CYANOBACTERIAL PEPTIDE TOXINS

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1. Exposure data

1.1 Introduction

Cyanobacteria, also known as blue-green algae, are widely distributed in fresh, brackish and marine environments, in soil and on moist surfaces. They are an ancient group of prokaryotic organisms that are found all over the world in environments as diverse as Antarctic soils and volcanic hot springs, often where no other vegetation can exist (Knoll, 2008). Cyanobacteria are considered to be the organisms responsible for the early accumulation of oxygen in the earth's atmosphere (Knoll, 2008). The name 'blue-green' algae derives from the fact that these organisms contain a specific pigment, phycocyanin, which gives many species a slightly blue-green appearance.

Cyanobacterial metabolites can be lethally toxic to wildlife, domestic livestock and even humans. Cyanotoxins fall into three broad groups of chemical structure: cyclic peptides, alkaloids and lipopolysaccharides. Table 1.1 gives an overview of the specific toxic substances within these broad groups that are produced by different genera of cyanobacteria together, with their primary target organs in mammals. However, not all cyanobacterial blooms are toxic and neither are all strains within one species. Toxic and non-toxic strains show no predictable difference in appearance and, therefore, physicochemical, biochemical and biological methods are essential for the detection of cyanobacterial toxins.

The most frequently reported cyanobacterial toxins are cyclic heptapeptide toxins known as microcystins which can be isolated from several species of the freshwater genera *Microcystis*, *Planktothrix* (*Oscillatoria*), *Anabaena* and *Nostoc*. More than 70 structural variants of microcystins are known. A structurally very similar class of cyanobacterial toxins is nodularins (< 10 structural variants), which are cyclic pentapeptide hepatotoxins that are found in the brackish-water cyanobacterium *Nodularia*.

Table 1.1. General features of the cyanotoxins

Toxin group[a]	Primary target organ in mammals	Cyanobacterial genera[b]
Cyclic peptides		
Microcystins	Liver	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis*
Nodularin	Liver	*Nodularia*
Alkaloids		
Anatoxin-a	Nerve synapse	*Anabaena, Planktothrix (Oscillatoria), Aphanizomenon*
Aplysiatoxins	Skin	*Lyngbya, Schizothrix, Planktothrix (Oscillatoria)*
Cylindrospermopsins	Liver[c]	*Cylindrospermopsis, Aphanizomenon, Umekazia*
Lyngbyatoxin-a	Skin, gastrointestinal tract	*Lyngbya*
Saxitoxins	Nerve axons	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*
Lipopolysaccharides	Potential irritant; affects any exposed tissue	All

From Sivonen & Jones 1999

[a] Many structural variants may be known for each toxin group.

[b] Not all species of the particular genus produce toxins.

[c] Whole cells of toxic species elicit widespread tissue damage, including damage to kidney and lymphoid tissue.

1.2 Chemical and physical properties

Cyclic peptides are comparatively large natural products that have a molecular weight in the range of 800–1100 but are relatively small compared with many other cell oligopeptides and polypeptides (proteins) (molecular weight, > 10 000). Nodularins and microcystins contain either five (nodularins) or seven (microcystins) amino acids; the two terminal amino acids of the linear peptide are condensed (joined) to form a cyclic compound.

The common structure of microcystins is cyclo(D-alanyl–L-*X*–D-erythro-β-methylaspartyl(iso-linkage)-L-*Z*-ADDA–D-glutamyl(iso-linkage)-*N*-methyldehydroalanyl) where ADDA stands for the β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid, which is unique to microcystins and nodularins. The

main structural variation in microcystins is observed in the L-amino acid residues 2 (*X*) and 4 (*Z*), which are indicated by a two-letter suffix; for example, the common microcystin-LR contains leucine (L) in position 2 and arginine (R) in position 4 (Carmichael *et al*., 1988a; Falconer, 2005).

The ADDA side chain is a key structural element that is necessary for biological activity. Separation of the ADDA component from the cyclic peptide renders both components non-toxic (Carmichael, 1992). L Amino acids vary among toxins and a large number of combinations can be formed. To date, more than 70 microcystins have been discovered including non-toxic geometric isomers of microcystins-LR and -RR (Sivonen *et al*., 1992; Sivonen & Jones, 1999; Codd *et al*., 2005). Microcystins are stable at high temperatures for extended periods and are not denatured by boiling. They are non-volatile, resistant to changes in pH and are soluble in water, ethanol and acetone.

The common structure of nodularins is cyclo(D-methylaspartyl^{1}-L-arginine^{2}-ADDA^{3}-D-glutamate^{4}-Mdhb^{5}), in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid. A few naturally occurring variations of nodularin have been found: two demethylated variants, one with D-aspartyl^{1} instead of D-methylaspartyl^{1} and the other with DMADDA^{3} instead of ADDA^{3}, and the non-toxic nodularin which has the 6(*Z*)-stereoisomer of ADDA^{3} (Namikoshi *et al*., 1994; Chorus & Bartram, 1999). The key difference between microcystins and nodularins is that the former usually occur as a mixture of several structural variants, whereas the variants of nodularins (i.e. demethylated or with modified ADDA) are rarely found.

1.2.1 *Nomenclature*

Microcystins

Nomenclature, Chemical Abstracts Service Registry (CAS) number and synonyms of the individual microcystins including microcystin-LR are listed in Table 1.2.

Nodularin

Chem. Abst. Serv. Reg. No.: 118399–22–7

CAS Name: Cyclo[(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoyl-D-γ-glutamyl-(2*Z*)-2-(methylamino)-2-butenoyl-(3*S*)-3-methyl-D-β-aspartyl-L-arginyl]

Synonyms: Cyclo[(*Z*)-2,3-didehydro-*N*-methyl-2-aminobutanoyl-erythro-3-methyl-D-β-aspartyl-L-arginyl-(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-4,5,6,7-tetradehydro-9-methoxy-2,6,8-trimethyl-10-phenyl-3-aminodecanoyl-D-γ-glutamyl]; nodularin R; 1,4,8,11,15-pentaazacyclo-nonadecane, cyclic peptide derivative

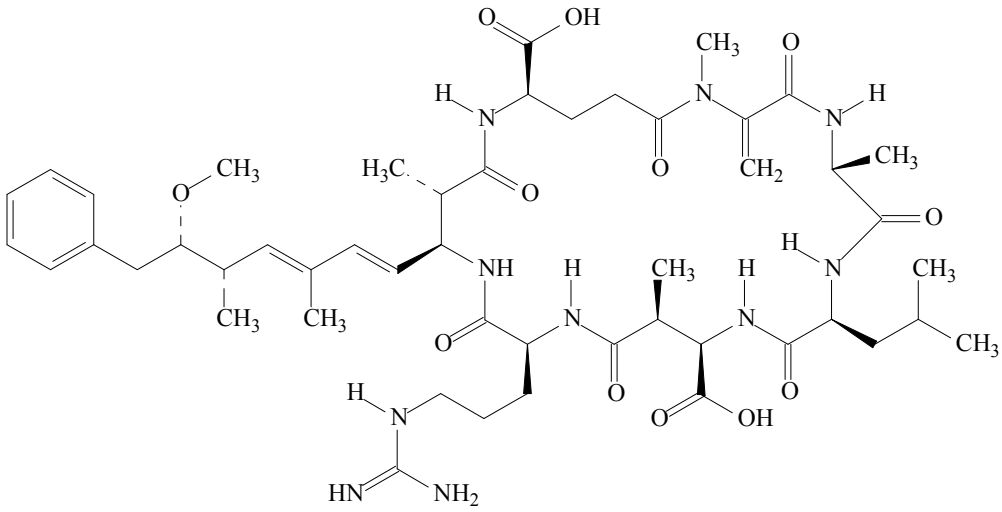
Table 1.2. Nomenclature of individual microcystins including microcystin-LR

CAS Name	CAS Registry number	Synonyms
Microcystin-LR	101043-37-2 Deleted CAS numbers: 847664-11-3; 128657-50-1	5-l-Arginine-microcystin LA; cyanoginosin-LA, 5-l-arginine; cyanoginosin LR; cyclo[2,3-didehydro-*N*-Me-ala-d-ala-l-leu-erythro-3-Me-d-β-asp-l-arg-(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-4,5,6,7-tetradehydro-9-methoxy-2,6,8-trimethyl-10-phenyl-3-aminodecanoyl-d-γ-glu]; Toxin I (*Microcystis aeruginosa*); Toxin T17 (*Microcystis aeruginosa*)
Microcystin	77238-392	Cyanoginosin; Fast-Death Factor
Microcystin-LA	96180-79-9	Cyanoginosin-LA; Toxin BE 4
Microcystin-YM	101043-35-0	Cyanoginosin-LA, 3-l-tyr-5-l-met; cyclo(ala-tyr-Me-asp-met-ADDA-glu-MDHA); cyclo(ala-tyr-Me-asp-met-3-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid-glu-methyldehydrolanyl)
Microcystin-YR	101064-48-6	Cyanoginosin-LA, 3-l-tyrosine-5-l-arginine
Microcystin-RR	111755-37-4	Cyanoginosin-LA, 3-l-arginine-5-l-arginine
Microcystin-FR	111982-70-8	Cyanoginosin-LA, 3-l-phenylalanine-5-l-arginine
Toxin III (*Microcystis aeruginosa*)	118389-26-7	Cyanoginosin-LA, 3-l-arginine-4-d-β-aspartic acid-5-l-arginine; 3-desmethylmicrocystin RR; microcystin D
Toxin II (*Microcystis aeruginosa*)	120011-66-7	Cyanoginosin-LA, 4-d-β-aspartic acid-5-l-arginine; cyclo-ala-leu-isoasp-arg-ADDA-isoglu-*N*-MDHA; 3-desmethylmicrocystin LR; microcystin-A; toxin T16 (*Microcystis aeruginosa*)
Microcystin-LY	123304-10-9	Cyanoginosin-LA, 5-l-tyr
Microcystin-WR	138234-58-9	Cyanoginosin-LA, 3-l-tryptophan-5-l-arginine
Microcystin-AR	138258-91-0	Cyanoginosin-LA, 3-l-alanine-5-l-arginine
Microcystin-LL	154037-67-9	Cyanoginosin-LA, 5-l-leucine
Microcystin-LF	154037-70-4	Cyanoginosin-LA, 5-l-phenylalanine
Microcystin-LW	157622-02-1	Cyanoginosin-LA, 5-l-tryptophan

ADDA, β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid; ala, alanyl; arg,arginyl; asp, aspartyl; CAS, Chemical Abstracts Services; glu, glutamyl; leu, leucyl; met, methionine; MDHA, methyldehydroalanyl ; Me, methyl; tyr, tyrosine

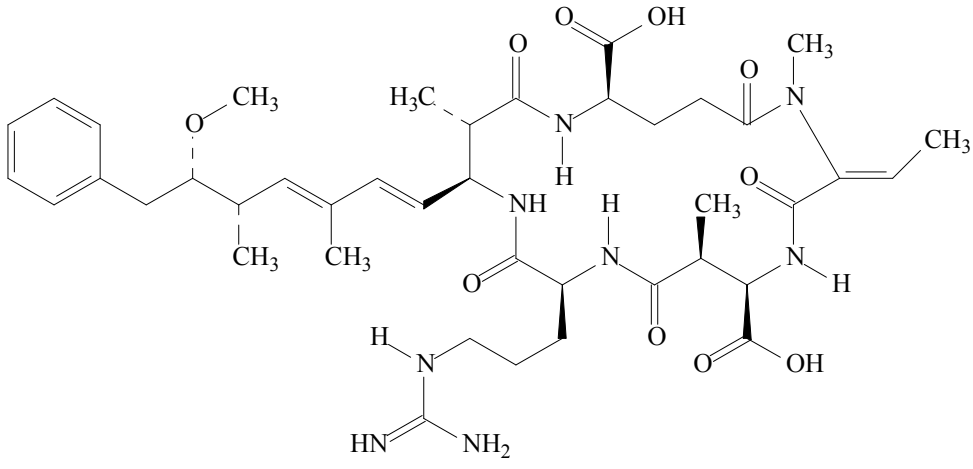
1.2.2 *Structural and molecular formulae*

Microcystin-LR



$C_{49}H_{74}N_{10}O_{12}$

Nodularin



$C_{41}H_{60}N_8O_{10}$

1.3 Analysis

A wide range of laboratory methods have been used to detect and identify cyanotoxins in water and solid matrices (including biomass); there is no single method that will provide adequate monitoring for all cyanotoxins.

Methods for determining microcystins and nodularins include: (a) physicochemical analysis by chromatographic separation (high-performance liquid chromatography [HPLC], gas chromatography, liquid chromatography) and detection either by ultraviolet absorbance (photodiode array detector) or mass spectrometry; (b) an immunoassay (enzyme-linked immunosorbent assay [ELISA]) for which several kits are commercially available; and (c) an enzyme assay that uses inhibition of protein phosphatase (Falconer, 2005).

An International Standards Organization (2005) method for the analysis of microcystin by HPLC is available, although currently no certified standard microcystins are available (McElhiney & Lawton, 2005). While chemical analysis differentiates between the structural variants of microcystin, immuno- and enzyme assays detect the sum of all microcystins in a sample. The systematic errors that are associated with the immuno- and enzyme assays are due to differences in reactivity between variants, but these assays are usually more rapid, require less elaborate equipment and may be cheaper when large numbers of samples are analysed (Falconer, 2005; McElhiney & Lawton, 2005).

ELISAs are widely used for the detection of microcystins and nodularins because of the ease of the procedure and the fact that they only require equipment that is readily available. Because of the many variants of microcystins and nodularins and the possible presence of metabolites and toxin covalently bound to protein phosphatases, antibodies give different results according to their respective affinities. This complicates the interpretation of quantitative data. ELISAs are therefore best used on simple well-defined samples. Many investigators have raised antibodies to microcystins and nodularins and have developed immunoassays that are more or less specific and vary largely in their reactivities to the different microcystin and nodularin variants or in their capabilities to detect non-cyclic degradation products (Chu *et al*., 1989; An & Carmichael, 1994; Bourne *et al*., 1996; Nagata *et al*., 1999; Baier *et al*., 2000; Fischer *et al*., 2001; Mikhailov *et al*., 2001; Zeck *et al*., 2001; McElhiney *et al*., 2002; Hilborn *et al*., 2005).

Inhibition of protein phosphatase measures free microcystins and nodularins and any metabolites that may still retain inhibitory activity. Conversely the method cannot detect or measure any microcystin that is covalently bound to cellular protein phosphatase or any metabolite that is not active. It is an assay that requires specific care, particularly in complex matrices such as cell or tissue extracts (Tencalla & Dietrich, 1997; Runnegar *et al*., 1999).

Other ways of determining toxicity are by bioassays in mice or other whole animals or in cells (Falconer, 2005).

HPLC or more advanced combinations of liquid chromatography and mass spectrometry may require complex steps of cleaning and concentrating but are best for the identification of microcystin and nodularin variants and the quantitation of toxins and metabolites. The disadvantage is that they require sophisticated equipment and expertise for reliable results (Azevedo *et al*., 2002; Hilborn *et al*., 2005).

1.4 Occurrence

Cyanobacteria are ubiquitous in water bodies with a great range of salinity and temperature, and occur in and on the soil as well as on rocks and in their fissures. In general, they are most abundant in nutrient-rich waters. Their growth is particularly favoured in lakes or water reservoirs where eutrophication occurs. Lake 'ageing' or eutrophication occurs primarily as a result of an increase in nutrients, in biological activity (productivity) and in sediments and organic matter from the watershed that fill the water basin. It is now accepted that human activities (e.g. domestic, industrial and agricultural wastes) play a significant role in the eutrophication or ageing process of the world's water bodies. In the seasonal cycle of freshwater phytoplankton that occur in temperate lakes, the appearance of cyanobacteria is probably due to the increased light and temperature at the end of spring. In water bodies that have a eutrophic to hypereutrophic nutrient status, the intensity and duration of cyanobacterial blooms are increased (Carmichael, 1996).

In addition to their wide range of social, economic and environmental impacts, cyanobacterial waterblooms which produce biotoxins are of particular concern for animal and human health; the more commonly occurring hepatotoxic biotoxins are microcystins and nodularins (Carmichael, 1996). In aquatic environments, these toxins usually remain contained within the cyanobacterial cells and are only released in substantial amounts during cell lysis or after cell death. This may occur naturally, although such events are short-lived, or through water treatment, e.g. by the application of copper sulfate to reservoirs to kill algae and cyanobacterial mass development. Release during some processes of drinking-water treatment may also be of concern (Falconer *et al*., 1983). Together with the high chemical stability and water solubility of microcystins, this containment has important implications for their environmental persistence in bodies of surface water and consequent human exposure (Sivonen & Jones, 1999).

1.4.1 *Concentrations of microcystin and nodularin in water bodies*

An increasing number of surveys worldwide have addressed the frequency of occurrence and the concentrations at which microcystins are found. Compilations of data (e.g. Sivonen & Jones, 1999; Fastner *et al*., 2001; Kardinaal & Visser, 2005) as well as overviews of case reports (e.g. Chorus, 2001) have been published, which cover a wide range of geographical regions and types of water body. More recently, survey results have also emerged from tropical settings (e.g. Morocco, Oudra *et al*., 2001; Kenya, Ballot *et al*., 2003; Bangladesh, Welker *et al*., 2005), most of which found that microcystins were

present in more than half of the samples tested, and that both the likelihood of the occurrence of microcystins and their concentrations increase with the abundance of cyanobacterial taxa.

Fewer data are available on the occurrence of nodularins. Results have focused on the Baltic Sea (e.g. Sivonen *et al*., 1989) and the Australian and New Zealand coastal areas (e.g. Carmichael *et al*., 1988b; Jones & Orr, 1994).

Table 1.3 (adapted from Sivonen & Jones, 1999) gives examples of concentrations of microcystin and nodularin reported in cyanobacterial bloom and water samples worldwide. Microcystin concentrations range from non-detectable to several milligrams per litre, and extremes of 10–25 mg microcystin-LR equivalents per litre have been reported in scum samples. Concentrations of micrograms per litre are often found, particularly when *Microcystis* spp. or *Planktothrix* spp. (syn. *Oscillatoria*) proliferate. Even in the absence of scum formation, these can cause levels of around 100 µg/L when both population density and toxin content per cell are high. For example, in a survey of Bangladeshi ponds in 2002 (Welker *et al*., 2005), microcystins were found in 39/79 ponds, 26 of which contained more than 10 µg/L and 18 more than 100 µg/L. Where cells accumulate, particularly in surface scums of *Microcystis* spp., concentrations in the range of milligrams per litre are not uncommon. With very few exceptions, these findings relate to cell-bound microcystin and, when the fraction dissolved in water was measured, it was usually very low (from < 1 to 5% of cell-bound microcystin; see Fastner *et al*., 2001 for a compilation of data from five studies).

Some structural microcystin variants are typically produced by certain genera or species, and the profile of microcystin variants can be quite typical for a given population of—for example—*P. agardhii* or *Microcystis* spp. (Fastner *et al*., 1999a). However, there is also overlap between taxa of microcystins; different strains of the same species show somewhat different microcystin profiles, and specific microcystin variants cannot be allocated unambiguously to certain cyanobacterial species.

In contrast, the production of nodularins appears to be species-specific. Laamanen *et al*. (2001) tested 345 single filaments from six different locations in the Baltic Sea using molecular methods for the allele to indicate nodularin production, and the results suggested that nearly all planktonic *Nodularia* (97%) in the Baltic sea produce this toxin. Interestingly, a close correlation was reported between the biomass concentration of *N. spumigena* and the concentration of nodularin which indicated a genetically very stable population of *Nodularia* that produces constant levels of nodularin (Chorus, 2001). In several Australian localities, Bolch *et al*. (1999) demonstrated that nodularin blooms within a water body tend to be clonal, which confirms the stability of toxin production.

Furthermore, the data of Laamanen *et al*. (2001) in the Baltic Sea suggest that *N. baltica* and *N. litorea* may both belong to the species *N. spumigena*, which may produce different morphotypes. If these results can be generalized and also hold true for other ecoregions, this would mean that nodularin is produced by only one species and that the large majority of strains of this species produce the toxin.

Table 1.3. Concentrations of toxin reported in cyanobacterial bloom or water samples worldwide

Location	Period of study	No. of positive samples (total no. of samples)	Toxins identified	Range of total concentrations (μg/g dry weight, unless otherwise indicated)
Microcystins				
Australia	1991	4	Microcystins, 24 unidentified	2100–4100[a]
Canada, Alberta	1990	37 (50)	Microcystin-LR	4–610
Canada, Alberta (3 lakes)	1990–93	168 (226)	Microcystin-LR	1–1550
China	1988	5 (10)	Microcystin-RR,-LR	200–7300
Czech and Slovak Republic	1995–96	(63)	Microcystin-LR	4–6835
Denmark	1992–94		Microcystin-RR,-LR	3–2800
Denmark	1993–95	198 (296)	Microcystins	5–1900
Finland	1994–95	17 (20)	Microcystin-LR	>10–800
France	1994	16 (22)	Microcystins	70–3970
France, Lake Grand-Lieu	1994	19 (30)	Microcystins	30–230
Germany	1992	8 (15)	Microcystin-LR	36–360
Germany	1993	17 (18)	Microcystins	0.15–36[a,b]
Germany	1995–96	385 (533)	Microcystins	1–5000
Germany	1997	34	Microcystins, several	1–25 000[b]
Japan	1990	12 (14)	Microcystin-RR, -YR, -LR	160–950
Japan	1988–92	11 (19)	Microcystin-RR, -YR, -LR	70–1610
Japan, Lake Suwa	1980–91	13	Microcystin-RR, -YR, -LR	30–2100
Japan	1986–88	4 (4)	Microcystin-RR, -YR, -LR	100–860
Japan	1992–95	18 (22)	Microcystin-RR, -YR, -LR	0.04–480[b]
Japan	1993–95	46 (57)	Microcystins	0.05–1300[a,b]
Japan	1993–94	12 (17)	Microcystins	0.06–94[a,b]
Japan	1989–94	10 (10)	Microcystins	300–15 600[a,b] 330–19 500[a,b]
Portugal	1989–92	12 (12)	Microcystin-LR plus six known and three unidentified microcystins	1000–7100
Portugal	1994–95	28 (29)	Microcystins	0.1–37[a,b]
South Africa	1985–86		Microcystin-FR, -LR, -YR, -LA, -YA	5–420
South Africa	1988–89	9 (9)	Microcystin-YR, -LR, -FR, -YA, -LA	40–630
United Kingdom	1992	3 (3)	Microcystins	17–131[a,b]
USA, Wisconsin	1993	9	Microcystins	1900–12 800[a]
Nodularins				
Australia, Tasmania, Orielton Lagoon	1992–93	7 (9)	Nodularin	2000–3500
Baltic Sea	1985–87	17 (23)	Nodularin	<100–2400
Baltic Sea	1990–91	6 (16)	Nodularin	300–18 000

Adapted from Sivonen & Jones (1999)
[a] Microcystin-LR
[b] Given as μg/L

Concentration per unit biomass of nodularin appears to be higher than that reported for microcystins: up to 18 mg/g dry weight of biomass were found in the Baltic Sea (Sivonen *et al*. 1989; Sivonen & Jones, 1999). While scum accumulations of *Nodularia* are likely to contain high concentrations of nodularin, concentrations in the open sea are rarely above a few micrograms per litre (e.g. Repka *et al*., 2004), merely because *Nodularia* filaments are less likely to accumulate.

In summary, microcystins are most liable to occur where cyanobacteria of the genera *Microcystis* or *Planktothrix* are found, and field populations of these genera that do not produce microcystins are rarely found. Nodularin is most liable to occur where *N. spumigena* is found. Microcystins are also found in populations of *Anabaena* spp., although less regularly (Fastner *et al*., 1999b), but have been reported less frequently in populations of other microcystin-producing taxa. However, it is currently unclear whether this is because such populations occur less frequently at sufficiently high levels to cause concern or whether they are less likely to contain microcystins. Some mat-forming cyanobacteria (e.g. *Phormidium* spp.) may contain microcystins, and, since such mats may become detached, exposure to microcystins may occur through this phenomenon (Mohamed *et al*., 2006). Microcystins and nodularins mainly occur as cell-bound entities. Extracellular concentrations greater than 1–5% of the intracellular concentrations have rarely been reported and are observed only under conditions that are detrimental for cell survival which trigger cell lysis and thus release microcystin. The consequences for risk assessment are that exposure is highest when cells are ingested or aspired.

Microcystins are very stable chemically (Harada *et al*., 1996). Although their photodegradation has been demonstrated (Tsuji *et al*., 1995; Welker & Steinberg, 1999), this process is usually of minor relevance, because water bodies that typically contain elevated concentrations of microcystin are usually quite eutrophic and consequently rather turbid. Microbial degradation of microcystins dissolved in water can be rapid (Jones *et al*., 1994). Lag phases are sometimes observed before degradation occurs, probably because bacteria that can degrade microcystins are not always present in sufficient numbers or need to adapt. However, once degradation begins, half-lives have been reported to be in the range of a few days and often only 1–2 days (Welker & Steinberg, 1999).

1.4.2 *Factors that influence concentrations of microcystin and nodularin*

The initial data obtained in molecular and physiological studies suggested that environmental factors as well as composition and dynamics of the cyanobacterial population are involved in the total concentration of the toxins in the water bodies. Clearly, however, more field experiments need to be conducted to assess fully the real impact of these two factors and to elaborate efficient water management (Dittmann & Börner, 2005).

(a) Population composition

Microcystins are produced by bloom-forming species of *Microcystis*, *Anabaena*, *Oscillatoria (Planktothrix)* and *Nostoc* (see Table 1.1), by a species of *Anabaenopsis* and by a soil isolate of *Haphalosiphon hibernicus*.

Nodularins have been found, with the exception of the marine sponge *Theonella*, only in *N. spumigena* (Table 1.1).

Cyanobacterial populations may be dominated by a single species or be composed of a variety of species, some of which may be not toxic. Even within a single-species bloom, there may be a mixture of toxic and non-toxic strains. Some strains are much more toxic than others, sometimes by more than three orders of magnitude. This means that one highly toxic strain, even when it occurs in small amounts among larger numbers of non-toxic strains, may render the bloom sample toxic (Sivonen & Jones, 1999; Janse *et al*., 2005).

Whether or not a strain produces the peptide toxins depends on its possession of the gene cluster that encodes for the multienzyme complex which is necessary for microcystin production (Kurmayer *et al*., 2003; Via-Ordorika *et al*., 2004). Strains both with and without these genes have been found for all potentially peptide-producing taxa known to date. Field populations of microcystins typically consist of a mixture of genotypes, i.e. with and without genes for microcystin production. The relative distribution of these genotypes, as well as the microcystin content of the respective clones, are major determinants of the concentrations of microcystin caused by a given cyanobacterial population. Gene probes are available to assess whether or not a given culture strain has the potential to produce microcystin (Tillett *et al*., 2000).

In contrast, for nodularins, populations of *N. spumigena* may contain only producer genotypes.

(b) Physiological responses

Numerous laboratory experiments with microcystin- or nodularin-producing strains of different cyanobacterial taxa have addressed the extent to which their net microcystin or nodularin production is affected by environmental conditions, i.e. availability of light, concentrations of nutrients and temperature (reviewed in Sivonen & Jones, 1999; Kardinaal & Visser, 2005). Contrary to earlier working hypotheses, levels of cyanobacterial toxins in field populations are not determined primarily by variations in environmental conditions that impact on the production rates of the cells but depend directly on the population sizes of cyanobacterial species and the relative distribution of genotypes with or without genes for microcystin production.

In some water bodies, the ratios of microcystins to biomass appear to vary rather rapidly, and there is some indication that levels of *Microcystis* spp. are higher at the beginning of the growing season (Kardinaal & Visser, 2005). In some water bodies, particularly those dominated by *P. agardhii*, the ratios remain stable throughout most of a growing season or even for several years (Janse *et al*., 2005). This may also be the case

for nodularin and *Nodularia*. Once this has been established for a given water body and particularly where cyanobacteria dominate the phytoplankton or form blooms, biomass estimates of the microcystin-producing cyanobacterial taxa may be useful site-specific surrogates for the approximation of concentrations of microcystin (Kardinaal & Visser, 2005).

1.5 Human exposure

Pathways of exposure to microcystins and nodularin in most settings are largely through water. In deriving its provisional guideline value for microcystin-LR, WHO (2003a) assumed an allocation factor of 80% to water as an exposure pathway. However, in specific settings, other pathways may gain major significance or be dominant. Therefore, to assess human health risks from microcystins, all potential exposure pathways should be considered. These include: recreational exposure, particularly to scums and in situations of high turbidity due to dispersed cyanobacterial cells, drinking-water, particularly in settings where particle removal is poor, haemodialysis during which surface water is used and treatment fails, occupational exposure to aerosols when surface water that contains cyanobacterial cells is used, e.g. in irrigation or for cooling water, 'health food' or dietary supplement tablets produced from cyanobacteria, and fish and mussels.

An important aspect of pathways of human exposure is that microcystins and nodularins do not appear to enter the human body through dermal exposure but chiefly through active transport mechanisms (see Section 4.1). Consequently, exposure requires ingestion or aspiration of water or food that contains cyanobacterial cells that have these peptides and/or dissolved cyanopeptides.

It is probable that the same human populations are exposed repeatedly to microcystins as a result of on-going contamination of freshwater sources, e.g. by *M. aeruginosa, P. agardhii* or *P. rubescens*, or brackish water sources that contain *N. spumigena.* Natural lakes and drinking-water reservoirs that are affected by these organisms regularly develop seasonal or perennial water blooms. Rural and less developed country populations that use surface water without treatment are also vulnerable to exposure. However, few studies on chronic exposure have been carried out either experimentally in animals or epidemiologically in human populations (WHO, 2003a).

1.5.1 *Recreational exposure*

Recreational exposure is the most probable pathway for ingestion of a high dose of microcystins or nodularins. Any water sport that involves immersion of the head invariably leads to some oral uptake or aspiration. Swimmers—if alerted to the hazard—might control their action to reduce ingestion and aspiration. Activities such as sailboarding, sailing in bad weather conditions or water skiing may lead to substantial uptake of water, and aerosol uptake through the spray generated by coastal wave action

may lead to exposure to nodularin. Children who play in shallow bays in which cyanobacterial scums tend to accumulate are particularly liable to swallow water.

Acutely lethal human intoxications through microcystins or nodularins appear to be improbable. [The provisional WHO tolerated daily intake (TDI) for microcystin-LR (0.04 µg/kg bw) may easily be exceeded through recreational exposure. This was illustrated by Chorus and Fastner (2001) using data from the Havel River in Berlin during a heavy, but moderately toxic bloom; half of the 28 samples taken on four occasions at 13 different sites contained more than 100 µg/L microcystins (as sum of all variants), four contained more than 1000 µg/L and two contained more than 10 000 µg/L. A re-calculation of their data for adult exposure (Table 1.4) shows that an adult would very probably ingest more than the TDI. If the cells contained five- to 10-fold more microcystin, swallowing only a few millilitres would already reach the TDI.] Similar concentration ranges of microcystins (mainly microcystin-LR) were detected in 25% of the 155 lakes in southwestern Germany that were monitored (Frank, 2002).

Table 1.4. Ingestion of scum material that would cause a dose above the WHO provisional tolerated daily intake for microcystin-LR (0.04 µg/kg bw): derivations from concentrations measured along the Havel River in July and August 1997

At 100 µg/L (0.1 µg/mL)		At 1000 µg/L (1 µg/mL)		At 25 000 µg/L (25 µg/mL)	
per kg	for a 100-kg adult	per kg	for a 100-kg adult	per kg	for a 100-kg adult
0.4 mL	40 mL	0.04 mL	4.0 mL	0.0016 mL	0.16 mL

From Chorus & Fastner (2001)

In many European cultures, permanently leased campsites or datchas are regularly used during holidays, on week-ends and, if sufficiently close to city flats, also on late afternoons and evenings. These are frequently located next to very eutrophic water bodies that harbour toxic cyanobacterial populations from July until late September or along the Baltic Sea coast; these water bodies are regularly used for swimming. Exposure thus occurs regularly over periods of several months. In subtropical and tropical settings, the cyanobacterial season and the period of recreational exposure may be substantially longer.

In summary, the estimation of recreational exposure requires a good understanding of the patterns of water use and occurrence of microcystins and nodularin. In view of the substantial, but hardly measurable, health benefits that populations often derive from the use of these settings, such exposure assessments should be carried out with care before interventions that curtail recreational use are implemented.

1.5.2 *Exposure through drinking-water and haemodialysis*

If the water is not treated to remove cyanobacterial cells, exposure scenarios for drinking-water can be similar to those outlined under Section 1.5.1 for recreational use. Disinfection in such situations probably does not degrade microcystins sufficiently, as chlorine is consumed by the high level of organic material. Such settings exist in many parts of the world, and a recent published example is ponds used in Bangladesh (Welker *et al*., 2005).

Where drinking-water is treated to remove particles, some break-through of microcystins may occur, although most of the microcystins are removed with the cells. When drinking-water is treated by initial oxidation (e.g. chlorine or ozone), microcystins are released from cells the process but may not be sufficient to oxidize all of the liberated microcystin (Hoeger *et al*., 2005). Examples of concentrations of cyanobacteria and toxins reported in drinking-water plants worldwide (Table 1.5) show that cyanobacterial toxin levels are usually well below 1 μg/L and rarely substantially above a few micrograms per litre.

Overall, exposure to microcystin through drinking-water can be assumed to be significant in settings where poorly treated surface water sources are used, whereas it is probably low or at least usually within the range of the provisional WHO guideline value in communities that are served by larger utilities that perform well-managed particle and organic contaminant maintenance, particularly when followed by an oxidation step.

Exposure through haemodialysis involves much larger amounts of water, i.e. approximately 120 L per treatment, which are effectively equivalent to an intravenous dose. This explains the severe impact of cyanotoxins on haemodialysis patients in Caruaru, Brazil, (Jochimsen *et al*., 1998), and highlights the importance of both the choice of the water source as well as excellent treatment of the water used in dialysis clinics.

1.5.3 *Occupational exposure*

Very few published data exist to demonstrate occupational exposure. However, scenarios can be estimated from the understanding of pathways of uptake and occurrence in surface waters. These would include any situation that leads to substantial ingestion or inhalation. Anecdotal evidence has been proposed from spray irrigation in agriculture and from aerosols produced by cooling the water used for mine drilling. Exposure would appear to be probable during large-scale and commercial harvesting and processing of cyanobacteria (e.g. for food supplements—see Section 1.5.4—and production of cosmetics). Estimates of uptake are hampered by the difficulty of estimating the volumes of water inhaled with such aerosols.

1.5.4 *Exposure through cyanobacterial dietary supplements*

Several regions in the world, e.g. Mexico, northern Africa and China, have a documented history of use of blue-green algae (*Spirulina* and *Nostoc* spp.) as a food source

Table 1.5. Examples of concentrations of cyanobacteria and/or cyanobacterial toxins in drinking-water before and after treatment in water plants worldwide

Location and source	Water treatment	Cyanobacteria	Raw water	Final water
Argentina, Bahía Blanca	NR	*Anabaena*/*Microcystis*	48×10^3–84×10^3 cells/ mL	276–2.5×10^3 cells/ mL
Australia, Queensland, reservoir	Flocculation/sedimentation, particulate activated carbon, slow filtration, chlorination	*Anabaena*, *Microcystis*	<2.200×10^3 cells/ mL <8 µg/L (microcystins)	<11×10^3 cells/ mL 0–0.5 µg/L (microcystins)
Bangladesh, lakes, ponds, reservoirs	NR	*Microcystis*	Samples positive for microcystins	samples positive for microcystins
Brazil, Itaparica Dam	Copper sulfate	*Anabaena*, *Microcystis*	NR	NR
Canada, Alberta, Camrose plant	Flocculation/sedimentation, slow filtration, chlorination, particulate activated carbon	NR	0.15–0.87 µg/L	0.09–0.18 µg/L
Canada, Alberta, Ferintosh plant	Flocculation/sedimentation, slow filtration, chlorination, granular activated carbon	NR	0.27–2.28 µg/L	0.05–0.12 µg/L
Czech Republic	NR	NR	≤8.7 µg/L	0–7.79 µg/L
China	NR	NR	0.28–35.3 µg/L	≤1.4 µg/L
Finland	Bank filtration Particulate activated carbon	*Planktothrix/Oscillatoria* NR	0.1–1.9 µg/L NR	0.01–0.1 µg/L ≤0.001 µg/L
France, Saint-Caprais reservoir	 Particulate activated carbon Particulate activated carbon	*Aphanizomenon* NR NR	63 µg/L NR NR	NR 33.2 ± 8.0 ng/L ≤0.001 µg/L
France, Lake Bourget	Ozonation, slow filtration	*Planktothrix/Oscillatoria*	<18×10^3 cells/ mL <5 µg/L	<6×10^3 cells/ mL <1 µg/L

Table 1.5 (contd)

Location and source	Water treatment	Cyanobacteria	Raw water	Final water
Germany, Dörtendorf, Weida Reservoir	Microsieve, flocculation/sedimentation, slow filtration	*Planktothrix/Oscillatoria*	7.5–10 µg/L	0–0.1 µg/L
Germany, Rostock, Warnow River	Ozonation, flocculation/sedimentation, slow filtration Ozonation, activated carbon filtration	*Microcystis* *Planktothrix/Oscillatoria*	10–28 µg/L 0.4–8.0 µg/L	0–0.2 µg/L 0.07–0.11 µg/L
Germany, Radeburg Reservoir	Bank filtration	*Aphanizomenon*/*Microcystis*	2–19 µg/L	≤0.06 µg/L
Israel, Lake Kinneret	Flocculation/sedimentation, chlorination	*Aphanizomenon*	$\leq 150 \times 10^3$ cells/ mL	NR
Italy, Lake Simbirizzi, Lake Flumendosa, Lake Mulargia	NR	*Planktothrix/Oscillatoria*	480 and 220 µg/g dry weight	NR
Korea, Republic of, Lakes and reservoirs	Mostly only rapid sand filtration	*Microcystis* (60%), *Anabaena* (30%), *Planktothrix/Oscillatoria* (10%)	0.6–171 µg/L	NR
Latvia, Baltezers, Lake Mazais	Slow filtration/bank filtration	*Aphanizomenon*, *Anabaena*, *Microcystis*	19–1229 µg/g dry weight; lake: ≤0.63 µg/L; infiltration basin: ≤0.25 µg/L	≤1.47 µg/L
Poland, Sulejów Reservoir	Flocculation/sedimentation, particulate activated carbon, rapid sand filtration, ozonation, chlorination	*Microcystis*	2.1–2.3 µg/L	0.5–0.8 µg/L

Table 1.5 (contd)

Location and source	Water treatment	Cyanobacteria	Raw water	Final water
Portugal, Crestuma-Lever reservoir	NR	*Aphanizomenon*, *Microcystis*	$\leq 12\times10^{3}$ cells/ mL (*Microcystis*) 4.7 μg/g dry weight (*Aphanizomenon*)	NR
Thailand	Partly without treatment	*Anabaena*, *Cylindrospermopsis*, *Microcystis*	NR	<1.0 μg/L
USA, Florida	NR	*Cylindrospermopsis*, *Microcystis*	NR	≤90 μg/L

Adapted from Hoeger *et al.* (2005); data come from field studies between 1980 and 2003
NR, not reported

(Carmichael *et al*., 2000; Jensen *et al*., 2001). In the twentieth century, blue-green algae supplements, which were primarily products that consisted entirely or partially of *Aphanizomenon flos-aquae* and *Spirulina* spp., represented an important economic activity (Carmichael *et al*., 2000), and were sold mainly in industrialized countries.

Blue-green algae supplements that consist of A. flos-aquae are specifically marketed and consumed for their putative beneficial health effects, e.g. increased alertness, increased energy, 'detoxification', elevated mood and weight loss (Jensen *et al*., 2001). More importantly, these supplements are marketed in some instances as a replacement for or alternative to the pharmacological therapy of 'attention deficit hyperactivity disorder' (Lindermann, 1995), and thus directly target the parents whose children present this disorder, providing a highly specific route of exposure to microcystins in small children.

Although producers and retailers of blue-green algae supplements maintain that batches that contain levels of microcystins above 1 μg/g dry weight are not marketed (Carmichael *et al*., 2000), independent investigations of microcystin contamination in these publicly available products have demonstrated toxin concentrations of up to 35 μg microcystin-LR equivalents/g dry weight. Although samples with toxin contamination greater than 10 μg microcystin-LR equivalents/g dry weight are the exception, several independent analyses detected more than 1 μg microcystin-LR equivalents/g dry weight in 50–100% of the blue-green algae products tested (Gilroy *et al*., 2000; Fischer *et al*., 2001; Lawrence *et al*., 2001; Dietrich & Hoeger, 2005; Bruno *et al*., 2006). Several studies (Lawrence *et al*., 2001; Bruno *et al*., 2006) have shown differences in detectable amounts of toxin when different detection methods were used. These differences appear primarily to stem from differences in the cross-reactivity of the microcystin congener of some of the ELISAs used but are also attributed to the lack of certified standards for five to 10 of the microcystin congeners that are commonly detected in blue-green algae supplements. Despite the latter findings, not all of these products contain high levels of microcystin (above 1 μg microcystin-LR equivalents/g dry weight). However, the levels of microcystin in a given brand can vary extensively from batch to batch (Gilroy *et al*., 2000) which does not allow for a proper assessment of human exposure, and specifically that of children, to microcystins.

Gilroy *et al*. (2000) calculated a TDI of 0.04 μg microcystin-LR equivalents/kg body weight (bw) per day based on a no-observed-adverse-effect level for microcystin-LR in mice of 40 μg/kg bw per day that was defined by Fawell *et al*. (1999); the application of a total 1000-fold uncertainty factor resulted in a provisional tolerable level for microcystins in blue-green algae supplements of 1 μg microcystin-LR equivalents/g dry weight. This level was adopted by the Oregon Health Division as a provisional regulatory standard for these products in 1997. This safe level translates into 2 μg microcystin-LR equivalents per adult per day. However, extrapolation of these daily doses to children (5–20 kg bw) shows that they would actually be exposed to three- to 12-fold higher daily doses than adults (Dietrich & Hoeger, 2005). Moreover, when assuming the worst case, i.e. blue-green algae supplements contaminated with 35 μg microcystin-LR equivalents/g dry

weight, the actual daily exposure of children could exceed the TDI by a factor 88–350, based on a maximum daily consumption of 2 g per day.

Contrary to the situation for food or water intake, in which a natural limitation of consumption can be assumed, daily consumption of blue-green algae supplements is largely dependent on the individual. Thus, overzealous parents may potentially severely increase the daily exposure of their child (Dietrich & Hoeger, 2005). The latter scenario is not improbable, as consumption of up to 20 g per day has been reported in the case of an adult (Schaeffer *et al*., 1999; Gilroy *et al*., 2000). Furthermore, contrary to water and food that are usually consumed together, e.g. during a meal, supplements are more probably treated as pharmaceuticals and are thus ingested on an empty stomach which may lead to higher potential uptake of microcystins from the gastrointestinal tract. Thus, the uptake from blue-green algae supplements should be treated entirely differently from the usual risk calculations for food and water.

1.5.5 *Exposure through food*

The order of importance of the individual food sources of exposure to microcystins varies between countries and largely depends on factors such as climatic conditions and irrigation practices, conditions for and traditions in agriculture and aquaculture (e.g. availability of cyanobacteria-free versus contaminated surface water), eating habits of the local population and, most of all, the affluence of the population in question. Indeed, a lack of regular income and the consequent discontinuity of sustenance forces poorer families to consume cyanobacteria-contaminated shellfish, crayfish or fish due to the inability to afford better quality food. Microcystins (and other cyanobacterial toxins, e.g. nodularin) accumulate in fish, crayfish and shellfish (Vasconcelos, 1999; Magalhães *et al*., 2001, 2003; Mohamed *et al*., 2003) at maximum concentrations of 300 µg/kg in the edible parts of fish, 2700 µg/kg in crayfish and 16 000 µg/kg in mussels (for discussions, see Falconer, 2005).

Microcystins have been reported to be taken up by commercially cultivated plants such as lettuce (*Lactuca sativa*) (Codd *et al*., 1999) and common beans *(Phaseolus vulgaris)* (Abe *et al*., 1996) when the toxins are present in the irrigation water or the growth media. The central leaves of lettuce were contaminated with 2.5 microcystin-LR equivalents/g dry weight that were not removable by washing, but no data were available on the actual level of contamination of beans with microcystins, as only inhibition of photosynthesis was determined following spray irrigation with microcystin-contaminated water. As some cyanobacteria fix nitrogen from the atmosphere and provide a valuable source of nitrogen to growing rice plants, cyanobacteria are welcome in rice fields (Rahman *et al*., 1996). However, although some of these cyanobacteria are presumably producers of microcystin, little is known about the mechanism(s) of uptake of microcystins into plants or the concentration of toxic cyanobacterial compounds in rice fields.

Few data are available on the accumulation of microcystins in livestock (e.g. cattle, swine, sheep), although these animals may frequently be exposed to microcystins and other cyanobacterial toxins through consumption of water contaminated with cyanobacteria (Beasley *et al*., 1983, 1989a,b). No carry-over of microcystins into milk (Orr *et al*., 2001) or meat (Orr *et al*., 2003) was observed in cows following administration of toxic *M. aeruginosa* in the drinking-water.

The actual exposure of humans (adults and children) to microcystins from food is difficult to estimate, especially since there is no general rule as to how much fish, shellfish, salad or rice is consumed daily per 'international adult or child'. It is possible that, in some regions of the world, children consume more than 0.1 kg fish or shellfish per day (Mohamed *et al*., 2003), especially when local populations are largely dependent on one type of food source (e.g. fish, shellfish or crustaceans, rice). Using the data from actual contamination of fish and shellfish reported by Vasconcelos (1999), Magalhães *et al*. (2001, 2003) and Mohamed *et al*. (2003) of up to 300 µg/kg edible fish, 2700 µg/kg crayfish and 16000 µg/kg mussels, there is a distinct possibility that exposure (subacute and chronic) of children occurs through microcystins in fish and shellfish (Dietrich & Hoeger, 2005). Daily consumption of 200 g fish contaminated with 300 µg microcystin-LR equivalents/kg edible fish by a 20-kg child would result in a total exposure of 60 µg microcystin-LR equivalents per day or 3 µg microcystin-LR equivalents/kg bw per day. Such an exposure would exceed the TDI of 0.04 µg microcystin-LR equivalents/kg bw per day proposed by the WHO by a factor of 75. In the worst case of highly contaminated mussels (16 000 µg microcystin-LR equivalents/kg mussel) and assuming the same daily consumption of 200 g, the TDI in a child would be exceeded by a factor 4000. Both of the latter calculations are, however, based on the assumption that all of the microcystin in the fish and shellfish is biologically and thus also systemically available to the exposed child.

1.6 Regulations, guidelines and preventive measures

1.6.1 *Drinking-water*

In 1997, WHO derived a provisional guideline value for microcystin-LR in drinking-water of 1 µg/L, based on a TDI of 0.04 µg/kg bw (WHO, 2004). The guideline is provisional because of the limitations of the database, particularly with regard to studies on long-term exposure and carcinogenicity. Moreover, it is limited to only one of several structural variants that occur as frequently in the same concentration range or even exclusively. In a supporting document to the *WHO Guidelines for Drinking-water Quality* (Chorus & Bartram, 1999), Falconer *et al*. (1999) recommended the use of concentration equivalents that include the other variants. An increasing number of countries are converting this WHO guideline into national regulations. Their approaches vary in dealing with the problem of the numerous microcystin variants that are typically found as mixtures in samples. While some (e.g. Canada) explicitly set the level to accommodate

for the presence of microcystins on the basis of general assumptions on their occurrence, others (e.g. Spain) simply refer to 'microcystin' without further specification.

In the third edition of the *WHO Guidelines for Drinking-water Quality* (WHO, 2004), the 1998 provisional guideline value for microcystin-LR was not changed, but is relevant to any hazard with emphasis on the need to consider national and regional conditions when converting any WHO guideline values into national standards and regulations. These may include issues of implementation, such as institutional capacity, and in particular the importance of a hazard for public health in relation to other prevalent hazards.

The publication of the WHO provisional guideline value for microcystin-LR in 1998 has led to national regulations on microcystins in drinking-water in several countries, and—in line with WHO's explicit emphasis on the need to use WHO guideline values in a nationally and locally adequate way to optimize the protection of public health—some countries have adapted their standard. Table 1.6 provides examples of national regulations and guidelines. In 2003, the European Union began discussions on whether microcystin-LR should be included in the forthcoming revision of its Drinking-water Directive.

Explicit guidelines for nodularin in drinking-water are known only for Australia. The Australian Government (2004) states that, due to the lack of adequate data, no guideline value is set for concentrations of nodularin. However, given the known toxicity of nodularin, the relevant health authority should be advised immediately if blooms of *N. spumigena* are detected in sources of drinking-water. Since there are some similarities between the toxicity of nodularin and microcystins, the guideline for microcystins could be used to derive cell numbers of *N. spumigena* that represent a preliminary indication of the potential hazard. It is recommended that notification and further assessment be made when cell numbers of *N. spumigena* exceed 40 000 cells/mL.

Table 1.6. Examples of regulatory approaches to cyanobacteria and microcystins in drinking-water

Country	Regulatory approach
Approaches motivated by compliance to a standard or guideline value	
Australia	Federal Drinking-water Guideline for total microcystins of 1.3 µg/L, expressed as microcystin-LR equivalents.
Brazil	Monthly monitoring of cyanobacteria in drinking-water resources; if cell counts exceed 10 000 cells/mL or biovolumes (determined from cell counts) exceed 1 mm^3 cell volume, weekly monitoring and analyses of toxins or toxicity testing are required; standard value for microcystins (variants not specified), 1 µg/L.
Canada	Maximum acceptable concentration for microcystin-LR in drinking-water, 1.5 µg/L; intended to be protective of human health against exposure to other microcystins that may also be present.

Table 1.6 (contd)

Country	Regulatory approach
Czech Republic	Mandatory monitoring of tap-water for microcystin-LR; limits, 1 µg/L; an update of the ordinance will include alternatives to analysis of microcystins such as quantification of cyanobacterial biomass in raw water or bioassays in conjunction with cell counts, and analyses of toxins only if thresholds for cyanobacterial biomass are exceeded.
France	Drinking-water Decree maximum limit is 1 µg/L microcystin-LR; analyses required if cyanobacteria proliferate in raw water.
Poland	Limit of 1 µg/L for microcystin-LR in drinking-water
Spain	Drinking-water Decree includes a limit for 'microcystin' (variants not specified) of 1 µg/L; sampling regimes specified in relation to size of population served; to be reviewed at 5-year intervals.
USA	In February 2005, the Environmental Protection Agency included cyanobacteria, other freshwater algae and their toxins on its 'contaminant candidate list' of unregulated contaminants, for which research is to be prioritized and data collected to determine whether regulation is necessary.
Indirect or implicit inclusion in drinking-water regulations	
Germany	National Drinking-water Ordinance stipulates drinking-water should contain no substances at concentrations that may be harmful to health, and the provisional WHO value for microcystin-LR defines such concentrations. The prerequisite for this approach is that drinking-water suppliers that use surface water usually monitor and acknowledge the phytoplankton in their resource and have effective treatment in place (as part of best practice and technical rules) and thus are aware of the cyanotoxin hazard.
Italy	No limit value has been implemented, but the national Drinking-water Decree considers 'algae' as an accessory parameter to be monitored when local authorities suspect a risk to human health; the provisional WHO Guideline of 1 µg/L for microcystin-LR is used as the basis for this assessment.
Hungary	The national Decree on Drinking-water Quality and the ordinance on monitoring include the number of cyanobacteria cells as a biological parameter to be monitored by microscopy, although no limit is given for cyanotoxins.
'Risk-based' approaches in regulations	
Australia	Fact sheets for each of the four cyanotoxins (microcystins, nodularin, saxitoxins and cylindrospermopsin) include the guideline value of 1.3 µg/L for the sum of all microcystin variants or a cell density of 6500 cells/mL for a highly toxic population of *Microcystis aeruginosa*. These values are not mandatory legally enforceable standards, but guidelines within a framework for analysing hazards and assessing risks for individual water supply systems and are being adopted by water authorities as agreed quality targets or as contract conditions for water supply, e.g. as targets and performance indicators for audits of process performance.

Table 1.6 (contd)

Country	Regulatory approach
New Zealand	Individual water safety plans are developed for each drinking-water supply system, following a comprehensive multi-barrier approach, in which process control is central. Hazard priorities are assigned by the Medical Officer of Health, with Priority 1 usually being assigned to pathogens and their indicators (i.e. *Escherichia coli*, *Giardia* and *Cryptosporidium*) and Priority 2 to cyanotoxins, when present at concentrations above 50% of the maximum acceptable value. Water safety plan development includes identification of barriers to contamination and eutrophicating nutrients in the catchment and in water treatment for the removal of cells and/or destruction of toxins. A 'Barriers to Contamination' guide assists suppliers to assess performance of barriers and to estimate risk of cyanotoxin occurrence. Plans include reporting and communication pathways, i.e. who receives which information and how often, and documentation.
Low regulatory level approaches including cyanotoxins in an understanding of good practice	
Denmark	Administrative units and research institutions collaborate to collate information, and the Danish National Environmental Research Institute posts a national overview of the occurrence of toxic cyanobacterial on its website.
Finland	Starting in the late 1980s, waterworks have been advised to monitor cyanobacteria microscopically, and, if cyanobacterial cells occur in raw or treated water, to analyse toxins. The Finnish Drinking-water Decree further stipulates that drinking-water should contain no substances harmful to health.

From Chorus (2005)

1.6.2 *Recreational water use*

Guidance on recreational water safety provided by WHO (Falconer *et al*., 1999; WHO, 2003b) is largely based on the occurrence of cyanobacteria as such, because it is at present unclear whether all important cyanotoxins have been identified, and the health outcomes observed after recreational exposure—particularly irritation of the skin and mucous membranes—are probably related to cyanobacterial substances other than well-known toxins. In addition, the WHO approach considers the particular hazard of liver damage by microcystins at high concentrations. This approach uses three levels of alert that are associated with incremental severity and probability of health effects.

The newly revised Bathing Water Directive (European Union, 2006) follows a risk-based approach and Article 8 of the Directive explicitly addresses toxic cyanobacteria. It stipulates that

(i) "When the bathing water profile indicates a potential for cyanobacterial proliferation, appropriate monitoring shall be carried out to enable timely identification of health risks" and

(ii) "When cyanobacterial proliferation occurs and a health risk has been identified or presumed, adequate management measures shall be taken immediately to prevent exposure, including information to the public".

The Bathing Water Profile describes the risk of pollution and explicitly includes an assessment of the potential for proliferation of cyanobacteria. Actions and frequency of monitoring should be related to the history and classification of the bathing water and to regional climatic conditions, and emphasis placed on bathing waters where risks may occur.

Several countries have regulations or guidelines that address cyanobacteria and/or cyanotoxins at recreational sites, and some include approaches to address the capacity of a water body to sustain large cyanobacterial populations. Examples of national regulations and guidance are included in Table 1.7.

Table 1.7. Examples of regulatory approaches to cyanobacteria and microcystins in water for recreational use

Country	Recreational sites
Australia	The monitoring of cell densities is often preferred to toxin limits because cell counting is widely available, cost-effective and is performed rapidly. The Federal Recreational Water Guideline provides values for three different parameters: 10 µg/L total microcystins or ≥ 50 000 cells/mL toxic *M. aeruginosa*; biovolume equivalent of ≥ 4 mm^3/L for the combined total of all cyanobacteria where a known toxin producer is dominant in the total biovolume; or 10 mm^3/L for total biovolume of all cyanobacterial material where known toxins are not present. A new approach is to assess the susceptibility for cyanobacterial growth from general monitoring data and historical information, including the scoring of water bodies as 'good, fair or poor'.
Denmark	Bathing Water Instruction requires when massive blooms occur that the material is investigated, the risk assessed and the authority alarm groups trigger posting of warning signs at the waterfront as well as dissemination of information particularly to local water body-user groups.
Finland	Health authorities were provided with guidelines in the late 1980s; a cost-effective monitoring network of nuisance algae occurrence is based on long-term data on occurrence collected since 1967, and now also includes the involvement of private citizens for visual monitoring.
France	Three levels of cyanobacterial cell density trigger management responses up to prohibition of water contact sports. Information on cell numbers is published on the internet within not more than 5 days of sampling.
Germany	Three-step guideline based on visual inspection and assessment of the nutrient capacity for blooms and assessment of cyanobacterial biomass, with thresholds for warning or closure. Sites may remain open if microcystin levels are low even when cyanobacterial levels are high.
Hungary	Addresses cyanobacterial blooms indirectly through a limit for chlorophyll-a.

Table 1.7 (contd)

Country	Recreational sites
Italy	Decree on Quality of Bathing Water addresses cyanobacteria indirectly: derogations above its limit for dissolved oxygen is granted only if not due to excessive proliferation of toxic algae. In 1998, the Ministry of Health provided a list of toxic algae and cyanobacteria of concern and analytical methodologies, and recommended a limit value of 5×10^6 cells/L for toxic algae species as a safe level for bathing activities.
Netherlands	Guideline of: 10 µg/L for issuing warnings; and 20 µg/L and scums for closure of bathing sites and continued monitoring

From Chorus (2005), Australian Government (2008)

1.6.3 *Measures to control human exposure*

The prevention of cyanobacterial proliferation in the water source is largely achieved through the reduction or prevention eutrophication, i.e. 'fertilization' of water bodies with plant nutrients, in most cases phosphorus and in some settings also nitrogen. It may require substantial reductions of concentrations within the water body and, where multiple and diffuse sources contribute to the total nutrient load of a water body, success may be slow. In such situations, other water-body management approaches that render growth conditions less favourable for cyanobacteria may be useful. These largely include physical measures, i.e. changes in the thermal mixing regime or flushing rate of the water body, and are possible only in some settings. When cyanobacterial proliferation cannot be prevented, other barriers against human exposure are necessary (see Chorus & Bartram, 1999, for a more detailed overview).

For drinking-water and dialysis units, control measures include offtake strategy and treatment: offtakes may be located away from surface scums or deeper horizons where cells may accumulate, or may occur through banks drilled close to the river, using the subsurface as a filter (bank filtration). Other commonly used particle removal techniques have often proven very successful and include flocculation combined with sedimentation and rapid filtration, dissolved air flotation, microfiltration and slow sand filtration (see Table 1.5). For some of these techniques, it is important that cells accumulated on filters be removed before they lyse and release their toxin content. When elevated concentrations of dissolved microcystins occur, these can be removed by oxidation (ozone or chlorination) and treatment with activated carbon. Comprehensive overviews of the state of the art of microcystin removal may be found in Falconer (2005).

For recreational exposure, no further barriers other than the prevention of cyanobacterial proliferation are available and keeping people out of the water under high-

risk conditions. Effective surveillance and public information strategies are key to achieving this (see Section 1.6.2).

For dietary supplements, tight monitoring of contamination with microcystins may be required, e.g. by the State of Oregon in the USA and the Food and Health Authorities of Switzerland. However, this cannot rule out the occurrence of other bioactive and potentially harmful cyanobacterial metabolites or other contaminants when cell material is harvested from scums on water bodies and pressed into tablets.

For occupational exposure through aerosols, either filtration of the water before use or application techniques to avoid generation of the aerosol may prevent exposure.

1.7 References

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2. Studies of Cancer in Humans

The incidence of hepatocellular carcinoma (HCC) in Southeast China is among the highest in the world, with annual rates in some counties reaching over 100/100 000 (Ferlay *et al.*, 2004). Risk factors in this region include infection with hepatitis B and C viruses and intake of aflatoxin B_1 from food items such as corn. Several epidemiological investigations have also suggested a role for cyanobacterial toxins, in particular microcystin, as a contributor to the overall risk for HCC. Three types of study have been conducted to evaluate the relationship between HCC and microcystin: comparisons of HCC mortality rates among groups who have different types of water source (ecological studies), cohort studies and case–control studies. In addition to studies of HCC, the incidence of colorectal cancer was investigated in an ecological study. Certain studies that the Working Group evaluated provided some data on levels of microcystins, and reported relative risks among persons who consumed water from sources that had elevated levels compared with those who consumed low levels. In other studies, the contrast used to calculate relative risk was between persons who had different types of water source, typically water from ponds, ditches or small rivers compared with well-water, that was sometimes characterized as being from deep or shallow wells. The Working Group did not have access to original publications of many studies of HCC, and only those studies for which summary information was available are reported here (see Section 5.2).

2.1 Hepatocellular carcinoma

2.1.1 *Ecological studies*

In a review, Yu (1995) presented a summary of findings from several studies. In Qidong County of Jiangsu Province, China, an endemic area for primary liver cancer, mortality rates of less than 20 deaths per 100 000 were observed in some districts compared with more than 60 deaths per 100 000 in adjacent locations. Mortality rates were higher in areas where drinking-water was drawn from ponds and ditches compared with rates in areas with deep wells. Six studies in Nantong City by different authors were cited. All showed that people who consumed water from ponds or ditches had higher rates of mortality from HCC (approximately 100 deaths/100 000) than people who drank well- or deep well-water (mortality rate, < 20 deaths/100 000). Yu (1995) also tabulated results from six evaluations of mortality from HCC, four from Qidong County for overlapping periods during 1972–83, one from Haimen County (1968–72) and one from Nanhui County (1981–84). Mortality rates for consumers of pond and ditch water were higher (range, approximately 60–140/100 000/year) than those for well-water users (range, approximately 0–15/100 000/year). In this Province, microcystin-producing cyanobacteria are abundant in surface waters, and significant amounts of microcystin were detected in

pond-ditch waters whereas no detectable levels were found in deep well-water; this provides supportive evidence that microcystins in drinking-water were partially responsible for the higher incidence of HCC (Chen *et al.*, 1996).

In a Chinese county that displayed high mortality from primary liver cancer, consumption of pond water with low microcystin concentrations (160 ng/L; $n = 27$) was correlated with a higher mortality rate than consumption of deep well-water with no detectable levels of microcystin (< 50 ng/L; $n = 25$) ($P < 0.01$ for deep well- in relation to pond or river water) (mortality rate, 115.05/100 000 for pond water versus 20.00/100 000 for deep well-water; $P < 0.01$) (Ling, 2000).

2.1.2 *Cohort studies*

In a cohort study of 77 682 persons in Nanhui County, who were followed from 1986 to 1991 (Yu *et al.*, 1995), a total of 202 deaths from HCC yielded relative risks of 1.16 (95% confidence interval [CI], 1.02–1.32) for consumption of pond and ditch water and 1.25 (95% CI, 1.09–1.43) for consumption of river water, using a case–cohort approach. The relative risk for history of hepatitis was 1.03 (95% CI, 1.02–1.04). The consumption of shallow and deep well-water (including tap-water) was protective and gave relative risks of 0.65 (95% CI, 0.59–0.73) and 0.20 (95% CI, 0.16–0.25), respectively. [The Working Group noted that the relative risk for surface water was approximately five times that for deep well-/tap-water].

A cohort study reported in the review by Yu *et al.* (1995) found rates of mortality from HCC of 121.96/100 000 for consumers of pond-ditch water (12 299 person–years), 77.81 for consumers of river water (5141 person–years) and 0 for consumers of well-water (1333 person–years) (Yu & Chen, 1994).

2.1.3 *Case–control studies*

Zhao *et al.* (1994) conducted a pooled analysis of 10 Chinese case–control studies of HCC, six from southern China and four from northern China, with a total of 920 cases and 920 controls. Water source (drinking pond-ditch water) was a risk factor in the pooled studies from southern China, but not in those from northern China. Additive and multiplicative models were used to evaluate risk and test for interaction of risk factors. In analyses of the data from southern China, the adjusted odds ratio was 1.60 (95% CI, 1.19–2.13) for consumption of pond water (multiplicative model), with similar findings from the additive model. Among users of non-pond water, the odds ratio for hepatitis antigen-positivity was 10.68 (95% CI, 7.94–14.37). The odds ratio for persons who used pond water and were positive for the hepatitis antigen was 17.04 (95% CI, 12.75–22.77). [A *P*-value for the interaction between HCC and pond water was not provided.]

A population-based case–control study of 99 incident cases of HCC diagnosed between October 1988 and October 1989 and 99 age- and sex-matched controls was undertaken in Fusui County, Guangxi Autonomous Region, China, using data from

interviews of study subjects (Zhang, 1993). Conditional logistic regression showed associations with drinking pond-ditch water (odds ratio, 3.70; 95% CI, 1.25–10.96) continuously for more than four decades relative to never-users of pond water. The findings were adjusted for ever having had a hepatitis B virus infection. [The authors did not present measurements or estimates of microcystins in the drinking-water.]

In the review by Yu *et al.* (1995), several case–control studies were mentioned. In Haimen County, the odds ratio for drinking pond-ditch water was 1.91 (95% CI, 1.01–4.74). Microcystin was found in several ponds and ditches of the high-endemic areas for HCC at levels of 0.061±0.086 μg/L; levels in well-water were 0.036±0.022 μg/L. [The Working Group noted that the levels of microcystin were relatively low.]

Yu *et al.* (2001) performed a meta-analysis of six case–control studies and calculated an odds ratio of 2.46 (95% CI, 1.69–2.59) for primary liver cancer following consumption of pond-ditch water, with a population-attributable risk of 30.39% (95% CI, 23.30–37.47%). In this study, much higher levels of microcystins were observed in pond-ditch water than in well-water.

2.2 Colorectal cancer

Four hundred and eight colon and rectal carcinomas diagnosed from 1977 to 1996 in eight townships were identified from the cancer registry of Haining City of Zhejiang Province, China. The type of drinking-water consumed by each patient was ascertained by interview with each case or a family member if the case was deceased. In addition, in June to September 1997, 640 samples were taken from the four types of water source in each of the eight townships and were analysed for levels of microcystins. The incidence of colorectal cancer was significantly higher among people who drank river water (relative risk, 7.94; 95% CI, 6.11–10.31) or pond water (relative risk, 7.70; 95% CI, 5.75–10.30) than among those who consumed well-or tap-water. The maximum levels of microcystins measured in well-, tap-, river and pond water were 9.13 ng/L, 11.34 ng/L, 1083.43 ng/L and 1937.94 ng/L, respectively ($P < 0.01$ for the contrast in levels of microcystins in river or pond water versus those in well-and tap-water). The incidence of colon cancer in each of the eight townships showed a significant positive correlation ($r = 0.881$; $P < 0.01$ Spearman rank) with the average concentration of microcystins in the township. The authors mentioned that findings for men and women were similar (Zhou *et al.*, 2000). [Cancer incidence was ascertained for the 20-year period of 1977–96 and the survey of concentrations of microcystins was conducted in 1997, after the period of diagnosis. Although this may lead to some misclassification of exposure, it was the opinion of the Working Group that levels of microcystins in 1997 were probably generally representative of levels over the previous 20-year period. The authors did not describe how they estimated denominators of population size for calculating the water source-specific estimates, and it was not stated whether incidence rates of colorectal cancer by township were sex- or age-adjusted.]

2.3 References

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3. Studies of Cancer in Experimental Animals

3.1 Pure microcystin-LR (see Table 3.1)

3.1.1 *Mouse*

A group of 13 male ICR mice, 5 weeks of age, received 100 intraperitoneal injections of 20 μg/kg bw microcystin-LR (five times a week) over 20 weeks and were killed after the end of the treatment (five mice) or after a 2-month withdrawal period (eight mice). Three non-treated mice were used as controls. Liver foci which were, according to the authors, probably benign tumours were induced in all 13 treated mice (Ito *et al.*, 1997). [The Working Group noted the small number of animals, the use of a single dose exposure regimen, inadequacies of statistical evaluations and the lack of results for the control animals.]

3.1.2 *Rat*

Groups of 10–16 male Fischer 344 rats, 7 weeks of age, were given a single intraperitoneal injection of 0 or 200 mg/kg bw *N*-nitrosodiethylamine (NDEA) in saline followed 2 weeks later by intraperitoneal injections of 0, 1 or 10 μg/kg bw pure microcystin-LR twice a week for 6 weeks and partial hepatectomy at the end of week 3. Phenobarbital (0.05%) was used as a positive control. In a second experiment, groups of 14–19 rats were initiated with NDEA followed 2 weeks later by two intraperitoneal injections of 0 or 10 μg/kg bw pure microcystin-LR, partial hepatectomy at the end of week 3 and intraperitoneal injections of 10, 25 of 50 μg/kg bw microcystin-LR twice a week for 5 weeks. The tumour-promoting activity was estimated by induction of glutathione *S*-transferase placental form-positive (GST-P) foci in rat liver. In the first experiment, rats treated with NDEA plus 10 μg/kg bw microcystin-LR had an increased incidence of GST-P foci per liver compared with NDEA-treated rats (26.0±8.1 versus 16.5±3.9/cm^2; $P < 0.005$). In the second experiment, the three groups of rats treated with NDEA plus 10 and 10 μg/kg bw microcystin-LR, NDEA plus 10 and 25 μg/kg bw microcystin-LR or NDEA plus 10 and 50 μg/kg bw microcystin-LR had an increased incidence of GST-P foci per liver compared with NDEA-treated rats (17.4±3.8 ($P < 0.01$), 32.7±11.1 ($P < 0.01$) and 44.4±10.3 ($P < 0.001$) versus 13.4±4.2/cm^2,respectively) (Nishiwaki-Matsushima *et al.*, 1992).

Groups of male Fischer 344 rats [initial number unspecified], 7 weeks of age, received a single intraperitoneal injection of 0 or 200 mg/kg bw NDEA in saline followed 2 weeks later by intraperitoneal injections of 0 or 25 μg/kg bw microcystin-LR. The tumour-promoting activity of microcystin-LR was evaluated on the basis of an increase in

Table 3.1. Summary of the liver tumour-promoting activity and/or carcinogenicity of intraperitoneal injection of microcystin-LR

Initiator	Microcystin-LR (μg/kg × times)	Species	Partial hepatectomy	Biomarker	Estimation	Reference
–	20 × 100 times	Male ICR mice	–	Neoplastic nodules	Weak carcinogen	Ito *et al.* (1997)
NDEA	10 × 12 times	Male Fischer 344 rats	+	GST-P foci	Tumour promoter	Nishiwaki-Matsushima *et al.* (1992)
NDEA	10–50 × 12 times	Male Fischer 344 rats	+	GST-P foci	Tumour promoter	Nishiwaki-Matsushima et al. (1992)
NDEA	25 × 20 times	Male Fischer 344 rats	–	GST-P foci	Tumour promoter	Ohta *et al.* (1994)
NDEA + aflatoxin B_1	10 × 12 times	Male Fischer 344 rats	+	GST-P foci	Tumour promoter	Sekijima *et al.* (1999)
Aflatoxin B_1	10 × 12 times	Male Fischer 344 rats	+	GST-P foci	Tumour promoter	Sekijima *et al.* (1999)

GST-P, glutathione *S*-transferase placental form-positive; NDEA, *N*-nitrosodiethylamine

three parameters: the number of GST-P-positive foci per liver (no./cm^2), the area of foci per liver (mm^2/cm^2) and the volume of foci per liver (v/v%) (see Table 3.2; Ohta *et al.*, 1994).

Groups of male Fischer 344 rats, 6 weeks of age, received an intraperitoneal injection of 0 or 200 mg/kg bw NDEA in saline 2 weeks before intraperitoneal injections of 0, 1 or 10 μg/kg bw microcystin-LR twice a week for 6 weeks. Other groups were also treated with aflatoxin B_1 (0.5 mg/kg bw) and NDEA, or aflatoxin B_1 alone, followed by partial hepatectomy (for dosing regimes and results, see Table 3.3; Sekijima *et al.*, 1999).

Table 3.2. Induction of liver GST-P foci by intraperitoneal administration of microcystin-LR or nodularin to rats initiated with NDEA

NDEA	Toxin (μg/kg) 20 times	Effective no. of rats	No. of foci/liver (no./cm^2)[a]	Area of foci/liver (mm^2/cm^2)[a]	Volume of foci/liver (v/v %)[a]
+	–	20	10.0±2.9	0.18±0.07	0.37±0.18
–	–	5	0	0	0
+	Microcystin-LR (25)	18	95.7±27.9*	4.74±2.23*	8.55±4.04*
–	Microcystin-LR (25)	17	1.6±1.4	0.02±0.02	0.04±0.03
+	Nodularin (25)	20	106.0±22.6*	39.87±10.51*	71.75±18.78*
–	Nodularin (25)	16	6.3±7.3	0.49±0.89	0.92±1.58

From Ohta *et al.* (1994)
GST-P, glutathione *S*-transferase placental form-positive; NDEA *N*-nitrosodiethylamine;
[a] Mean±standard deviation
* $P < 0.005$ versus NDEA control

Table 3.3. Induction of liver GST-P foci by microcystin-LR in rats initiated with NDEA and/or aflatoxin B_1

NDEA	Aflatoxin B_1	Microcystin-LR	No. of rats	No. of foci/liver[a]	Area foci/liver[a]
+	–	–	5	2.46±1.86	0.39±0.29
+	+	–	15	8.34 ± 3.60*	1.69±0.79*
+	+	1 μg/kg bw	13	10.72±6.74*	2.26±1.75*
+	+	10 μg/kg bw	13	9.16±4.70	1.96±1.03**
–	+	–	10	1.61±0.74	0.76±0.51
–	+	1 μg/kg bw	16	3.46±1.14***	2.24±1.35***
–	+	10 μg/kg bw	16	3.50±1.74	2.75±2.86

From Sekijima *et al.* (1999)
GST-P, glutathione *S*-transferase placental form-positive; NDEA, *N*-nitrosodiethylamine
[a] Mean±standard deviation
**P* <0.01 versus NDEA control
***P* <0.001 versus NDEA control
****P* <0.01 versus aflatoxin B_1 control

3.2 Microcystis extracts

Mouse

Groups of five male and five female Swiss mice, 3 weeks of age, were given extracts of *Microcystis aeruginosa* (1/4, 1/8 and 1/16 dilutions; equivalent to 28.3, 14.1 and 7 μg microcystin/mL) in the drinking-water or drinking-water alone (controls) for periods up to 1 year, during which mice were killed at various intervals. The incidence of tumours in mice was: 4/71 (one abdominal carcinoma, two lung carcinomas and one thoracic lymphosarcoma) in the high-dose exposure group, 0/150 for the mid- and low-dose exposure groups, and 2/73 (one uterine adenocarcinoma and one thoracic lymphosarcoma) in the control group (Falconer *et al.*, 1988). [The Working Group noted that details about examination of tumours was not reported.]

Groups of 20 female Swiss mice, 3 months of age, received a single dermal application of 0 or 500 μg 7,12-dimethylbenz[*a*]anthracene (DMBA) in acetone onto the shaved skin and, 1 week later, were given extracts of *Microcystis* (1/2 dilution; equivalent to 40 μg microcystin/mL) in the drinking-water or drinking-water alone. After 52 days of exposure, a significant increase ($P < 0.05$) in the weight of skin papillomas/mouse was reported [DMBA plus extract, 16 mg/mouse; DMBA, 2 mg/mouse; read from Figure]. No tumours were reported in the groups treated with the extract only or drinking-water alone [2.5 tumours/mouse and four tumours/mouse were reported in the groups treated with DMBA and DMBA plus extract, respectively; numbers of tumours were not significantly different] (Falconer & Buckley, 1989; Falconer, 1991). [The Working Group noted that the number of animals was not given.]

The possibility of promotion of growth of tumours initiated by two doses of 40 mg/kg bw *N*-methyl-*N*-nitrosourea at 1-week intervals was investigated in 115 C57 black mice [sex and age unspecified] exposed to 0, 10 or 40 μg/mL *Microcystis* toxins in the drinking-water (0, 29 or 89 μg/day/mouse). After 154 days, tumours of the duodenum, liver and lymphoid system were assessed. No effect of exposure to microcystin on the growth of these tumours was observed (Falconer & Humpage, 1996). [The Working Group noted the absence of a group treated with microcystins alone.]

To investigate the possibility that microcystin promotes colon tumours, a total of 176 male C57Bl/6 mice, 13 weeks of age, were given three weekly intraperitoneal injections of 0 ($n = 20$) or 5 mg/kg bw azoxymethane ($n = 156$) in saline. Three weeks later, azoxymethane-treated mice were exposed for 212 days to 0 ($n = 61$), 6 ($n = 53$) or 12 ($n = 42$) mg microcystin-LR equivalents/L (0, 382 or 693 μg/kg bw per day) in the drinking-water. Mice from each group were killed at 13, 22, 28 or 36 weeks after the first azoxymethane treatment. Examination of hypertrophic crypts in the colon showed a dose-dependent increase in the area of aberrant crypt foci after exposure to microcystin. Results were as follows: azoxymethane-only group (291 foci/57 mice), 25×10^3 μm^2; azoxymethane plus low-dose microcystin (280 foci/49 mice), 26×10^3 μm^2; azoxymethane plus high-dose microcystin (195 foci/38 mice), 29×10^3 μm^2 ($P < 0.05$). Only 1/16 untreated

mice had one foci and the rest had none (Humpage *et al.*, 2000). [The Working Group noted the absence of a group treated with microcystin alone.]

3.3 Nodularin

Rat

Groups of male Fischer 344 rats [initial number unspecified], 7 weeks of age, received an intraperitoneal injection of 0 or 200 mg/kg bw NDEA in saline followed 2 weeks later by intraperitoneal injections of 10 or 25 μg/kg bw nodularin twice a week for 10 weeks. Saline was injected into control animals. Nodularin showed strong tumour-promoting activity in rat liver on the basis of parameters such as the number of GST-P foci per liver (no./cm^2), the area of foci per liver (mm^2/cm^2) and the volume of foci per liver (v/v%). Treatment with NDEA followed by 25 μg/kg bw nodularin (20 rats) induced 106±22.6 foci per liver ($P < 0.005$), 39.87±10.51 mm^2/cm^2 foci per liver ($P < 0.005$) and 71.75±18.78% volume of foci per liver ($P < 0.005$) compared with 10.0±2.9 foci per liver, 0.18±0.07 mm^2/cm^2 foci per liver and 0.37±0.18% volume of foci per liver in NDEA alone-treated animals (20 rats). Treatment with NDEA followed by 10 μg/kg bw nodularin (19 rats) induced 0.25±0.09 mm^2/cm^2 foci per liver ($P < 0.025$). Treatment with 25 μg/kg bw nodularin alone (16 rats) induced GST-P foci at the same potency as NDEA alone (6.3±7.3 foci per liver, 0.49±0.89 mm^2/cm^2 foci per liver and 0.92±1.58% volume of foci per liver; statistically non-significant). The results suggest that nodularin is a stronger tumour promoter than microcystin-LR and has initiating activity equal to that of NDEA (Ohta *et al.*, 1994).

The tumour-promoting activity of nodularin was confirmed in groups of male Fischer 344 rats [initial number unspecified], 7 weeks of age, that received a single intraperitoneal injection of 0 or 200 mg/kg bw NDEA in saline and received intraperitoneal injections 2 weeks later of 0 or 25 μg/kg bw nodularin twice a week for 10 weeks. Animals were maintained up to experimental week 22 but were killed periodically. Results are presented in Table 3.4. GST-P foci decreased significantly after the cessation of intraperitoneal injections of nodularin. Following treatment with NDEA plus nodularin, GST-P foci displayed two types of hyperplastic nodules: homogenously stained dense nodules and heterogeneously stained pale nodules. The results suggest that nodularin is a promotor that induces hepatocyte proliferation (Song *et al.*, 1999).

Table 3.4. Induction of liver GST-P foci by nodularin in rats initiated with NDEA

Duration of the experiment	GST-P foci (no./cm^3)[a]			
	Nodularin only	NDEA only	NDEA + nodularin	
			Dense nodules	Pale nodules
Untreated, 8 weeks	0	0	0	0
8 weeks	6.4±2.0	11.4±2.0	71.0±43.0	0
10 weeks	6.8±2.0	10.0±2.1	87.8±16.2	0
12 weeks	8.5±2.4	9.5±2.0	124.5±38.3	0
15 weeks	7.5±1.8	6.9±0.9	83.7±6.8	14.5±2.9
18 weeks	5.2±1.0	6.4±1.8	60.0±11.4	13.2±4.5
22 weeks	7.2±3.6	7.6±2.2	59.4±17.8	18.0±4.5

From Song *et al.* (1999)
GST-P, glutathione *S*-transferase placental form-positive; NDEA, *N*-nitrosodiethylamine
[a] Mean±standard deviation

3.4 References

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4. Mechanistic and Other Relevant Data

A number of reviews that deal with various aspects of the toxicity of microcystins and nodularin have been published (Metcalf & Codd, 2004; Falconer & Humpage, 2005; Dietrich & Hoeger, 2005).

Over 70 variants of microcystins have been identified; most studies have used microcystin-LR, -RR, -YR and -YM, for which toxicity data are summarized in Section 4.4 (Gupta *et al.,* 2003).

Most work on nodularin has been carried out with the arginine pentapeptide, although the best structural work has been performed with motuporin (a more hydrophobic congener in which Arg is replaced by Val) (Bagu *et al*., 1995; Goldberg *et al*., 1995; Maynes *et al*., 2006).

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

There are only two reports in which the absorption of microcystin was documented by direct measurement of its association to tissues. Both reports referred to the same accident that occurred in Brazil, in patients who underwent haemodialysis in 1996 (see Section 4.4) (Pouria *et al.*, 1998; Carmichael *et al.*, 2001).

There are really no quantitative data on tissue distribution, metabolism or excretion in humans. A common finding in agreement with most animal studies is the accumulation and persistence of microcystins in the liver (Azevedo *et al*., 2002; Soares *et al.,* 2006). Although concentrated in the liver, microcystin was also detected in the sera of patients up to 2 months or longer after exposure (Hilborn *et al.*, 2005; Soares *et al.*, 2006).

Little is known about the metabolism of microcystins; indirectly, it probably occurs but nothing is known about any intermediates or products. The finding of unidentified peaks by mass spectrometry analysis could represent metabolic products of microcystins (Soares *et al.*, 2006).

The mode and extent of excretion in humans is not known either. It is unlikely to occur through the kidneys; many animal studies have shown that secretion in urine, when it occurs, is very limited.

There are no published records that directly and unequivocally link absorption of nodularin and its toxicity in humans. Many different animals are susceptible to the toxicity of nodularin (see Section 4.1.2); it is therefore most probably toxic in humans and this toxicity may be similar to that found in animals. In addition, nodularin shares some properties with the microcystins. Since microcystins have caused death in haemodialysis patients exposed to microcystin-contaminated water (Jochimsen *et al.*, 1998), nodularin is probably toxic to humans.

In a recent publication, Fischer *et al.* (2005) showed uptake of radiolabelled [^{3}H]dihydromicrocystin-LR in frog (*Xenopus laevis*) oocytes that express the human organic anion transporters polypeptides (OATPs) OATP1B1, OATP1B3 and OATP1A2, whereas no uptake was obtained in cells that express OATP2B1. All of these transporters are found in the liver and brain. [From calculations, the radioactivity taken up was very small] (Fischer *et al.* 2005). [If this pattern of differential species-specific uptake holds, then uptake of microcystin by the brain would be a property of this toxin that is peculiar to humans. This needs to be investigated in order to validate extrapolations between experimental models and humans.]

The uptake of nodularin has not been investigated (Fischer *et al.*, 2005).

4.1.2 *Experimental systems*

(a) Absorption and distribution

(i) *Whole animals*

Microcystins

Administration of microcystins to many species including mice, rats, cattle, sheep, swine and fish results in liver toxicity (Jackson *et al*., 1984; Falconer *et al*., 1986; Runnegar *et al*., 1986; Brooks & Codd, 1987; Galey *et al*., 1987; Robinson *et al.*, 1989, 1991; Williams *et al.,* 1995; Stotts *et al.*, 1997a,b; Tencalla & Dietrich, 1997). Most studies to investigate the uptake and tissue distribution of microcystins have used the intraperitoneal or intravenous routes, although a few have used oral administration.

Numerous studies used radiolabelled peptides but, depending on the radioactive labelling method used for microcystins or nodularin, the results may vary since the labelled products obtained may present different characteristics with both advantages and disadvantages (Table 4.1). A disadvantage of using different preparations with widely varying specific activities is that it is difficult to deduce whether or not variations between studies result in part from the choice of the labelled toxin. On the positive side, it reinforces any common findings that result from experiments that used these differently labelled tools.

Kinetic studies on the absorption and distribution of microcystin are described in Table 4.2.

In studies that used intravenous or intraperitoneal administration in different experimental models, with different protocols and variously labelled microcystins, the one common finding in almost all of them was the accumulation of microcystin in the liver and low concentrations in the kidney in animals as diverse as salmon, pigs and rodents. The intestinal concentration of microcystin was much more varied and probably reflects the different dosing and degrees of metabolism and secretion.

Not unexpectedly, the oral dose required to induce microcystin toxicity is higher than the intravenous or intraperitoneal dose. In most oral studies, microcystin is found in the liver. The pathology following oral administration of microcystin parallels broadly that

Table 4.1. Advantages and disadvantages of the different radioactive labelling methods for microcystins (MC) and nodularin (NOD)

Isotope	Toxin	Method	Advantages	Disadvantages	Specific activity (mCi/mmol)	Reference
^{14}C	MC-LR	Biosynthesis with $^{14}[C]NaHCO_3$	• Most stable labelled product • Structure closest to native peptide	• Time consuming labelling method • Low specific activity of the labelled product	2.6 1.38 > 0.9 0.08	Brooks & Codd (1987) Williams *et al.* (1997) Craig *et al.* (1996) Pflugmacher *et al.* (1998)
^{3}H	MC-LA MC-LR	Exchange with $^{3}H_2O$	• The two isomers obtained retain toxicity of the native peptide	• Dihydromicrocystins unable to form covalent bond to PP1 and PP2A • Potential differences of interaction, stability and metabolic properties with the native peptide	NR 194	Botes *et al.* (1984) Robinson *et al.* (1989)
^{3}H	MC-LR	Reduction with $^{3}[H]NaBH_4$	• The two isomers obtained retain toxicity of the native peptide	• Dihydromicrocystins unable to form covalent bond to PP1 and PP2A • Potential differences of interaction, stability and metabolic properties with the native peptide	170–310 22 700 32.6 1039 247	Meriluoto *et al.* (1990) Nishiwaki *et al.* (1994) Williams *et al.* (1995) Stotts *et al.* (1997a,b) Bury *et al.* (1998)

Table 4.1 (contd)

Isotope	Toxin	Method	Advantages	Disadvantages	Specific activity (mCi/mmol)	Reference
^{125}I	MC-YM MC-YM MC-YR	$Na^{125}I$ with lactoperoxidase/H_2O_2 $Na^{125}I$ with lactoperoxidase/H_2O_2 Iodogen	• Easy labelling procedure • High specific activity • Retain toxicity of the native peptide	• Limited to variants that contain tyrosine	NR NR NR	Falconer *et al.* (1986) Runnegar *et al.* (1986) Moorhead *et al.* (1994)
^{3}H	NOD	Reduction with $^{3}[H]NaBH_4$	• Retain toxicity of the native peptide		669–678	Spoof *et al.* (2003)

Adapted from Spoof *et al.* (2003)
H_2O_2, hydrogen peroxide; MC, microcystin; $NaBH_4$, sodium borohydride; $NaHCO_3$, sodium bicarbonate; NOD, nodularin; NR, not reported; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A

Table 4.2. Absorption and distribution of microcystins and nodularin

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Microcystin *Mammals*									
							µg/kg tissue		Stotts *et al.*
Female Landrace cross specific pathogen-free swine (18–24 kg)	3 per dose	[^{3}H]Dihydro-MC-LR	iv; 25 µg/kg bw	4 h	Liver	64.6	633	3.7, 134 min[a]	(1997a,b)
					Kidneys	1.2	121		
					Lungs	1.75	62		
					Heart	0.22	17		
					Ileum	0.13	11		
					Spleen	0.04	9		
					Bile	4 (n = 1)			
			iv; 75 µg/kg bw	4 h	Liver	46.99	1110	3, 270 min[a]	
					Kidneys	2.19	654		
					Lungs	0.55	59		
					Heart	0.23	54		
					Ileum	0.20	57		
					Spleen	0.07	41		
					Bile	5.9 (n = 1)			
			Ileal loop; 75 µg/kg bw	5 h	Liver	49.6	1408		
					Kidneys	1.04	31		
					Lungs	0.65	69		
					Heart	0.81	19		
					Ileum	33.94	9165		
					Spleen	0.16	94		
					Bile	5.26 (n = 2)			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Female albino rats (207–249 g)	10 (5 per time interval)	[^{125}I]MC-YM	iv; ~10 μg/kg bw	30 min	Liver Gut contents Kidney	21.7±1.1 7.0±0.3 5.6±0.2 0.9±0.5		2.1, 42 min[a]	Falconer *et al.* (1986)
				120 min	Urine (total) Liver Gut contents Kidney Urine (total)	19.2±0.3 9.4±1.1 5.3±0.4 1.9±0.2			
Male Swiss Webster mice (25–37 g)	25 (1–3 per dose)	[^{125}I]MC-YM mono- and di-iodinated peptides, results here for [^{125}I]MC-YM similar to [^{125}I]$_2$MC-YM	ip; ~80–240[b] μg/kg bw	At death (2–15 h) (lethal dose)	Liver Kidney	43–63 1–2			Runnegar *et al.* (1986)
				(24 h) (sublethal dose)	Liver Kidney	24–57 0.5–3			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Male Han:NMRI mice (30±2 g)	4	[^{3}H]dihydroMC-LR epimers	iv; 15 µg/kg bw (0.5 µg/mouse)	45 min			**Relative concentration in counts (min × mass unit) plasma = 1**		Meriluoto *et al.* (1990)
					Liver		35		
					Intestine		7		
					Kidney		5		
					Spleen		1.5		
					Muscle		1		
					Brain		1		
					Plasma		1		
Male VAF/plus CD-1 mice (19–25 g)	5 per time interval	[^{3}H]MC-LR	ip; 45–101 µg/kg bw	At death or 6 h (surviving mice)	Liver	>50			Robinson *et al.* (1989)
					Intestine	~10			
					Carcass	~10			
					Kidney	~1			
					Heart, spleen, lung, skeletal muscle	All <1			
		[^{3}H]MC-LR	ip; 70 µg/kg bw	60 min	Liver	60		29 min	
					Kidney	1			
					Intestine	9			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Male VAF/plus CD-1 mice (20–27 g)	6	[^{3}H]MC-LR	iv; 35 µg/kg bw	1 min	Liver	23±5			Robinson *et al.* (1991)
					Kidney	2.0±0.2			
					Intestine	5.2±0.9			
					Carcass	30±3			
					Plasma	25±4			
				60 min	Liver	67±4			
					Kidney	0.8±0.1			
					Intestine	8.6±0.7			
					Carcass	6±2			
					Plasma	Trace			
Female ICR mice	2–3 mice per time interval	[^{3}H]dihydroMC-LR	ip; ~80 mg/kg bw[c]	5 min	Liver	17±4.1			Nishiwaki *et al.* (1994)
				15 min		38.0±7.1			
				30 min		57.3±4.1			
				60 min		71.5±6.9			
				60 min	Small intestine	1.4			
					Large intestine	0.5			
					Kidney	0.5			
					Gall bladder	0.5			
					Lungs	0.4			
					Stomach	0.3			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose		Concentration of MC in tissue	Half-life	Reference
Female ICR mice	NR	[^{3}H]dihydroMC-LR	Oral; ~70 mg/kg bw[e]	6 h 6 days 6 h	Liver Liver Small intestine Large intestine Caecum Kidney Stomach Brain	0.68 0.41 0.2 0.2 0.15 0.05 0.05 0.01				Nishiwaki *et al.* (1994) (contd)
Male ICR mice (20–27 g)	2 per time interval	(1) [^{3}H]dihydroMC-LR and (2) ELISA with polyclonal anti-MC antibody	ip; 35 µg/kg bw			**[^{3}H]MC-LR %**	**ELISA %**			Lin & Chu (1994)
				15 min	Liver	16.3	16			
					Serum	5.3	5.7			
				12 h	Liver	89	89			
					Serum	23	23			
				24 h	Liver	71	71			
					Serum	15	21.5			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Fish									
Atlantic salmon (80–160 g)	4 per time interval, only 1 at 46 h	[^{3}H]dihydroMC-LR	ip; 1 mg/kg bw				**µg/g tissue**		Williams *et al.* (1995)
				2 h	Liver	2.8±0.4	3.5		
				5 h		4.9±0.4	5.3		
				22 h		4.2±0.6	4.4		
				46 h		2.4	2.5		
				2 h	Muscle		0.1 or less		
				5 h	Muscle		0.1 or less		
				22 h	Muscle		0.3		
				46 h	Muscle		0.1 or less		
Atlantic salmon (100–130 g)	4 per time interval	[^{14}C]MC-LR	ip; 1 mg/kg bw				**µg/g tissue**		Williams *et al.* (1997)
				2 h	Liver	11.93±0.17	9.29±0.06		
				5 h		16.55±0.85	9.84±0.56		
				24 h		6.53±0.54	4.35±0.34		
				43.5 h		2.70±0.31	2.15±0.24		
				2 h	Muscle		0.15±0.02		
				5 h			0.26±0.04		
				24 h			NR		
				43.5 h			0.24±0.04		
				2 h	Carcass	40.7±3.5	0.43±0.04		
				5 h		39.3±3.0	0.42±0.03		
				24 h		13.2±1.5	0.14±0.02		
				43.5 h		6.1±0.7	0.07±0.01		

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Rainbow trout (60 g)	3 per time interval	(1) PP activity inhibition and (2) extractable MC-LR by PP activity inhibition	Gavage; freeze-dried cyanobacteria equivalent to 5700 µg/kg bw MC-LR*; dose toxic at 96 h			**PP inhibition (% of control)**	**Extractable MC-LR (ng/g liver, ng/mL plasma)**	3.3 h	Tencalla & Dietrich (1997); Fischer *et al*. (2000)
				1 h	Liver	60	144		
					Plasma		24		
				3 h	Liver	4	524		
					Plasma		520		
				12 h	Liver	20	226		
					Plasma		180		
				24 h	Liver	42	51		
					Plasma		62		
				48 h	Liver	41	40		
					Plasma		0		
				72 h	Liver	52	44		
					Plasma		0		

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Little skate (*Raja erinacea*) (0.6–1 kg)	2–3 skate per dose	(1) PP activity inhibition and (2) free MC-YM in plasma by PP activity inhibition				**% of dose/PP inhibition (% of control)[d]**			Runnegar *et al.* (1999)
			iv; 125 μg/kg bw	24 h	Liver	NR/93			
					Plasma	8.6/NR			
				48 h	Liver	NR/93			
				72 h	Liver	NR/93			
				7 days	Liver	NR/93			
			iv; 250 μg/kg bw	24 h	Liver	NR/93			
					Plasma	9.6/NR			
				48 h	Liver	NR/93			
				72 hr	Liver	NR/93			
				7 days	Liver	NR/95			
			iv; 500 μg/kg bw	24 h	Liver	NR/98			
					Plasma	10.2/NR			
				48 h	Liver	NR/94			
				72 h	Liver	NR/87			
				7 days	Liver	NR/99			
			iv; 32 μg/kg bw	24 h	Liver	NR/10			
			iv; 63 μg/kg bw		Plasma	2.45/NR			
				24 h	Liver	11/89			
					Plasma	4.2/NR			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Nodularin									
BALB/c mice (20–25 g)	8	[^{3}H]dihydro-NOD	iv; 20 µg/kg bw	2 h			**cpm/mg**		Spoof *et al.* (2003)
					Liver	[1.6][e]	~15		
					Blood	[0.5]	~5		
					Intestine	[0.5]	~5		
					Kidney	[0.1]	~1		
					Spleen		trace		
					Bone		trace		
					Brain		trace		
					Muscle		trace		
					Lung		trace		

ELISA, enzyme-linked immunosorbent assay; ip, intraperitoneal; iv, intravenous; LD_{50},dose that is lethal to 50 % of animals; MC, microcystin; MC-LR*: corresponds to a conformational variant of microcystin-LR which is equally toxic; NOD, nodularin; NR, not reported; PP, protein phosphatase

[a] Biphasic disappearance curve of blood radioactivity

[b] Note: the LD_{50} of the peptide was 110 µg/kg for male mice in this study

[c] The doses given in the original article were: 2.4 µmol/mouse [~80 mg/ kg bw] and 2.1 µmol/mouse [~70 mg/ kg bw]. The Working Group noted that these reported doses are in great excess of the LD_{50} of [^{3}H]dihydromicrocystin-LR for mice (~110 µg/ kg bw). It is reasonable to deduce that the unit was misreported and that the real doses were 1000 times lower.

[d] > 90% inhibition of PP activity considered to be fully inhibited

[e] % of dose calculated by the Working Group

found after intravenous or intraperitoneal administration, although the amount of microcystin or microcystin-containing bloom that is needed for oral toxicity varies with species and on whether or not the animal is fasted. For fasted mice, the ratio between the oral and intraperitoneal dose required is ~30; for sheep, it is ~150; and for chickens, it is ~125 (Jackson *et al.*, 1984). In BALB/c mice, Yoshida *et al.* (1997) found that the intraperitoneal LD_{50} (dose that is lethal to 50% of animals) of microcystin-LR was 65.4 μg/kg bw and the oral LD_{50} was 10.9 mg/kg bw. In many of these studies, uptake of microcystin or microcystin-containing bloom was inferred by the resulting toxicity. The accumulation of microcystin in the liver can be very fast, within minutes after injection as was shown in mice (Robinson *et al.*, 1991; Nishiwaki *et al.*, 1994; Lin & Chu, 1994) and can last several hours and even several days as shown in salmon (Williams *et al.*, 1995, 1997).

Nodularin

A number of studies have demonstrated the toxicity of nodularin and, by inference, its absorption, both in field cases and in laboratory animals. In field cases, nodularin is taken up as cyanobacterial bloom that contains toxic strains of *Nodularia*. Laboratory animals are most frequently administered purified nodularin. Field cases attributed to nodularin poisoning include the deaths of a dog in South Africa (Harding *et al.*, 1995), sea birds in the Baltic Sea (Sipiä *et al*., 2004) and sheep in Australia (Main *et al.*, 1977). Laboratory animals tested include mice (Carmichael *et al.*, 1988; Eriksson *et al.*, 1988; Runnegar *et al.*, 1988), guinea-pigs and sheep (Main *et al.*, 1977). The primary target organ was the liver.

There is only one study in mammals of the distribution of nodularin (Spoof *et al.*, 2003; Table 4.2). Two hours after intravenous injection of a non-toxic dose of [^{3}H]dihydronodularin isomers (20 μg/kg bw) into mice, the toxin (and possibly its metabolites) was found concentrated in the liver. Intestine and blood also retained significant amounts of the labelled compound. [The Working Group noted that the experimental details were not sufficiently clear, the reported total activity was low and only a single time point was reported.]

Other studies have investigated the effect of nodularin in aquatic animals: from sea-ducks to zooplankton (Sipiä *et al.*, 2001a,b; Kankaanpää *et al.*, 2002; Sipiä *et al.*, 2002; Kankaanpää & Sipiä, 2003; Lehtonen *et al.*, 2003; Sipiä *et al.*, 2004; Kankaanpää *et al.*, 2005a,b). Adsorption and distribution in aquatic species are reported here (Table 4.3) because of the potential for bioaccumulation that would result in contaminated food sources. In fish from the Baltic Sea (such as flounders and cod), nodularin has also been reported to accumulate mainly in the liver. In flounders, the concentration of toxin was shown to increase strongly between May and August during the blooming period of *Nodularia*. However, when measured, none or only trace amounts of nodularin were detected in the muscle of fish even when the concentration in the liver was significant (Sipiä *et al.*, 2001a,b, 2002; Karlsson *et al.*, 2003).

Table 4.3. Detection and distribution of nodularin in aquatic animals

Species	Origin	Method of detection	Tissue	Quantitation	Reference
Flounders (*Platichthys flesus*)	Baltic Sea, 1995	LC–MS MALDI-TOF-MS	Liver	100–600 µg/kg wet wt	Karlsson *et al.* (2003)
Eiders (*Somateria mollissima*)	Baltic Sea, 2002	ELISA LC–MS	Liver	3–180 µg/kg dry wt	Sipiä *et al.* (2004)
Flounders (*Platichthys flesus*)	Baltic Sea, 1996–98	ELISA, HPLC, PP1 inhibition	Liver Muscle	25–140 µg/kg dry wt None detected	Sipiä *et al.* (2001a)
Cod (*Gadus morhua*)	Baltic Sea, 1996–98	ELISA, HPLC, PP1 inhibition	Liver Muscle	~55 µg/kg dry wt None detected	Sipiä *et al.* (2001a)
Flounders (*Platichthys flesus*)	Baltic Sea, 1999–2000	ELISA LC–MS MALDI-TOF-MS	Liver Muscle	Up to 400 µg/kg None detected	Sipiä *et al.* (2002)
Clams (*Macoma baltica*)	Baltic Sea, 2000	ELISA LC–MS MALDI-TOF-MS		100–130 µg/kg dry wt (one site, 1490 µg/kg)	Sipiä *et al.* (2002)
Mussels (*Mytilus edulis*)	Baltic Sea, 1999–2000	ELISA LC–MS MALDI-TOF-MS		40–130 µg/kg dry wt (one site, 1490 µg/kg)	Sipiä *et al.* (2002)

ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PP, protein phosphatase

Clams and mussels can also accumulate nodularin. Blue mussels collected from the northern Baltic Sea in 2000 contained about 40–130 µg/kg dry wt and, in the Gulf of Finland, toxin concentrations were up to 1490 µg/kg dry wt. Total hepatotoxin levels in mussels decreased from August to September, indicating at least partial detoxification/depuration of the toxin. However, in deeper-living wreck mussels, hepatotoxin levels continued to increase from August to September, indicating that portions of cyanobacterial hepatotoxins reach the sea floor (Sipiä *et al.*, 2002).

(ii) *In-vitro absorption*

Many studies have investigated the uptake of microcystins in isolated hepatocytes and its consequences. Most frequently, the source of hepatocytes is rats but cells from any other species would be expected to respond to microcystin in qualitatively the same way. It has been known for more than 20 years that uptake in hepatocytes is carrier-mediated, dose-dependent and saturable and that it can be inhibited (Runnegar *et al.*, 1981; Eriksson *et al.*, 1990a). Other cells (even non-parenchymal liver cells) are much less sensitive to the toxicity of microcystin and require much higher doses and longer incubation times for toxicity (Runnegar *et al.*, 1995a). [This finding of preferential uptake of microcystins in

hepatocytes may explain why the liver is the primary target of its accumulation and toxicity.]

(iii) *In-vitro and in-vivo inhibition of uptake*

A number of earlier studies on the toxicity of microcystin and/or *Microcystis* described the partial protection of the co-administration of a number of compounds. Runnegar *et al.* (1981) showed that at least some of these compounds protect rat hepatocytes *in vitro*: sodium cholate, deoxycholate, rifampicin (rifamycin SV) and bromosulfophthalein all inhibited the cytoskeletal changes (blebbing of the cells) induced by microcystin. With the introduction of the use of radiolabelled microcystins, this protection in rat hepatocytes was shown to result from inhibition of uptake (Runnegar *et al.*, 1991, 1995b). Runnegar *et al.* (1999) found a similar pattern of protection in skate hepatocytes which indicated the related nature of transporters in the uptake of microcystins in these very divergent species.

Fischer *et al.* (2005) recently expressed the human OATP family that catalyses the uptake of the cell-impermeable microcystins in *Xenopus laevis* oocytes (Section 4.1.1). The activity of these transporters is inhibited by bile acids and dyes which concords with the findings in hepatocytes by Runnegar *et al.* (1981). These transporters are present in the brain, as well as the liver, and may mediate uptake of microcystins across the blood–brain barrier, although this has not yet been demonstrated experimentally. However, some data suggest the possible transfer of microcystins across the blood–brain barrier in mice (Falconer *et al.*, 1988; Maidana *et al.*, 2006).

Hermansky *et al.* (1990) showed that 25 mg/kg bw rifampin given intraperitoneally before 100 µg/kg bw microcystin-LR protected mice from microcystin-LR toxicity. Runnegar *et al.* (1993) confirmed this in mice dosed intraperitoneally with 84 µg/kg bw microcystin-YM. This dose, when injected alone, inhibited protein phosphatase (PP) activity and [^{125}I]microcystin-YM accumulated in the liver of mice within 15 min of treatment. By 30 min, the liver had significantly increased in weight because of haemorrhage. Intraperitoneal injection of the antibiotic rifamycin (5 µmol/mouse) 5 min before microcystin-YM prevented the uptake of [^{125}I]microcystin-YM, the inhibition of PP activity and toxicity (increase in liver weight). This protection was still seen 24 h after treatment. Injection of rifampin (Hermansky *et al.*, 1990) or rifamycin (Runnegar *et al.*, 1993) after treatment with microcystins did not protect against toxicity.

No studies have reported factors that may inhibit uptake of nodularin.

(b) Metabolism

The proposed pathway for excretion of both microcystins and nodularin is conjugation to the thiol of glutathione (GSH), which may be excreted as such or processed to the γ-glutamyl cysteine conjugate and finally to the cysteine conjugate and then excreted.

(i) *Microcystins*

The metabolic reactions of microcystins have been shown to occur enzymatically *in vitro* with cell extracts from many sources (see Pflugmacher *et al.*, 1998). Formation of a GSH conjugate is the most probable pathway *in vivo* in view of the observed changes in GSH peroxidase and GST activities following treatment with microcystins in mice and *in vitro* (Pflugmacher *et al.*, 1998; Gehringer *et al.,* 2004).

Only one report identifies microcystin–GSH and –cysteine adducts *in vivo* following treatment with microcystin. Kondo *et al.* (1996) identified an HPLC peak that corresponded to a microcystin–GSH standard (chemically synthesized) as a microcystin-RR–GSH adduct. Nucleophilic reaction of the thiol group of GSH with the α-β unsaturated carbonyl of the methyl dehydroalanine moiety of microcystin results in the microcystin–GSH adduct. *In vivo*, the reaction would be catalysed by liver GSTs. Three hours after treatment, this HPLC metabolite constituted a small, unspecified percentage of the dose in mice injected intraperitoneally with microcystin-RR (20 μg/mouse, equivalent to about 600 μg/kg bw). Another peak was identified as the microcystin-LR–cysteine conjugate in the liver cytosol of rats 24 h after injection with 4 μg microcystin-LR/rat. Experimentally, the authors stated that results were at the limit of detection in a concomitant liquid chromatography–mass spectrometry analysis. A number of other unidentified metabolites that were more hydrophilic than microcystin-RR were also formed as reported previously for microcystin-LR (Robinson *et al.*, 1991).

The major mechanism that explains the high toxicity of microcystins is the ability of these molecules to bind covalently to and inhibit the Ser/Thr protein phosphatases 1 and 2A (see below). The bond between microcystins and protein phosphatases is very stable and influences metabolism (Runnegar *et al.*, 1995c).

The metabolism of microcystins would result from a balance between the two types of reactions described above.

Microcystin-LR, -YR and -RR–GSH adducts have been shown to retain some toxicity *in vivo*. Microcystin-LR–GSH retained 6% of the toxicity of the parent compound when injected intravenously into mice, while microcystin-LR–cysteine retained 14% of the toxicity (Kondo *et al.*, 1992). This indicates that GSH and cysteine adducts might themselves inhibit PP activity. [However, the possibility that the adducts convert back in part to the native microcystin *in vivo* and that this native toxin is the cause of toxicity was not considered. Whether the GST can catalyse the conjugation of GSH to microcystin when it is associated (non-covalently) with PPs has not been investigated either. The inhibitory binding between most PP inhibitors, including microcystins, is very tight and renders the interaction kinetically nearly irreversible (Takai *et al*., 1995).]

[In addition to GSH conjugation, many other factors may influence metabolism. The methyl dehydroalanine moiety could react with cytochrome P450 enzymes to form more soluble metabolites that retain, lack or have increased toxicity. Other possibilities include glucoronidation and compartmentation within the cell that could lead to the sequestration of some of the toxin. Decreased or increased activity or membrane localization of uptake and/or export of microcystin and its metabolites that may result from changes in

phosphorylation could also impact metabolism. Even catabolism of PP could lead to increases in active toxin within the cell. Metabolism of microcystin by endogenous enzymes and/or intestinal flora is a further possibility.]

(ii) *Nodularin*

Nodularin readily forms adducts with GSH. As for microcystins, it has not yet been fully demonstrated that the enzymatically formed GSH conjugate pathway applies to the metabolism and detoxication of nodularin. GSTs catalyse the conjugation of nodularin at the *N*-methyl dehydrobutyric residue to the thiol of GSH. These adducts can then be excreted as such or processed to the γ-glutamyl cysteine conjugate and finally to the cysteine conjugate. To date, the only evidence of this pathway of nodularin metabolism has been in aquatic animals.

In-vitro assays using the substrate 1-chloro-2,4-dichlorobenzene (Beattie *et al.*, 2003) showed that extracts from cysts, nauplii and adult brine shrimp (*Artemia salina*) contained significant amounts of GST. This GST activity was inhibited when tissue extracts from nodularin-treated *A. salina* were added to the assay. The partially purified GSTs catalysed the formation of nodularin–GSH efficiently. Feeding a culture of algae that contained nodularin to Australian black tiger prawns resulted in a change in the classes and distribution of GST enzymes (Pflugmacher *et al.*, 2005)

Few studies have reported the occurrence of nodularin–GSH adducts in tissue from animals exposed to nodularin. Sipiä *et al.* (2002) showed by mass spectrometry the presence of adduct in mussels. This is the only report that identified nodularin–GSH in animals exposed to nodularin. [Although no quantitation was given, by comparison of peaks in the measurements of the soft tissue of mussels, it is possible to estimate that they represent 20–30% of the native nodularin.] Other, unidentified nodularin-like compounds have been found (Lehtonen *et al.*, 2003) in clams (*Macoma baltica*).

[On balance, the metabolism of microcystin and nodularin is probably, at least partially, catalysed by hepatic GSTs. Because of the varied specificities and activities of GSTs between species and between individuals, the consistency in the response to microcystin and nodularin is remarkable, and similar toxicities are found across species as diverse as fish, pigs and mice.]

(c) Excretion

There is some evidence of biliary excretion of microcystin and its metabolites (Sahin *et al.*, 1996). In trout orally dosed with *M. aeruginosa* bloom containing microcystin-LR (equivalent to 4.6 mg microcystin-LR), free microcystin or its metabolites that retained the ability to inhibit PP1 were found in the bile by the PP inhibition assay. Cumulative excretion was not reported, but maximal inhibition was exerted by two samples 3 h after gavage, the concentration that inhibited PP was equivalent to 3.5 mg/mL of bile; another sample contained 475 ng/mL 48 h after gavage. Enterohepatic circulation could lead to the cycling of microcystin and its metabolites between the bile, intestine and liver.

It is not clear what role biliary excretion would play in acute microcystin intoxication. Studies in perfused rat liver (Pace *et al.*, 1991; Runnegar *et al.*,1995a) showed that bile formation decreased shortly after exposure of the liver to a toxic dose of microcystin. Bile flow was reduced by 50% within 15 min of perfusion; the amount of bile produced between 45 and 60 min was less than 5% of that in control livers treated in exactly the same way and, by 60 min, it had stopped completely (Runnegar *et al.*, 1995a). Pace *et al.* (1991) calculated that, after 60 min of perfusion, 1.7% of [^{3}H]microcystin-LR was found in the bile while liver-associated radiolabel was 16.8% of the total. The cessation of bile flow paralleled liver damage. With non-toxic doses of microcystin, excretion through the bile may play a significant role.

No radioactivity was detected in the urine of pigs that had been dosed intravenously or into the ileal loop with [^{3}H]dihydromicrocystin-LR and killed 4–5 h after injection (Stotts *et al.*, 1997b).

Mice treated intravenously with 35 μg/kg bw [^{3}H]microcystin-LR had excreted 9.2% of the dose within 6 days; most of the 9.2% was excreted within 24 h of treatment (only 6% at 6 h) (Robinson *et al.*, 1991). In the same study, radioactivity in the faeces of mice was maximal 6 h after injection and cumulative faecal excretion after 6 days was 14.5% of the dose. The parent toxin constituted about 60% of the 23.7% of the dose excreted in faeces and urine. A number of other more hydrophilic peaks constituted the remaining 35–40%.

From the above results, it can be concluded that microcystins are primarily excreted in the faeces as the parent compound or as yet unidentified hydrophilic metabolites.

Orr *et al.* (2001) gave drinking-water containing *Microcystis* cultures to four lactating Holstein-Friesian dairy cattle. The cattle consumed a total of 15 mg (1.21 μg/kg bw per day) microcystin over a period of 21 or 28 days. The milk produced was analysed by ELISA and HPLC and no microcystin was detected (detection limit, 2 ng/L of milk).

No data were available on the excretion of nodularin. The presence or absence of nodularin or its metabolites has not been determined in faeces or urine after dosing.

4.2 Genetic and related effects

4.2.1 *Genotoxicity* (see Table 4.4 for details and references)

The genotoxic properties of microcystin-LR have been studied extensively in a variety of test systems and the results have been contradictory.

Microcystin-LR was not mutagenic in *Salmonella typhimurium* or in *Bacillus subtilis* in the multigene sporulation test in the presence or absence of exogenous metabolic activation.

No cell transformation was observed in microcystin-LR-exposed Syrian hamster embryo cells and no chromosomal aberrations were observed in Chinese hamster ovary K1 cells exposed to microcystin-LR, although an increased frequency of polyploid cells was reported, which suggests that it is aneugenic.

Table 4.4. Genetic and related effects of microcystins and nodularins

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Microcystin-LR				
Salmonella typhimurium TA98, TA100, reverse mutation	–	NT	10000	Grabow *et al*. (1982)
Salmonella typhimurium TA98, TA100,TA102, reverse mutation	–	–	900	Repavich *et al*. (1990)
Bacillus subtilis, multigene sporulation test	–	–	900	Repavich *et al*. (1990)
Chromosomal aberrations, Chinese hamster ovary-K1 cells *in vitro*	–	–	100	Lankoff *et al*. (2003)
Cell transformation, Syrian hamster embryo cells *in vitro*	–	NT	10	Wang *et al.* (1998)
Polyploidy, Chinese hamster ovary-K1 cells *in vitro*	+	+	25	Lankoff *et al*. (2003)
Comet assay, human lymphocytes *in vitro*	+[c]	NT	1	Lankoff *et al*. (2004)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	25	Lankoff *et al.* (2004)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	0.5	Lankoff *et al*. (2006a)
Nodularin				
Micronucleus assay, human hepatoma HepG2 cells *in vitro*	+	–	2.5	Lankoff *et al.* (2006b)

[a] +, positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, μg/mL
[c] Complementary results revealed negative clastogenic properties

Microcystin-LR gave positive results in the comet assay in human cells, which suggests that it may be genotoxic. However, complementary experiments with a modified comet assay in human hepatoma HepG2 cells revealed an accumulation of unrepaired DNA strand breaks which indicates that DNA fragmentation reflected the intermediates of the cellular repair of oxidized purines and pyrimidines resulting from the action of reactive oxygen species. This suggests that microcystin-LR might induce formation of reactive oxygen species that cause DNA damage rather than have a direct clastogenic effect (Zegura *et al.*, 2003, 2004). [Due to inappropriate experimental design and statistical evaluation of the data, and because HepG2 cells do not, or very weakly, express the transporters necessary for microcystin uptake (Le Vee *et al.*, 2006), the Working Group raised serious doubt about the validity of the findings reported by Zegura *et al.* (2003, 2004).] However, a comparison of the positive results from the comet assay, the negative results from the chromosomal aberration assay and the positive results for apoptosis in human lymphocytes (Fladmark *et al.*, 1999) showed that the microcystin-LR-induced DNA damage observed in the comet assay might be related to apoptosis due to cytotoxicity rather than to genotoxicity (Lankoff *et al.*, 2004, 2006a).

[The Working Group raised doubts about the validity of positive results for the genotoxicity of microcystin-LR (Suzuki *et al.*, 1998; Ding *et al.*, 1999; Mankiewicz *et al.*, 2002; Maatouk *et al.*, 2004; Zhan *et al.*, 2004; Bouaïcha *et al.*, 2005) due to inappropriate experimental design, statistical evaluation and interpretation of the data.]

The aneugenic effect of nodularin was tested in a single study that reported a dose-dependent induction of centromere-positive micronuclei in HepG2 cells.

In conclusion, no evidence was provided for the mutagenic or clastogenic properties of microcystins or nodularins in non-mammalian or mammalian test systems. However, an increased frequency of polyploid cells as well as centromere-positive micronuclei were observed, which possibly suggests that both microcystins and nodularin are aneugenic.

4.3 Mechanisms associated with the tumour promotion of microcystin-LR and nodularin

4.3.1 *Relationship between structure and inhibition of protein phosphatases (PP)*

Although the structures of microcystin-LR and nodularin are not related to that of okadaic acid (Fujiki & Suganuma, 1993), Uemura and Hirata (1989) proposed that the okadaic acid molecule also has a cyclic structure with the formation of a hydrogen bond between the carbonyl group at C1 and the hydroxyl group at C24, which may provide evidence that microcystin-LR and nodularin act similarly to okadaic acid as inhibitors of PP1 and PP2A (Yoshizawa *et al*., 1990; see also MacKintosh *et al.*, 1990; Honkanen *et al*., 1991). Microcystin-LR and nodularin inhibited PP activity in a cytosolic fraction of mouse liver *in vitro*, with an IC_{50} (concentration that leads to 50% inhibition) of 1.6 nM for microcystin-LR and 0.7 nM for nodularin. In addition, both inhibited specific [^{3}H]okadaic acid binding to PP1 and PP2A in the cytosolic fraction of mouse liver, with

an IC_{50} of 1.3 nM for microcystin-LR and 2.3 nM for nodularin, and in the particulate fraction of mouse liver, with an IC_{50} of 11.0 nM for microcystin-LR and 8.0 nM for nodularin (Yoshizawa *et al*., 1990). Microcystin-LR and nodularin both show a planarity of peptide rings and similar relative spatial alignments of the ADDA and arginine side chains (Taylor *et al*., 1992). Furthermore, Quinn *et al*. (1993) used molecular modeling and identified common regions of microcystin-LR, okadaic acid and calyculin A. Moreover, the crystal structure of mammalian PP1 complexed with microcystin was determined by X-ray crystallography (Goldberg *et al.*, 1995; Maynes *et al.*, 2006). Microcystin was reported to bind to a PP1 catalytic subunit through the interaction with three distinct regions: the metal-binding site, the hydrophobic groove and the edge of the C-terminal groove near the active site. In contrast to microcystin-LR, Bagu *et al*. (1997) reported that nodularin does not bind covalently to PP1 or PP2A, and that microcystin-LR and motuporin (nodularin-V) are strikingly similar to okadaic acid and calyculin A. Maynes *et al.* (2006) recently elucidated that the crystal structures of dihydromicrocystin-LR or motuporin (nodularin-V) binds to human PP1c (γ isoform). Comparisons of the structures of the toxin:PP1 complexes explain why microcystins but not nodularins permanently modify PP by covalent binding to an active cysteine residue.

4.3.2 *Enzymatic inhibition of PP1 and PP2A*

Microcystin-LR strongly inhibited PP1 and PP2A with inhibition constant values below 0.1 nM, but inhibited PP2B 1000 times less potently (MacKintosh *et al*., 1990); microcystin-LR inhibits both PP1 and PP2A with similar potency, whereas nodularin and okadaic acid inhibit PP2A much more strongly than PP1 (Table 4.5; Honkanen *et al.*, 1991; Suganuma *et al*., 1992). Seven inhibitors of PP1, including microcystin-LL, -LV, -LM, -LF and -LZ (Z represents an unknown hydrophobic amino acid), were purified from blooms of *Microcystis aeruginosa* and inhibited PP1 with inhibitory concentration 50% (IC_{50}) values of 0.06–0.4 nM (Craig *et al*., 1993). However, the structure and functional relationships between 6*(E)*-ADDA microcystin-LR and -RR and 6*(Z)*-ADDA microcystin-LR and -RR were significantly different: the IC_{50} values of 6*(E)*-ADDA microcystin-LR and -RR for PP2A activity were 0.28 and 0.78 nM, respectively, whereas those of 6*(Z)*-ADDA microcystin-LR and -RR were 80 nM in both cases, indicating that 6*(Z)*-ADDA microcystins inhibited PP2A about 100 times more weakly than the parent microcystin-LR and -RR compounds (Nishiwaki-Matsushima *et al*., 1991). Runnegar *et al*. (1995c) raised antibodies specific for PP1 and PP2A and found that, in hepatocytes, microcystin forms secondary covalent bonds with the C-terminal of PP1 and PP2A catalytic subunits. This covalent binding of microcystin to PP1 and PP2A catalytic subunits was confirmed by Bagu *et al*. (1997). Microcystin-LR has been shown to inhibit nuclear protein phosphatases (Guzman *et al.*, 2003) and to be present in the nuclei (Guzman & Solter, 2002) of mouse hepatocytes following in-vivo administration.

Table 4.5. Inhibition of the protein phosphatases (PP) 1 and 2A compared with that of protein tyrosine phosphatase (PTP)

Agent	PP1 (IC_{50} nM)	PP2A (IC_{50} nM)	PTP (IC_{50} nM)	Reference
Microcystin-LR	0.1	0.10	>10 000	Suganuma *et al.* (1992)
Nodularin	1.8	0.027	NR	Honkanen *et al.* (1991)
Okadaic acid	3.4	0.07	>10 000	Suganuma *et al.* (1992)

NR, not reported; IC_{50}, concentrations that lead to 50% inhibition

4.3.3 *Cellular effects*

The biochemical consequence of the inhibition of PP1 and PP2A by microcystin-LR and nodularin is the accumulation of hyperphosphorylation of intracellular proteins in hepatocytes (Eriksson *et al.*, 1990b; Falconer & Yeung, 1992; Fujiki, 1992). These alterations in phosphorylation have been shown to result in major morphological changes in isolated human and rodent hepatocytes exposed to low (nM) concentrations of microcystins. The major consequence observed is the disaggregation of intermediate filaments composed of cytokeratins 8 and 18, which leads to the collapse of the cytoskeleton of the hepatocytes. This disaggregation secondarily results in detachment of the actin microfilament structure and contraction of the fibres. The characteristic blebbing of exposed hepatocytes is the result of this microfilament contraction in cells (Runnegar *et al.*, 1981; Falconer & Runnegar, 1987; Eriksson *et al.*, 1989; Yoshizawa *et al.*, 1990; Falconer & Yeung, 1992; Ohta *et al.*, 1994; Toivola *et al.*, 1997; Batista *et al.*, 2003).

At concentrations up to 9.6 μM, microcystin-YR did not induce any effects in human fibroblasts; it only induced morphological changes similar to those induced by okadaic acid following microinjection of concentrations of 670 μM (Matsushima *et al.*, 1990). This suggests that microcystin and nodularin do not easily penetrate into fibroblasts, and supports the finding that microcystin requires active uptake through OATPs (see Section 4.1). Similarly, the uptake of [^{3}H]dihydromicrocystin-LR was shown to be specific for freshly isolated rat hepatocytes, since its uptake in the human hepatocarcinoma HepG2 cell line, the human neuroblastoma SH-SY5Y cell line and the mouse fibroblast NIH/3T3 cell line was negligible (Eriksson *et al.*, 1990a).

4.3.4 *Apoptosis*

A number of studies have demonstrated that microcystins and nodularins cause apoptosis in cell cultures. At picomolar concentrations, microcystin-LR stimulated cytokinesis in primary mouse hepatocytes and reduced the rate of apoptosis, whereas

higher concentrations (nanomolar) inhibited cytokinesis and induced cell death (Humpage & Falconer, 1999).

Fladmark *et al.* (1999) reported that both microcystin-LR and nodularin induced caspase-3-dependent apoptosis in an ultra rapid manner in toxin-microinjected Swiss mice 3T3 fibroblasts, rat promyelotic (IPC-81) cells, normal rat kidney cells and human embryo kidney HEK 293 cells. It was also proposed that apoptosis induced by microcystins and nodularins correlates with PP inhibition and requires Ca^{2+}/calmodulin-dependent protein kinase II (Fladmark *et al.*, 2002).

The induction of apoptosis was also shown *in vitro* in microcystin-LR-treated rat hepatocytes and human lymphocytes (Mankiewicz *et al.*, 2001), Chinese hamster ovary K1 cells (Lankoff *et al.*, 2003) and colon carcinoma CaCo2 cells (Botha *et al.*, 2004) as well as in nodularin-treated primary rat hepatocytes (Herfindal *et al.*, 2005).

4.3.5 *Gene expression*

Sueoka *et al.* (1997) demonstrated that microcystin-LR and nodularin modulate the expression of oncogenes and tumour-suppressor genes and revealed a strong induction of tumour necrosis factor-α, and *c-jun*, *jun B, jun D*, *c-fos*, *fos B* and *fra-1* gene expression in primary cultured rat hepatocytes *in vitro*.

A two-stage carcinogenesis experiment that used initiation with NDEA and promotion with nodularin in Fischer 344 rats increased the incidence of liver preneoplastic foci. Increased transforming growth factor-β1 protein expression was seen to co-localize with GST-P expression in the preneoplastic foci (Lim *et al.*, 1999).

In summary, a number of studies have reported the involvement of microcystin-LR in epigenetic processes. These include stimulation of gene expression, cell survival/apoptosis and cell division. There is also evidence of the inhibitory effects of microcystin-LR on DNA repair (Lankoff *et al.*, 2004, 2006a). It was also found that cytokinesis was stimulated and the rate of cell division was increased by picomolar concentrations of microcystin-LR in primary mouse hepatocytes whereas nanomolar concentrations inhibited cytokinesis and induced apoptosis (Humpage & Falconer, 1999). These findings were supported by a combined transcriptomic and proteomic analysis with gene expression profiling in the liver of mice treated with microcystin-LR which demonstrated a modification of 61 of 96 apoptosis-related genes. At low concentrations, microcystin-LR increased the expression of the anti-apoptotic *Bcl-2* gene more that 4000-fold. At high concentrations of microcystin-LR, expression of the *Bcl-2* gene dropped markedly (Chen *et al.*, 2005).

4.4 Other relevant toxic effects

4.4.1 *Humans*

(a) Acute toxicity

The most relevant example of acute toxicity from cyanobacterial toxins was provided by exposure through renal dialysis. In Caruaru, Brazil, following renal dialysis during a single week in 1996, 116 of 131 dialysed patients developed disturbance of vision, vomiting, nausea, headache, muscle weakness and epigastric pain. Of these, 100 developed liver failure and 76 died. The deaths of 52 patients could be attributed directly to liver failure. Serum enzyme analysis showed an eightfold increase in aspartate transaminase (AST) and a fourfold increase in total bilirubin in patients with clinical symptoms (Jochimsen *et al*., 1998; Pouria *et al*., 1998; Carmichael *et al*., 2001). Examination of the cation filters in the dialysis unit showed the presence of microcystins-YR, -LR and -AR. The carbon filters also contained cylindrospermopsin; this compound is an alkaloid toxin from freshwater cyanobacteria, which is also responsible for human poisoning that results predominantly in liver damage, but through a different mechanism (Hawkins *et al*., 1985). Microcystins were present in the blood of patients at an average level of 2.2 ng/mL and in the liver at an average level of 223 ng/g. It was calculated that 19.5 μg/L microcystins were present in the dialysis water, and that the patients were probably exposed to 120–150 L (Carmichael *et al*., 2001).

Another report of human injury related to acute exposure to *M. aeruginosa* was among army recruits who carried out canoeing exercises in a lake that carried a heavy water-bloom of this cyanobacterium (Turner *et al*., 1990). The exercises involved swimming with a pack and also going underwater and coming back up while canoeing, both of which probably resulted in the inhalation and oral ingestion of water. Ten of the recruits reported symptoms of abdominal pain, nausea, vomiting, diarrhoea, sore throat, dry cough, blistering of the lips and mouth and headache. Two were hospitalized with pneumonia that was considered to be due to aspiration of bloom material. Serum enzymes indicative of liver damage, alanine transaminase (ALT) and aspartate transaminase (AST), were elevated in the most severe of the two cases investigated. Microcystin-LR was identified in the cyanobacterial bloom material from the lake (Turner *et al.*, 1990).

No data on nodularin were available to the Working Group.

(b) Subacute toxicity

Among the causes of human illness that have been associated with cyanotoxins in drinking-water sources, the following three show plausibility.

In Harare, Zimbabwe, a paediatrician noted that children whose homes were supplied with water from one reservoir developed seasonal acute gastroenteritis in the autumn, whereas children whose drinking-water came from other municipal reservoirs were unaffected. The reservoir that was the source of the water for the affected children had developed a summer water-bloom of *Microcystis*, which broke down in the early winter

at the time of the gastroenteritis. The paediatrician concluded that the lysed cyanobacteria in the water supply could be responsible for the illness (Zilberg, 1966).

A larger and more severe outbreak of gastroenteritis in children occurred in Brazil after a new hydroelectric dam was filled and was the source of drinking-water for several towns, including Paulo Alfonso that had a population of about 200 000 (Teixeira *et al*., 1993). Overall, approximately 2000 cases were recorded at the hospital, and 88 deaths resulted. Very high concentrations of cyanobacterial cells were detected in the water at the intakes of the drinking-water treatment plant, including *Microcystis.* The authors considered cyanobacterial toxins to be the probable cause of the outbreak, especially as patients who used only boiled water were among those affected.

A less damaging but more closely investigated occurrence of human injury from microcystins was reported in Australia in 1983 (Falconer *et al*., 1983). The laboratory in Armidale, New South Wales, that monitored reservoirs of drinking-water supplies for cyanobacterial blooms and regional cases of cyanobacterial poisoning of livestock observed a water-bloom of highly toxic *M. aeruginosa* growing in the city drinking-water reservoir. The water offtake for the treatment plant was located in a narrow bay that accumulated wind-drift of cyanobacterial scums. The drinking-water at Armidale was the subject of complaints of off-flavours and odours caused by *Microcystis*, to which the operating authority responded by applying copper sulfate to the reservoir by air. This caused rapid lysis of the cyanobacteria, and resulted in the liberation of free toxin into the water. A retrospective cohort study of serum indicators of liver damage was carried out. The data on liver enzymes from the regional pathology laboratory over a period of 18 weeks were sorted into analyses of samples taken during the 6 weeks of bloom development and termination by treatment with copper sulfate, the 6 weeks before that time and the 6 weeks after. Significantly increased levels of serum γ-glutamyl transferase were observed in the samples collected from the population who had drunk water from the affected reservoir only in the period when *Microcystis* was present and lysed. No increase was observed in the cohort population or during the periods before and after the water-bloom in either population. A smaller increase which was not statistically significant was seen in ALT, but no increase was seen in AST or alkaline phosphatase. While the average increase in γ-glutamyl transferase activity was approximately twofold, some samples showed a considerably larger increase. Serum enzyme activities had returned to normal by 6 weeks after the bloom. This evidence of liver damage was attributed to microcystins in the drinking-water supply. Unfortunately, analytical techniques that were sensitive enough to measure microcystins in drinking-water had not been developed at that time, so no actual exposure data were available to allow a dose–response determination.

(c) Immunotoxicity

No data were available to the Working Group.

4.4.2 *Experimental systems and natural exposure*

(a) Acute and subacute toxicity

Most of the structural variants of microcystins and nodularin are highly toxic in mice within a comparatively narrow dose range: LD_{50} following intraperitoneal injection were largely in the range of 50–300 μg/kg bw (Sivonen & Jones, 1999). Only a few non-toxic variants have been identified. In general, any structural modifications to the ADDA-glutamate region of the toxin molecule, such as a change in isomerization of the ADDA-diene (6*(E)* to 6*(Z)*) or acylation of the glutamate, renders microcystins and nodularin non-toxic. Linear microcystins and nodularin are more than 100 times less toxic than the equivalent cyclic compounds. Linear microcystins are thought to be microcystin precursors and/or bacterial breakdown products (Sivonen & Jones, 1999).

Microcystins and nodularin are primarily hepatotoxins. After acute or subacute exposure by intravenous or intraperitoneal injection of microcystins in mice or pigs, severe liver damage is characterized 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, lungs and intestines (for an extensive review of microcystin toxicity, see Sivonen & Jones, 1999).

(b) Chronic toxicity

Swiss albino mice were exposed to microcystin in the drinking-water for 1 year or longer in two studies (Falconer *et al*., 1988; Ueno *et al*., 1999). In one study, female BALB/c mice were exposed continuously to 20 μg/L microcystin-LR in the drinking-water for 18 months (mean cumulative toxin intake estimated at 35 μg/mouse for 18 months). No adverse effects of treatment were recorded, including no clinical changes, no observed liver histopathology or dysfunction and no liver tumours (Ueno *et al*., 1999).

In the other study, an extract of *M. aeruginosa* was administered in the drinking-water at six dose levels to male and female Swiss albino mice. Animals were killed at intervals up to 1 year. The highest dose administered was 56.6 μg/mL (approximately equivalent to 10 mg/kg bw per day), lower doses were 1/2, 1/4, 1/8 and 1/16 dilutions of this dose. Only the highest dose resulted in a reduced growth rate in both sexes of mice. Male mice were more adversely affected than females; at the 1/2 dilution, livers were significantly heavier in males, with elevated ALT levels, whereas female livers were heavier and serum contained significantly increased ALT only with the undiluted extract. Mortality clearly increased with dose throughout the range. Histopathological examination of the livers showed chronic active liver injury with hepatocyte necrosis, leukocyte infiltration and fibrosis in livers from mice receiving undiluted and 1/2 diluted extract. At lower concentrations of toxin, increased hepatic infiltration with neutrophils was seen. Histopathological examination of other tissues showed a significantly increased incidence of bronchopneumonia with age and dose, and a small number of cases of kidney damage at 1/4 and 1/8 dilutions from 31 weeks onwards (Falconer *et al.*, 1988).

In rats, daily intakes of lower doses of approximately 50 and 150 μg/kg bw pure microcystin-LR administered in the drinking-water for 28 days also led to increased liver weight, altered enzyme activities and histological injury to the liver (Heinze, 1999).

Intraperitoneal injections of microcystin-LR and -YR at a concentration of 10 μg/kg bw every other day for 8 months increased the numbers of TUNEL-positive cells in the cortex and medula of the kidney in rats (Milutinović *et al*., 2003).

It is apparent that microcystin-LR is predominantly hepatotoxic, whether administered acutely, subchronically or chronically, in all species investigated. However, toxic events due to consumption of or exposure to *M. aeruginosa* have been recorded in human and large animal populations with a wider range of clinical symptoms than hepatic injury alone, some of which are probably secondary to liver damage. It is also possible that poisoning by naturally occurring cyanobacterial water blooms leads to exposure to a range of toxic compounds, including several other microcystins and other cyanobacterial toxins with different toxic potentials.

No studies on the chronic toxicity of nodularin were available to the Working Group.

(c) Immunotoxicity

Only limited effects of microcystin or nodularin on the immune system of whole animals have been recorded. In rats administered microcystin-LR orally for 28 days, the number of leukocytes and lymphocytes in blood increased significantly (Heinze, 1999).

In a study of mice exposed to extracts of *M. aeruginosa* in the drinking-water for up to 1 year, the increased mortality could largely be attributed to pneumonia, not to liver dysfunction, which may reflect impaired immune function (Falconer *et al*., 1988).

In a more recent study, mice were injected intraperitoneally with a *M. aeruginosa* extract for 14 days and changes in the immune system were observed (Shen *et al*., 2003). At the highest dose (20 μg microcystin equivalents/kg bw), both spleen and thymus weights were decreased. Inhibition of lipopolysaccharide-induced B lymphocyte proliferation and a dose-dependent decrease in antibody-forming cells in mice that were immunized by sheep red blood cells were seen. [It should be noted that intraperitoneal injection of extracts may cause responses to other compounds, e.g. lipopolysaccharide endotoxins.] The proliferation of T cells following concanavalin A stimulation was not affected by treatment.

Nodularin administered intraperitoneally to mice decreased humoral immune responses to sheep red blood cells; the effect was heightened in animals given rifampicin to inhibit nodularin uptake into the liver (Yea *et al*., 2001).

Similar results were reported in an in-vitro study of mouse splenocytes, which demonstrated inhibition of lipopolysaccharide-stimulated lymphoproliferation in response to exposure to microcystin-LR and -YR and nodularin at concentrations of 10–50 μM. Concanavalin A-stimulated lymphoproliferation was suppressed by microcystin-YR and nodularin but not by microcystin-LR (Yea *et al*., 2001). [These concentrations of toxin in an in-vitro incubation are probably 10^3–10^4 times higher than those that occur in the blood during acute toxicity *in vivo*.]

A range of other in-vitro assessments of microcystin activity on immune cells have been undertaken, largely with the aim of clarifying the mechanism of toxicity. Macrophages incubated with microcystin-LR at approximately 100 nM (~0.1 μg/Ml) have been shown to respond by synthesis of tumour necrosis factor-α and interleukin-1β. These factors resulted in stimulation of intestinal secretory activity *in vitro* of rabbit ileum by supernatants from the incubation (Rocha *et al*., 2000).

Substrate adherence by polymorphonuclear leukocytes is an essential first step in the response to inflammation and phagocytosis of bacterial pathogens. Very low concentrations of microcystin-LR and nodularin (0.01–1.0 nM; ~0.01–1ng/mL) *in vitro* enhanced the early spontaneous adherence of polymorphonuclear leukocytes, but had no effect on late adherence or adherence of cells already stimulated by the peptide formyl-methionyl-leucyl-phenylalanine (Hernandez *et al*., 2000).

These limited results on immunomodulation by microcystin indicate that lower doses or concentrations may enhance immune response in some systems, but that higher doses are probably toxic, particularly to B lymphocytes.

4.5 References

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5. Summary of Data Reported

5.1 Exposure data

Microcystins and nodularin are cyclic peptide toxins that have a ring structure of seven and five amino acids, respectively, which comprise one unique phenyl deca-dienoic acid, four invariable D-amino acids and, in microcystins, two variable L-amino acids. There are approximately 70 variants of microcystin and several variants of nodularin. These peptides are produced naturally by cyanobacteria, an evolutionarily very ancient group of photosynthetic prokaryotic organisms. The bacteria occur in filamentous and coccoid forms (blooms) in free suspension in water or form layers (scums) on surfaces; they are distributed worldwide in water and soils, and on rock and plant surfaces.

Microcystin-LR (lysine-arginine) is the most extensively investigated cyanobacterial peptide toxin because it is frequently present in blooms in rivers and lakes. Nodularin occurs primarily in brackish waters. The peptide toxins are contained primarily within the cyanobacterial cells and are rarely released before the cells die. The concentration of toxin in water therefore depends on the cell content of microcystins or nodularin and the concentration of cells in the water. Due to widespread eutrophication in many settings, these toxins can occur at unnaturally high frequency and concentration. In natural water bodies and in water storage reservoirs, the concentrations of toxin vary widely from undetectable to several milligrams per litre in cyanobacterial scums.

A number of analytical techniques are used for the quantitation of cyanobacterial peptide toxins, and include high-performance liquid chromatography, liquid chromatography–mass spectrometry with a number of sophistications, enzyme inhibition assays and the enzyme-linked immunosorbent assay. Genetic probes are also available for the detection of the genes involved in the synthesis of these toxins.

Human exposure to these toxins occurs most frequently through the ingestion of water, i.e. through drinking or during recreational activities in which water is swallowed. Furthermore, cyanobacterial dietary supplements (blue-green algae supplements) are now on sale, and consumption of these toxins occurs from this source. Marine products such as fish, shellfish and crustaceans also accumulate nodularin, which remains stable and unchanged during cooking; this leads to their ingestion by humans.

WHO has adopted a provisonal guideline value for drinking-water supplies of 1 μg/L microcystin-LR, based on its subacute toxicity in mice. The toxicity of this microcystin variant is representative of that of other variants and of nodularin, and therefore provides a reasonable approximation of the toxicity of naturally occurring mixtures of these variants in water bodies. Many European countries and countries such as Australia, Brazil and Canada have adopted similar guideline values for microcystins, some of which are based on their total concentration in water samples.

Guidelines for recreational exposure have also been proposed by WHO; they are derived from the drinking-water guideline but are related to the concentrations of cyanobacterial cells found in water. They have also been implemented internationally, and include the emission of warnings at or the closure of recreational water sites when the specified levels of cyanobacterial contamination are reached.

5.2 Human carcinogenicity data

The Working Group reviewed several reports on ecological, cohort and case–control studies of the risk for hepatocellular carcinoma and source of drinking-water, some of which contained information on concentrations of microcystins in the water source. As access to the original publications was limited, the Working Group relied in many instances on summary information in review articles that lacked detailed descriptions of study methods and results. The studies of hepatocellular carcinoma included several ecological studies (12 from a review), two cohort studies (one in the review) and several case–control analyses (one meta-analysis, one pooled analysis and one additional case–control study). All studies conducted in the area of Southeast China that is endemic for hepatocellular carcinoma showed a positive association between the risk for hepatocellular carcinoma and water source; surface waters (pond, ditch or river waters) were associated with higher risks in contrast with either shallow or deep wells. Exposure assessment was limited in all studies to the use of these categorical measures. The few studies that reported concentrations of microcystins indicated that levels were much higher in surface waters than in well waters, but no study estimated the level of microcystins on an individual basis. In an analysis of pooled data from six case–control studies, the relative risk was 1.59; estimates of relative risk from other studies were generally in the range of 1.5–4, which raised the possibility that the observations were a consequence of confounding factors. Exposure to aflatoxin was not generally considered, and other contaminants or organisms in surface waters or factors related to water source were not evaluated. Some studies controlled for hepatitis B viral antigen or a history of hepatitis, which decreased the likelihood of confounding from this strong risk factor. An ecological study of colorectal cancer showed an association with concentration of microcystins, but confounding by other factors that are common to surface water sources in which the levels of microcystins were highest could not be ruled out. In summary, although many studies of hepatocellular carcinoma and one study of colorectal cancer found intriguing, positive associations with consumption of surface waters, in light of the quality of the published material available to the Working Group, it was not possible to associate the excess risk specifically with exposure to microcystin.

5.3 Animal carcinogenicity data

In one limited study in male mice, repeated intraperitoneal injections of microcystin-LR induced liver foci, which were probably benign tumours.

In three experiments in male rats that were initiated with *N*-nitrosodiethylamine and one experiment in male rats that were initiated with aflatoxin B_1, multiple intraperitoneal injections of microcystin-LR increased the incidence in the liver of glutathione *S*-transferase placental form-positive foci, which are considered to be preneoplastic lesions.

In one experiment in female mice that were initiated by skin application of 7,12-dimethylbenz[*a*]anthracene, *Microcystis* extracts given in the drinking-water increased the weight of skin papillomas per mouse.

One study in male mice that were initiated with *N*-methylnitrosourea and given *Microcystis* extracts in the drinking-water gave negative results.

In one experiment in male mice that were initiated by intraperitoneal injection of azoxymethane, exposure to *Mycrocystis* extracts in the drinking-water resulted in an increase in the area of aberrant crypt foci in the colon.

In two studies in male rats that were initiated with *N*-nitrosodiethylamine, multiple intraperitoneal injections of nodularin increased the incidence of glutathione *S*-transferase placental form-positive foci in the liver.

5.4 Other relevant data

Studies on the distribution of microcystins and nodularin have been carried out after intravenous and intraperitoneal administration to mice, rats and pigs of ^{125}I-, ^{14}C- or ^{3}H-labelled microcystins or nodularin. Kinetic studies showed rapid distribution into the liver and low accumulation in other tissues.

The cyanobacterial toxin microcystin does not permeate into cells. It is hepatotoxic because hepatocytes express transporters, which are organic anion-transporting polypeptides that permit the uptake of the toxin. A number of chemicals are known to compete for these transporters and inhibit the uptake of microcystins. In-vitro studies in hepatocytes have shown competitive inhibition of microcystin uptake by endogenous transporter substrates (e.g. bile acids) and xenobiotics (e.g. antibiotics).

The toxic action of microcystins is a consequence of their profound inhibition of cellular Ser/Thr protein phosphatases, which results in altered phosphorylation homeostasis. This impacts cell functions and structures that are controlled by changes in phosphorylation. The cellular metabolism of microcystins has not been elucidated but probably involves, at least in part, glutathione conjugation. Some data suggest that the conjugation of microcystins and possibly nodularins by glutathione occurs. It has been proposed, but not shown, that glutathione- and cysteine- adducts are excreted either in bile or back into the circulation together with native microcystin. No evidence was available of the involvement of cytochrome P450 in the detoxification of microcystins or nodularin.

Excretion of microcystins occurs primarily in faeces. A number of studies have shown that renal excretion, when it occurs, clears insignificant amounts of microcystins.

A common mechanism in the toxicity of microcystins and nodularin is the specific inhibition of Ser/Thr protein phosphatases 1 and 2A at picomolar concentrations in the cytoplasm and the nucleus *in vitro*. This inhibition results in hyperphosphorylation of

intracellular proteins, which is shown by the rapid disaggregation of intermediate filaments (cytokeratins) that form the cellular scaffold in human and rodent hepatocytes. Microfilaments become detached from the cytoplasmic membrane, which results in cell cytoskeletal deformation and bleb formation. Cell lysis and apoptosis follow, depending on the dose. Death results from dissolution of the liver structure and intrahepatic pooling of blood, which lead to overall haemorrhagic shock. Doses that are not immediately lethal can result in death from liver failure in large animals and humans several months after the initial exposure to microcystin.

The acute toxicity of microcystins in humans was shown unequivocally in the intoxication of haemodialysis patients in Caruaru, Brazil, who were exposed to microcystins in the dialysis water; this resulted in the death of more than 50 patients. Significant amounts of microcystin were detected in the livers and sera of these patients, and several incidents of hepatic disease and/or gastroenteritis have been reported after subacute intoxication with microcystins.

The toxicity of microcystins and nodularin has been described in rodents after intraperitoneal administration. The main injury was to the liver. Similar toxicity was also demonstrated in sheep and pigs in which hepatic damage was sustained for long periods after exposure.

Administration of cyanobacterial extracts that contain microcystins in the drinking-water to mice over a period of 1 year leads to liver damage. There are indications that chronic administration of microcystins results in immunotoxicity.

Nodularin has been studied less extensively than microcystin.

The mechanism(s) associated with the suspected carcinogenic activity of microcystins and nodularin is the enzymatic inhibition of protein phosphatases, which leads to downstream hyperphosphorylation of intracellular proteins.

There is no clear evidence that microcystins or nodularin are mutagenic or clastogenic in non-mammalian, mammalian or human cell systems. However, other mechanistic data indicate that both toxins are involved in epigenetic processes such as the modulation of oncogene and tumour-supressor gene expression, cell survival and/or apoptosis and the inhibition of DNA repair. In addition, an increased frequency of polyploid cells and centromere-positive micronuclei was observed, which suggests that both microcystins and nodularin are possibly aneugenic.

6. Evaluation and Rationale

There is *inadequate evidence* in humans for the carcinogenicity of microcystin-LR.

There is *inadequate evidence* in humans for the carcinogenicity of nodularin.

There is *inadequate evidence* in experimental animals for the carcinogenicity of microcystin-LR.

There is *inadequate evidence* in experimental animals for the carcinogenicity of *Microcystis* extracts.

There is *inadequate evidence* in experimental animals for the carcinogenicity of nodularins.

Overall evaluation

Microcystin-LR is *possibly carcinogenic to humans (Group 2B)*.

In three experiments in rats, Microcystin-LR promoted preneoplastic lesions of the liver. In a study in mice, microcystins promoted preneoplastic foci in the colon and a limited subchronic study with microcystin-LR resulted in persistent neoplastic nodules in mouse liver.

Strong evidence supports a plausible tumour promoter mechanism for these liver toxins. This mechanism is mediated by the inhibition of protein phosphatases 1 and 2A, an effect observed in rodents as well as in primary hepatocytes *in vitro*. The resulting hyperphosphorylation of intracellular protein leads to disruption of intermediate filaments that form the cellular scaffold in human and rodent hepatocytes. These toxins modulate the expression of oncogenes, early-response genes and of the cytokine, tumour necrosis factor α, and affect cell division, cell survival and apoptosis.

Microcystis extracts are *not classifiable as to their carcinogenicity to humans (Group 3)*.

Nodularins are *not classifiable as to their carcinogenicity to humans (Group 3)*.