MICROCYSTINS

A BRIEF OVERVIEW OF THEIR TOXICITY AND EFFECTS, WITH SPECIAL REFERENCE TO FISH, WILDLIFE, AND LIVESTOCK

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Integrated Risk Assessment Branch
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Introduction

Cyanobacteria, also known as blue-green algae, are a family of single-celled algae that proliferate in water bodies such as ponds, lakes, reservoirs, and slow-moving streams when the water is warm and nutrients are available. Many cyanobacteria species produce a group of toxins known as microcystins, some of which are toxic. The species most commonly associated with microcystin production is *Microcystis aeruginosa* [1]. Upon ingestion, toxic microcystins are actively absorbed by fish, birds and mammals. Microcystin primarily affects the liver, causing minor to widespread damage, depending on the amount of toxin absorbed. People swimming, waterskiing, or boating in contaminated water can be exposed to microcytins. Microcystins may also accumulate in fish that are caught and eaten by people. Finally, pets and livestock have died after drinking water contaminated with microcystins.

Microcystins have been measured in several water bodies in California including the Salton Sea [2], the Klamath River and its reservoirs [3, 4], several lakes in southern California (Lake Mathews, Lake Skinner, Diamond Valley Lake, and Lake Perris) [5] and the delta region above San Francisco Bay up into the Sacramento and San Joaquin Rivers [6, 7]. In some areas, microcystin concentrations have reached high levels, although the amount can vary drastically between water bodies and times of the year. In California, one dog death has been attributed to microcystin poisoning [8]. Cattle and wildlife mortalities have been linked to microcystin poisoning in other areas [9]. While there have been impacts on human health, no human deaths from ingestion of microcystins have been reported in the scientific literature. In this report, an emphasis is placed on the effects of microcystins in fish, wildlife and livestock.

Chemistry of Microcystin

Microcystins are cyclic peptides, containing seven amino acids. They are the most numerous of the cyanotoxins, comprising over 80 analogs. Figure 1 shows the general structure shared by all microcystins. The seven amino acids are numbered with variable portions shown as X, Z, R^1 and R^2.

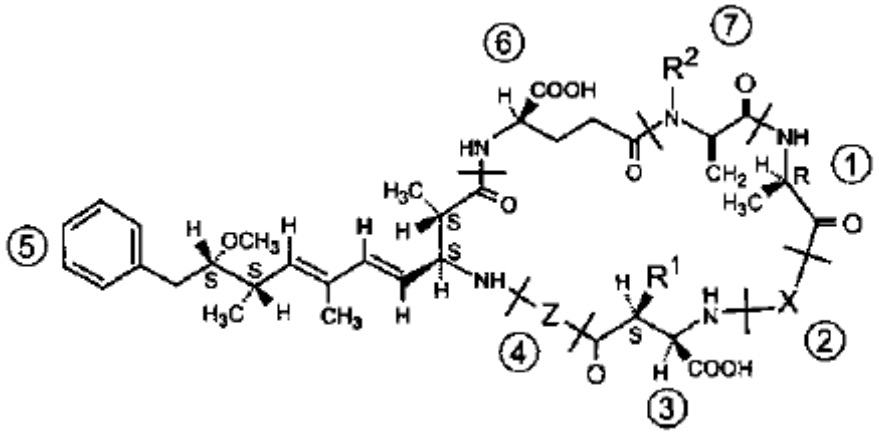

Figure 1. General structure of microcystins

The four microcystins that are the subject of this review have different amino acids in the X and Z positions in the Figure 1, but are otherwise identical [both R^1 and R^2 are methyl groups]. Microcystins are named using the one letter abbreviation for the amino acids substituted at the X and Z positions, respectively. The table below shows the amino acids that would appear in the structure above for the named microcystins.

Name	**X-position Amino Acid**	**Z-position Amino Acid**	**Molecular Weight**
Microcystin LA	Leucine (L)	Alanine (A)	910.06
Microcystin YR	Tyrosine (Y)	Arginine (R)	1045.19
Microcystin RR	Arginine (R)	Arginine (R)	1038.2
Microcystin LR	Leucine (L)	Arginine (R)	995.17

The most extensive toxicological information is available for the microcystin LR congener. However, the LA, RR and YR congeners have similar toxicological effects. The toxic effects of microcystins on animals have been studied with both purified microcystins and unpurified cyanobacterial extracts. In these unpurified cyanobacterial extracts, the microcystins isomers are sometimes inferred by the species of cyanobacteria from which the extracts were prepared.

Microcystins are produced by the cyanobacterial cells. When the algae dies, the cell walls burst, releasing the toxin into the water. Microcystins are extremely stable and resist common chemical breakdown such as hydrolysis or oxidation under conditions found in most natural water bodies. These toxins can break down slowly at high temperature (40 °C or 104 ^{o} F) at either very low (<1) or high (>9) pH [10]. The half-life, the time it takes for one-half of the toxin to degrade, at pH 1 and 40 ^{o}C is 3 weeks; at typical ambient conditions half-life is 10 weeks. Microcystins break down slowly in full sunlight especially when water-soluble pigments are present [11]. Although microcystins can be broken down by some bacterial proteases, in many circumstances these bacteria are not present so the toxin persists for months or even years once released into cooler, dark, natural water bodies [12-15]. Microcystins can even persist after boiling, indicating that cooking is not sufficient to destroy the toxins [1].

I. Toxicology of Microcystins

A. Human Mortality and Morbidity

Although no reports of human deaths occurring from the ingestion of microcystins could be found, there are numerous reports of a variety of health effects after exposure to cyanotoxins in drinking water or from swimming in water in which cyanobacteria were present. The most common sign of human poisoning with microcystins is liver damage [16]. In 1999, the World Health Organization (WHO) convened a panel of international experts and produced what remains the most comprehensive review in the field. "In comparing the available indications of hazards from cyanotoxins with other water-related health hazards, it is conspicuous that cyanotoxins have caused numerous fatal poisonings of livestock and wildlife, but no human fatalities due to oral uptake have been documented". [1]

In February 1996, 116 of 131 patients in Caruaru, Brazil experienced visual disturbances, nausea, vomiting, and muscle weakness following routine dialysis. One hundred of those affected then developed acute liver failure and 52 eventually died from symptoms of what is now called "Caruaru Syndrome" [17]. The cause of this syndrome was determined to be cyanotoxins from reservoir water that had not been treated, filtered, or chlorinated [16]. Microcystins were found in the water as well as the blood and livers of the patients. A related cyanobacterial toxin, cylindrospermopsin, was also found in the water.

B. Liver Toxicity

Microcystins in general are liver toxins. Most of the understanding about the toxicity of microcystins is based on studies with mice and rats that received intra-peritoneal (IP) injections of microcystin LR, i.e. injections directly into the abdominal cavity. In these studies the injection of microcystins caused death within a few hours. Early manifestations of liver damage include an increase in serum of liver enzymes, a sign of liver cell death, and increased liver weight. Liver damage and cell death can be seen microscopically as soon as 20 minutes following injection of a lethal dose of microcystin LR. Within an hour, the liver cells (hepatocytes) die, losing their connection to each other and disrupting the normal architecture of the liver [18, 19]. For example, two mice given oral doses of 16.8 and 20 mg/kg were dead within 160 minutes [20].

Microcystins inhibit a class of enzymes known as protein phosphatases. This enzyme removes phosphate from a protein, a common step in many biochemical pathways. This inhibition, with subsequent build up of phosphorylated proteins, is believed to be a mechanism by which microcystins destroy livers. Hepatocytes from animals treated with microcystins appear to die by a process of programmed cell death or cell suicide called apoptosis [21]. Cells undergoing apoptosis disappear in a characteristic fashion, cannibalizing their own cellular organelles [22]. There is some evidence that microcystin LR increases other proteins in pathways leading to apoptosis but this has not been as extensively studied as the inhibition of phosphatases [23]. Microcystins LA, RR and YR inhibit the same phosphatases and induce histological changes in rodent liver similar to microcystin LR [24]. Therefore, the toxicity criteria computed for microcystin LR are also used for microcystins LA, RR and YR.

C. Liver tumor promotion

Some published studies suggest that microcystins might act as tumor promoters, agents that do not cause cancer, but stimulate the proliferation of cancer cells. In June, 2006, the International Agency for Research on Cancer (IARC), a branch of the WHO, convened a panel of international experts to evaluate the toxicity of *Microcystis* extracts, microcystin LR, and another algal toxin, nodularin [25, 26]. The committee determined that "There is inadequate evidence in experimental animals for the carcinogenicity of *Microcystis* extracts." The committee also found inadequate evidence for microcystin LR to cause cancer in either laboratory animals or humans. The IARC committee concluded that "microcystin LR is possibly carcinogenic to humans," but that "*Microcystis* extracts are not classifiable as to their carcinogenicity to humans," noting that the studies were all short term exposures. In summary, the IARC did not find sufficient evidence to conclude that microcystin extracts cause cancer.

However, while microcystin-LR does not cause cancer, microcystin may stimulate the growth of cancer cells. *Microcystis* extracts in the drinking water increase the number and weight of skin tumors in mice topically treated with the carcinogen dimethylbenzanthracene [27, 28]. A short-term liver tumor promoter assay was conducted with microcystin LR. Rats treated with diethylnitrosamine develop liver tumors that are preceded by pre-cancerous foci of liver cells that express a number of enzymes atypical for liver. *Microcystis* extracts caused a dose-dependent increase in the percentage of the livers with these foci [29]. Interestingly, *Microcystis* extracts decreased duodenal tumors in mice [30].

The National Toxicology Program (NTP), a branch of the National Institutes of Health that oversees animal testing of chemicals or substances, conducts 24-month bioassays in rats and mice to evaluate carcinogenicity. The NTP Web site indicates that they are planning to expose F344 rats to intravenous mixtures of microcystins LA and LR. The results of these studies will not be available until 2011 at the earliest.

II. Health-Based Criteria for Safe Exposure to Microcystin

Prior to the 2006 IARC evaluation, the WHO conducted an evaluation of the Tolerable Daily Intake (TDI) level, based on a non-can.cer endpoint [1]. This value, 0.04 micrograms per kilogram body weight (µg/kg/d), is based on the results of liver toxicity studies in mice [31, 32]. A TDI is the maximum daily dose of microcystins that is considered safe. Using this TDI, WHO also developed a drinking water concentration limit of 1.5 µg/L for microcystin LR. They assumed that a 60 kg (132 lbs.) person drinks two liters of water each day and that 80% of the two liters is from a contaminated source. Their calculation was as follows:

0.04 µg microcystin/kg body weight/day x 60 kg person / (2 L water/day x 0.80) = 1.5 µg/L

The most recent publication [33] cites the 1998 provisional guideline of 1 µg/L based on the above equation and rounded to one significant digit (rounding down to be health-protective). WHO also categorized swimming risk levels as mild, moderate, high, or very high based on the water concentration of microcystins. These water concentrations are related to whether a swimmer, weighing 60 kg and ingesting 100 ml of water, would exceed the TDI.

III. Domestic Animal Poisonings

The majority of reported cyanotoxin poisonings have occurred in domestic animals that drink freshwater containing cyanobacterial blooms [see reviews by 9, 34-36]. Worldwide, thousands of livestock fatalities and numerous poisonings in dogs have been linked to the ingestion of cyanobacteria [reviewed by 9, 35, 36]. Animal poisonings have even occurred under environmental conditions considered unfavorable to cyanobacteria blooms such as cold lakes with low nutrient levels [37].

In North America, domestic animal poisonings have been linked to blooms of *Microcystis sp.* in California [8], Colorado [38], Georgia [39], Michigan [40], Mississippi [41], Oklahoma [42], Wisconsin [43], and Saskatchewan, Canada [44, 45]. Most of the poisonings were fatal and were associated with visible scum of cyanobacteria. Symptoms of microcystin poisoning in domestic animals include diarrhea, vomiting, weakness and recumbency [8, 9].

Unfortunately, some animals appear to be attracted to cyanobacteria in water and dried crusts of algae on top of the water [reviewed by 35]. Livestock and dogs have been observed to drink infested water while clean water was plainly accessible, and to avidly consume crust and mats [46-49]. Lopez-Rodas and Costas [47] found that mice showed a clear preference for *Microcystis aeruginosa* scum (1,000 and 15,000 cells/ml) over clean drinking water. These mice did not prefer non-cyanobacterial phytoplankton over clean drinking water and did not differentiate between toxic and non-toxic strains of the cyanobacteria. These observations and experiments indicate that at least some animals preferentially consume cyanobacteria. Domestic animals should be prevented from drinking or entering untested bloom waters and from eating crust or mats on the shoreline.

IV. Effects of Microcystins on Fish and Wildlife

A. Fish

Microcystins are toxic to fish at concentrations as low as a few micrograms per liter (µg/L) or possibly even fractional µg/L [reviewed by 50, 51, 52]. Considering that microcystins has been measured in concentrations up to 25,000 µg/L in waters with cyanobacterial blooms [reviewed by 1], it is not surprising that potential impacts on fish are receiving increased attention. Fish typically either ingest cyanobacteria or prey that have fed on cyanobacteria [53-55]. To a lesser extent, they can absorb the toxins directly from the water [56].

As with mammals, microcystins are actively taken up by the liver in fish where they disrupt normal cellular activity by inhibiting protein phosphatases [54, 57-63]. Inhibition of these enzymes in fish can ultimately result in widespread cellular death and loss of liver structure [reviewed by 50]. Protein phosphatases are particularly important during fish embryonic development because they regulate critical developmental processes [64]. Due to the limited capacity of fish to detoxify microcystins, they easily succumb to the toxic effects of increased microcystin concentrations [65-72].

Field observations of impacts on fish coincide when blooms are abundant. However, aquatic ecosystems are complex and it can be very difficult to discern the exact cause of the impacts. For example, fish kills following a bloom could be caused by microcystin released from dying cells, but are more likely due to the decreased oxygen and pH levels caused by the decaying

bloom [see 51]. Consequently, the toxic effects of microcystins in fish have been studied experimentally using several different fish species and exposure routes.

Like small mammals, most studies on the immediate (acute) lethality of microcystins in fish have utilized IP injections of extracted microcystins to determine the dose that is lethal to half the test population (LD_{50}). Reported LD_{50} values of microcystins in fish range from 20 to 1500 µg microcystin LR/kg body weight [reviewed by 50]. The large range of values could reflect variation between fish species, or differences in toxin extraction, purification, or measurement methods. As a group, mature fish are less sensitive to acute microcystin toxicity than mammals [1, 50]. Data from these acute studies are useful to make general comparisons between species. However, IP injections of microcystins are not analogous to field exposures since the toxin is absorbed faster and metabolized differently when administered into the abdominal cavity (as with the IP route) as compared to oral administration [see 51]. For example, IP injection of 50 µg MC/kg in carp killed all test fish while an oral dose of 250 µg MC/kg in similar carp resulted in no lethality and minimal liver damage [73]. No oral LD_{50} values were found for microcystins in fish. When developing loach were immersed in solutions of isolated MC-LR (over multiple days), the median lethal concentrations (LC_{50}) were 164.3 µg/L in embryos and 593.3 µg/L in small hatched juveniles [74].

In nature, fish are most likely subject to sublethal impacts resulting from exposure to microcystins over days or weeks. Several studies have observed severe liver damage in fish following oral administration of microcystins, usually in the form of freeze-dried cyanobacterial cells. The sublethal microcystin concentrations shown below are commonly found in food items of fish during blooms. For example, a diet containing greater than 130 to 2,500 µg MC/kg diet wet weight (ww) for two or more weeks may result in sublethal effects in carp (based on 5 kg fish consuming 2% body weight/day). Microcystin concentrations in cyanobacterial blooms commonly reach 20,000 µg MC/kg algae and have been reported as high as 129,000 µg MC/kg algae [ww, converted from dry weight, 1]. Mussels, snails and zooplankton collected from areas with blooms have contained microcystin concentrations up to 2,500, 2,900 and 13,700 µg MC/kg body weight (bw), respectively [ww, converted from dw, reviewed by 50]. These estimates indicate that fish exposed to typical microcystin producing blooms may be experiencing sublethal toxic effects (i.e., liver damage). This is in agreement with Carbis et al. [75], where the majority of common carp sampled from a lake with 22,000 – 40,000 µg MC-LR/kg bloom material (ww, converted from dry) exhibited widespread liver damage consistent with microcystin toxicity.

Examples of the effects of sublethal oral microcystin doses in fish

Fish	Dose (µg MC/kg)[1]	Number of Doses	Exposure Time (days)	Total Dose (µg MC/kg)	Sublethal Effect	Ref.
Carp (adult)	2.5	16	16	40	Widespread liver damage	[76]
Carp (adult)	50	28	28	1,400	Severe liver damage	[77]
Carp (juvenile)	400	1	1	400	Severe liver and kidney damage	[78]
Trout	550	8	4	4,400	Severe liver damage	[53]
Perch	1,150	8	4	9,200	Severe liver damage	[79]
Tilapia	1,200	21	21	25,200	Significant oxidative stress in liver	[80]

[1] **MC-LR equivalents in administered cyanobacteria cells**

Additional sublethal effects of microcystins have been described in fish including effects on kidney, gill, growth, immune status and cardiac function [73, 81-84].

Developing fish appear to be very sensitive to chronic exposures to microcystins [reviewed by 50]. In general, exposure of embryos and larvae to environmentally relevant concentrations of microcystins has resulted in oxidative stress, reduced growth, developmental defects, and lethality, as well as the lack of significant impacts. Fish embryos can take up significant levels of dissolved microcystins from the surrounding water [85]. Exposures as low as 0.25 µg/L resulted in oxidative stress to zebrafish embryos [86]. Immersion of embryos and larvae in solutions of 0.5 - 50 µg MC/L for up to 30 days resulted in interferences with hatching, developmental defects, liver damage and/or increased mortality in several species including chub, carp, loach, trout and zebrafish [reviewed by 50]. Reported concentrations of microcystins in water (not cells) during blooms range from trace amounts to 1,800 µg/L [median was 2 ug/L, 1].

Maternal transport of microcystins from the female to developing eggs may be an additional exposure route to developing fish. Although this route has not been demonstrated for microcystins, experiments indicate that developing fish embryos would be more sensitive to maternal transport of microcystins compared to uptake from water [87, 88]. Microinjection of minute amounts of microcystin into medaka embryos significantly reduced survival rates [87]. Similar experiments in zebrafish resulted in significant disruption of development and reduced survival [88]. These studies reveal potential impacts of microcystin maternal transport. The precise mechanisms of exposure and effects in fish embryos have not been fully determined.

Extracts from cyanobacteria, with or without microcystins present, disrupt development and growth of fish [89, 90]. Most studies have utilized purified cyanotoxins to isolate specific

toxicity thresholds and effects. However, most natural blooms contain more than one cyanobacteria species, many of which produce more than one toxin [reviewed by 1]. Typically, crude extracts of cyanobacteria elicit more severe effects in fish embryos and larvae than purified microcystins. Observed effects of exposure to crude extracts include increased oxidative stress, liver damage, gross malformations, osmoregulatory imbalance, and decreased survival [86, 91-94].

B. Birds

Bird deaths have been linked to cyanobacterial blooms in Canada and the United States since the early 1900s [reviewed by 9, 34]. Blooms of cyanobacterial species that produce microcystins and/or anatoxin-a have coincided with the deaths of ducks, gulls, songbirds, pheasants and hawks, as well as several other bird species. The severity of such bird kills have ranged from a few individuals to several thousand birds per incident. In California, high mortality in birds wintering at the Salton Sea has been linked to microcystins [2]. Levels of microcystins found in many of the dead birds were similar to those in mice exposed to lethal levels of this toxin. Microcystin poisoning has also been linked to the mortality and illness of great blue heron from Chesapeake Bay [2, 95].

In other countries, microcystins have also been specifically implicated in bird poisonings. In Japan, approximately 20 spot-billed ducks died at a pond containing a bloom of *M. aeruginosa* [96]. Bloom material contained high levels of microcystins and produced acute toxicity in a mouse bioassay that was consistent with the effects of the toxin. Waterfowl and other animals died at a reservoir containing an extensive *Microcystis sp.* bloom in South Africa [reviewed by 35]. Examined individuals showed liver damage consistent with acute and chronic microcystin toxicity. Furthermore, water from the reservoir was used to recreate the same effects in experimental animals.

Little experimental work has been completed in birds. Takahashi [97] reported an IP LD_{50} of 256 µg microcystin RR/kg in quail, which is low compared to that of mice [600 ug/kg, see 1]. Skocovska et al. [98] administered a daily oral dose of up to 46 µg microcystins, as *Microcystis sp.* biomass, to quail for up to 30 days. No mortality was observed during the experiment. However, histopathological lesions were observed in livers. More work is needed to better understand the impacts of microcystins on birds.

V. Conclusions

The blue-green algae *Microcystis aeruginosa* can produce a family of toxins known as microcystins. They can cause liver damage that can lead to death in dogs and livestock. No known deaths have been reported in humans from the ingestion of microcystins. Fish and birds are also at risk for microcystin toxicity. Regardless of species, the mechanism of action is the same – the inhibition of protein phosphatase which causes primarily liver damage, but also affects other organs. Microcystins also act as a tumor promoter.

While microcystins are not as toxic as many natural toxins, they are becoming more and more ubiquitous in California, leading to greater opportunities for exposures. Microcystis blooms occur in quiet, warm waters that are nutrient-rich; the type of conditions that are found in lakes, reservoirs, dammed rivers, and even agricultural drainage ditches throughout the state.

Microcystins have also been detected in the Delta. Steps are being taken to begin to address this problem. In 2008, the Klamath River was added to the Clean Water Act's 303d list as an impaired waterbody as a result of microcystis blooms. It appears that some dams on this river will be removed along the Klamath, which should reduce the frequency or possibly eliminate toxic blooms. Affirmative steps such as these will help reduce the risk of exposure and adverse effects associated with microcystins.

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