any** ingestion of microcystis from Upper Klamath Lake is toxic. As you can see by referring to our letter to Ms. Youngs as well as to Dr. Bell's report and recommendations with respect to this matter, further study needs to be performed in order to determine whether microcystis is toxic for human ingestion and, if so, at what level. We have urged adoption of Dr. Bell's recommended interim standard for microcystis levels per gram algae pending the development of further information with respect to this issue. See Attachment 1 to Laurna Youngs letter, p. 7.

We would appreciate it if you would respond to this letter and let us know what the status is, if any, with respect to any FDA review of this issue. It is our right to have this information as soon as possible. Further, we would hope that the FDA will be enlisting industry cooperation if it is indeed planning to set an acceptable level of microcystis for blue green algae product. I look forward to hearing from you with respect to the status of any work you are doing on this issue.



Sincerely yours,



Fred G. Brown, President
KAHA

cc: Robert Westley
Enclosures as stated

FILE COP

Klamath Algae Harvesters
ASSOCIATION
P.O. Box 1070 • Keno, Oregon 97627-1070
(541) 885-8975 • Fax (541) 885-9586

CLIENT COPY

April 29, 1997

Via Facsimile and U.S. Mail

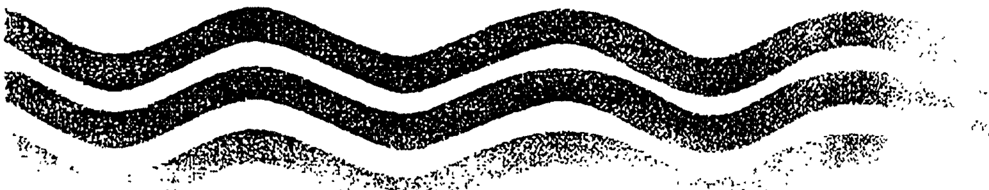
Laurna Youngs, Deputy Director
Oregon Department of Agriculture
635 Capitol Street, N.E.
Salem, Oregon 97310

Dear Ms. Youngs:

During one of our recent telephone conversations you advised that Duncan Gilroy of the Oregon Department of Health will be issuing a standard of acceptable parts per million microcystin in lyophilized algae product by the end of this week. This letter is to let you know what our Association believes would be an acceptable interim standard of parts per million microcystin in the lyophilized algae product until necessary further testing can be accomplished. We support an interium standard of 10 parts per million (ppm) microcystin in the lyophilized algae product based on projected human ingestion of 3 grams of algae per day. Attachment 1 - Dr. Bell's Report, p. 7.

It is not clear why Dr. Gilroy is insisting on a rush to judgment to propound a final standard when many questions exist with respect to whether microcystin is toxic when ingested by human beings, and if it is toxic, at what level it becomes toxic. Further, testing by MicroForest, Inc. shows that Klamath Lake Algae is free of saxitoxins, neo-saxitoxins, and microcystins. Attachment 2 - MicroForest Results for Evaluation of Klamath Lake Microcystis aeruginosa. Any decision other than to adopt interim measures by the Oregon Department of Health would be arbitrary and capricious, especially in light of existing knowledge.

Competitors in our industry met on March 26, 1997, with various experts in the field of microcystin, in order to discuss whether microcystin is toxic when ingested by humans, and further, at what level microcystin becomes toxic if it is toxic by ingestion.



What grew out of our meeting is the attached report by Dr. Robert A. Bell, PhD. In his report Dr. Bell comments on the toxicity of microcystins and its potential implications for human health. Dr. Bell discusses the existing literature, criticizes that literature as applied here, makes recommendations for future action, and proposes and interim standard pending further testing.

In his summary, Dr. Bell clearly states:

I. Little or no evidence exists to support the conjecture that toxic microcystins are either teratogenic or carcinogenic.

II. No statistical evidence of liver tumors has been seen in any of the acute or long-term mouse, pig or rat studies on microcystins. The most recent tumor promotion study on mice has shown no difference between controls and mice receiving microcystins at 1.2 mg/Kg body weight per day.

III. Animal studies have shown that oral administration of toxic microcystins produces far less acute toxicity (about a factor of 100) than i.p. injection. Thus, proper assessment of potential human hazard should recognize this factor if toxins are ingested rather than injected.

IV. Over 50 microcystins have not been identified. These yield i.p. LD50's from about 40 µg/Kg body weight to nondetectable (in excess of 1200 µg/KG). Thus, simply identifying Microcrocystis or Anabaena is not sufficient to determine the potential toxic hazard if any.

V. Present ELISA tests are known to be insensitive to some toxic microcystins.

Dr. Bell then recommends future action as follows:

"1. Determine strains of Microcystis, Anabaena, Nostoc and Oscillatoria which may exist in Klamath Lake. These should be grown as axenic cultures.

II. Conduct an oral mouse toxicity test on the actual strain of Microcystis which is known to grow in Klamath Lake.

III. Prepare a thorough and documented review of the literature on Microcystis to address any questions which the State of Oregon may raise.

IV. Determine a second method of microcystin detection which can be used to verify findings of the ELISA test (presumes the ELISA will be the test required by the State of Oregon). This is not only useful to verify findings but also as a hedge should the ELISA be found deficient in some way in the future.

V. Encourage work claiming tumor promotion by microcystin be done using methods other than the formation of GST-positive foci in the liver. To date, this is the only evidence of potential long-term health hazard. It is important to verify this finding or to demonstrate its inaccuracy."

KAHA agrees with Dr. Bell's assessment of the current state of literature, his recommendations for future testing, as well as his proposal of an interim standard. It is clear that we need to identify the microcystin strain of the Upper Klamath Lake. There are strains of microcystin that register false positives on the ELISA test. It is important to determine if any one of the suite of microcystins in the local Upper Klamath strain of microcystis reads a false positive. Further, it is important for us to identify what strains of microcystins exist in Upper Klamath Lake.

In his report, Dr. Bell discusses the LD-50 tests. It is clear that all LD-50 tests are not applicable to the Blue-Green algae industry in Klamath Falls, Oregon, as Blue-Green algae is ingested and not injected. An LD-50 of oral ingestion has not been performed using the strain of microcystis found in Upper Klamath Lake. There is a <u>discernible</u> difference in terms of levels of toxins among strains of microcystins - See Attachment 3. Because there are 50 strains of microcystis and each strain has a different suite of microcystins in it, it is important that double blind study been conducted from the microcystis from Upper Klamath Lake.

KAHA represents three of the major producers of Blue-Green algae for human consumption. As an industry, we need to be responsible and reasonable. It is unreasonable to expect us to depend solely on a tool such as the ELISA which clearly provides false positive information upon testing. Attachment 3. Further the use of the ELISA test for identification of microcystin is not standardized for identification or quantification. Attachment 3. Paradoxically, Dr. Gilroy expects us to accept the ELISA test as a standard for our industry whose consumers are against animal testing. Immunological testing is not the preferred methodology of the industry. The specific peaks for the HPLC have not standardized for uniform identification of the Upper Klamath Lake strain of microcystin. KAHA believes that it is reasonable to expect that the test for evaluation of toxins be standardized with approved methodologies by AOAC.

It would be arbitrary, capricious, and patently unreasonable for the Oregon Department of Health to determine a final standard short of receiving necessary scientific data. For all of the reasons cited in Dr. Bell's report, as well as the additional reasons cited in this correspondence, KAHA requests that an interim standard be established until further investigation is conducted and scientific data secured. Further, if public funding cannot be had to study concerns with respect to microcystis, it is important for all algae harvesters on Upper Klamath Lake to participate jointly with the Department of Agriculture in the funding arrangements for costly studies. We are willing to cooperate with Celltech and any other harvester to locate and obtain funding for scientific studies needed for further evaluation.

In order to satisfy any interim concerns you have with respect to microcystins in Klamath Lake Algae, KAHA harvesters will agree to use, on an interim basis, the newly developed ELISA test for this season's batch testing. Dr. F.S. Chu is developing a quick screening ELISA for this year's harvest, which KAHA harvesters plan to use.

It is only after Dr. Bell's recommendations and our recommendations to conduct further study on Upper Klamath Lake algae have been accomplished that the final standard should be established for the industry by the Oregon Department of Health.

It is my understanding that you will be providing us with the standards proposed by Celltech and the Council, as well as any scientific data submitted by Celltech and the Council to support their positions. I also understand that you will be providing us with any other information upon which Dr. Gilroy relies to finalize his opinion with respect to this matter. We have agreed that you will give us an opportunity to respond.

Sincerely yours,

Fred Brown, President
KAHA

Attachments as stated

Comments on the Toxicity of Microcystins and Its Potential Implications for Human Health

A Presentation by Robert A. Bell, PhD at the
Klamath Algae Harvesters Association meeting, March 26, 1997

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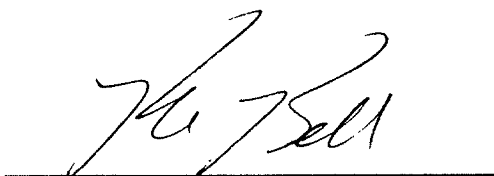

Robert A. Bell, PhD

Comments on the Toxicity of Microcystins and Its Potential Implications for Human Health

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INTRODUCTION:

Some blue-green algae (also known as cyanobacteria) produce a group of toxic cyclic heptapeptides known as microcystins. Such algal blooms have been implicated in the deaths and poisonings of animals for over one hundred years. In the past ten years extensive information about microcystins has been amassed. The reported toxicity of some congeners and their occurrence in some public water supplies has prompted inquiries into possible short-term and long-term hazards for humans. It is clear that the liver is the primary site of tissue damage but relatively large dosages are needed by ingestion. Of particular interest are birth defects, carcinogenicity and tumor promotion. Such effects may be delayed from initial toxin exposure or may result from repeated low doses over an extended period. In establishing guidelines for microcystin content it is vital that regulatory agencies base their conclusions on high quality research and not conjecture. It is the purpose of this contribution to review portions of the literature in an effort to separate facts from theories. Methods of assessing microcystin content and toxicity are also reviewed.

MICROCYSTINS:

Microcystins are a class of substituted cyclic peptides produced by a variety of blue-green algae including some strains of the genera *Microcystis*, *Anabaena, Nostoc* and *Oscillatoria*[1]. However, not all strains appear to produce toxins. Those blue-greens which do generate these materials produce a diversity of microcystins, over 50 of which have now identified. Additional complicating factors are the variable toxicities of the different microcystins which yield i.p. LD_{50}'s in mice from about 40 µg/Kg body weight to nondetectable[1] (in excess of 1200 µg/Kg)[3]. Thus, simply identifying *Microcystis* or *Anabaena* is not sufficient to determine potential toxic hazard.

The microcystin ring structure is composed of seven peptides, one of which contains an apparently unique side chain abbreviated ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). A similar class of materials known as Nodularins (ring of five peptides with the ADDA side chain) is produced by the genus *Nodularia* which grows in brackish water. As nodularins do not appear to be present in Klamath Lake, no further consideration of them is taken in this paper.

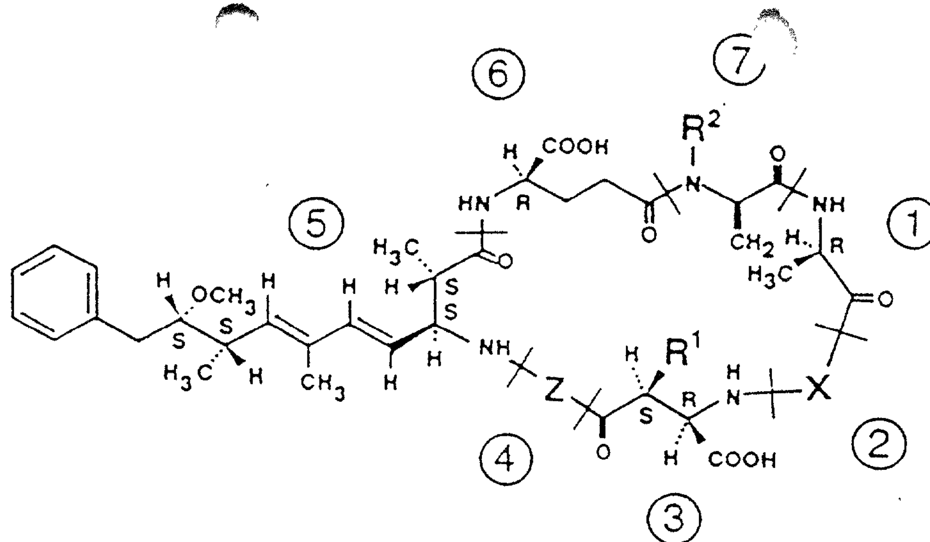

FIGURE 1. Generalized structure of microcystins

One can portray the microcystin structure, seen in figure 1, as a coonskin cap (picture borrowed from reference 1). The circular heptapeptide is the "cap" and the ADDA side chain is the "tail". It is important to note that microcystin requires both cap and tail to be dangerous. Apart, neither portion shows apparent toxicity[2]. According to the literature two chemical structures also appear to be critical to toxicity, the *trans* diene (area 5) and the carboxylic group on the glutamate peptide (area 6)[3]. If the diene is isomerized to contain a *cis* configuration or if the glutamate carboxylic acid is esterified (derivative other than hydrogen) virtually all toxic behavior disappears.

ACUTE TOXICITY:

The large majority of acute toxicity studies have been done via intraperitoneal injection (i.p.). As the product from Klamath Lake is taken orally, not by injection, i.p. does not appear to be the proper method to assess potential hazard for human exposure. For example, one can drink rattlesnake venom but the same amount injected would produce grievous injury. Three studies have established oral toxicities for microcystins. Prior to these the purified form of the most common microcystin (LR) was shown to have an i.p. LD_{50} of ~ 50 µg/Kg in mice[4,5]. The first[6] used *Microcystis* extract ("toxin" content ~ 57µg/ml) which gave an i.p. LD_{50} of 9mg/Kg and an oral LD50 of 560 mg/Kg in mice. The authors note that similar large variations between i.p. and oral values were seen in sheep and chickens. A later study by the same group[7] used dried *Microcystis* bloom which had been freeze-thawed. The i.p. LD_{50} in mice was 28.5 mg/Kg and the oral LD_{50} was in excess of 2600 mg/Kg. The a third comparison[8] used the most common congener, purified microcystin-LR, and found the i.p. LD_{50} was between 50 and 158 µg/Kg while the oral LD_{50} was approximately 5000 µg/Kg. All studies demonstrate a consistent variation between i.p. and oral values on the order of 100. Thus, proper assessment of potential human hazard should recognize this factor if toxins are <u>ingested</u> rather than injected.

BIRTH DEFECTS AND CARCINOGENICITY:

Although there was an early report of possible teratogenicity[6] the most recent study does not find that microcystins produce birth defects[8]. The lack of tumors at even very high dosage in mice and pigs has been interpreted as a lack of carcinogenicity in these compounds [6,7,8,15]. Also, microcystins are reported to be negative by Ames testing.

TUMOR PROMOTION:

Early work demonstrated that some compounds can enhance the rate of tumor production of known carcinogens[9]. These materials were termed "promoters" as they did not cause the cancer but in some fashion increased its likelihood. For example, phenol in tobacco tar is not carcinogenic but is a promoter[10]. One must be cautious in experiment design to properly show actual effects beyond the normal variations which may be experienced (statistical verification). An additional concern is any unconscious bias or predisposition workers may have to find a particular result. For this reason proper design often includes "blinded" evaluation of tissue samples in which the evaluator is not aware if samples come from treated animals or from controls.

An early study of the promotion of skin tumors using methylbenzanthracene on mice indicated a greater weight of tumors in a group treated with a *Microcystis* extract compared to controls[11]. However, a later study was not able to confirm this effect using a toxic Anabaena extract[12]. The most cited work in this area involves the formation of foci of enhanced GST liver enzyme activity which some have interpreted as a pre-tumor stage[13,14]. I find a number of problems with this work. First, the enhanced GST enzyme activity (glutathione S-transferase) is a normal biological response to reduce the activity of compounds with electrophilic carbons. Second, the evaluators were not blinded and could not, therefore, offer an unbiased review of the tissue samples. Third, no statistical evidence of liver tumors has been seen in any of the acute or long-term mouse, pig or rat studies on microcystins. A later tumor promotion study on mice has shown no difference between controls and mice receiving microcystins at 1.2mg/Kg body weight per day[15]. Indeed, higher doses of microcystins (4.2 mg/Kg/day) actually produced smaller and fewer tumors than found in controls. Although duodenal and lymphoid tumors were present, this study found no primary liver tumors in any mice. All of this is not to say that microcystins are not promoters. They may be. But it is important that if true, it should be established in a truly scientific manner.

ASSESSMENT OF TOXICITY LITERATURE:

It is clear that certain microcystins are potent toxins and that the primary site of attack is the liver. It is also apparent that some microcystins are virtually non-toxic and that not all strains of *Microcystis*, *Anabaena*, *Nostoc* or *Oscillatoria* produce toxins. Animal studies have shown that oral administration of toxic microcystins produces far less acute toxicity (about a factor of 100) than i.p. injection. Little or no evidence exists to support the conjecture that toxic microcystins are either teratogenic or carcinogenic. There is evidence that they may function as tumor promoters but this work may need to be duplicated in a truly objective manner to verify results.

REVIEW OF DETECTION TECHNIQUES:

The ability to detect and quantify microcystins is the foundation for any potential regulatory action. Below I have briefly described the advantages and limitations of the methods now available.

High Performance Liquid Chromatography (HPLC): Good quantitation and reliability down to about 10ng/ml. Requires relatively sophisticated equipment and operation. Can only be used for those microcystins where standards are available. Requires extensive "clean-up" of material prior to analysis. Usually considered the "gold standard" for quantitation.

Mouse assay (i.p.): Can be used on unprocessed material as well as purified toxins. Requires sacrifice of lab animals (esthetically problematic) and is relatively insensitive (detection limit is about 1 µg/ml for purified materials or 10 mg/Kg body weight for gross material). Does not prove microcystin involvement.

ELISA: Enzyme Linked ImmunoSorbant Assays have been developed from rabbit antibodies[16] and from chicken egg antibodies[17]. They appear to be very sensitive and repeatable for most of the toxic microcystins but do not provide actual identification of the individual congeners (sensitive but not specific). Relatively easy to administer and applicable to gross or purified samples. Current detection limit is about 0.1ng/ml. Greatest drawback is the fact that the present ELISA's appear to be insensitive to some toxic microcystins[18].

Phosphatase Assay: Assesses inhibition of Protein Phosphatase 1 activity on p-nitrophenol phosphate. Detection limit is about 3ng/ml. As for ELISA above it is sensitive but not specific.

MMPB-GC: Gas chromatography can be used to quantitate the amount of MMPB (2-methyl-3-methoxy-4-phenlybutyric acid) from chemically clipping a portion of the ADDA "tail" which all microcystins contain. Can potentially detect very small levels (<0.1ng/ml) but cannot determine which individual microcystins are present. Technique needs some development and verification. Potentially applicable to unprocessed material.

Summary:

I. Little or no evidence exists to support the conjecture that toxic microcystins are either teratogenic or carcinogenic.

II. No statistical evidence of liver tumors has been seen in any of the acute or long-term mouse, pig or rat studies on microcystins. The most recent tumor promotion study on mice has shown no difference between controls and mice receiving microcystins at 1.2 mg/Kg body weight per day[15].

III. Animal studies have shown that oral administration of toxic microcystins produces far less acute toxicity (about a factor of 100) than i.p. injection. Thus, proper assessment of potential human hazard should recognize this factor if toxins are ingested rather than injected.

IV. Over 50 microcystins have now been identified. These yield i.p. LD_{50}'s from about 40 μg/Kg body weight to nondetectable[1] (in excess of 1200 μg/KG)[3]. Thus, simply identifying *Microcystis* or *Anabaena* is not sufficient to determine the potential toxic hazard if any.

V. Present ELISA tests are known to be insensitive to some toxic microcystins[18].

RECOMMENDATIONS FOR FUTURE ACTIONS:

I. Determine strains of *Microcystis*, *Anabaena*, *Nostoc* and *Oscillatoria* which may exist in Klamath Lake. These should be grown as axenic cultures.

II. Conduct an oral mouse toxicity test on the actual strain of *Microcystis* which is known to grow in Klamath Lake.

III. Prepare a thorough and documented review of the literature on *Microcystis* to address any questions which the State of Oregon may raise.

IV. Determine a second method of microcystin detection which can be used to verify findings of the ELISA test (presumes the ELISA will be the test required by the State of Oregon). This is not only useful to verify findings but also as a hedge should the ELISA be found deficient in some way in the future.

V. Encourage work claiming tumor promotion by microcystin be done using methods other than the formation of GST-positive foci in the liver. To date, this is the only evidence of potential long-term health hazard. It is important to verify this finding or to demonstrate its inaccuracy.

INTERIM STANDARD: Based on the above information and other references which pertain to microcystins I feel an interim standard of 5 to 10 parts-per-million (ppm) microcystin in the lyophilized algae product is reasonable. Presuming the normal algae consumer ingests 1 gram of algae per day, 10ppm represents 10μg microcystin which translates into less than 0.2μg/Kg body weight for a typical adult. Since the oral LD_{50} in mice is on the order of 5000 μg/Kg[8] this level represents a safety factor of greater than 10,000. A more restrictive standard may have implications not only for the Klamath Lake algae harvesters but also for communities which obtain drinking water from lakes where algae may exist. For example, in a study of 46 sites across the state of Wisconsin it was found that microcystins were detectable by ELISA in all but six. The levels in the other 40 ranged from 0.5ppm to 200ppm. Recent work has also indicated that granular activated carbon filtration, the typical method used in water treatment to remove many pollutants, may remove only 80% of microcystins and that toxic residue on the order of 0.1 to 0.5ppm may pass through the filter[19]. As more research clarifies the actual degree of hazard which microcystins represent, the interim standard can be adjusted up or down as is appropriate.

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[1] Sivonen, K. (1996) Phycologia 35: 12-24.
[2] Dahlem, A.M., et al. (1989) Pharmacology Toxicology 63: 1-5.
[3] Rinehart, K.L., et al. (1994) J. Appl. Phycology 6: 159-176.
[4] Krishnamurthy, T., et al. (1986) Toxicon 24: 865-873.
[5] Elleman, T.C., et al. (1978) Aust. J. Biol. Sci. 31:209-218.
[6] Falconer, I.R., et al. (1988) J. Toxicol. Env. Health 24:291-305.
[7] Falconer, I.R., et al. (1994) Env. Toxicol. Water Qual. 9: 131-139.
[8] Fawell, J.K., et al. (1994) Report # FR 0359/2/DoE 3358/2, WRc Foundation for Water Research.
[9] Berenblum, I. (1974) Frontiers of Biology, Vol. 34. North-Holland Publishing Co., Amsterdam.
[10] Weisberger, J.H. and Williams, G.M. (1975) Toxicology, J. Doull, C.D. Klassen and M.O. Amdur, editors; Macmillan Publishing Co., New York, page 111.
[11] Falconer, I.R. and Buckley, (1989) T.H., Med. J. Austral. 150: 351.

[12] Falconer, I.R. (1991) Env. Toxicol. Water Qual. 6:177-184.
[13] Nishiwaki-Matsushima, R. et al. (1992) J. Cancer Res. Clin. Oncol. 118: 420-424.
[14] Ohta, T., et al. (1994) Cancer Res. 54: 6402-6406.
[15] Falconer, I.R. and Humpage, A.R. (1996) Phycologia 35: 74-79.
[16] Chu, F.S. et al. (1990) J. Assoc. Off. Analyt. Chem. 73:451-456.
[17] McDermott, C.M., et al. (1995) Toxicon 33: 1433-1442.
[18] An, J. and Carmichael, W. (1994) Toxicon 32:1495-1507.
[19] Lambert, T.W., et al. (1996) Wat. Res. 30: 1411-1422.