Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management
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Chapter 4. HUMAN HEALTH ASPECTS

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Evidence for adverse human health effects from cyanotoxins derives from three principal sources: epidemiological evidence including human poisonings, animal poisonings, and toxicological studies.

Epidemiological evidence results from studies of human populations that have shown symptoms of poisoning or injury attributed to the presence of cyanotoxins in drinking water or other sources of water. This type of evidence depends upon good case definition, good characterisation of exposure and a reporting system that enables these data to be compared. Such evidence, discussed later, includes the fatal intoxication by microcystin of 50 dialysis patients in Brazil, and the hospitalisation of 140 children supplied with water from a dam containing *Cylindrospermopsis raciborskii* in Australia. Reports of gastro-enteritis after the appearance of cyanobacterial blooms in drinking water sources come from North and South America, Africa and Europe. Most cases of human injury attributed to cyanobacterial toxins have been studied retrospectively, and complete epidemiological data, especially regarding exposure (number of organisms, type and concentration of cyanotoxins), are rarely available. Nevertheless, epidemiological evidence is of special importance in directly demonstrating the link between toxin exposures and human health outcomes, which otherwise cannot be derived directly from animal experiments.

A large number of animal poisonings have been recorded, some of which have been reported in detail, while others are anecdotal. These are important in the overall body of knowledge on cyanobacterial toxicity, because they demonstrate effects under natural conditions. However, cyanotoxin exposure is rarely quantified and cannot be directly extrapolated to human populations.

Although animal toxicity tests are not performed under conditions of natural exposure and are undertaken with different species, they are of particular importance because they are conducted under controlled laboratory conditions. They provide plausibility for the role of cyanotoxins in human and animal poisonings, and provide information on their comparative toxicity. Oral toxicity testing is especially relevant, and has been carried out on large animals as well as on rodents.

The overall case for the human health relevance of exposure to cyanotoxins can only be assessed properly by combining all available evidence and understanding its strength and its weaknesses.

Of all the cyanobacterial toxins currently known, the cyclic peptides represent the greatest concern to human health because of the potential risk of long-term exposure to comparatively low concentrations of the toxins in drinking water supplies. As will be discussed in sections 4.2.1 and 4.2.2, the cyclic peptide microcystins and nodularins are specific liver poisons in mammals. Following acute exposure to high doses, they cause death from liver haemorrhage or from liver failure, and they may promote the growth of liver and other tumours following chronic exposures to low doses.

The alkaloid neurotoxins (anatoxins and saxitoxins/PSP toxins) have shown only acute effects in mammals. Risk assessment will, therefore, be limited to acute exposure. However, there are a number of complicating factors. Firstly, while there is a great deal of published information on the mammalian and human toxicity and toxicology of saxitoxins/PSP toxins, the animal data for anatoxins is rather sparse, and completely lacking for human exposure. Secondly, some of the alkaloid toxins, PSP toxins in particular, are known to accumulate to high concentrations in marine and freshwater biota (see section 3.4.5). In many areas of the world, the local human population will be consuming fish, shellfish and animals that have grown in, or nearby, water bodies contaminated with toxic cyanobacteria. Thus there may be several sources of oral exposure for neurotoxins in addition to drinking water or recreational water. The potential for transfer of PSP toxins, and possibly other neurotoxins, to humans via these routes may be significant in freshwaters.

The toxicity of pure cylindrospermopsin has recently been studied following the identification of the toxin in 1992 (Ohtani *et al.,* 1992). These studies have helped to characterise the histopathology of cylindrospermopsin after intraperitoneal (i.p.) injection (Hawkins *et al.,* 1997; Falconer *et al.,* 1999; Seawright *et al.,* 1999). It is not known whether cylindrospermopsin will elicit histopathological damage following chronic (long-term) exposure at low concentrations.

Lipopolysaccharides (LPS) from cyanobacteria can elicit both allergic and toxic responses in humans, although little is known about their acute or chronic effects. The lipid part of the molecule is believed to cause the deleterious response in humans, while the sugar moiety is important in determining LPS solubility and micelle properties, and hence is important in modulating toxicity. A lack of knowledge regarding the occurrence and toxicity of cyanobacterial LPS, combined with the diversity of LPS structures within the cyanobacteria, should be considered by health officials and water managers, particularly when gastrointestinal and respiratory symptoms are reported from exposed humans.

4.1 Human and animal poisonings

Water supplies have been associated with gastrointestinal illness throughout human history with cholera, dysentery, and typhoid responsible for much human misery and death. The epidemiological evidence for human illness due to cyanobacterial toxins therefore has to be viewed against a background of alternative causes, with bacterial, viral or protozoal infections being the first causes to be investigated.

4.1.1 Short-term effects in humans

The recorded cases of gastrointestinal and hepatic illness that can be reliably attributed to cyanobacterial toxins in water supplies have all been coincident with either the breakdown of a natural cyanobacterial bloom or with the artificial lysis of a bloom by application of copper sulphate. Both mechanisms lead to cyanotoxin release from decomposing cells. Whereas treatment procedures might have removed cyanotoxins bound in intact cells, they were not effective in removing the dissolved cyanotoxins in these cases. The earliest reported cases of gastro-enteritis from cyanobacteria were in the population of a series of towns along the Ohio River in 1931. In these cases low rainfall had caused the water of a side branch of the river to develop a cyanobacterial bloom which was then washed into the main river. As this water moved downstream a series of outbreaks of illness were reported, which could not be attributed to infectious agents (Tisdale, 1931). In Harare, Zimbabwe, children living in an area of the city supplied from a particular water reservoir, developed gastro-enteritis each year at the time when a natural bloom of *Microcystis* was decaying in the reservoir. Other children in the city with different water supplies were not affected and no infectious agent was identified (Zilberg, 1966). The most lethal outbreak attributed to cyanobacterial toxins in drinking water occurred in Brazil, when a newly flooded dam developed an immense cyanobacterial bloom. Eighty-eight deaths, mostly children, were reported to have occurred (Teixera *et al.,* 1993) (Box 4.1).

Examples of illness following the use of copper sulphate to destroy a cyanobacterial bloom in a water storage reservoir have been described in the USA and in Australia. In each of these instances the cyanobacterial genera involved were identified, either at the time or subsequently. In one incident, 62 per cent of the population connected to a filtered, chlorinated water supply developed symptoms of gastro-enteritis within a period of five days. No pathogens were found, and it was concluded that abloom of cyanobacteria in an open storage reservoir which had over 100,000 cells per ml of *Schizothrix calcola, Plectonema, Phormidium* and *Lyngbya* was responsible. The reservoir had just been treated with copper sulphate (Lippy and Erb, 1976).

Box 4.1 Gastro-enteritis epidemic in the area of the Itaparica Dam, Bahia, Brazil

A severe gastro-enteritis epidemic in the Paulo Afonso region of Bahia State in Brazil followed the flooding of the newly constructed Itaparica Dam reservoir in 1988. Some 2,000 gastro-enteritis cases, 88 of which resulted in death, were reported over a 42-day period.

Clinical data and water sample tests were reviewed, blood and faecal specimens from gastro-enteritis patients were subjected to bacteriological, virological and toxicological testing and drinking water samples were examined for micro-organisms and heavy metals. The results demonstrated that the source of the outbreak was water impounded by the dam and pointed to toxin produced by cyanobacteria present in the water as the responsible agent. No other infectious agent or toxin was identified, and cases occurred in patients who had been drinking only boiled water. The cases were restricted to areas supplied with drinking water from the dam.

Cyanobacteria of the *Anabaena* and *Microcystis* genera were present in untreated water at 1,104 to 9,755 units per ml (conversion of colony units to cells per ml depends on colony size, but a minimum of 100 cells per colony is likely in a mixed bloom of these genera).

In Armidale, Australia, the water supply reservoir had been monitored for blooms of toxic *Microcystis* for several years, when a particularly dense bloom occurred. Within three weeks the water supply authority treated the reservoir with 1 ppm (part per million) of copper sulphate, which killed the bloom. An epidemiological study of the local population indicated liver damage occurring simultaneously with the termination of the bloom (see Box 4.2).

A more severe outbreak of cyanobacterial toxicity in a human population occurred on an island off the north-eastern coast of Australia. Due to complaints of bad taste and odour in the water supply, which were attributed to a cyanobacterial bloom, the authorities treated the reservoir with copper sulphate. Within a week numerous children developed severe hepato-enteritis, and a total of 140 children and 10 adults required hospital treatment (Byth, 1980). *Cylindrospermopsis raciborskii* was identified as the cyanobacterium responsible for this episode (see Box 4.3).

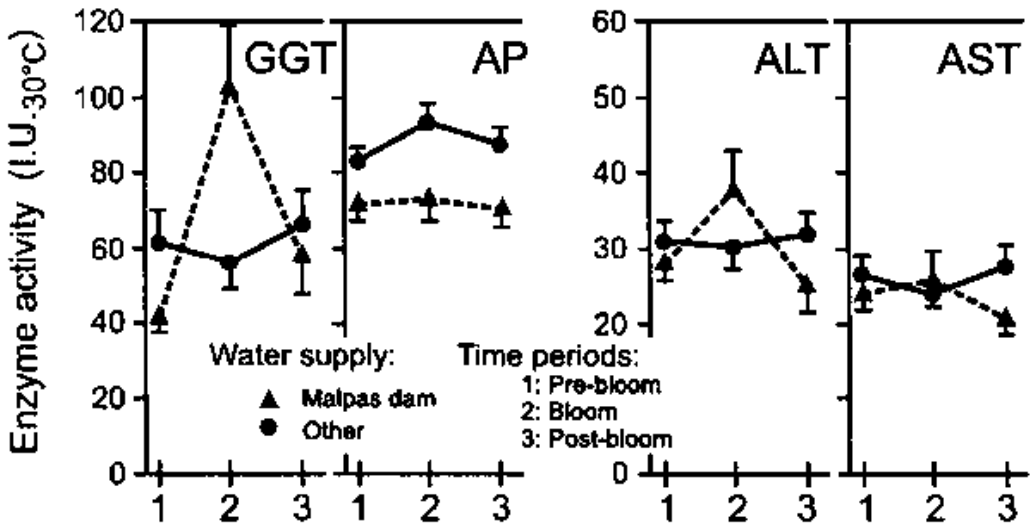
Within human populations, for a variety of reasons, there will be individuals who are at a much greater risk of injury from cyanotoxins than the population as a whole. Children are the most obvious example, because they drink a higher volume of water in proportion to their body weight than adults. Individuals who already have injury to organs susceptible to cyanobacterial toxins are also at increased risk, such as people with hepatitis, liver cirrhosis, toxic liver injury from other sources, or kidney damage. Kidney dialysis patients, if exposed to microcystins in the water used for dialysis, are especially vulnerable because treatment exposes them intravenously to large volumes of water. In a disastrous incident in Caruaru, Brazil, 117 patients developed cholestatic liver disease and at least 47 deaths were attributed to dialysis with water containing cyanobacterial toxins. Examination of the carbon filter from the dialysis unit demonstrated microcystin-LR, as did the blood and liver tissue of deceased patients (Jochimsen *et al.,* 1998) (see Box 4.4).

Box 4.2 Toxic *Microcystis* in the Armidale water supply reservoir and public health

The city of Armidale, New South Wales, Australia has a drinking water supply from a eutrophic reservoir which has experienced repeated blooms of *Microcystis aeruginosa* from the early 1970s to the present day. The reservoir, Malpas Dam, is on a plateau 150 m higher than the treatment plant to which it is connected by about 20 km of pipeline. The plant treated water by pre-chlorination, alum flocculation, sedimentation, rapid sand filtration and post chlorination and fluoridation. The geography of the water storage results in toxic scums accumulating around the offtake area through wind-drift. The local water authority use copper sulphate dosing to control blooms, with aerial distribution of 1 ppm of copper in the top metre of water. The cyanobacterial blooms have been monitored for toxicity, and the toxin has been characterised as microcystin-YM (Botes *et al.,* 1985).

In 1981, a particularly extensive toxic bloom of *Microcystis* was monitored during its development and subsequent termination with copper sulphate. At the time of this bloom, complaints of bad taste and odour were received, leading to the copper sulphate treatment of the reservoir. This event was used as the basis for a retrospective epidemiological study of liver function in the population consuming the water, compared with a population in the same region supplied from other reservoirs. The data for the activity of plasma enzymes measuring liver function were obtained for patients having blood samples at the Regional Pathology Laboratory for the six weeks prior to the bloom, the six weeks of peak bloom and its termination, and for six weeks after

that. The data were then separated into analyses from patients having the Malpas drinking water supply, and those using other supplies.



Serum enzymes measuring liver function in patients consuming drinking water from Malpas Dam or from other supplies during a heavy bloom of *Microcystis aeruginosa* and its termination with copper. GGT = gamma glutamyl transferase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; AP = alkaline phosphatase (From The Medical Journal of Australia, 1983; 1511-1514)

As shown in the figure above, a statistically significant increase in gamma glutamyl transferase in the blood was seen with the group using the Malpas water supply during the peak of the bloom and its lysis with copper sulphate, compared with the same population before and after, or the other population on independent water supplies. The clinical record gave no evidence of an infectious hepatitis outbreak or disproportionate alcoholism (Falconer *et al.*, 1983). While the mean increase in enzyme activity was indicative of minor liver toxicity, individuals within the population studied had highly elevated enzyme activity, indicating substantial liver damage. The serum enzyme showing this change has also been used as an indicator of *Microcystis* toxicity in experimental studies with pigs and rodents, where it is an effective marker for liver injury (Fawell *et al.*, 1993; Falconer *et al.,* 1994).

4.1.2 Chronic effects in humans

While acute toxicity is the most obvious problem in cyanobacterial poisoning, a long-term risk may also be present. Short exposures to toxins may result in long-term injury, and chronic low-level exposure may cause adverse health effects. Animal experiments have shown chronic liver injury from continuing oral exposure to microcystins. In particular the possibility of carcinogenesis and tumour growth promotion need careful evaluation, because both have been shown in animal experimentation.

The incidence of human hepatocellular carcinoma in China is one of the highest in the world, and studies have explored whether cyanobacterial toxins are part of a complex of risk factors which increase the incidence of this disease. The distribution of hepatocellular carcinoma varies geographically. In south-east China, rates of less than 15 incidents per 100,000 people are seen in some districts, compared with over 60

incidents per 100,000 people in adjacent localities. Two proven risk factors are infection with hepatitis B virus and intake of aflatoxin B_1 from food items, such as corn. The third significant element of association was the source of drinking water. On a village basis, lower cancer mortality rates were seen when the water was drawn from deep wells, compared with much higher rates when the water came from ponds and ditches. Cyanobacteria are abundant in surface waters in south east China where the incidence of hepatocellular carcinoma is highest, and it has been proposed that microcystins in the drinking water are responsible for the higher incidences of cancer among drinkers of pond and ditch water (Yu, 1989, 1995). Very low levels of cyanotoxins were found in one study with limited sampling of some drinking water sources in China (Ueno *et al.,* 1996), but a more representative study would be needed to investigate this association. At present, all three risk factors are being reduced concurrently in China and liver cancer rates appear to be decreasing (Yu, 1995) (see also Box 5.3).

Box 4.3 Palm Island Mystery Disease

In 1979, there was a major outbreak of hepato-enteritis among the children of an Aboriginal community living on a tropical island off the coast of Queensland, Australia. Altogether 140 children and 10 adults required treatment, which was provided by the local hospital for less severe cases and by the regional hospital on the mainland for severe cases possibly requiring intensive care. Diagnostic information included a detailed clinical examination showing malaise, anorexia, vomiting, headache, painful liver enlargement, initial constipation followed by bloody diarrhoea and varying levels of severity of dehydration. Urine analysis showed electrolyte loss together with glucose, ketones, protein and blood in the urine, demonstrating extensive kidney damage. This was the major life-threatening element of the poisoning. Blood analysis showed elevated serum liver enzymes in some children, indicating liver damage. Sixty-nine percent of patients required intravenous therapy and in the more severe cases the individuals went into hypovolaemic/acidotic shock. After appropriate treatment all the patients recovered (Byth, 1980).

Examination of faecal samples and foods eliminated a range of infectious organisms and toxins as possible causes for the outbreak, hence the name "Palm Island Mystery Disease". The affected population, however, all received their drinking water supply from one source, Solomon Dam. Families on alternative water supplies on the island were not affected by the disease.

Prior to the outbreak of the illness an algal bloom occurred in Solomon Dam. The bloom discoloured the water and gave it a disagreeable odour and taste. When the bloom became dense, the dam reservoir was treated with 1 ppm of copper sulphate (Bourke *et al.*, 1983). Clinical injury among consumers on that water supply was reported the following week.

The organisms from the dam were cultured and administered to mice, following which the mice slowly (over several days) developed widespread tissue injury involving the gastrointestinal tract, the kidney and liver (Hawkins *et al.,* 1985). The widespread tissue damage and delayed effects are quite different to those following *Microcystis aeruginosa* administration (Falconer *et al.,* 1981; see section 4.2.1).

Subsequent monitoring of the algal blooms in the dam identified the cyano-bacterium *Cylindrospermopsis raciborskii* as the cause of the blooms, with seasonal cell concentrations of up to 300,000 cells per ml of water. This organism does not form scums, and has the highest cell concentrations well below the water surface. In order to reduce bloom formation, the responsible authorities later introduced destratification of the reservoir (Hawkins and Griffiths, 1993).

Subsequent research on toxins produced by *Cylindrospermopsis raciborskii has* identified the cytotoxic alkaloid cylindrospermopsin which is toxic after i.p. and oral administration. It is possible that other toxins will be isolated from this organism (Hawkins *et al.,* 1997, see also section 4.2.7).

Box 4.4 Outbreak of severe hepatitis following haemodialysis in Caruaru, Brazil

In February 1996, an outbreak of severe hepatitis occurred at a Brazilian haemodialysis centre in Caruaru, Brazil, located 134 km from Recife, the state capital of Pernambuco. At this clinic 117 of 136 patients (86 per cent) experienced visual disturbances, nausea, vomiting, muscle weakness and painful hepatomegaly, following routine haemodialysis treatment. Subsequently, 100 patients developed acute liver failure and 50 of these died. As of October 1997, 49 of the deaths could be attributed to a common syndrome now called "Caruaru Syndrome". This syndrome includes:

- *Symptoms.* Painful huge hepatomegaly, jaundice and a bleeding diathesis manifested by ecchymosis, epistaxis and metrorrhagia.
- *Laboratory picture.* Elevated transaminases, variable hyperbilirubinemia, prolonged prothrombin time and severe hypertriglyceridemia.
- *Histopathology.* Light microscopy - disruption of liver plates, liver cell deformity, necrosis, apoptosis, cholestasis, cytoplasmic vacuolisation, mixed leukocyte infiltration and multinucleated hepatocytes; electron microscopy - intracellular oedema, mitochondrial changes, rough and smooth endoplasmic reticulum injuries, lipid vacuoles and residual bodies.

The pattern of liver plate disruption was identical to that found with previous laboratory animal experiments involving microcystin exposure.

Initial reports, at the beginning of March 1996, from the medical and public health profession in Pernambuco, implicated several xenobiotic or microbial sources as possibly responsible for a cluster of human fatalities. However, comparison with previous knowledge about cyanotoxicosis, from other parts of the world, suggested that cyanobacteria toxins might be responsible. Since phytoplankton counts were not being made by the city's water utility at the time of the outbreak, the presence of toxic cyanobacteria or microcystin could not be confirmed or denied. Examination of previous years' phytoplankton counts showed that cyanobacteria had been dominant in the reservoir during the summer months since at least 1990, with the most common genera present being *Microcystis, Anabaena* and *Anabaenopis (Cylindrospermopsis).* Samples collected on March 29, 1996 showed that the most common cyanobacteria present were *Aphanizomenon, Oscillatoria* and *Spirulina* (average number 24,500 cells per ml).

Carbon from the dialysis centre's in-house water treatment system showed chromatogram peaks that had characteristics of microcystins when analysed using HPLC. One of these peaks had a retention time corresponding to microcystin-LR, the most commonly found of the microcystin family. Further examination of carbon, sand and cation/anion exchange filters showed microcystin in the ppm range.

Blood sera, provided by state health officials in Pernambuco and sent through the Centers for Disease Control in Atlanta Georgia, USA, from affected and control patients, and liver tissue from deceased patients, showed a microcystin content of up to 10 ng ml^{-1} for sera and 0.1 to 0.5 ng mg^{-1} in liver tissue. All exposed patient sera and tissue were positive for microcystins. Chemical analysis of the most abundant microcystins revealed the presence of microcystins -YR, -LR and -AR. The average microcystin concentration in liver samples from 33 victims compares closely with that reported in laboratory test animals receiving acute exposure to microcystin-LR. When the analyses are completed, the resulting information on dose and related effects will be important in the hazard characterisation and risk assessment of microcystins in humans. A clinic for survivors set up by Brazilian health authorities will help monitor their progress and serve as a data base to assess long-term health effects, including cancer rates, because the microcystins are known to be potent liver tumour promoters in experimental animals.

The available biological and chemical evidence supports microcystins from the reservoir water as being the major factor in the deaths of patients at the dialysis clinic. The occurrence of microcystin in the water used for dialysis was due to insufficient treatment of the water obtained by truck from the city's water treatment system, as well as to insufficient functioning and maintenance of the clinic's dialysis water treatment system.

4.1.3 Injury from recreational exposure

There have been repeated descriptions of adverse health consequences for swimmers exposed to cyanobacterial blooms. Even minor contact with cyanobacteria in bathing water can lead to skin irritation and increased likelihood of gastrointestinal symptoms (Pilotto *et al.,* 1997), Some severe skin reactions have been reported, particularly from contact with the marine cyanobacterium, *Lyngbya majuscula,* which causes deep blistering when trapped under the bathing suit of swimmers (Grauer, 1961). In this case the organism contains a powerful dermal toxin which is further described in section 4.2.8.

Individual sensitivity to cyanobacteria in bathing waters varies greatly, because there can be both allergic reactions and direct responses to toxins. The cyanobacterial pigments can cause severe allergic reactions in sensitive individuals (Cohen and Reif, 1953). Cyanobacteria have features in common with general airborne allergens and surveys have shown allergic responses to cyanobacteria in patients with naso-bronchial allergy (Mittal *et al.,* 1979). More extensive discussion on this aspect can be found in Ressom *et al.* (1994). Illnesses from accidental swallowing of cyanobacteria during swimming are described in Box 4.5.

4.1.4 Animal poisonings

Numerous cases of animal poisoning (often lethal) substantiate the concern of health hazards for humans exposed to cyanobacteria. Therefore, the most important incidents are reported below. For impact of cyanotoxins on aquatic biota, see section 3.5.

The first report of cyanobacterial poisoning was of the deaths of cattle, sheep, dogs, horses and pigs after drinking a scum of *Nodularia spumigena* in Lake Alexandrina, Australia (Francis, 1878). Francis confirmed the source of poisoning by dosing a calf with the scum from the lake, which subsequently caused the death of the animal. Since that time there have been frequent instances of farm animal poisonings from cyanobacterial water blooms, and also deaths of pet dogs after swimming in, or eating, cyanobacteria. Other affected animals range in size from ducks, coots and other waterfowl, to skunks and mink, and even up to rhinoceros (see Carmichael, 1992). Besides the consumption of cyanobacteria from the water, it has been suggested that an additional source of intoxication for terrestrial animals is cyanotoxins that have bioaccumulated in the food chain. For example, freshwater mussels accumulate both microcystins (Prepas *et al.,* 1997) and saxitoxins (Negri and Jones, 1995), and mussels are an important food source for water rats, musk rats and birds.

An extensive list of poisoning incidents, and discussion of them, is included in Ressom *et al.* (1994). A selected group is given in Table 4.1. As with cases of suspected human poisoning by cyanobacterial toxins, in the earlier cases much of the evidence necessary for proof of poisoning is unavailable. Ideally, for such proof, the toxic organisms or toxins

need to be identified in the body of the victim, together with the evidence of access to toxic material (see also section 3.5). One of the most convincing mammalian poisonings has been the recorded deaths of sheep drinking from a farm dam contaminated with the neurotoxic *Anabaena circinalis* in Australia (Negri *et al.,* 1995). The authors recovered high concentrations of saxitoxins from cyanobacteria in the farm dam and from the rumen fluid from the dead sheep.

Box 4.5 Examples of health effects due to recreational exposure

1959 Saskatchewan, Canada. In spite of recreational use warnings and deaths in livestock, people swam in a lake infested with cyanobacteria. Thirteen persons became ill (headaches, nausea, muscular pains, painful diarrhoea). In the excreta of one patient (a medical doctor who had accidentally ingested 300 ml of water), numerous cells of *Microcystis* spp. and some trichomes of *Anabaena circinalis* were identified (Dillenberg and Dehnel, 1960).

1989 United Kingdom. Ten of 20 army recruits showed symptoms indicating intoxication (e.g. vomiting, diarrhoea, central abdominal pain, blistering of the lips, sore throats) after swimming and canoe training in water with a dense bloom of *Microcystis* spp. Two of the recruits developed severe pneumonia attributed to the aspiration of a *Microcystis* toxin and needed hospitalisation and intensive care (Turner *et al.,* 1990). The severity of illness appeared to be related to the swimming skills and amount of water ingested.

1995 Australia. Epidemiological evidence of adverse health effects after recreational water contact was established in a prospective study involving 852 participants. Results showed an elevated incidence of diarrhoea, vomiting, flu symptoms, skin rashes, mouth ulcers, fevers, eye or ear irritations within seven days following exposure (Pilotto *et al.*, 1997). Symptoms increased significantly with duration of water contact and cell density of cyanobacteria.

The post mortem symptoms of toxicity can also be characteristic of the poison involved. Animal deaths from cyanobacterial toxicity have been reported from North and South America, Europe, Australia and Africa. The major injury reported is hepatotoxicosis, i.e. liver poisoning. The cyanobacteria responsible have been *Microcystis aeruginosa, Nodularia spumigena* and *Oscillatoria (Planktothrix) agardhii.* In the most recent cases, post mortem examination has shown evidence of cyanobacterial ingestion as well as characteristic tissue injury in the liver.

Table 4.1 Selected examples of animal poisoning associated with cyanobacteria

Country	Species killed	Pathology	Organism	Reference
Argentina	Cattle	Hepatotoxicity	*Microcystis aeruginosa*	Odriozola *et al.*, 1984
Australia	Sheep	Hepatotoxicity	*Microcystis aeruginosa*	Jackson *et al.,* 1984
Australia	Sheep	Neurotoxicity, PSPs	*Anabaena circinalis*	Negri *et al.,* 1995
Canada	Cattle	Neurotoxicity, anatoxin-a	*Anabaena flos-aquae*	Carmichael and Gorham, 1978
Canada	Waterfowl	Neurotoxicity, anatoxin-a	*Anabaena flos-aquae*	Pybus and Hobson, 1986
Finland	Dogs	Hepatotoxicity, nodularin	*Nodularia spumigena*	Perrson *et al.,* 1984
Finland	Waterfowl, fish, muskrats	Hepatotoxicity, gill damage	*Planktothrix agardhii*	Eriksson *et al.,* 1986
Norway	Cattle	Hepatotoxicity, microcystin	*Microcystis aeruginosa*	Skulberg, 1979
UK				
England	Shepherd dogs	Hepatotoxicity, microcystin	*Microcystis aeruginosa*	Pearson *et al.,* 1990
Scotland	Dogs	Neurotoxicity, anatoxin-a	*Oscillatoria* spp.	Gunn *et al.,* 1992
Scotland	Fish (trout)	Gill injury, microcystin	*Microcystis aeruginosa*	Bury *et al.,* 1995
USA	Dogs	Neurotoxicity, anatoxin-a(S)	*Anabaena flos-aquae*	Mahmood *et al.,* 1988

PSPs Paralytic shellfish poisons

The other main cause of livestock and pet deaths due to cyanobacterial toxins has been from acute neurotoxicity leading to respiratory failure, with no post mortem indications of organ injury. In one case (Gunn *et al.,* 1992) the neurotoxin, anatoxin-a, was isolated from the stomach contents of a dog. Henriksen *et al.,* (1997) demonstrated lethality in wild ducks due to anatoxin-a(S). The cyanobacteria associated with deaths from neurotoxicity are *Anabaena flos-aquae, Anabaena circinalis, Aphanizomenon flos-aquae* and *Oscillatoria* spp. The toxins anatoxin-a, anatoxin-a(S) and saxitoxins have been implicated in different cases.

While the reported deaths have usually occurred shortly after the animals have ingested cyanobacterial scums, lasting injury with progressive mortality has also been seen in animal poisoning by *Microcystis aeruginosa.* The characteristic symptoms are those of liver failure with secondary photo-sensitisation, i.e. severe sunburn-like reactions (McBarron and May, 1966; Carbis *et al.,* 1995).

4.2 Toxicological studies

In order to set safe levels of toxicants or contaminants in food or drinking water, it is first necessary to determine the dose level in humans that is considered to be without adverse effects when taken daily over a lifetime; this is known as the Tolerable Daily Intake (TDI). Ideally, this value is derived from human studies, but often such studies are inadequate or non-existent. Alternatively, when there is an adequate experimental database, it can be derived from animal studies. One of the major shortcomings of animal studies is that differences in sensitivity between animals and humans vary, so that safety factors need to be incorporated to deal with this uncertainty (see below).

A further important extrapolation problem associated with animal studies relates to routes of exposure. Many toxins are more toxic when given by the intravenous (i.v.) or i.p. route of administration than by the oral route. This difference is evident when LD_{50} values (single dose level that will cause death in 50 per cent of the exposed animals within 7-14 days) are examined for various routes of exposure (see Chapter 3 and Table 4.2). Studies using the i.p. or i.v. route of administration require much less toxin and can be used to indicate relative acute toxicity and may provide information on the mechanism of toxicity. Such studies are used in bioassays (see section 13.3.1), but they have little use in predicting toxicity after oral administration. Another problem with many natural toxins, including cyanotoxins, is obtaining sufficient pure toxin to conduct controlled experiments. Several studies, to be described, have used algal extracts rather than pure toxin. An advantage of this is that the extract more closely mimics the real environmental situation. A disadvantage is that unless the extract is fully characterised for all the toxins that may be present and their concentrations, the assay may have limited predictive value for scenarios involving other conditions.

Several steps are involved in determining the TDI from appropriate human or animal studies. The highest dose associated with the absence of adverse health effects (the NOAEL or No Observed Adverse Effect Level) is selected or, when no NOAEL is available, the lowest dose associated with adverse effects (the LOAEL or Lowest Observed Adverse Effect Level) is selected. In order to extrapolate from animal data to the human situation, the next step involves the application of a number of uncertainty factors. In the absence of reliable data on inter- and intraspecies differences in sensitivity to a chemical substance, standard default uncertainty factors of 10 are generally used to give a composite uncertainty factor of 100. Additional uncertainty factors may be used to deal with inadequacies in the database and the severity of effects noted (WHO, 1993; IPCS, 1994). When extrapolating from human data, it is also necessary to use some of these factors. With human data there are, in addition, often problems in determining exposure accurately and thus the dose causing adverse effects.

The expert review of the pertinent literature in April 1997, which led to the production of this book, revealed that information currently available is insufficient for calculation of a TDI for most of the cyanotoxins. For microcystin-LR more data exist and a provisional value could be derived. The following sections summarise the present health-related information on each of the cyanotoxins, in order to provide research data to health officers and others, and in order to provide a basis for the assessment of the health hazards of cyanotoxins.

4.2.1 Microcystins

In the various reported incidents of poisoning in humans and livestock caused by cyanobacteria or their toxins, *Microcystis* is the most frequently cited organism. As a consequence, extensive experimental studies have been carried out on this cyanobacterium and its toxins. Although there is qualitative evidence of cause and effect, indicating human susceptibility, these data have so far been inadequate to establish a dose response relationship that can be used for assessing human risk in a variety of exposure scenarios. The animal studies described below have been conducted in an attempt to address these issues.

Metabolic disposition

There have been no pharmacokinetic studies with orally administered microcystins. After i.v. or i.p. injection of sublethal doses of variously radiolabelled microcystins in mice and rats, about 70 per cent of the toxin was rapidly localised in the liver (Falconer *et al.,* 1986; Runnegar *et al.,* 1986; Brooks and Codd, 1987; Robinson *et al.,* 1989, 1991; Meriluoto *et al.,* 1990; Lin and Chu, 1994a; Nishiwaki *et al.,* 1994). Plasma half-lives of microcystin-LR, after i.v. administration, were 0.8 and 6.9 minutes for the first and second phases of elimination, but the concentration of radioactive (^{3}H-microcystin-LR) label in the liver did not change throughout the later part of the six-day study period (Robinson *et al.,* 1991). In this study, about 9 per cent of the dose was excreted early via the urinary route, with the remainder being excreted slowly (~1 per cent per day) via the faecal route. Based on the protective effect of microsomal enzyme inducers, it is evident that the liver plays a large role in the detoxification of microcystins (Brooks and Codd, 1987). Time-dependent appearance and disappearance of additional chromatograph peaks, thought to represent detoxification products, were seen in urine, faeces and liver cytosol fractions (Robinson *et al.,* 1991). Three metabolic products have since been identified, a glutathione conjugate, a cysteine conjugate and a conjugate with the oxidised ADDA diene (Kondo *et al.,* 1996).

Microcystin-LR does not readily cross cell membranes, and hence does not enter most tissues. After oral uptake it is transported across the ileum into the bloodstream through a bile acid type transporter (the multispecific organic ion transport system) present in hepatocytes and cells lining the small intestine (Runnegar *et al.,* 1991; Falconer *et al.,* 1992) and is concentrated in the liver as a result of active uptake by hepatocytes (Runnegar *et al.,* 1981). It is covalently bound to a 40 kdalton protein (protein phosphatase 2A and possibly protein phosphatase 1) in the hepatocyte cytosol (Holmes *et al.,* 1994; Bagu *et al.,* 1997). Some other microcystin congeners are more hydrophobic than microcystin-LR and may cross cell membranes by other mechanisms, including diffusion.

Acute and subacute toxicity

Microcystin-LR is highly toxic. The LD_{50} by the i.p. route ranges from 25 to 150 µg kg^{-1} body weight (bw) in mice (a value of 50 or 60 µg kg^{-1} bw is commonly accepted). The oral LD_{50} (administered by gavage, i.e. dosing directly into the stomach through the mouth) is 5,000 µg kg^{-1} bw in one strain of mice (Fawell *et al.,* 1994), 10,900 µg kg^{-1} bw in a different strain of mice (Yoshida *et al.,* 1997), and higher in rats (Fawell *et al.,* 1994). This indicates that, even by the oral route, microcystin-LR displays acute toxicity in

rodents. There is no evidence of hydrolysis of microcystins by peptidases in the stomach and it is apparent that a significant amount of microcystin-LR passes the intestinal barrier and is absorbed. Similarly, the oral LD_{50} of *Microcystis* extracts in mice was 50- to 170-fold higher than the i.p. LD_{50} of the same extracts (Falconer, 1991; Kotak *et al.,* 1993).

The i.p. LD_{50}s of several of the commonly occurring microcystins (microcystin-LA, -YR, and -YM) are similar to that of microcystin-LR, but the i.p. LD_{50} for microcystin-RR is about tenfold higher (see Table 3.1).

However, because of differences in lipophilicity and polarity between the different microcystins, it cannot be presumed that the i.p. LD_{50} will predict toxicity after oral administration.

The microcystins are primarily hepatotoxins. After acute exposure by i.v. or i.p. injection of microcystin, severe liver damage is characterised by a disruption of liver cell structure (due to damage to the cytoskeleton), a loss of sinusoidal structure, increases in liver weight due to intrahepatic haemorrhage, haemodynamic shock, heart failure and death. Other organs affected are the kidneys and lungs (Hooser *et al.,* 1990) and the intestines (Falconer, 1994; Falconer and Humpage, 1996).

In a recent study, a single oral dose of microcystin-LR at 500 µg kg^{-1} bw was given to 5 and 32 week old mice, and liver pathology was examined 2-19 hours later. In 62 per cent of the older mice, there was clear microscopic evidence of liver injury. Furthermore, microcystin-LR and a metabolite were detected in the livers. On the contrary, none of the young mice developed liver injury. In untreated control mice, an examination of gastric mucosa and small intestine revealed age-related disruption of surface cell structure. The authors suggested that this disruption may markedly influence gastro-intestinal absorption of microcystin-LR and hence explain the observed age-dependent liver toxicity in exposed mice (Ito *et al.,* 1997a).

Intranasal installation of microcystin-LR in mice resulted in extensive necrosis of the epithelium of the nasal mucosa of both the olfactory and respiratory zones, progressing to destruction of large areas of tissue down to levels of deep blood vessels (Fitzgeorge *et al.,* 1994). The LD_{50} by this route of administration was the same as the i.p. LD_{50}, and dose-dependent liver lesions were observed. The same authors also demonstrated cumulative liver damage after repeated dosing. While a single dose of 31.3 µg kg^{-1} bw did not result in an increase in liver weight, repeated daily administration over a period of seven days caused a 75 per cent increase in liver weight, which was almost as high as the effect of a single intranasal dose of 500 µg kg^{-1} bw.

Repeated oral administration

Pure microcystin-LR. For assessing possible chronic human health effects, studies involving repeated oral administration of pure microcystins at various dose levels are most desirable. In a 13-week gavage study in mice, conducted under good laboratory practice (GLP), pure microcystin-LR was administered orally to groups of 15 male and female mice at 0, 40, 200, or 1,000 µg kg^{-1} bw per day for 13 weeks (Fawell *et al.,* 1994). The NOAEL was 40 µg kg^{-1} bw per day. At the next highest dose level there were slight liver tissue changes in some male and female mice. At the highest dose level, all male

and most female mice showed liver changes, including chronic inflammation, focal degeneration of hepatocytes and haemosiderin deposits. In male mice at the two highest dose levels, serum transaminases were significantly elevated, serum gamma glutamyl transferase was significantly reduced, and there were small but significant reductions in total serum protein and serum albumin. In female mice, at the highest dose level, only the changes in transaminases were observed (Fawell *et al.,* 1994).

Microcystis extract. In an oral dosing study, *Microcystis aeruginosa* extract was supplied to mice at five concentrations (equivalent to 750 to 12,000 µg kg^{-1} bw per day of microcystin-YM) in their drinking water for up to one year. At the higher concentrations there was increased death, increased bronchopneumonia (which was endemic in the colony), and chronic liver injury. There was no evidence of tumourigenic changes in the liver, despite the liver injury caused by chronic oral exposure to microcystins. No clear NOAEL was established (Falconer *et al.,* 1988).

In another study in pigs, *Microcystis aeruginosa* extract was given to groups of five pigs in their drinking water for 44 days at dose levels calculated from potency estimates using the mouse i.p. bioassay to be equivalent to 280, 800 or 1,310 µg kg^{-1} bw per day of microcystins (assuming an average i.p. LD_{50} for microcystins of 100 µg kg^{-1} bw). The extract contained at least seven microcystin variants, with microcystin-YR tentatively identified by high pressure liquid chromatography (HPLC) as the major constituent. A LOAEL of 280 µg kg^{-1} bw per day of toxins was identified, with general liver injury (evident from histopathology and changes in serum enzymes) observed at the two higher dose levels. At the lowest dose level, one pig was affected. The authors determined the potency of their extract by the mouse i.p. LD_{50} bioassay, by HPLC analysis and by the *in vitro* phosphatase inhibition assay (Falconer *et al.,* 1994). Summation of the peak areas from the HPLC identification of microcystin variants, standardised against microcystin-LR (see Chapter 5), indicated that the LOAEL equated with 100 µg microcystin-LR equivalents per kg bw per day.

Developmental effects

In an investigation on the effects of microcystin-LR on embryonic and foetal development of the mouse, groups of 26 time-mated female mice were dosed once daily by gavage with aqueous solutions of pure microcystin-LR from days 6 to 15 of pregnancy, at dose levels of 0, 200, 600, or 2,000 µg kg^{-1} bw per day. Maternal clinical signs, body weights and food consumption were recorded. Only treatment at 2,000 µg kg^{-1} bw per day was associated with maternal toxicity and mortality. Seven of the 26 females died and two were sacrificed because of distress during the dosing period. On day 18 of pregnancy the remaining females were killed. At the highest dose level, there was a retardation of foetal weight and skeletal ossification, but no foetal deaths. Apart from this there was no effect of microcystin-LR on sex ratio, implantation, post-implantation loss, or on external or visceral or skeletal abnormalities. The NOAEL for any aspect of developmental toxicity was 600 µg kg^{-1} bw per day (Fawell *et al.,* 1994).

In a scoping study, eight 20-week old male and female mice which had received an extract of *Microcystis aeruginosa* in their drinking water since weaning were mated; extract exposure continued throughout pregnancy. Examination of litters showed no effects on weight, number or sex ratio of offspring, compared with control litters. However, there was some evidence of hippocampal injury and reduced brain size in 7

out of 73 of the five-day old young from parents who had received the *Microcystis* extract, and in none of the controls (Falconer *et al.,* 1988).

Carcinogenicity

Microcystin administered alone. In a recent study, microcystin-LR, administered i.p. to mice, induced neoplastic liver nodules. Animals were given the toxin at 20 µg kg^{-1} bw, 100 times over 28 weeks. At autopsy, nodules up to 5 mm in diameter were observed in the livers of all exposed mice. Some mice were kept a further two months after cessation of dosing, and autopsy showed that liver nodules persisted (Ito *et al.,* 1997b). In the same study, mice orally administered microcystin-LR, at a dose level of 80 µg kg^{-1} bw, 100 times over the same time period showed no evidence of liver injury or nodule formation. Previous work had shown that microcystin-LR given 20 times i.p. to mice, at 25 µg kg^{-1} bw, over 10 weeks did not induce liver nodules (Ohta *et al.,* 1994).

Microcystin interaction with tumour initiators. It is generally understood that some chemicals (usually DNA-damaging) can initiate the cancer process while other classes of chemicals are able to promote the appearance of cancer after initiation has occurred (Boutwell, 1974; Yamasaki, 1988; Fitzgerald and Yamasaki, 1990). Microcystins have been tested for tumour promoting activity.

In vivo animal experiments. There has been some evidence of tumour promotion in animal studies (see also section on mechanism of action). In a modified, two-stage carcinogenesis mouse skin bioassay, a single dose of 7,12-dimethyl benzanthracene (DMBA, 500 µg) was applied to the dorsal skin of groups of 20, three-month old, female mice. After one week, groups of treated and control mice received either water alone, water with *Microcystis* extract (80 mg microcystin-YM per litre; equivalent to 50 mg microcystin-LR per litre), or croton oil (as a tumour-promoting phorbol ester-containing positive control) applied to the skin (0.5 per cent in 0.1 ml acetone twice a week). At 52 days after DMBA exposure, there was a 1.6-fold increase in the number and a sevenfold increase in the mean weight of skin papillomas (a type of benign tumour) per mouse in animals given the cyanobacterial extract compared with those given water alone. It was concluded by the authors that oral consumption of *Microcystis* extract from drinking water may act directly or indirectly as a tumour promoter (Falconer, 1991). The mechanism of action is not clear because microcystin-LR, at least, has difficulty penetrating epidermal cells (Matsushima *et al.,* 1990). In this study, there was considerable liver damage, which could affect the interpretation of these findings (Falconer, 1991). In a short-term two-stage carcinogenicity bioassay, groups of 10-19 seven-week old male F344 rats were initiated by i.p. injection with 200 mg kg^{-1} bw diethylnitrosamine (DEN), followed by partial hepatectomy (performed to stimulate cell division and thus increase the sensitivity of the assay). In two separate experiments, twice-weekly i.p. doses of 1 and 10 µg kg^{-1} microcystin-LR and of 10,25 and 50 µg kg^{-1} microcystin-LR were then administered during eight weeks and this resulted in a dose-dependent increase in the number (up to threefold) and area (up to 11-fold) of GST-P-positive liver foci (GST-P is the placental form of glutathione-S transferase, a biomarker for preneoplastic changes in liver (Sato *et al.,* 1984)). The i.p. NOAEL in this study was 1 µg kg^{-1} (Nishiwaki-Matsushima *et al.,* 1992). Microcystin-LR given without DEN initiation showed no induction of liver foci. Macroscopic nodules, histologically diagnosed as neoplastic nodules, were seen in this study when DEN was followed by microcystin-LR

at 10 µg kg^{-1} (before) and 50 µg kg^{-1} (after) partial hepatectomy (Nishiwaki-Matsushima *et al.,* 1992).

In another tumour initiation and promotion assay aimed at evaluating possible tumour promoting effects in the upper small intestine, two doses of 40 mg kg^{-1} bw of the initiator N-methyl-N-nitrosourea (MNU) were orally administered to C57 black mice, one week apart, followed by drinking water containing various levels of *Microcystis* extracts, estimated to be equivalent to 0, 1.2 or 4.2 mg kg^{-1} bw per day of microcystins for up to 22 weeks. Time to 50 per cent survival was about 17 weeks in controls and 15 weeks in extract-exposed mice. No primary liver tumours were seen in any group and there was no evidence of microcystin-induced promotion of lymphoid or duodenal tumours (Falconer and Humpage, 1996).

In vitro studies on interaction of microcystins and tumour initiators are discussed in the section on *in vitro* studies below.

Genotoxicity

No mutagenic response has been observed for purified toxins (exact nature not determined) derived from *Microcystis* in the Ames *Salmonella* assay (strains TA98, TA100 and TA102) with or without S9 activation. The *Bacillus subtilis* multigene sporulation test was also negative with regard to mutagenicity using both the 168 and hcr-9 strains (Repavich *et al.,* 1990). An earlier study with purified *Microcystis* extract also elicited a negative response in the Ames test (Runnegar and Falconer, 1982).

The purified toxins from a *Microcystis* species tested against human lymphocytes suggested that the toxins may be clastogenic as indicated by dose-related increases in chromosomal breakage (Repavich *et al.,* 1990).

In vitro studies

Extracts from *Microcystis aeruginosa* blooms containing mainly microcystins (no analytical data presented) from a lake in China were tested in a two-stage cell-transformation assay using Syrian hamster embryo (SHE) cells. In this assay, the microcystin extract had no initiating activity when followed by the tumour promoter TPA (12-*O*-tetradecanoyl phorbol 13-acetate). With methylcholanthrene as the initiator (0.5 µg ml^{-1}), followed by bloom extract, a dose-related (up to sevenfold) increase in transformation frequency was observed (Wang and Zhu, 1996).

Primary hepatocyte cultures in the presence of picomolar and nanomolar concentrations of microcystin-LR showed selective cell toxicity and selective cell proliferation depending on the ploidy (chromosome copy number) of the cells (Humpage and Falconer, 1999).

Mechanism of action

Microcystin-LR was found to be a potent inhibitor of eukaryotic protein serine/threonine phosphatases 1 and 2A both *in vitro* (Honkanen *et al.,* 1990; MacKintosh *et al.,* 1990) and *in vivo* (Runnegar *et al.,* 1993), and this effect has become the basis of one of the bioassays to detect its presence. Substances that inhibit these protein phosphatases are considered to be non-phorbol ester (TPA)-type tumour promoters. Other substances that

act in a similar way to microcystins are okadaic acid, nodularin, tautomycin and calyculin (for a review see Fujiki and Suganuma, 1993). The protein phosphatases serve an important regulatory role to maintain homeostasis in the cell (Cohen, 1989). Protein phosphatase inhibition results in a shift in the balance towards higher phosphorylation of target proteins, such as tumour suppresser proteins. This is a major post-translational modification which can result in excessive signalling and may lead towards cell proliferation, cell transformation and tumour promotion (Fujiki and Suganuma, 1993). In liver cells, intermediate filaments of the cytoskeleton are hyperphosphorylated leading to cellular disruption (Falconer and Yeung, 1992). In monolayer cell cultures this leads to detachment from adjacent cells, and involves actin filament contraction (Hooser *et al.,* 1991, Ghosh *et al.,* 1995). The inhibition of protein phosphatase 2A by microcystin-LR can be effectively reversed in the presence of polyclonal antibodies against microcystin-LR (Lin and Chu, 1994b); such antibodies can also protect *in vivo* against microcystin-LR toxicity as shown with i.p. co-administration studies in mice (Nagata *et al.,* 1995).

The implications of protein phosphatase inhibition in humans, due to low level chronic exposure to microcystins, are not known.

Additional study requirements

Further short-term studies are needed to understand better the genotoxic and tumour promoting potential of microcystins. Such studies would also be useful as a preliminary to the design of appropriate chronic or other *in vivo* studies to assess their carcinogenic potential. Regarding the possible role of microcystins in tumour promotion, further studies are especially needed to establish a dose-effect relationship for nodule induction with microcystin alone using various routes of administration.

Derivation of a tolerable daily intake (TDI)

Most of the relevant data on microcystin toxicity have come from either reported human injury related to consumption of drinking water containing cyanobacteria (see section 4.2) or from limited work with experimental animals (see above). Although the cyanotoxins have not been reviewed by the International Agency for Research on Cancer (IARC), their standard evaluation procedures (IARC, 1995) lead to the conclusion that, at present, the human evidence for microcystin carcinogenicity is inadequate and the animal evidence is limited. In such instances, the current practice for deriving a TDI is to use a LOAEL or NOAEL divided by appropriate safety or uncertainty factors as described in the Addendum to the *Guidelines for Drinking Water Quality* (WHO, 1998).

A 13-week mouse oral (by gavage) study with pure microcystin-LR has been considered the most suitable for the derivation of a guideline value for microcystin-LR. In a study by Fawell *et al.* (1994) a NOAEL of 40 µg kg^{-1} bw per day was determined, based on liver histopathology and serum enzyme level changes. By applying a total uncertainty factor of 1,000 (10 for intra-species variability, 10 for inter-species variability and 10 for limitations in the database, in particular a lack of data on chronic toxicity and carcinogenicity), a provisional TDI of 0.04 µg kg^{-1} bw per day has been determined for microcystin-LR. This TDI is supported by the results of a 44-day pig study, in which pigs were given *Microcystis* extract in their drinking water, resulting in a LOAEL of 100 µg microcystin-LR equivalents per kg bw per day (Falconer *et al.,* 1994; see above). To this LOAEL an overall uncertainty factor of 1,500 was applied, arrived at by using 10 for

intra-species variability, 3 rather than 10 for inter-species variability (because pigs physiologically resemble humans more closely than rodents), 5 for extrapolating from a LOAEL to a NOAEL (10 was considered inappropriate due to the low incidence of effects in the lowest dose group and the deduced shape of the dose-response curve) and 10 for the less-than-lifetime exposure. This resulted in a provisional TDI of 0.067 µg kg^{-1} bw per day. The lower of these two values, 0.04 µg kg^{-1} bw per day, has been used in deriving a provisional guideline value (see Chapter 5).

4.2.2 Nodularin

Compared with *Microcystis* and microcystins, there have been fewer reported incidents of human and livestock disease involving *Nodularia* spp. and nodularin (see section 4.1).

Experimental animal data

The toxicity and liver pathology induced by nodularin is similar to that caused by microcystins, with hepatocyte necrosis and haemorrhagic diathesis (Runnegar *et al.*, 1988). Nodularin inhibits protein phosphatases 1 and 2A with the same potency as microcystin-LR (Yoshizawa *et al.,* 1990). In a two-stage liver carcinogenesis experiment in male F344 rats initiated with DEN and without partial hepatectomy, repeated i.p. administration of 10 µg nodularin per kg bw induced GST-P-positive foci more effectively than microcystin-LR. Nodularin alone also induced some foci. In addition, nodularin was capable of activating several proto-oncogenes of the *fos* and *jun* family, which are considered to play a role in tumour promotion (Ohta *et al.,* 1994).

Derivation of a tolerable daily intake

There are no studies available that use oral administration of nodularin, thus a TDI cannot be set. Nevertheless, several experimental studies cited above indicate that nodularin has similar toxicity to microcystin-LR. It may be appropriate, therefore, to consider nodularin in an analogous fashion to microcystins in terms of human health risk assessment.

4.2.3 Anatoxin-a

The potent neurotoxin, anatoxin-a, from *Anabaena flos-aquae* has frequently been involved in animal and wildfowl poisoning (Ressom *et al.,* 1994) (Table 4.1).

Experimental studies

Anatoxin-a is a nicotinic (cholinergic) agonist that binds to neuronal nicotinic acetylcholine receptors. It has been suggested that the activation of presynaptic nicotinic acetylcholine receptors by anatoxin-a results in an influx of Na^{+}, producing sufficient local depolarisation to open voltage sensitive Ca^{++} and Na^{+} channels. The latter may then amplify the response, activating further Ca^{++} channels (Soliakov *et al.,* 1995). As a result of this depolarisation there is a block of further electrical transmission, and at sufficiently high doses this can lead to paralysis, asphyxiation and death (Carmichael *et al.,* 1975; Carmichael, 1997). Anatoxin-a is more potent than nicotine or acetylcholine in evoking type 1A or type 2 current responses in rat hippocampal neurones (Alkondon and Albuquerque, 1995), and it is more potent than nicotine in its ability to evoke the

secretion of endogenous catecholamines from bovine adrenal chromaffin cells through their neuronal-type nicotinic receptors (Molloy *et al.,* 1995). Similar to nicotine, anatoxin-a was more potent than noradrenaline in releasing dopamine from striatal nerve terminals from rat superfused hippocampal synaptosomes (Clarke and Reuben, 1996). *In vivo* studies in the rat showed that the toxin stimulates the sympathetic system through the release of catecholamines from nerve endings (Dube *et al.,* 1996).

Acute toxicity

In mice, the i.p. LD_{10} (lowest dose causing death) of anatoxin-a was 250 µg kg^{-1} bw (Stevens and Krieger, 1989) and the i.p. LD_{50} of anatoxin-a is 375 µg kg^{-1} bw (Fitzgeorge *et al.,* 1994). The i.v. LD_{50} is less than 100 µg kg^{-1} bw (Fawell and James, 1994). The oral LD_{50} for anatoxin-a is greater than 5,000 µg kg^{-1} bw, and the intranasal LD_{50} in mice is 2,000 µg kg^{-1} bw (Fitzgeorge *et al.,* 1994).

Subacute toxicity

In two studies, anatoxin-a was administered to rats orally in the drinking water for 54 days at 0.51 or 5.1 ppm (equivalent to 51 and 510 µg kg^{-1} bw per day, respectively) or by i.p. injection at 16 µg per rat per day for 21 days (equivalent to 80 µg kg^{-1} bw per day; Astrachan *et al.,* 1980). No toxicity was observed (no deaths due to treatment; no significant changes in body weight gain, haematology or clinical chemistry).

In a dose range-finding study for a four-week oral toxicity experiment, groups of 2 male and 2 female mice were administered anatoxin-a hydro-chloride by gavage at dose levels of 1,500, 7,500 or 15,000 µg kg^{-1} per day for five days. All mice at the highest dose, and one female at the intermediate dose, died within three minutes of their first dose. Males at the intermediate dose were hyperactive after their third dose. All other animals survived, and no treatment-related signs of clinical toxicity, or changes in body weight were observed. No treatment-related changes were observed at necropsy (Fawell and James, 1994).

In a 4-week study, groups of 10 male and female mice were administered anatoxin-a hydrochloride by gavage at dose levels of 0, 120, 600 or 3,000 µg kg^{-1} bw. In the course of the experiment, one male receiving 600 µg kg^{-1} bw per day and one female receiving 3,000 µg kg^{-1} per day died. No signs of clinical toxicity or histopathological abnormalities were observed in these two animals, and no cause of death could be identified. It was concluded by the authors that the possibility that these deaths were treatment-related could not be excluded, although this was considered to be unlikely. There were no other treatment-related findings. Although the NOAEL for anatoxin-a hydro-chloride in this study was determined to be 120 µg kg^{-1} bw per day, equivalent to 100 µg kg^{-1} bw per day of anatoxin-a, the toxicological database was considered to be inadequate to use these results for setting a formal TDI.

Reproductive effects

Doses of 200 or 125 µg kg^{-1} bw anatoxin-a were given i.p. to hamsters one or three times per day, respectively, at days 12 to 14 of pregnancy (after organo-genesis), and the dams were sacrificed at day 15. The treatment given three times per day caused foetal malformation (hydrocephaly) in all foetuses in one of 10 litters, and stunted growth in

almost all litters; treatment given once per day resulted in stunted growth. No maternal toxicity was observed (Astrachan *et al*., 1980).

Groups of 10 and 12 time-mated female mice were given anatoxin-a hydrochloride by gavage at 0 or 3,000 µg kg^{-1} bw per day (equivalent to 2,460 µg kg^{-1} bw per day anatoxin-a) respectively, on days 6 to 15 of pregnancy. The anatoxin-a dose was considered the maximum dose that could be tolerated. No treatment-related effects were observed in the dams or offspring, although there was a slight decrease in foetal weight compared with controls (Fawell and James, 1994). No data on the genotoxic potential of anatoxin-a are available.

Derivation of a tolerable daily intake

Anatoxin-a is a potent acute neurotoxin. The available data indicate that significant concern with regard to chronic toxicity (Fawell and James, 1994) is unlikely, but the data base is insufficient for derivation of a TDI.

4.2.4 Homoanatoxin-a

Homoanatoxin-a is a potent neuromuscular blocking agent with an i.p. LD_{50} in mice of 250 µg kg^{-1} bw. Toxicosis in the lethal dose range leads to severe body paralysis, convulsions and death by respiratory arrest in 7-12 minutes. Experiments with rat phrenic nerve hemidiaphragm preparations demonstrated that the physiological effects of homoanatoxin-a are related to those observed for *d*-tubocurarine (Skulberg *et al.* 1992). Recent studies have shown that homoanatoxin-a enhances the influx of Ca^{++} ions in the cholinergic nerve terminals (Aas *et al.,* 1996). No TDI can be derived.

4.2.5 Anatoxin-a(S)

Anatoxin-a(S) is an organophosphate produced by the cyanobacteria *Anabaena flos-aquae* and *A. lemmermannii* (see Chapter 3). This toxin blocks acetylcholinesterase activity in a manner analogous to organophosphate insecticides. The i.p. LD_{50} in mice is 20 µg kg^{-1} bw with muscle weakness, respiratory distress (dyspnea) and convulsions (effect on seizure threshold) preceding death (Mahmood and Carmichael, 1986a; Matsunaga *et al.* 1989). There are no oral toxicity studies for this toxin.

Derivation of a tolerable daily intake

A lack of adequate experimental data or human data precludes the derivation of a TDI.

4.2.6 Saxitoxins

Effects in humans

Saxitoxin and some of its analogues are produced by *Anabaena circinalis* in Australian freshwaters and *Aphanizomenon flos-aquae* in the USA (Sawyer *et al.,* 1968; Mahmood and Carmichael, 1986b; Humpage *et al.,* 1994). The saxitoxin group has been the cause of paralytic shellfish poisoning (PSP) in people. Several species of dinoflagellates produce PSP toxins that accumulate in molluscs which filter-feed on these organisms.

People who have consumed shellfish containing high levels of PSP toxins may suffer from this acute illness. The signs and symptoms of PSP in humans may range from a slight tingling and numbness about the lips to complete paralysis and death from respiratory failure (Meyer *et al.,* 1928; Medcof *et al.,* 1947; McFarren *et al.,* 1958). More than 1,000 cases of PSP have been reported since the early 1900s in North and Central America and there have been 109 deaths. In a recent epidemic in Guatemala, the mortality rate in children was 50 per cent, while for adults it was 5 per cent (Rodrigue *et al.* 1990; for a review see Kao, 1993). No PSP-like illnesses have been reported in humans from the consumption of drinking water containing saxitoxins, although there have been fatalities in sheep after ingesting a PSP toxin-containing bloom of *A. circinalis* (Negri *et al.,* 1995).

Experimental studies

Mode of action. Of the various PSP toxins, only saxitoxin has been studied in detail for pharmacological effects, partly because the other toxins are usually not available in sufficient quantities for such studies. Nearly all the systemic actions of saxitoxin can be explained by its pharmacological effect on nerve axon membranes. This involves a wide spread blockage of sodium ion channels of the excitable membranes of nerves, thereby affecting (partially or completely, depending on dose) impulse generation in peripheral nerves and skeletal muscles (Catterall, 1980). This results in generalised nerve dysfunction as measured by electromyography (Easthaugh and Shepherd, 1989). In mammals, these effects lead to paralysis, respiratory depression and respiratory failure. Direct cardiac effects are usually minimal (see review by Kao, 1993).

Metabolism. No data are available on PSP toxin absorption, distribution, metabolism and excretion.

Table 4.2 LD_{50} values following a single dose of PSP toxin extract in the mouse in relation to the route of administration

Route of administration	**LD_{50}[1] (µg PSP kg^{-1} body weight)**	
	Male	**Female**
Intravenous	3.4 (3.2-3.6)	
Intraperitoneal	10.0 (9.7-10.5)	8.0 (7.6-8.6)
Oral	263.0 (251-267)	

PSP Paralytic shellfish poison

Source: IPCS, 1984; adapted from Wiberg and Stephenson, 1960

[1] The 95% confidence limits are given in parentheses

Acute toxicity

The principle of the standardised mouse bioassay developed by Sommer and Meyer (1937) is measurement of time to death after i.p. injection. In that study, the authors suggested that signs characteristic of PSP, such as dyspnea, could be observed after the i.p. administration of toxin. Hypotensive effects have been observed to accompany

the respiratory depression, implicating both central and peripheral nervous system actions (Watts *et al.,* 1966).

Acute toxicity studies have been conducted in several species with extracts containing PSP toxins obtained from the Alaskan butter clam (Genenah and Shimizu, 1981). Using this preparation, Wiberg and Stephenson (1960) determined the LD_{50} values for male mice, using three routes of administration (i.v., i.p. and oral). In addition, the i.p. LD_{50} was determined in female mice (Table 4.2). The PSP toxin extract was much less toxic when administered by the oral route than by the i.v. or i.p. routes (Table 4.2). Increasing the pH of the injection medium (> 3.8) or the addition of sodium ions affected i.p. toxicity, while the addition of sodium ions did not influence oral or i.v. toxicity. Similar comparative LD_{50}s for different routes of administration were obtained in rats; in addition new-born rats were about tenfold more susceptible than adults after oral administration (Watts *et al.,* 1966) (Table 4.3). For a number of animal species tested, the oral LD_{50} for PSP toxin ranged from 128 µg kg^{-1} bw PSP toxin in guinea pigs to 420 µg kg^{-1} bw in mice (IPCS, 1984).

Table 4.3 LD_{50} values following oral or intraperitoneal administration of a single dose of PSP toxin extract to rats of different ages

Age	LD_{50}[1] (µg PSP kg^{-1} body weight)	
	Oral	Intraperitoneal
New-born (24 hours)	64 (51-80)	5.5 (4.7-6.5)
Weanling (21 days)	270 (204-356)	8.3 (7.7-9.0)
Adult (60-70 days)	531 (490-576)	10.0 (8.5-11.8)

[1] The 95% confidence limits are given in parentheses

Source: IPCS, 1984; adapted from Watts *et al.,* 1966

Prior exposure to non-lethal doses of PSP toxin seems to lower the susceptibility of rats to lethal doses of PSP toxin. In a study using Sprague-Dawley rats (sex not indicated), the oral LD_{50} value for the purified PSP extract was determined (McFarren *et al.,* 1958). One group of rats was given a non-lethal dose of PSP toxin (about one-third of the LD_{50}) 14 days before the test. The LD_{50} for the pre-treated rats was about 50 per cent higher than that for untreated rats. This finding corroborates the fact noted by Prakash *et al.* (1971) that fishermen who habitually eat shellfish containing low levels of PSP toxins appear to be less susceptible to developing PSP.

There is a wide range in i.p. toxicity of the various PSP toxins (i.e. saxitoxin, neosaxitoxin, the gonyautoxins and C toxins) as tested following the Association of Official Analytical Chemists (AOAC) mouse bioassay (AOAC, 1984). Potencies of these toxins are usually expressed in mouse units (MU) per µmol of toxin. Saxitoxin is one of the most toxic of the PSP toxins (2,483 MU per µmol) and the C toxins are the least toxic (15-143 MU per µmol) (Oshima, 1995). Potencies may also be expressed as saxitoxin equivalents. Inter-conversions during storage, cooking or digestion may modify the i.p. and oral toxicity (Humpage *et al.,* 1994; see also section 3.4.2). No subchronic or chronic animal studies with PSP toxins are available.

Derivation of a tolerable daily intake
The animal toxicity data for the saxitoxins are inadequate to set a TDI.

4.2.7 Cylindrospermopsin

This cyanotoxin was initially isolated from a culture of *Cylindrospermopsis raciborskii* obtained from a water supply reservoir in tropical northern Australia. The organism was identified as a result of an outbreak of acute hepato-enteritis and renal damage among an Aboriginal population on Palm Island, off the coast of North Queensland (Hawkins *et al.,* 1985) (see Box 4.2). Intraperitoneal injection of the lysed organism to mice resulted in widespread and progressive tissue injury, with cell necrosis in the liver, kidneys, adrenals, lung, heart, spleen and thymus (Hawkins *et al.,* 1985, 1997). In mice, the i.p. LD_{50} at 24 hours was 52 mg dry weight (dw) of cells per kg bw, equivalent to 300 µg kg^{-1} bw of cylindrospermopsin, whereas the i.p. LD_{50} at seven days was approximately 32 mg cells per kg bw, equivalent to 180 µg kg^{-1} bw of toxin (Hawkins *et al.,* 1997). Administration of the pure toxin to mice showed this delayed toxicity more clearly, with the 24-hour i.p. LD_{50} being 2,100 µg kg^{-1} bw and the 5-6 day i.p. LD_{50} being 200 µg kg^{-1} bw (Ohtani *et al.,* 1992). *In vitro* studies with pure cylindrospermopsin have shown that it inhibits glutathione synthesis and protein synthesis in general (Runnegar *et al.,* 1994, 1995; Terao *et al.,* 1994). In mouse liver after i.p. administration, major changes were seen in hepatocytes, with progressive proliferation of the smooth endoplasmic reticulum and accumulation of lipid over five days (Terao *et al.,* 1994). No data on the oral toxicity of pure cylindrospermopsin are available, but studies with aqueous extracts of *Cylindrospermopsis* provide a preliminary indication. After administering to mice a single oral dose of an aqueous extract of freeze-dried *Cylindrospermopsis* cells, a median lethal dose in the range of 4.4 to 6.9 mg kg^{-1} toxin equivalent was determined (Seawright *et al.,* 1999). Because the i.p. LD_{50} is 0.2 mg kg^{-1} and the LD_{100} is likely to be double that, the oral toxicity appears to be over tenfold lower than i.p. toxicity.

At present it is not known if cylindrospermopsin is the only toxin in *Cylindrospermopsis,* because the major kidney damage seen on i.p. dosing of some toxic extracts is not similarly observed after administration of pure toxin.

Derivation of a tolerable daily intake
On the basis of present data it is not possible to derive a TDI.

4.2.8 Other cyanotoxins produced by marine cyanobacteria

Swimmers off Hawaii and Okinawa who have come into contact with the marine cyanobacterium *Lyngbya majuscula* have contracted acute dermatitis, causing "swimmers' itch" (Moikeha and Chu, 1971; Hashimoto *et al.,* 1976). The effect is a cutaneous inflammation with signs of erythema, blisters and desquamation within 12 hours of exposure to the cyanobacterium. The inflammatory activity of *Lyngbya* is caused by aplysiatoxins and debromoaplysiatoxin (Mynderse *et al.,* 1977). These toxins are potent animal skin tumour promoters and protein kinase C activators (Fujiki *et al.,* 1990). Aplysiatoxins are toxic to mice at a minimum lethal dose of about 0.3 mg kg^{-1} (Moore, 1977). Debromoaplysiatoxin, along with other toxic compounds, has also been isolated from other Oscillatoriaceae such as *Schizothrix calcicola* and *Oscillatoria nigroviridis.* The chemically different lyngbyatoxin-a (Cardellina *et al.,* 1979), found in another shallow water strain of marine Lyngbya *majuscula, has* caused dermatitis and

severe oral and gastrointestinal inflammation (Moore et al., *1993).* Its toxicity to mouse corresponds to aplysiatoxins (Moore, 1977) and it has skin tumour promoting activity similar to the well known tumour promoter, TPA (Fujiki *et al.,* 1981, 1984). *Lyngbya majuscula* sometimes grows epiphytically on edible algae, such as *Acanthophora spicifera* which is eaten in Indonesia and in the Philippines. Cyanobacteria have also been suspected to be a source of toxins in the ciguatera food chain that may lead to human poisoning (Hahn and Capra, 1992; Endean *et al*., 1993).

4.2.9 Cyanobacterial lipopolysaccharides

Lipopolysaccharides, cell wall components of gram-negative bacteria, are pyrogenic (fever-causing agents) and toxic (Weckesser and Drews, 1979). In Sewickley, Pennsylvania, an outbreak of gastro-enteritis is suspected to have been caused by cyanobacterial LPS (Lippy and Erb, 1976; Keleti *et al.,* 1979). The few studies carried out on cyanobacterial LPS indicate that they are less toxic than the LPS of other bacteria such as *Salmonella* (Keleti and Sykora, 1982; Raziuddin *et al.,* 1983). Lack of axenic cyanobacterial strains has previously hindered detailed studies on structures and toxicities of cyanobacterial LPS.

4.2.10 Other bioactive compounds in cyanobacteria

Cyanobacteria produce a wide variety of bioactive compounds in addition to the cyanotoxins described in this chapter. They include anti-tumour (cytotoxic), anti-viral, and anti-fungal compounds, antibiotics and protease inhibitors (Moore *et al.,* 1996; Namikoshi and Rinehart, 1996). Further screening of these biomedically interesting compounds is underway and is likely to lead to the discovery of many new compounds in the future, some of which may be toxic. Bioassays of cyanobacterial cell extracts have often revealed a higher toxicity than expected from the content of known toxins in the extract.

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