NCI NOMINATION

Blue-Green Algae

SUBMITTED TO THE NTP

SEPTEMBER 2000

SUMMARY OF DATA FOR CHEMICAL SELECTION

Blue-Green Algae

BASIS OF NOMINATION TO THE NTP

The lack of information on the chronic toxicity, genotoxicity, and carcinogenicity of cyanobacteria is brought to the attention of the CSWG.

This problem first came to the attention of the National Cancer Institute's Division of Cancer Biology as the result of a study of dietary supplements in the US market. This study identified blue-green algae tablets and capsules prepared from *Aphanizomenon* algae as a potential candidate for nomination. These dietary supplements can become contaminated with microcystin-LR, a potent hepatotoxin and suspected liver carcinogen, and *Anabaena*, which contains possible neurotoxins. Although standards and guidelines have been set by the state of Oregon to control the amount of microcystin-LR in the tablets and capsules, no information on the long-term safety of the supplements, themselves, was found in a review of the available literature.

Blue-green algae supplements containing *Aphanizomenon* algae are believed to be taken by over a million consumers.

A review of the information on blue-green algae supplements revealed a much larger gap in knowledge regarding the toxicity of cyanobacteria in general. Cyanobacteria are found throughout the world and contaminate many water supplies. Several genera are known to produce neurotoxins or hepatotoxins. However, nearly all studies on the toxicity of cyanobacteria have been acute animal experiments or accidental animal and human poisonings. The genotoxicity and chronic toxicity of cyanobacteria, in general, are not well characterized.

INPUT FROM GOVERNMENT AGENCIES/INDUSTRY

A joint US Environmental Protection Agency (EPA)/National Institute for Environmental Health Sciences (NIEHS) committee interested in drinking water contaminants recently nominated microcystin-LR to the National Toxicology Program (NTP). According to the joint committee, it is crucial to determine the effects of long-term exposure to microcystins in the drinking water. The committee suggested the following tests: toxicokinetic studies and cancer, including range

finding studies, in mice and rats. The committee also noted that additional studies using initiator/promoter rodent models may be indicated. This nomination is scheduled for review by the NTP in October 2000.

SELECTION STATUS

ACTION BY THE CSWG: September 28, 2000
Studies requested: Complete genotoxicity profile (Ames *Salmonella*, mouse lymphoma, chromosome aberrations, SHE cell transformation, and micronucleus assays) of common blue-green algae dietary supplements and identified active metabolites of cyanobacteria, including but not necessarily limited to microcystin-LR, other microcystins, nodularin, cylindrospermopsin, anatoxin-a, anatoxin-a(s), saxitonin, and neosaxitoxin.

Based on the results of the genotoxicity profile, reconsider possible need for initiator/promoter studies in rodent models.

Priority: High

Rationale/Remarks:
Widespread exposure of consumers, including children, to blue-green algae supplements.

Measurable levels of microcystins have been found in blue-green algae supplements evaluated by independent laboratories.

High suspicion that some toxic metabolites found in cyanobacteria may be tumor promoters.

A lack of information on the chronic toxicity of cyanobacteria metabolites that are not acutely toxic.

A need to develop a short-term test protocol which can be applied to other situations in which water supplies are contaminated with cyanobacteria.

NCI will test commercially available dietary supplements containing blue-green algae and cyanobacterial metabolites in the Ames *Salmonella* and mouse lymphoma assays.

CSWG members expressed their support for the nomination of microcystin-LR for testing by the NTP.

CHEMICAL IDENTIFICATION

CAS Registry No.:	Blue-green algae – no CAS No. Microcystin-LR – 101043-37-2, 128657-50-1
Chemical Abstracts Service Name:	None assigned
Synonyms and Tradenames:	Blue-green bacteria; Cyanchloronta, Cyanophyta; Myxo-phyta (SWCS, 1997)
Structural Class:	Cyanobacteria
Botanical Names:	Common species of blue-green algae include the heterocystous filamentous forms, *Anabaena*, *Aphanizomenon*, *Nostoc*, *Gloeotrichia*, and *Nodularia*; the nondifferentiated filamentous forms, *Oscillatoria*, *Spirulina*[1], and *Lyngbya*, and the unicellular-colonial form, *Microcystis aeruginosa*. Others *Cylindrospermopsis raciborskii*, *Hapalosiphon*, *Schizothrix*, *Synechocystis*, and *Umezakia* (Carmichael, 1995; Gupta, 1998; SWCS, 1997; WHO, 1996).

Description: The blue-green algae used in dietary supplements are cyanobacteria, organisms with some characteristics of bacteria and some of algae. They are similar to algae in size and contain blue-green or green pigments and thus perform photosynthesis. They are prokaryotes (without nuclei) having cell walls composed of peptidoglycan and lipopolysaccharide layers. (Carmichael & Falconer 1993; WHO, 1998).

Freshwater cyanobacteria may accumulate in surface water supplies as “blooms” and concentrate on the surface as blue-green “scums” (WHO, 1998).

Cyanobacterial blooms have a wide range of social, economic, and environmental impacts. The biomass contributes to aesthetic problems and affects the ability of many aquatic animals to survive. Of particular concern for human health is the production of bioactive metabolites. These metabolites include potent neurotoxic alkaloids (anatoxin-a, anatoxin-a(s), saxitoxins), the hepatotoxic alkaloid, cylindrospermopsin, and hepatotoxic cyclic

[1]The dietary supplement, Spirulina is not discussed in this Summary Sheet.

peptides (microcystins and nodularins) (Carmichael, 1995; Falconer, 1994).

The hepatotoxins are produced by various species within the genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Nodularia*, *Nostoc*, *Cylindrospermopsis*, and *Umezakia*, although not all strains do so. At least 50 congeners of microcystins are known. The microcystins are produced by both filamentous (*Anabaena*, *Oscillatoria*, *Nostoc*, *Hapalosiphon*) and unicellular-colonial (*Microcystis)* cyanobacteria. The nodularins are produced by *Nodularia*, a filamentous cyanobacterium. Cylindrospermopsin is produced by the filamentous cyanobacterium *Cylindrospermopsis raciborskii* (Carmichael, 1995; Gupta, 1998).

Neurotoxins are produced by species and strains of *Anabaena*, *Aphanizomenon*, *Oscillatoria*, and *Trichodesmium*. Five chemically defined neurotoxins are known to be produced by species within these genera, including saxitoxin and neosaxitoxin, the primary toxins of red tide paralytic shellfish poisoning, which have been identified in *Aphanizomenon.* Neurotoxins are not considered as widespread in water supplies, and they do not appear to pose the same degree of risk from chronic exposure as microcystins (Carmichael, 1992; Fawell *et al*., 1993; Gupta, 1998).

Toxins are either membrane-bound or occur free within cells. Most of the toxin release occurs as cells age and die and passively leak their cellular contents, although active release of toxins can occur from young growing cells. Copper sulfate added to the water to control the algal blooms also induces cell death (Falconer, 1994; Gupta, 1998).

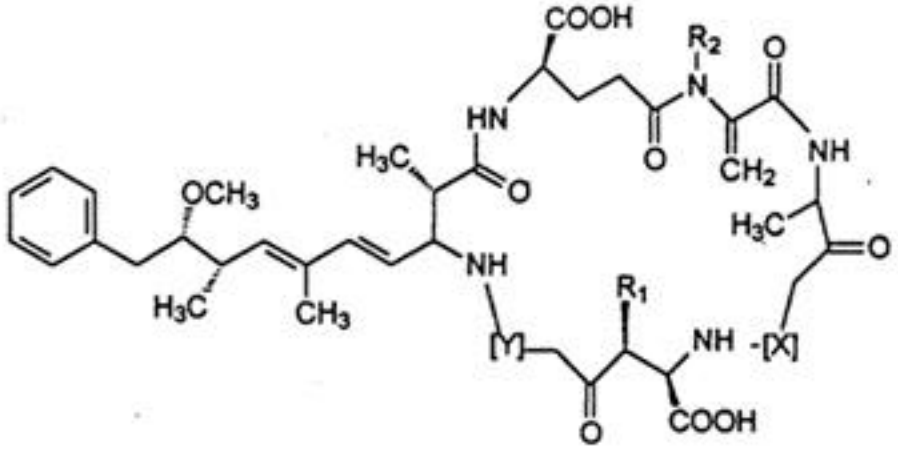
One of the puzzling elements of toxic cyanobacteria is the presence of different toxins in samples drawn from a single genus (or even species) in different geographical locations. Conversely, the presence of the same toxin in widely different genera is observed. For example, anatoxin-a was first isolated in *Anabaena* and has since been identified in *Oscillatoria*. Other *Anabaena* neurotoxins include anatoxin-a(s), saxitoxin, and neosaxitoxin (Falconer, 1996).

Technical Products and Impurities: Blue-green algae dietary supplements contain *Aphanizomenon* algae, much of which is harvested from Klamath Lake in Oregon. Contaminants from the Klamath Lake harvest may include *Microcystis*, *Anabaena*, and other types of

toxic algae (Cell Tech, 1999).

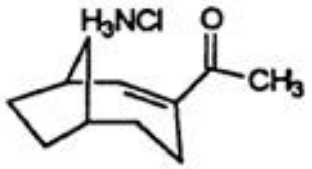
Chemical Composition: The chemical structures of the most common hepatotoxins and neurotoxins released from the cyanobacteria cell have been characterized (Falconer, 1996; Fawell *et al*., 1993; Hitzfeld *et al*., 2000).

The chemical structure of microcystins includes an unusual aromatic amino acid, ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), containing a substituted phenyldecadienoic acid. About 60 structural analogues of microcystin are known. They vary with respect to methyl groups and two amino acid groups within the ring (see structure below). This has consequences for the tertiary structure of the molecule and results in pronounced differences in toxicity as well as in hydrophobic/hydrophilic properties. Microcystin-LR, with a relative molecular mass of about 1,000, contains a leucine in the X position, alanine in the Y position, and methyl groups in both R positions. The hepatotoxic cyclic peptide nodularin has a structure similar to that of microcystin but contains only five amino acids (Carmichael, 1992; Falconer, 1996; Gupta, 1998; WHO, 1996).

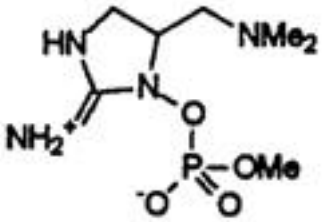


Microcystin

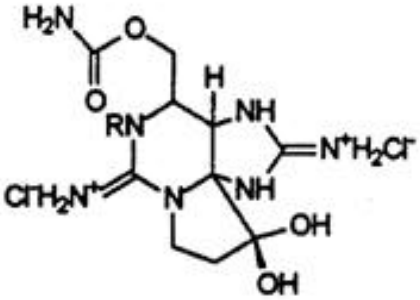
As seen below, the neurotoxic compounds (anatoxins and saxitoxins) are simpler structures (Falconer, 1996; Fawell *et al*., 1993).



Anatoxin-a hydrochloride



Anatoxin-a(s)



R=H;saxitoxin dihydrochloride
R=OH;neosaxitoxin dihydrochloride

EXPOSURE INFORMATION:

Production and Producers: About 10 companies harvest *Aphanizomenon* from Klamath Lake, Oregon, to produce dietary supplements (Cell Tech, 1999). Generally, the product is harvested off the lake, put in 5 gallon plastic containers, cleaned, and frozen, and then shipped off to be freeze-dried (ODA, 1997; Rossha Enterprises, Inc.,1999). Research quantities of highly purified microcystin-LR are available from major suppliers, including Sigma-Aldrich (1999) and Fisher Scientific (1999).

Use Pattern: In some countries, blue-green algae are used as fertilizers, and several types are served as side dishes (SWCS, 1997). The primary use of blue-green algae in the United States is as a dietary supplement. As a dietary supplement, blue-green algae has been promoted for use in children to treat Attention Deficit Disorder (Health Canada, 1999). Some manufacturers also encourage its use for pets (Eat on Algae Network, 1999).

Human Exposure: The occurrence of toxic water blooms of cyanobacteria has been reported widely in many regions of the world (Ding *et al*., 1999). Toxic blooms affect a wide range of communities, from village and rural dwellers in undeveloped areas drinking surface

water to towns in developed countries with reservoir and water treatment plants (Falconer, 1994).

Cyanobacterial poisonings of farm livestock and animal pets have been reported in the scientific literature since 1878. All of the major bloom-forming cyanobacterial genera are potentially toxic, and humans and all major groups of animals can be affected, so experts recommend viewing all cyanobacterial blooms with caution. Many blooms are apparently not hazardous, however, due to low concentrations of toxin within strains and species comprising the water bloom, low concentration of biomass in the water bloom, species sensitivity, amount consumed, age and sex, and amount of other food in the gut (Carmichael & Falconer, 1993; Falconer, 1991; Falconer, 1996).

The major route of human exposure to cyanobacteria is the consumption of drinking water (Gupta, 1998).

Humans and animals will avoid drinking water that tastes or smells of cyanobacteria unless there is no alternative supply. Urban populations, however, largely depend on water supplies that are chlorinated for disinfection but not treated to remove cyanobacterial toxins. In every instance of a suspected cyanobacterial poisoning of communities, the water supply was from an urban drinking water system. The supply reservoir had a recorded water-bloom of cyanobacteria, which in several instances had been treated with copper sulfate (Carmichael & Falconer, 1993; Falconer, 1996).

Recreational exposure to cyanobacteria occurs through inadvertent swallowing of water and skin and intranasal contact through water sports. An additional minor route of exposure is through inhalation while taking showers. Cyanobacterial toxins can also move up the food-chain (Falconer, 1996; Falconer *et al*., 1992; Gupta, 1998; WHO, 1998).

Some people are exposed to cyanobacteria through the consumption of dietary supplements. In the United States and Canada, it is estimated that more than one million people consume dietary supplements containing blue-green algae (Anon., 1999). In 1997, the state of Oregon estimated the economic value of Klamath Lake blue green algae to be $100 – $200 million (ODA, 1997). The major supplier of blue-green algae products in the US had estimated sales in 1995 at >$133 million, up from $18 million in 1993 (Winner,

1997).

Health agencies have examined the levels of microcystin-LR in dietary supplements. The Oregon Health Division found that 50 of 67 samples obtained from blue green algae harvesters, wholesalers, and retail outlets exceeded 1 ppm (Anon., 1999). Health Canada described findings of microcystin-LR at levels not considered safe for daily consumption in 9 randomly selected samples and of varying levels of microcystin in an additional 6 samples (Health Canada, 1999).

Environmental Status: Cyanobacteria are distributed worldwide in fresh, brackish, and marine waters (Falconer, 1989). Blooms of cyanobacteria are very common in lakes and reservoirs used for potable water supplies (WHO, 1996).

In one study, about 25 percent of 102 samples collected from sites in Wisconsin contained toxic algae (as defined by the mouse bioassay [see section on acute toxicity]) (Repavich *et al*., 1990).

The occurrence of a particular genus and species of cyanobacteria is influenced by regional differences in water chemistry and climate. *Microcystis* and *Anabaena* blooms occur widely in the temperate regions of the world. In general, 50 –75 percent of bloom isolates can produce toxins, often with more than one toxin being present. Toxic and non-toxic blooms of the same species can be found together (Gupta, 1998).

Unicellular and filamentous blue-greens are almost invariably present in freshwater lakes frequently forming dense planktonic populations or water blooms in nutrient rich waters. In temperate lakes, a seasonal succession of the bloom-forming species occurs. Usually the filamentous forms (e.g., *Anabaena*, *Aphanizomenon*, and *Gloeotrichia echinulata*) develop in late spring or early summer, while the unicellular-colonial forms (e.g., *Microcystis*) bloom in mid-summer or in autumn (SWCS, 1997).

Although toxic cyanobacteria are found world-wide in inland and coastal water environments and at least 46 species have been shown to cause toxic effects in vertebrates (WHO, 1998), very little information is available in the literature regarding the presence of toxic metabolites except for microcystin. Microcystins and alkaloid

toxins are degraded in natural waters, but there appears to be a lag phase of about 9 or 10 days before significant degradation takes place (Gupta, 1998).

According to the Oregon Health Division, microcystin levels in water can greatly exceed 1 ppb (1 µg toxin per liter of water) during algal blooms (Gilroy, 1998).

In a study of selected water utilities throughout the United States and Canada during 1996 and 1997, microcystins were found in 65 percent of the water samples that had not undergone treatment. A third of the positive samples registered levels of microcystins in excess of 1 µg/L. In a few cases, treated water samples contained microcystin at levels exceeding 1 µg/L (Anon., 1999).

In August 1993, microcystin at sub-µg/L levels was detected in Winnipeg's distributed water because of an algal bloom containing *Microcystis aeruginosa* in the water supply (Hrudey, 1994). Following this occurrence, in 1995, 160 surface water supplies, located mainly in southwestern Manitoba were studied. Treated water samples were analyzed if raw water supplies had detectable levels of toxins (**$** 0.1 µg/L); toxin was present in 68 percent of the treated water samples collected from both the municipal water supply and dugouts used for domestic and livestock consumption (Gupta, 1998).

The levels of microcystin-LR in lakes and dugout ponds of Alberta, Canada, ranged from 4 to 1500 µg/g dry weight of biomass. Between 1990 and 1992, more than 70 percent of over 380 bloom biomass samples from 19 Alberta lakes showed detectable levels (>1 µg of microcystin-LR per g dry weight of biomass) of toxin. For two Alberta drinking water supplies, the raw water intake levels of microcystin ranged from 0.15 to 4.3 µg/L. In treated water, levels ranged from 0.09 to 0.64 µg/L (Gupta, 1998).

Regulatory Status: The World Health Organization (WHO) has established a provisional value for total microcystin-LR (free plus cell bound) of 1 µg/L (rounded figure) in drinking water. According to the WHO, insufficient data exist to establish drinking water guidelines for other cyanobacterial toxins (Gupta, 1998; WHO, 1996; WHO, 1998).

The EPA has placed cyanobacteria (blue-green algae), other freshwater algae, and their toxins on the Drinking Water Contaminant Candidate List in accordance with

requirements of the Safe Drinking Water Act, as amended in 1996. These algae are listed as priorities for additional health research, treatment research, and analytical methods research (EPA, 1998).

Currently, there is no drinking water standard in the United States for microcystins. Canada, Australia, and Great Britain have developed a guideline level of 1 µg toxin per liter of water (1 ppb). The Oregon Department of Agriculture requires blue-green algae supplement manufacturers to ensure that no products sold to consumers contain microcystin at levels greater than 1.0 µg/g (1 ppm) (Cell Tech, 1999; Gilroy, 1998; Gupta, 1998; ODA, 1997).

Since 1994, dietary supplements have been regulated under the Dietary Supplement Health and Education Act (DSHEA). The DSHEA requires no proof of safety for dietary supplements on the market prior to October 15, 1994. Labeling requirements for dietary supplements allow warnings and dosage recommendations as well as substantiated "structure or function" claims. All claims must prominently note that they have not been evaluated by the FDA, and they must bear the statement "This product is not intended to diagnose, treat, cure, or prevent any disease" (FDA, 1995).

EVIDENCE FOR POSSIBLE CARCINOGENIC ACTIVITY

Human Data: *Illnesses from cyanobacterial contamination of water supplies.* Blue-green algae have been known to cause animal and human poisoning in lakes, ponds, and dugouts in various parts of the world for over 100 years (Gupta, 1998). The earliest public health report implicating cyanobacteria in cases of gastroenteritis affecting a population drawing water from a common source occurred on the Ohio River in 1931 (Carmichael & Falconer, 1993). More recently, a major outbreak of gastrointestinal illness attributed to contamination of the water supply by *Schizothrix, Plectonema, Phormidium, and Lyngbya* affected 5000 of 8000 residents of Sewickley, Pennsylvania (Falconer, 1994). Some *Lyngbya* metabolites are considered to be tumor promoters (Falconer, 1994).

An epidemiological survey for the causes of a high incidence of primary liver cancer in Haimen city, Jian-Su province and Fusui county, Guangxi province in China, found a close correlation between the incidence of primary liver cancer and the use of drinking water from ponds and ditches. The results of a mass screening for blue-green algae toxins in the drinking water supported a hypothesis that microcystin in the drinking water of ponds, ditches, and rivers, is one of the risk factors for the high incidence of liver cancer in China (Ueno *et al*., 1996).

In Australia, the Palm Island mystery disease, affecting about 140 people, largely children, occurred after a dense cyanobacterial bloom was treated with copper sulfate. Within a week, severe illness characterized by vomiting, hepatomegaly, and kidney dysfunction was seen; recovery took 1-3 weeks. The causative organism has been identified as *C. raciborskii* (Byth, 1980; Falconer, 1994; Gupta, 1998).

According to a WHO report, the most convincing evidence of algal toxins in drinking water supplies is an epidemiological study of an Australian community conducted by Falconer and his coworkers. In this study, raised serum enzymes indicative of mild, reversible liver damage were observed in hospital patients who drank water from a local reservoir with a very large toxic bloom of *Microcystis aeruginosa* (WHO, 1996).

Observations of human deaths caused by acute exposure to cyanobacterial toxins have been limited to liver failure in patients exposed to microcystins while being treated at a

renal dialysis center in Brazil (Jochimsen *et al*., 1998).

Illnesses from recreational exposure to cyanobacteria. Allergic reactions to cyanobacteria are relatively common and have been described after human contact while swimming in algal blooms (Carmichael & Falconer, 1993). The recreational use of water contaminated with blooms of *Microcystis, Anabaena*, and other genera has been linked to incidences of human illness in many countries. Symptoms include stomach cramps, vomiting, diarrhea, fever, headache, pains in muscles and joints, and weakness as well as skin, eye, and throat irritation and allergic responses (Gupta, 1998).

In the United Kingdom, 10 of 18 army recruits on a military exercise in a reservoir contaminated with a bloom of *Microcystis aeruginosa* suffered abdominal pains, vomiting, diarrhea, sore throat, dry cough, blistering at the mouth, and headache. Two were hospitalized with pneumonia. Serum enzymes indicating liver damage were elevated (Falconer, 1994; Gupta, 1998; Turner, 1990).

Information on adverse events following use of algal supplements. The FDA's Special Nutritionals Adverse Event Monitoring System reported 58 matches for blue-green algae dietary supplements in 43 adverse event reports as of October 20, 1998. The following effects were reported for 31 products apparently containing only blue-green algae as the active ingredient: diarrhea; nausea; vomiting; dizziness; headaches; sleeplessness; sweat; seizure, metallic taste, shakes, and felt faint; racing feeling; heart palpations/tachycardia; increased heart rate/elevated pulse; cardiac arrhythmia; crushing chest pain; myalgias, eosinophilia; swollen extremities; body numbness; tingling in fingers and feet; increase in neuropathy of feet and legs; saw bright color and images; extreme weakness; stomach pain, indigestion, and constipation; elevated liver function tests; abdominal pain; bacterial liver infection; intestinal infection; burning sensation in throat, stomach, and intestines; suicidal and extreme pain; vaginal hemorrhaging; gastrointestinal bleeding; "reactivated hypoglycemia"; low blood pressure, dizziness, nausea, coughing, and felt faint; hives; pimples. No information on preexisting conditions was available, and there is no certainty that a reported adverse event can be attributed to a particular product or ingredient (FDA, 1998).

Animal Data: *Acute toxicity.* Observations of lethal poisoning of animals drinking from water

contaminated with cyanobacteria are numerous and include sheep, cattle, horses, pigs, dogs, fish, rodents, amphibians, waterfowl, bats, zebras, and rhinoceri (WHO, 1998).

Most poisonings by cyanobacteria involve acute hepatotoxicity caused by microcystins and nodularin. First, the toxin is released from the cyanobacterial cells in the stomach causing injury to the intestinal cells. Second, the hepatocytes concentrate the toxin via bile acid carriers. Third, hepatotoxin-induced changes occur in the cell shape with loss of cell-cell adhesion. Finally, lethal intrahepatic hemorrhage (minutes to hours) or hepatic insufficiency (hours to days) occurs. At necropsy, animals show liver enlargement and often intrahepatic hemorrhage. Hepatic necrosis begins in the centrilobular region and proceeds periportally (Carmichael & Falconer, 1993).

Acute toxicity from the neurotoxins proceeds along different lines. Anatoxin-a is a potent post-synaptic depolarizing neuromuscular blocking agent that causes death within minutes to hours. Neurotoxicity has also been associated with anatoxin-a(s), a potent cholinesterase inhibitor. These neurotoxins are potentially lethal by causing suffocation through cramps (Carmichael & Falconer, 1993; Hyde & Carmichael, 1991; WHO, 1998).

Some strains of *Aphanizomenon* and *Anabaena* produce the potent paralytic shellfish poisons, saxitoxin and neosaxitoxin. These sodium channel blocking agents inhibit transmission of nervous impulses and cause death by respiratory arrest (Carmichael & Falconer, 1996).

Cylindrospermopsin is an alkaloid isolated from *Cylindrospermopsis raciborskii*. It is a general cytotoxin which blocks protein synthesis, the first clinical symptoms being kidney and liver failure. Crude extracts of the organism also cause injury to the lungs, adrenals, and intestine. Clinical symptoms may only become manifest several days after exposure (WHO, 1998).

The acute toxicities of cyanobacterial metabolites are frequently measured by intraperitoneal injection of samples of algal bloom into mice. Acute toxicities reported in this mouse bioassay for various cyanobacteria are listed in Table 1. Pure microcystin-LR is about two orders of magnitude less toxic in mice by the oral route than by intraperitoneal injection, and the rat is much less sensitive (Fawell *et al.*, 1993).

Microcystin toxicity is cumulative; a single oral dose showed no increase in liver weight, whereas the same dose applied daily over 7 days caused an 84 percent increase in liver weight (WHO, 1998).

Table 1. Cyanobacterial toxins and their acute toxicity

Toxin	LD_{50} (mouse, intraperitoneal)	Taxa known to produce toxin	Class	Mechanism of toxicity
Microcystin-LR	60 (25-125) µg/kg	*Anabaena, Anabaenopsis*	Cyanotoxin	Blocks protein phosphatases by covalent binding; causes liver haemorrhage
Microcystin-YR	70 µg/kg	*Hapalosiphon*		
Microcystin-RR	300-600 µg/kg			
Nodularin	30-50 µg/kg	*Nodularia sumigena*		
Anatoxin-a	250 µg/kg	*Anabaena, Oscillatoria, Aphanizomenon, Cylindrospermum*	Alkaloid neurotoxin	Blocks post-synaptic depolarization
Anatoxin-a(s)	40 µg/kg	2 species of *Anabaena*	Organo-phosphate	Blocks acetyl cholinesterase
Saxitoxins	10-30 µg/kg	*Aphanzomenon, Anabaena, Lyngbya,*	Carbamate alkaloids	Blocks sodium channels
Cylindrosperm-opsin	2100 µg/kg	*Cylindrospermopsis raciborskii*	Alkaloid	Blocks protein synthesis; cumulative toxin

Source: adapted from WHO, 1998

According to Hooser and coworkers, there are marked differences in the response of mice and rats to microcystin-LR even though the liver is the target organ in livestock, wildlife, and humans, as well as in mice and rats. In mice, massive centrilobular to midzonal hemorrhage and death occur within 60 to 90 minutes. In rats, although hepatic necrosis and hemorrhage occur within 60 minutes, the hemorrhage is not as severe as in mice, and rats survive 20 to 32 hours (Hooser *et al*., 1990).

In the past, dietary supplement manufacturers used the mouse bioassay to determine the safety of their blue-green algae products. They have replaced the mouse bioassay with enzyme-linked immunosorbent assays (ELISA) to detect microcystin, a protein phosphatase inhibition assay which measures hepatotoxins, and an anticholinesterase enzyme assay to detect neurotoxins (Cell Tech, 1999).

Some investigators have used rodent livers to assess the acute toxicity of blue-green algal blooms. *Microcystis* peptide toxin reduced quantities of cytochromes P-450 and b5 while increasing P-420, consistent with conclusion that *Microcystis* toxin interfers with membrane integrity and function (Brooks & Codd, 1987). Toxicity effects scored by means of morphological studies of rat hepatocyte cells and by measuring leakage of lactate dehydrogenase from the cells correlated well with the results of the traditional mouse bioassay in terms of distinguishing toxic and nontoxic extracts from *Microcystis aeruginosa* and *Oscillatoria agardhii* (Aune & Berg, 1986). In another study, *Microcystis aeruginosa* caused a significant leakage of cytosolic enzymes and histologic alterations in mouse liver cultures, even though five other algal species were inactive (Bhattacharya *et al.*, 1996).

Subchronic toxicity. Microcystin-LR was administered by gavage to groups of 15 male and 15 female mice at 0 -1000 µg/kg bw per day for 13 weeks. No treatment-related changes were noted at 40 µg/kg. At 200 µg/kg, slight liver pathology was observed in some animals. At the highest dose, all mice showed liver changes, which included chronic inflammation, focal degeneration of hepatocytes, and hemosiderin deposits. In male mice, serum transaminases were significantly elevated at 200 and 1000 µg/kg, serum gamma glutamyl transferase was significantly reduced, and small but significant reductions in total serum protein and serum albumin occurred. In female mice, changes in transaminases were observed, but only at 1000 µg/kg (Fawell *et al*., 1999; Gupta, 1998).

Extract from *Microcystis aeruginosa* containing at least seven microcystins was given to groups of five pigs in their drinking water for 71 days at doses equivalent to microcystin-LR doses of 0-1310 µg/kg bw per day. Dose-related liver injury was observed at 800 and 1310 µg/kg. One pig was affected at 280 µg/kg. Histopathological assessment showed cytoplasmic degeneration, hepatic cord disruption, single cell necrosis, periacinar degeneration, congestion, and Kuppfer cell proliferation. Liver function tests showing dose-related changes included γglutamyl transpeptidase, alanine aminotransferase, total bilirubin, and plasma albumin (Falconer *et al*., 1994; Gupta, 1998).

Chronic toxicity. No 2-year carcinogenicity studies of blue-green algae or its toxic metabolites were identified in the available literature. Microcystin-LR is thought to be a tumor promoter of the okadaic acid activity class (Fujiki *et al.*, 1997; Fujiki & Suganuma,

1999). Two metabolites of the blue-green algae, *Lyngbya majuscula*, aplysiatoxin and debromoaplysiatoxin are also tumor promoters (Fujiki *et al*., 1985).

An oral repeated-dose study was conducted with *Microcystis aerigonosa* extract supplied to male and female Swiss mice. A total of 320 weanling mice, in groups of 5, were allocated to different treatment groups (control, 1/16th dilution, 1/8th dilution, and 1/4th dilution) and administered the extract in their drinking water for 2 weeks to 1 year. Sections of liver from 40 mice (120 total) killed at 25, 31, and 37 weeks were examined. Amyloid material was deposited mainly between the hepatocytes and the sinusoidal endothelium and the portal veins, and substantial hepatic infiltration with neutrophils was observed, particularly around portal blood vessels. The prevalance of these pathological changes appeared greater in mice consuming the toxic extract, but the increase with concentration was not significant. No hepatic neoplasms were recorded (Falconer *et al.*, 1988).

Tumor promotion. In a two-stage carcinogenicity bioassay, 7,12-dimethylbenz[a]anthracene (DMBA) was applied to the skin of female Swiss mice. After 1 week, the DMBA-treated mice received drinking water, *Microcystis* extract in drinking water, croton oil applied to the skin plus drinking water, or croton oil plus *Microcystis* extract. Negative controls not treated with DMBA received drinking water or *Microcystis* extract. After 52 days from initiation, skin tumors and ulcers were visible on DMBA-treated mice consuming *Microcystis* extract and skin tumor weight per mouse was significantly greater in the microcystis/DMBA group than in any of the other groups. Negative *Microcystis* extract controls did not develop skin tumors. Tumor growth was appreciably slower in comparable groups given an *Anabaena* extract. Treatment in this case was continued for 86 days after initiation (Falconer, 1991).

A second tumor promotion study using *Microcystis* extracts was carried out in C57 black mice. *N*-methyl-*N*-nitrosourea was used as a tumor initiator, and the mice then received *Microcystis* extracts of 10 or 40 mg/L (average toxin consumption 0.29 and 89 µg/day). The experiment was terminated after 154 days of exposure to the toxins. No evidence of promotion of lymphoid or duodenal tumors by *Microcystin* toxins was observed even though a dose related elevation of serum enzymes (sorbitol dehydrogenase and alanine amino transferase) indicative of liver damage was recorded (Falconer & Humpage, 1996).

In a two stage carcinogenicity bioassay, male F-344 rats initiated with diethylnitrosamine followed by partial hepatectomy received daily intraperitoneal injections of microcystin-LR. An increase in glutathione S-transferase placental form (GST-P) positive liver foci was seen after 8 weeks. In a second experiment, the animals were dosed twice with microcystin-LR at 10 µg/kg bw, followed by partial hepatectomy and 5 weeks of microcystin-LR dosing at 10, 25, or 50 µg/kg bw per day twice a week. A dose-related increase in GST-positive foci was observed. Microcystin-LR had no effect when given to non-initiated rats or to rats not receiving promotion doses prior to partial hepatectomy (Nishiwaki-Matsushima *et al.*, 1992).

Short-TermTests: Eight Wisconsin lake extracts that contained acutely toxic cyanobacteria and pure anatoxin-a(s), hepatotoxin, and neosaxitoxin were analyzed using the Ames *Salmonella* assay and the *Bacillus subtilis* multigene sporulation test. A chromosome breakage test using human lymphocytes was conducted on anatoxin-a(s), hepatotoxin, and neosaxitoxin. No mutagenic response was observed in *Salmonella* strains TA98, TA100, and TA102 with or without S-9 activation. The *Bacillus subtilis* multigene sporulation test was also negative with regard to mutagenicity. The results of the chromosomal breakage test suggested the possibility that these algal toxins may be clastogenic (Repavich *et al.*, 1990).

The genotoxicity of microcytic cyanobacteria extract (MCE) from a water source in China was studied using the Ames test, Comet assay, and mouse micronucleus test. Results from the Ames test showed that MCE was mutagenic in *S. typhimurium* strains TA97, TA98, TA100, and TA102 with and without S-9. MCE also induced DNA damage in primary cultured rat hepatocytes and enhanced bone marrow micronucleated polychromatic erythrocytes in mice. Microcystin-LR, the main component of MCE, was also tested. Microcystin-LR was negative in the Ames assay but positive in the Comet assay and micronucleus test (Ding *et al.*, 1999).

Microcystis aeruginosa obtained from the water-supply reservoirs of the Dneiper river were reportedly administered orally to rats daily for 6 months. Dosing with 10 mg algal biomass/kg per day increased metaphase aberrations in bone marrow (Kirpenko *et al.*, 1981).

Microcystin-LR induced base substitution mutation at K-r*as* codon 12 in human RSA cells (Suzuki *et al*., 1998).

Addition of whole fresh-water blue-green algae *Aphanizomenon* marketed under the commercial name "Alpha Sun" before application of Nitrovin markedly reduced mutagenic activity in *S. typhimurium* strain TA102 but had no effect on TA97 or TA100 The presence of algae alone at concentrations >2 mg/plate showed an inhibitory effect on growth of the *Salmonella.* These tests were conducted without metabolic activation by S-9 (Lahitova *et al*., 1994).

Metabolism: No pharmacokinetic studies with orally administered microcystins were identified in the available literature.

After iv or ip injection of sublethal doses of toxins in mice and rats, microcystin appears to be carried by bile acid transporter in both the intestine and the liver. About 70 percent is rapidly localized in the liver. The kidney and intestine also accumulate significant amounts. Plasma half-lives of microcystin-LR, after iv administration, were 0.8 and 6.9 minutes for the alpha and beta phases of elimination, but the concentration of microcystin-LR in the liver did not change throughout a 6-day study period. Microcystin-LR was excreted rapidly, with 75 percent excreted within 12 hours. An additional 24 percent was excreted after 6 days, about 9 percent via the urinary route and 15 percent via the fecal route (Gupta, 1998).

No information on the metabolism, distribution, or excretion of other toxic metabolites produced by cyanobacteria was identified in the available literature.

Other Biological Effects: *Mechanistic data.* Microcystins and nodularin, isolated from toxic blue-green algae have been shown to be potent and selective inhibitors of protein phosphatases 1 and 2A in the livers of rainbow trout administered cyanobacteria orally, in a cytosolic fraction of mouse liver, and in rat liver. The increase of phosphoproteins observed in rat primary cultured hepatocytes was associated with morphological changes, which appeared to be a step in the process of hepatotoxicity (Nishiwaki-Matsushima *et al*., 1992; Sahin *et al*., 1995; Yoshizawa *et al*., 1990).

Reproductive and developmental toxicity: The effect of cyanobacteria contamination of drinking water with perinatal outcome was examined in a study involving 32,700 newborns from 156 Australian communities. Statistically significant differences between the proportion of time during the first trimester with cyanobacterial occurrence and percentage of births with low or very low birth weight were observed. Significant differences were also found among various categories of first trimester exposure based on average cell density and low birth weight, prematurity, and congenital defects. However, no clear dose-response relationships were observed, causing the authors to conclude that no clear evidence for an association between cyanobacterial contamination of drinking water sources and adverse pregnancy outcomes had been demonstrated (Pilloto *et al*., 1999).

The effects of orally administered microcystin-LR on the embryonic and fetal development of Cr1:CD-1(ICR) BR mice were examined. Doses of 200-2000 µg/kg bw per day were administered from days 6 to 15 of pregnancy. Treatment at 2000 µg/kg was associated with maternal toxicity and mortality; at necropsy, a number of females had abnormal livers and fetal weight and skeletal ossification were retarded. No evidence of embryolethality, teratogenicity, or embryonic growth retardation was observed. There was no apparent effect of treatment on litter size, post implantation loss, or the sex distribution of the live fetuses (Fawell *et al*., 1994).

In another study, Falconer investigated the potential teratogenic effects of *Microcystis* extracts. These extracts were administered in drinking water to mice, with both sexes of parents exposed. No evidence of organ or bone deformity was observed in the offspring. Of 73 neonatal mice born to parents given *Microcystis* extract, seven showed reduced brain size (Falconer *et al*., 1988; Falconer, 1989).

Structure-Activity Relationships: The chronic toxicity and genotoxicity of the metabolites produced by the thousands of cyanobacteria present in the environment are not sufficiently characterized to perform a structure activity analysis with the exception of the microcystins.

Microcystins LR, YR, and RR bind to and inhibit protein phosphatases 1 and 2A with

the same potencies as okadaic acid,. The 4(E),6(E)geometry in the ADDA portion of the microcystin structure appears to be important in the interaction with protein phosphatases, and hence with predictions regarding tumor-promoting potential (Nishiwaki-Matsushima *et al*., 1991).

Repeated topical applications of okadaic acid have promoted skin tumors on mice initiated with DMBA. Okadaic acid in drinking water also promoted glandular stomach tumors in rats initiated with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. Likewise, repeated intraperitoneal injections of microcystin-LR and nodularin promoted rat liver tumors initiated with diethylnitrosamine (Fujiki & Suganuma, 1999; Nishiwaki-Matsushima *et al*., 1992, Ohta *et al*., 1994).

The key difference between tumor promoters of the okadaic acid class and those of the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) class is that the former are potent inhibitors of protein phosphatases-1 and 2A, which dephosphorylate phosphoserine and phosphothreonine in proteins. However, the biochemical consequences of the okadaic acid class of tumor promoters are similar to those of TPA with regard to the accumulation of phosphoproteins: both induce signal transduction (Fujiki & Suganuma, 1999).

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