Review

Toxins of cyanobacteria

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Blue-green algae are found in lakes, ponds, rivers and brackish waters throughout the world. In case of excessive growth such as bloom formation, these bacteria can produce inherent toxins in quantities causing toxicity in mammals, including humans. These cyanotoxins include cyclic peptides and alkaloids. Among the cyclic peptides are the microcystins and the nodularins. The alkaloids include anatoxin-a, anatoxin-a(S), cylindrospermopsin, saxitoxins (STXs), aplysiatoxins and lyngbyatoxin. Both biological and chemical methods are used to determine cyanotoxins. Bioassays and biochemical assays are nonspecific, so they can only be used as screening methods. HPLC has some good prospects. For the subsequent detection of these toxins different detectors may be used, ranging from simple UV-spectrometry *via* fluorescence detection to various types of MS. The main problem in the determination of cyanobacterial toxins is the lack of reference materials of all relevant toxins. In general, toxicity data on cyanotoxins are rather scarce. A majority of toxicity data are known to be of microcystin-LR. For nodularins, data from a few animal studies are available. For the alkaloids, limited toxicity data exist for anatoxin-a, cylindrospermopsin and STX. Risk assessment for acute exposure could be relevant for some types of exposure. Nevertheless, no acute reference doses have formally been derived thus far. For STX(s), many countries have established tolerance levels in bivalves, but these limits were set in view of STX(s) as biotoxins, accumulating in marine shellfish. Official regulations for other cyanotoxins have not been established, although some (provisional) guideline values have been derived for microcystins in drinking water by WHO and several countries.

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1 Introduction

Blooms of blue-green algae (cyanobacteria) are found in many eutrophic to hypertrophic lakes, ponds and rivers throughout the world. In tropical countries, the ponds that are heavily polluted and exposed to high temperatures and intense sunlight provide optimum conditions for the growth of cyanobacteria which may multiply so prolifically in such conditions that they cause a striking green colour to the water which consequently appears as pea soup. Predictions of where and when blooms will be formed are difficult if not impossible [1, 2].

Timing and duration of the bloom season of cyanobacteria depend largely on the climatic conditions of the region. In temperate zones, blooms of cyanobacteria are most prominent during the late summer and early autumn and may last for 2–4 months. In regions with more Mediterranean or subtropical climates, the bloom season may start earlier and persist longer. In France, 4 months is not uncommon, and in Japan, Portugal, Spain, South Africa and southern Australia, blooms may occur for up to 6 months or longer. In dry years, in tropical or subtropical areas of China, Brazil and Australia, cyanobacterial blooms may occur almost all year around, perhaps waning only briefly during reservoir overturn (cited from Chorus and Bartram [3]).

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Abbreviations: **AChE**, acetylcholinesterase; **GST**, glutathione-*S*-transferase; **HCC**, hepatocellular carcinoma; **IACs**, immunoaffinity columns; **i.p.**, intraperitoneal; **i.v.**, intravenous; **n.d.**, no data; **PPIA**, protein phosphatase inhibition assay; **SPME**, solid phase microextraction; **TEFs**, toxicity equivalent factors

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It has been suggested that blooms are caused by a complex interaction of high concentrations of nutrients, sunlight, warm temperature, turbidity, pH, conductivity, salinity, carbon availability and slow-flowing or stagnant water. These blooms of cyanobacteria in fresh or brackish water result in an unpleasant odour and taste and the loss of recreational and fishing value (cited from Duy *et al*. [4]).

Of particular concern can be the production of toxins by these organisms. Cyanobacteria are Gram-negative bacteria capable of producing a wide range of potent toxins as secondary metabolites, *i. e.* the cyanotoxins. The class cyanobacteria includes 150 genera and about 2000 species. They are now placed within the group *Eubacteria* in the phylogenetic taxonomy [4]. There are distinct cyanotoxin producing and noncyanotoxin producing organisms. Organisms responsible for cyanobacteria toxin poisoning (CTP) include an estimated 40 genera, but the main ones are *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Microcystis*, *Nostoc* and *Oscillatoria* (*Planktothrix*) [5]. Toxin production of cyanobacteria appears highly variable, both within and between blooms. Toxin production and potency can also vary with time for an individual bloom (cited from Duy *et al*. [4]).

Cyanotoxins are biotoxins and responsible for acute and (sub)chronic poisonings of wild/domestic animals and humans. The fresh and brackish water biotoxic cyanotoxins fall into three broad groups of chemical structure, namely the cyclic peptides (including the hepatotoxic microcystins and nodularins), the alkaloids (including the hepatotoxic cylindrospermopsins, the neurotoxins anatoxin-a, anatoxin-a(S) (S means salivation factor) and saxitoxins (STX)) and the LPSs which are potentially irritant (can affect any exposed tissue). Furthermore, there are two marine cyanotoxins belonging to the group of alkaloids (aplysiatoxin plus debromoaplysiatoxin and lyngbyatoxin-a) which cause gastrointestinal and/or skin irritation [3, 5].

Cyanotoxins are responsible for almost all known cases of fresh and brackish water intoxication involving phycotoxins. Since the first report of stock deaths in South Australia in 1878, CTP in animals has been widely reported around the world in cattle, swine, dogs, fish and bats [6]. Cyanotoxins have even been identified in marine environments as a cause of liver disease in net-pen reared salmon, although it was not clear which organism in marine environments contained these toxins [3].

Human poisoning cases occurred in Australia after the exposure of individuals to contaminated drinking water and in the UK, where army recruits were exposed while swimming and canoeing. Only in one incident in Caruaru, Brazil, human fatalities were reported when exposure through renal dialysis led to the death of over 50 patients [3]. In European fresh water, cyanobacterial blooms had been reported in 16 European countries up to and including 1989 [1]. Water used for human consumption should be regularly monitored for cyanotoxins. Predicting where and when toxic blooms will be formed is difficult if not impossible. The enormous variability in the toxicity of blooms within and between years – and even within a lake on a single day – makes assessment of the potential risk a major problem [6].

Globally, the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters are the cyclic peptide toxins of the microcystin and nodularin family.

In the field, healthy bloom populations produce little extracellular toxin. The range of measured concentrations for dissolved cyanotoxins is 0.1–10 μg/L. Cell-bound concentrations are several orders of magnitude higher. In lakes or rivers, toxins liberated from cells are rapidly diluted by the large mass of water. However, concentrations of dissolved toxins may be much higher in ageing or declining blooms. Release of toxins from cells to the surrounding water, to form dissolved toxin, appears to occur mostly, if not exclusively, during cell senescence, death and lysis, rather than by continuous excretion, and is enhanced by chemical treatments for the eradication of cyanobacteria, especially the use of algicides (either copper sulphate or organic herbicides). Treatment of a bloom with copper sulphate, for example, may lead to complete lysis of the bloom population within 3 days and release all the toxins into the surrounding water (cited from Chorus and Bartram [3]).

Although cyanobacterial blooms remain occasional events, most emphasis is still placed on the protection of drinking water supplies through the preparation of contingency plans and their activation when appropriate. Early warning systems and predictive models can facilitate this and should be based upon available information on the conditions leading to cyanobacterial bloom development and on occurrence, localization and movement of scums (for areas in Europe that are monitored see Fig. 1, source:www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm). Unfortunately, the toxins produced by cyanobacteria are not removed by conventional water treatment processes such as flocculation, sedimentation, sand filtration and chlorination and treatment processes that include potassium permanganate or chlorine, may release the toxins from the cyanobacteria which may therefore reach the people through water supplies [1].

Epidemiological data are important in determining the true nature and severity of the health effects in man, but are generally lacking in relation to exposure data [3].

2 Cyclic peptides

2.1 Microcystins (MCYSTs)

2.1.1 Chemical structures and properties

Microcystins (MCYSTs) are cyclic heptapeptides and their M_rs are estimated to vary between 500 and 4000 Da. However, for most of the known members of this group, the M_rs

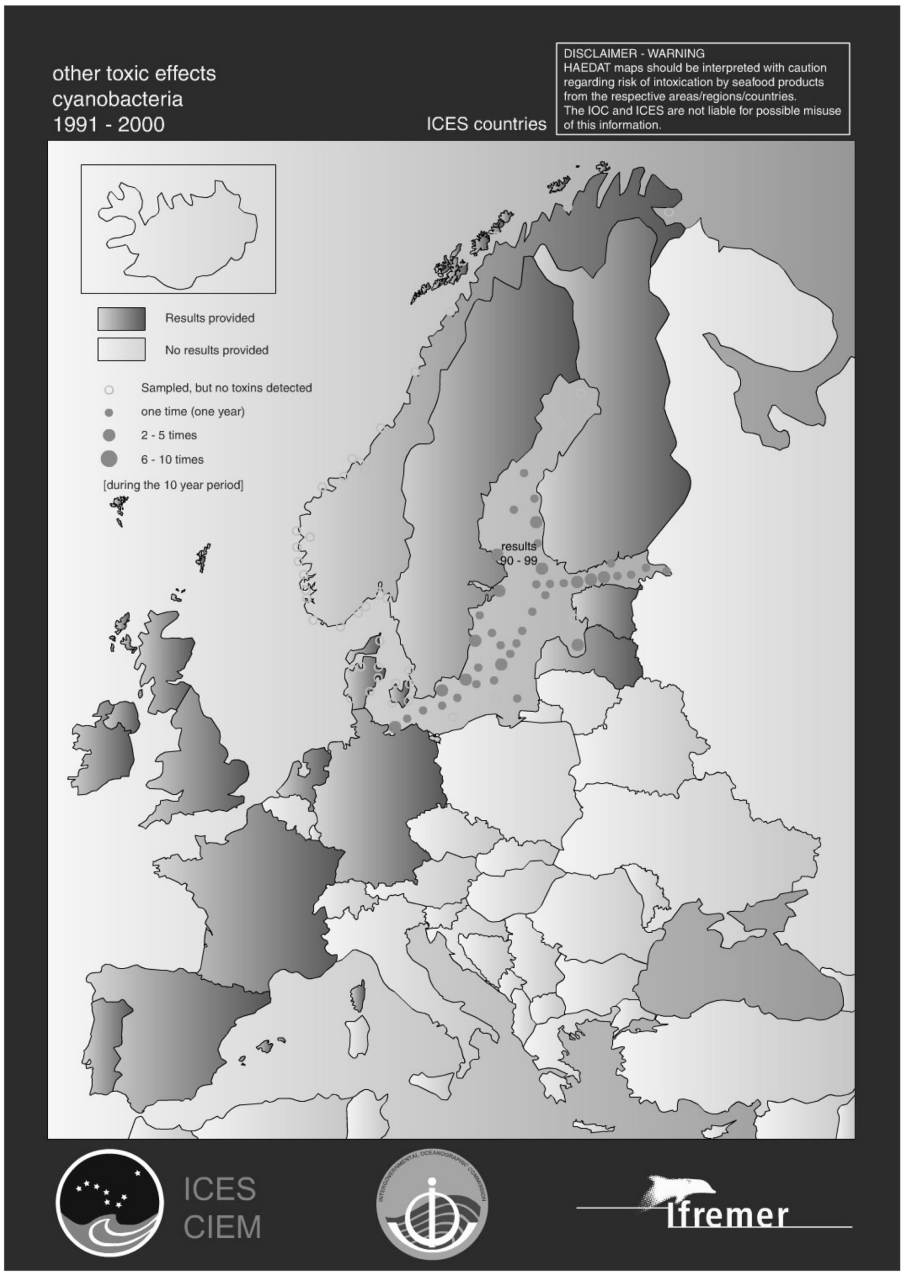
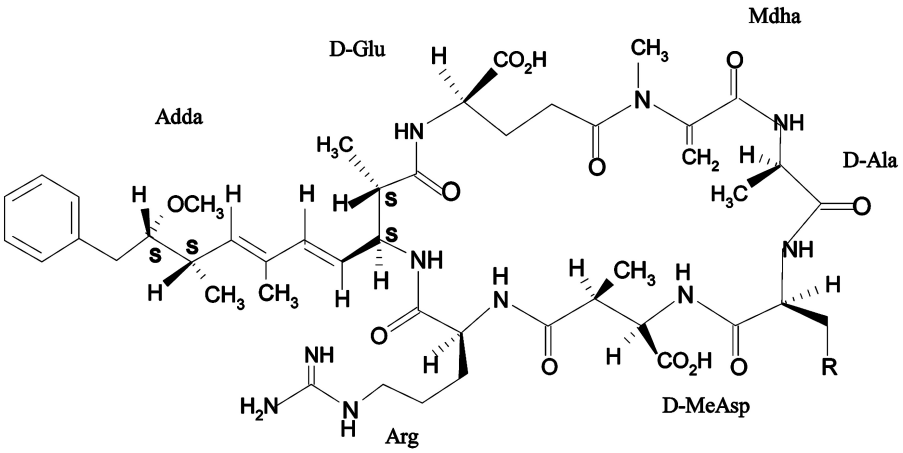


Figure 1. Occurrence of toxins of cyanobacteria in coastal waters of European ICES countries from 1991 to 2000 (source: http://www.ifremer.fr/envlit/documentation/dossiers/ciem/aindex.htm).

are between 900 and 1100 Da. Sixty-four variants are now described [5].

Microcystins contain seven peptide-linked amino acids, with the two terminal amino acids of the linear peptide being condensed (joined) to form a cyclic compound (see Fig. 2). The general structure is: cyclo-(D-alanine-*X*-D-MeAsp-*Z*-Adda-D-glutamate-Mdha) in which *X* and *Z* are variable L amino acids, D-MeAsp is D-*erythro*-β-methylaspartic acid, and Mdha is *N*-methyldehydroalanine. The amino acid Adda, (2S,3S,8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is the most unusual structure in this group of cyclic peptide toxins. Structural variations have been reported in all the seven amino acids, but most frequently with substitution of L-amino acid *X* (mostly *X* is leucine, arginine or tyrosine but also homotyrosine, alanine, phenylalanine, homophenylalanine, methionine-*S*-oxide or tryptophan is possible) and *Z* (*Z* is arginine, or alanine but it might be also aminoisobutyric acid, homoarginine or methionine-*S*-oxide) and demethylation of D-MeAsp and/or Mdha. Most of the microcystins contain β-methylaspartic acid, glutamic acid and alanine with methylamine attached to the glutamic



Microcystin LR R=$CH(CH_3)_2$
Microcystin RR R=$CH_2CH_2NHC(NH_2)$=NH
Microcystin YR R=C_6H_4-p-OH

Figure 2. Chemical structures of some common microcystins (from Duy *et al.* [4]).

acid. In some microcystins, Mdha is replaced by L-serine and D-Ala by D-serine. Some nontoxic variants have been identified for example the compound containing the 6Z-stereoisomer of Adda. In general, any structural modification to the Adda region or acylation of the glutamate renders microcystins less toxic or even nontoxic [3, 4]. Recently, two new microcystins, *seco*[D-Asp]microcystin-RR and [D-Asp,D-Glu(OMe)]microcystin-RR were isolated [6].

Some 60 congeners with the general structure D-alanine-*X*-D-MeAsp-*Z*-Adda-D-glutamate-Mdha have been characterized [8]. In a Canadian prairie lake a new microcystin, named [D-Leu] microcystin-LR, was detected. The amino acid composition resembled that of microcystin-LR except for the presence of D-Leu instead of D-Ala in microcystin-LR [9]. Microcystins are soluble in water, methanol and ethanol, and insoluble in acetone, ether, chloroform and benzene. Microcystins are stable in reservoir water for less than 1 wk, but are stable for much longer periods in filtered or deionized water. They are resistant to chemical hydrolysis or oxidation at near neutral pH. Microcystins are reported to withstand several hours of boiling. At high temperatures (40°C) and at elevated or low pH, slow hydrolysis has been observed with the times to achieve >90% breakdown being approximately 10 wk at pH 1 and greater than 12 wk at pH 9. Rapid chemical hydrolysis occurs only under laboratory conditions that are unlikely to be attained outside the laboratory, *e.g.* 6 M HCl at high temperature. Microcystins can be oxidized by ozone and other strong oxidizing agents [3]. Microcystins are very stable under natural sunlight, whereas UV light around the absorption maxima of microcystin-LR and microcystin-RR rapidly decomposed the toxins [8].

2.1.2 Methods of analysis

2.1.2.1 General

Among the range of techniques for the determination of microcystins in water, there is no single technique that can provide an accurate measure of the toxin concentration in microcystin-LR toxicity equivalents where complex mixtures of microcystins occur in water samples. Each technique generates a value that requires assumptions in order to derive a result in toxicity equivalents [10]. However, Wolf and Frank [11] proposed to use toxicity equivalent factors (TEFs) for the mixtures of cyanobacterial toxins in anology with TEFs used for polychlorinated dibenzo[*p*]-dioxins and dibenzofurans. The TEF for microcystin-LR was set as 1.0 by definition.

Techniques can be selected depending on the facilities and expertise available, coupled with the type of information required. Information obtained from simple, rapid screening methods such as microscopic examination can be used to make a decision on the type of bioassay or physicochemical technique which should be employed. When samples arrive in the laboratory, the type of analysis that will be used should have been anticipated previously. The type of information required prior to sample collection should be fully considered [3].

Irrespective of the detection method there are a number of criteria which must be satisfied if a toxin concentration in terms of microcystin-LR toxicity equivalents is to be obtained:

(i) the toxin must be unequivocally identified,

(ii) an analytical standard must be available for the specific toxin so that the concentration can be accurately determined,

(iii) an acute toxicity value (LD_{50}) must be available for that specific toxin so that a concentration in microcystin-LR equivalents can be calculated. Given these criteria, all methods have limitations [10].

Cyanobacteria are microscopic photosynthetic organisms. Appropriate and careful handling of samples both prior to and during analysis is extremely important to ensure an accurate determination of toxin concentration. Microcystins are readily degraded both photochemically (also during analytical procedures) and microbiologically [10]. Furthermore, toxins in water bodies at the time of a bloom will be present in both the water (free, dissolved or extracellular toxins) and the cyanobacterial cells (intracellular) [12].

2.1.2.2 Extraction/concentration/clean-up procedures

Effective extraction of microcystins from cyanobacteria is difficult without disrupting or perforating the cell wall structure. Most procedures use freeze-drying for this purpose alternatively combined with oven drying [12], prior to solvent extraction with 75% methanol. Other extractants for microcystins in cyanobacterial material include supercritical CO_2 [13]. This approach may be useful but requires evaluation with a wider range of toxins.

SPE is the most widely used technique in sample concentration and clean-up for HPLC of cyanobacterial toxins. SPE C_{18} cartridges as well as other cartridges (cyano, silica gel) or two-step combinations have been studied [14]. An example of the variety of choice of SPE is a study in which microcystins, nodularin, anatoxin-a and STX were simultaneously determined [15]. The procedure of this method is based on an ion-pair supported SPE enrichment and RP-LC coupled with MS/MS detection. Detection limits in surface water of all toxins were in the ng/L range. On-line trace enrichment with SPE has also been reported [16, 17]. Important advantages of this technique are its fast, time-saving and simple features. Micro-SPE-micro-LC-ES-MS (ES: electrospray) has detection limits of microcystins in all kind of waters at concentrations below the 0.05 μg/L. And even without ES-MS detection: SPE-LC coupled with (photo-DAD or PDA) comes up with detection limits in the same range.

A main disadvantage of SPE is that coeluting substances may interfere with the determination of the analyte(s) in question. A way to overcome is the use of immunoaffinity columns (IACs). These IACs utilize antibodies against microcystins. A two-step combination of SPE and IAC as clean-up prior to HPLC-UV was able to detect microcystins in lake water down to 5 ng/L [18]. Recently, SPE using immunoaffinity chromatography (in the form of SPE/IAC cartridges) was used as an extraction and a clean-up step in the analysis of microcystins in complex matrices [19]. The results obtained demonstrate the efficiency of SPE/IAC as a sample pretreatment strategy. Obviously, IACs can also be coupled to ELISA with good performances: detection limits of microcystins in tap water in the ng/L range [20]. To overcome difficulties in the production of large amounts of antibodies required, a reusable IAC (it could be used three times) was prepared by coupling antimicrocystin-LR monoclonal antibodies to activated immunoaffinity support formyl-cellulofine [21]. Further shortcomings of IACs are the variable crossreactivity/recovery of the different microcystins and the limited stability of the antibodies.

By synthesizing artificial receptors (polymers) for microcystin-LR using the technique of molecular imprinting [22], a step forward has been made to improve the selectivity of SPE. The use of the so-called MIP-SPE (molecularly imprinted polymer SPE) provided up to 1000-fold preconcentration of microcystin-LR in drinking water. MIP-SPE in combination with a piezoelectric sensor can detect microcystin-LR below 1 μg/L.

2.1.2.3 Determination/detection

HPLC in general. The most common instrumental analytical procedures for the determination of microcystins employ HPLC [10]. The stationary phases that are being used mostly (at least the last decades) are RP C18 and C8. Striking however is an excellent study in which a novel amide C16 column was compared with C18 columns [23]. The amide C16 column had the best overall performance and unique selectivity properties. It separates desmethylated toxins (encountered in field samples) well from the nondesmethylated toxins and it could be useful in the separation of certain stereoisomers.

The mobile phases suitable for the detection of microcystins with the above-mentioned columns are aqueous, containing methanol or ACN. More and more separation is achieved over a gradient of water and organic modifier. The gradient has to cover a sufficient range of polarities (*e.g.* 30–70% ACN) to allow the analysis of all microcystins which are known to vary considerably in their polarities [24]. Acidic mobile phases have been shown to be able to resolve more microcystins (and nodularins) than the neutral ones (often containing ammonium acetate). Examples of HPLC analyses are conveniently arranged in a manual and practical guide by Meriluoto and Codd [14].

HPLC coupled with a spectroscopic-based detector. Microcystins comprise an interesting family of closely related peptides for the separation and detection studies. The number of toxin analogues is high, and therefore the separations require high resolution and good selectivity. The molar absorbance of microcystins is strong, roughly between 220 and 240 nm [14]. In addition Ikawa *et al.* [25] showed that common additives in plastics could contaminate water samples, coelute with microcystins and have sufficient UV absorbance at 238 nm to produce erroneous results. Thus, plastic materials must be avoided, or their

suitability checked, in the determination of these microcystins using HPLC-UV. In order to improve specificity for the analysis of microcystins, a (photo-)DAD or PDA can be used. This technique probably offers the best means of determining microcystins for compliance with the proposed guidelines [10].

In a comparative study [26], the suitability of different methods (protein phosphatase inhibition assay (PPIA), HPLC-DAD and ELISA) to detect different toxin variants was evaluated. Using HPLC-DAD, individual microcystins can be separated and recognized on their retention times and their characteristic UV absorption spectra. However, the HPLC analysis is hampered by the large number of different microcystin variants and the lack of commercial standards.

LC-MS. Different advances in LC-MS have resulted in lowered detection limits, such as the application of microbore LC coupled to an IT MS [27] for the analysis of microcystins from environmental samples. And not in the last place, the use of LC-MS/MS (multiple reaction monitoring, MRM) as a tool for toxin identification and quantification for regulatory purposes [28]. LC-MS/MS is relatively expensive for routine screening, and therefore initial analyses of the samples are often performed by ELISA. Positive samples and selected negative samples are subsequently confirmed by LC-MS/MS after SPE.

Powerful hyphenated techniques like LC-ESI-MS are considered to be useful for sequence analysis of small peptides. Compared to ‚older' methods like ELISA and HPLC-UV, LC-ESI-MS in selected ion recording mode (SIR) has a greater selective power while the LOD (<0.2 μg/L in water) is not inferior to both conventional methods [29].

The selectivity that is put forward by MS/MS techniques can be used to obtain the fragmentation patterns of compounds suspected to be 'new' microcystins [30]. And, at least as important as that, MS/MS may be used as a multimethod for the simultaneous determination of the so-called HAB toxins (Harmful Algal Blooms is a collective term for all algal toxins). Within 30 min (chromatography), a wide range of HABs is determined with individual LODs at or below 1 μg/L after a single methanol–water extraction [30].

Concerning multimethods, the wide range of structures and charges of most cyanobacterial toxins sometimes necessitates the use of other stationary phases. Recently, it was shown that HILIC-MS (hydrophilic interaction LC-MS) could be a promising technique [31]. But more research has to be done in order to improve the chromatography of particularly the microcystins.

Few noncolumn techniques. Although most methods use conventional column-HPLC and absorption detection, other techniques may be promising, especially the combination of lateral-flow-type immunochromatography as a separation system with fluorescence detection [32]. Interesting features of this method are: its fastness, convenience, reproducibility, and its low detection limit (for microcystin-LR below 0.1 μg/L in drinking water).

Before the use of LC-MS for the detection of cyanobacterial toxins, fast atom bombardment (FAB) MS had become an important tool for identification [14]. A more recent application of MALDI-TOF MS to study microcystin variants directly from cell extracts has been reported [33]. MALDI-TOF MS machines can be equipped with a postdecay (PSD) option that allows fragmentation of a mass signal for further characterization and eventual identification of the compound [34]. MALDI-TOF MS is essentially an off-line technique which is a drawback, but the small amounts of sample required for a complete analysis is a plus for this technique.

The mouse bioassay is used to detect the toxicity of suspected bloom material and detects all cyanotoxins together. The toxicity is tested by intraperitoneal injection to male Swiss Albino mice. About 0.1–1.0 mL of a lysate of cyanobacteria prepared either by sonication or by freeze-thawing of a cell suspension which has been sterilized by membrane-ultrafiltration. Samples can be suspended in water or physiological saline solution which is preferred if the volume to be injected is 0.5 mL or greater. Mice should be observed for 24 h and then killed. The observation period must be extended to 7 days where cylindrospermopsin is suspected. During the observation period clinical symptoms and mortality are recorded. The mice that have died are further examined for gross macroscopical changes in organs and tissues. The observed symptoms and the results of the postmortem examination are used to determine which type of cyanotoxin is present. When more than one type of cyanotoxin is present, the more rapid acting toxin may mask other symptoms (as cited in Chorus and Bartram [3]). The mouse bioassay plays an important role as a screening tool and provides a measure of total toxicity (response), but the assay is not specific or sensitive. Furthermore, a license is needed to perform this test and in some countries the use of this test is not permitted [3, 10].

The brine shrimp (*Artemia salina*) assay is used as a routine bioassay for cyanotoxins. This assay has only been fully evaluated for microcystins, but there appears to be a correlation between anatoxin-a content and toxicity. Brine shrimps are available commercially as standardized test kits. Lee *et al*. [35] compared the sensitivity and time requirements of the *Artemia* bioassay with those of the mouse bioassay. The study showed that the *Artemia* bioassay required more time than the mouse bioassay and that the *Artemia* bioassay was less sensitive at observation intervals shorter than 24 h.

With the use of *Daphnia* sp., microcystins can be detected successfully. However, standardized culturing is very labour intensive [3].

The response and measurement of toxins in a PPIA is related to the mode of toxic action of the microcystins. The

data till date suggest an overestimation of toxin concentration in terms of microcystin-LR toxicity equivalents. Response data with a much larger range of microcystin variants need to be obtained in order to determine whether there is an underestimation of toxin concentration with some variants [10]. One version of this assay is based on the quantitation of ^{32}P-phosphate releases from a radiolabelled substrate by the activity of the PP enzyme (PP1 and PP2A). It is sensitive to subnanogram levels of microcystin and many samples can be analysed in a few hours. This assay is applicable for microcystin class compounds in the marine environment, to extracts of liver tissue taken from Atlantic salmon afflicted with netpen liver disease and to hydrophobic microcystins from freshwater cyanobacteria. The assay can also be used for quantitation of microcystins in drinking water before and after water treatment. The detected amounts in raw and treated water were estimated to be 0.12–0.87 and 0.09–0.18 μg/L, respectively. A disadvantage is the use of radioactive material which necessitates specialized laboratory equipment and regulations [3].

Another version is the colorimetric PP inhibition assay, which avoids the use of radioactive material. Mainly a substrate such as *p*-nitrophenyl phosphate is used, and the release of the coloured *p*-nitrophenol from the substrate in the presence of PP and microcystin is determined [10]. A similar assay using phosvitin, as substrate and also measuring the phosphate released colorimetrically, was reported (as cited by Nicholson and Burch [10]).

A firefly bioluminescence system for the detection of PP2A inhibitors is reported in which luciferin phosphate is hydrolysed to luciferin and inorganic phosphate by PP2A [3].

ELISAs using monoclonal and polyclonal antibodies have been developed. Commercial ELISA test kits are now available. These kits and ELISAs, in general, must be viewed as producing semiquantitative results. The cross-reactivity of the various microcystins depends on the similarity in chemical structure to the microcystin against which antibodies have been raised (usually microcystin-LR) and does not depend on toxicity. Therefore, the application of these antibodies to an unknown or complex mixture of microcystins may give rise to a poor reaction with some components. The accuracy will depend on the microcystins present, their crossreactivities relative to the standard used for quantification and susceptibility to interferences. ELISA techniques can greatly over- or underestimate the concentration with some variants of microcystins, but they are, however, useful screening tools. If only microcystin-LR, or microcystins with equivalent crossreactivities, were present, then accurate results in terms of microcystin-LR equivalents would be obtained (as cited in Nicholson and Burch [10]).

2.1.2.4 Conclusion

A summary of some general properties of analytical methods for microcystins (and nodularins) can easily be found. Many methods with their characteristics, like quickness, LOD, selectivity, *etc.*, are compared conveniently in tabular form by Meriluoto and Codd [14]. From a general point of view, one could also particularly be interested in the comparison of selectivity and LOD for different methods [24].

It is clear that bioassays and PPIAs for their lack of specificity and on the other hand for their simplicity, can be perfectly suitable to be used as screening methods for the determination of microcystins in water [10, 15]. LC, irrespective of the detection method, will only be at its most accurate where the toxins are well characterized in that particular source, and can therefore be readily identified. Standards must then be available for each toxin to determine identity and concentrations, and toxicity (LD_{50}) data must be available to convert toxin concentrations to microcystin-LR toxicity equivalents. Although the lack of standards and toxicity data will be a limitation involving HPLC, assumptions can be made in order to produce a result in microcystin-LR equivalents. Taking this into account, HPLC procedures are still the most appropriate for monitoring in compliance with the guidelines [10]. The method of aqueous methanol extraction and SPE prior to HPLC-UV (or DAD) has been developed by ISO as a standard method [14]. However, LC-MS systems become more and more common in routine analytical laboratories. Comparing UV with MS techniques, LC-UV offers analysts a cheap and apparently easily applicable and furthermore robust method for microcystins in environmental samples. And yet, there are only a few other analytical disciplines so eventful as the area of MS. With its great specificity and low LOD, and in addition its development towards a more applicable technique, LC-MS will be the most powerful analytical tool for the determination of microcystins within the next decade.

2.1.3 Source organism(s) and habitat/occurrence

2.1.3.1 Source organism(s)

Microcystins were first isolated from the NRC-1 (SS-17) strain from the cyanobacterium *Microcystis aeruginosa* and were named after this organism. Strains such as NRC-1 (SS-17) produce a single toxin, whereas others are capable of producing several toxins (strain WR 70 produces four microcystins and other strains can produce six types of microcystins) [3–5]. Microcystins are found in most populations of *Microcystis* spp., which frequently form scums. High microcystin content is further found in *Anabaena* spp. which may also form scums. However, *Planktothrix agardhii* never forms scums and *P. rubescens* usually does not form scums during the bathing season, and yet high microcystin levels are formed in blooms of these strains [3]. Eight microcystins were isolated from 13 strains of *Oscillatoria agardhii* collected from different Finnish lakes (all strains produced 2–5 microcystins). Nowadays, the occurrence of microcystins was shown in several cyanobacterial genera, namely *Microcystis* (*aeruginosa*, *wesenbergii* and *viridis*), *Anabaena*

(*flos-aquae*), *Nostoc*, *Oscillatoria* (*Planktothrix*) (*agardhii*, *rubescens* and *tenuis*), *Anabaenopsis*, a soil isolate of *Haphalosiphon* (*hibernicus*) and *Aphanocapsa* (*cumulus*) [3–5]. Recently, two new microcystins, *seco*[D-Asp]microcystin-RR and [D-Asp,D-Glu(OMe)]microcystin-RR, together with the known [D-Asp]microcystin-RR, were isolated from a *P. rubescens* toxic bloom in Lake Bled, Slovenia [7].

2.1.3.2 Habitat/occurrence

Microcystins occur world-wide in freshwaters. However, they have now been also identified in marine environments as a cause of a liver disease in net-pen reared salmon, although it is not clear which organism in marine environments contains these toxins. Environmental factors that may influence growth and microcystin production of cyanobacteria such as nutrients, light, pH and temperature, have been studied in batch and continuous cultures.

The amount of microcystin production by a cyanobacterial population in culture appears to be directly proportional to its growth rate, no matter what environmental factor was limiting the growth. During the logarithmic growth phase the amount of microcystin is increasing and the highest amount is produced during the late logarithmic phase. The amount of microcystin contained by a single cell of *M. aeruginosa* was shown to be constant within a narrow range (2–3-fold). While variants of microcystins produced by a particular strain are rather constant, the ratios of individual microcystins may change with time, temperature and light intensity. Environmental factors affect the toxin content but only within a range of less than an order of magnitude. Different cyanobacterial species have different requirements. For example *Planktothrix* prefers low light intensities for growth and *Anabaena* moderate light intensities. Temperature gradients cause 2- to 3-fold differences in toxin content. At low and high pH cyanobacterial cells are found to contain more toxin [3].

In a Turkish lake (Lake Sapanca), a bloom of cyanobacteria occurred in May 1997. No microcystins were detected in the water column above 10 m, but at 20 m depth 3.65 μg microcystin-LR eq/L were detected. Ninety percent of the microcystin pool was found between depths of 15 and 25 m. The principal microcystin detected was microcystin-RR. The depth at which the microcystins were detected coincided with the draw-off depth for the drinking water supply for the city of Sakarya [36].

At high phosphorus levels, hepatotoxic cyanobacterial strains produced more toxins; differences in toxin production between high and low phosphorus levels vary between 2- and 4-fold. Nonnitrogen fixing species such as *Microcystis* and *Oscillatoria* produce more toxins under nitrogen-rich conditions. The role of iron is contradictory. In batch cultures with *M. aeruginosa* only zinc was required for both optimal growth and toxin production [3]. Moisander *et al.* [37] reported that *Anabaena* grew up to 15 g NaCl/L under laboratory conditions, but the maximum tested level of 20 g NaCl/L was inhibitory. *Anabaenopsis* maintained similar growth rates in the full range of salinities tested (from 2 to 20 g/L).

2.1.4 Bioaccumulation in the environment

2.1.4.1 Accumulation in aquatic organisms

The ability of microcystins to accumulate in aquatic organisms, which are a food source for many kinds of fish, is known from literature. This bioaccumulation of microcystins in food chains may be of concern for public health. Microcystins have been found in phytoplankton, zooplankton and gastropods (*Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gynna*), as well as in copepods and crab larvae [3, 4].

At an exposure of the aquatic macrophyte *Ceratophyllum demersum* to ^{14}C-labelled microcystin-LR 11.2% of the applied radioactivity was taken up within 7 days of exposure [38].

In Lake Biwa, Japan, accumulation of microcystins in the resident snail, *Sinotaia histrica*, occurred (in hepatopancreas up to 1.7 μg microcystin-RR and 1.5 μg microcystin-LR/g dry weight and in intestine up to 12.8 μg microcystin-RR and 6.7 μg microcystin-LR/g dry weight). In the hepatopancreas of the edible clam, *Corbicula sandai*, from the same lake in Japan, no microcystins were detected. In a laboratory study the snail *S. histrica* was fed daily for 15 days to toxic *M. ichthyoblabe* cells (1.0×10^6 cells/mL) containing high amounts of microcystin-LR. At day 10 of the exposure period hepatopancreas contained 436 μg microcystin-LR/g dry weight. During the first 5 days of a 15-day depuration period a high value (200–297 μg/g dry weight) persisted in the hepatopancreas and thereafter a slow decrease was seen from day 10 to 15 of depuration until 85.8 μg/g dry weight. The biological half-life of microcystin-LR in the hepatopancreas was 8.4 days [39].

Furthermore, microcystins bioaccumulate in freshwater mussels (a.o. *Anodonta cygnea*), freshwater clams and fish. In mussels the highest microcystin concentration is found in the hepatopancreas, and in fish in the liver. Whether microcystins bioaccumulate to a level which may form a risk to humans, is uncertain. Common advice is not to eat the viscera of the fish [3, 4].

Microcystin bioaccumulation in three freshwater Unionid bivalves, *A. woodiana*, *Cristaria plicata*, and *Unio douglasiae*, was seen in the hypereutrophic Lake Suwa, Japan. Total microcystin (-MC, -RR and -LR) levels (determined by RP-HPLC) were high in the hepatopancreas of *C. plicata* and *U. douglasiae*, with maxima at 297 and 420 μg/g dry weight, respectively. The amounts and seasonal changes in total microcystin levels differed in all species. Total microcystin level in *A. woodiana* was always less than that in the other species with a maximum level of 12.6 μg/g dry weight. No correlation could be found between microcystin levels in tissues and extracellular microcystin [40].

M. galloprovincialis fed on a toxic *M. aeruginosa* strain for 16 days, accumulated microcystin up to levels of 10.5 μg/g dry mussel weight. After a 11-day withdrawal period microcystin was still detectable [41]. Yokayama and Park [42] carried out uptake and depuration experiments with microcystin-LR at 15 and 25°C in the freshwater bivalve *U. douglasiae*. Microcystin-LR accumulated rapidly in the hepatopancreas at the beginning of the uptake experiment (lasting 15 days) and reached steady-state conditions at day 5 at 130 and 250 μg/g dry weight at 15 at 25°C, respectively (exposure concentration of microcystin-LR was twice at high as at 15°C). Depuration of microcystin-LR was more rapid at 25°C than at 15°C; the concentration was halved in 3.1 days at 25°C and in 4.9 days at 15°C. At study termination after 15 days of depuration hepatopancreas still contained 17.1 μg microcystin-LR/g dry weight at 15°C and 8.4 μg/g at 25°C.

Magelhães *et al*. showed the accumulation of microcystins in muscle tissue of fish and crustaceans from a bay (Sepita Bay) at the coast of Brasil. The maximum level found in crab muscle tissue was 103.3 μg microcystin eq/kg and in fish muscle tissue 39.6 μg/kg (by ELISA). In an earlier study Magelhães *et al*. [43] already demonstrated the accumulation of microcystin in liver, viscera and muscle tissue of the fish, *Tilapia rendalli* caught in Jacarepaguá Lagoon in Brazil. Rapid uptake of microcystin was seen. The highest levels in muscle tissue, liver and viscera were 337.3, 31.1 and 67.8 μg/g, respectively. During the entire sampling period (August 1996–November 1999) 71.7% of fish muscle samples had microcystin levels close to or above the recommended TDI (0.04 μg/kg body weight (bw)) assuming that a 60 kg weighing person is eating 300 g fish muscle/day.

Adult crayfish (*Procambarus clarkii*) were fed twice a week for 11 days microcystin producing *M. aeruginosa* followed by a 12-day depuration period. Accumulation up to 2.9 μg microcystin/g dry weight was seen (at day 11). During depuration period there was a rapid decline of the toxin level by day 14, but an increase occurred on day 17. By day 23 no toxin was found. Most of the microcystin was found in the intestine and hepatopancreas [44].

Fish collected from Portuguese freshwater did not accumulate very high levels of microcystin-LR in their edible parts (carp, barbel and grey mullet contained 50–280, 0.7–120 and 8.5–110 ng microcystin-LR/g, respectively). In the edible parts of crayfish and mussels, on the other hand, maximum levels of 2.7 and 16.0 μg microcystin-LR/g, respectively, were found. In shellfish microcystin was readily accumulated and persisted for several days. In crayfish microcystin was accumulated mainly in the gut but is also cleared very slowly [41].

In three Greek lakes muscle tissue and viscera of seven species of fish, a frog, a mollusc and a gastropod collected during the warm period of 1999 and 2000 were examined for microcystins with two methods (PP1 inhibition and ELISA). In all the samples microcystins were found. In viscera, the levels were higher than in muscle tissue and were in the order of 200–600 ng microcystin-LR eq/g. Average microcystin-LR level in fish and frog muscle tissues were 225 and 125 ng/g, respectively [45].

Mohamed *et al*. [46] reported the accumulation of microcystins in the freshwater fish *Oreochromis niloticus* from an Egyptian fish farm containing every year in the warm season (April–December) heavy blooms of *M. aeruginosa*. Highest microcystin levels were found in the gut (821 ng/g fresh weight), followed by the liver (531.8 ng/g) and kidneys (400 ng/g). In the muscles 102 ng/g was detected (by ELISA).

In aquatic ecosystems, phytoplanktivorous fish play an important role as consumers of phytoplankton, as food fish, and as biological managers of algal blooms. Both silver carp and bighead carp were suggested to be able to suppress and graze out *M. aeruginosa* blooms. The grazing ability of silver carp on toxic cyanobacteria suggests an applicability of using phytoplanktivorous fish to counteract cyanotoxin contamination in eutrophic waters [47].

Little is known of the dynamics of microcystins in such phytoplanktivorous fish. Therefore, a subchronic feeding experiment was performed with the warm-water phytoplanktivorous cyprinid, silver carp. The fish was fed during 80 days with fresh *M. viridis* cells containing both microcystin-RR and microcystin-LR. No microcystin-LR was detected in blood and muscle tissue of the fish and in the liver only trace on one occasion despite the abundant presence of the toxin in the intestines. Microcystin-RR was detected in the fish at maximum levels of 49.7, 17.8 and 1.77 μg/g in blood, liver and muscle, respectively. Depuration of microcystin-RR levels occurred in the following order blood>liver>muscle. A depuration period of 20 days resulted in microcystin-RR declines from *ca.* 91% in blood, *ca.* 87% in liver and *ca.* 55% in muscle (depuration of microcystin-LR in rainbow trout is much faster: a depuration period of 3 days resulted in reductions of 100 and 96.1% in blood and liver, respectively) [47].

2.1.4.2 Accumulation in crops/plants

Lettuce plants (*Lactuca sativa*) were irrigated during growth, with water containing three microcystins totalling 3.23 μg microcystin-LR eq/mg dry weight of bloom and scum. Colonies and single cells of *M. aeruginosa* and microcystin itself were retained by the lettuce after growth. The mature leaf basal zone, mature leaf distal zone and central leaves contained 0.094, 0.883 and 2.487 μg microcystin-LR eq/g dry weight, respectively [48].

Rape and rice seedlings were exposed to water containing 0, 0.024, 0.12, 0.6 and 3 μg microcystin-LR eq/mL. Extracts of exposed rape and rice seedlings showed that except the extract of rice seedlings exposed to 0.024 mg/mL, all plant extracts contained microcystin levels which were correlated to the toxin levels in the water. The micro-

cystin levels in rape seedlings (2.61, 8.32, 123.57 and 651.0 ng microcystin-LR eq/g fresh weight at exposure levels of 0.024, 0.12, 0.6 and 3 μg/mL, respectively) were significantly higher than those in rice seedlings (0, 2.94, 5.12 and 5.40 ng/g fresh weight at exposure levels of 0.024, 0.12, 0.6 and 3 μg/mL, respectively). This study indicated that consumption of edible plants exposed to microcystins *via* irrigation might have a health risk [49].

Germinated runner bean (*Phaseolis vulgaris*) seeds (stems 20–30 cm) were exposed to microcystin-LR (1.12 μg/mL) for 18 days. After 3 days of exposure, a mean concentration of 11.55 μg/mL of plant extract was found which represented approximately 96% of the amount of microcystin-LR which had been taken up. After 6 and 12 days of exposure 6.82 and 16.35 μg/mL of plant extract, respectively, was found which represented 32 and 23%, respectively, of the amount taken up. After 18 days of exposure 19.10 μg/mL of plant extract was found representing 18% of the amount taken up. The decrease in recovery from the total amount of microcystin-LR taken up, may have occurred as a result of metabolism of microcystin-LR by plants exposed to microcystin-LR for a longer period. However, this phenomenon had not yet been adequately investigated [50].

Exposure of the emergent reed plant *Phragmites australis* to 0.5 μg ^{14}C-labelled microcystin-LR/L for 72 h (in a 300 mL beaker) demonstrated a rapid uptake of the toxin (sampling times 0.5, 1, 2, 4, 6, 12, 24, 48 and 72 h). The main uptake route appeared to be in the stem and rizome, from which the toxin is transported into the higher parts of the plant to the leaves. The uptake significantly increased from 0.5 to 72 h exposure: In the leaf from 2.6 to 22.5 pg/g fresh weight, in the stem from 5.9 to 77.0 pg/g fresh weight and in the rhizome system from 5.8 to 21.8 pg/g fresh weight. Uptake in the stem was 3.4- to 3.5-fold higher than in leaf and rhizome fractions. The uptake directly through the leaves may occur by direct contact of small plants or by the lowest leaves of a plant with surface water and with upper leaves by wave and spray contact. For microcystin-LR, a complete metabolism from the formation of a glutathione conjugate (no conjugation was seen with nodularin, neither chemical nor enzymatic) to the degradation of a cysteine conjugate in all cormus parts of the plant was reported [51].

`There are indicators that the scenario of exposure of crop plants to cyanobacterial toxins *via* irrigation is an area for concern, especially in countries with a high reliance on the irrigation of high value crops, and a high incidence of eutrophication and blooms, like the Netherlands' (G. A. Codd, Personal communication from a letter received in June 2004).

2.1.5 Biodegradation in the environment

Microcystins are intracellular toxins and, whilst contained within living cells, they are degraded only slowly. Microcystins are only released into the water by senescence or cell death or through water treatment processes such as prechlorination or algicide application. Once released into the water, microcystins can persist also for a relatively long period before being removed by biodegradation or photolysis [3, 4].

In full sunlight, microcystins undergo slow photochemical breakdown and isomerization, with the reaction rate being enhanced by the presence of water-soluble cell pigments, presumably phycobiliproteins. In the presence of pigments, the photochemical breakdown in full sunlight can last for only 2 wk for >90% breakdown, or for longer than 6 wk, depending on the concentration of pigment.

A more rapid breakdown under sunlight has been reported in the presence of humic substances (which can act as photosensitizers) in field concentrations ranging from 2 to 16 mg/L dissolved organic carbon (DOC). Approximately 40% of the microcystins was degraded *per* day under summer conditions of insolation. In deeper or muddy waters, the rate of breakdown is likely to be considerably lower [3].

Studies in natural waters of the UK indicated that 5 days were required for 50% destruction of microcystin-LR. Studies in Australia showed that microcystin-LR was present up to 21 days after treatment of an *M. aeruginosa* bloom with an algicide [52]. In natural waters, microcystins are susceptible to breakdown by aquatic bacteria. The bacterium capable of degrading microcystins is a new *Sphingomonas* species, previously known as *Pseudomonas*. These microorganisms have been found in sewage effluent, lake sediments and natural water bodies and initiated ring-opening of microcystin-LR to produce linear microcystin-LR as a transient intermediate, which was nearly 200 times less toxic than the parent compound [4]. There is usually an initial lag phase with little loss of microcystin and this period can be as short as 2 days or more than 3 wk, depending on the water body, climatic conditions, the concentration of dissolved microcystin and in some cases, although not at all, the previous bloom history of the water body. Once biodegradation commences, removal of microcystin can be >90% complete within 2–10 days depending on the water body, initial microcystin concentration and water temperature [3]. A thorough degradation in 4 days was reported by Park *et al*. [53] when *Sphingomonas* was added to microcystins in culture medium. The highest degradation rates of microcystin-RR and microcystin-LR were 13 and 5.4 $mg \cdot L^{-1}day^{-1}$, respectively. Degradation rates were strongly dependent on temperature and the maximum rate was seen at 30°C [53].

The breakdown of microcystin-LR may involve at least three enzymes. One enzyme which was called microcystinase, breaks the bond between two of the seven amino acids in the cyclic heptapeptide. The resulting linear chain of seven amino acids is said to be more than a 100 times less toxic than the ring structure. Two more enzymes cause further breakdown of the peptide to single amino acids [6].

2.1.6 Toxicity of microcystins

2.1.6.1 Mechanism of action

Microcystin-LR and most of its congeners are hydrophilic and generally not able to penetrate vertebrate cell membranes and therefore require uptake *via* an adenosine triphosphate (ATP)-dependent transporter. One thus far unidentified multispecific organic anion transporter (or bile acid transporter) has been described as the carrier of cyclic peptides (among which microcystins). As a result of this, toxicity of microcystin-LR is restricted to organs expressing the organic anion transporter on their cell membranes such as the liver [8]. *Via* the oral route, microcystin-LR in mammals is transported across the ileum into the bloodstream through the bile acid transport system present in hepatocytes and cells lining the small intestine and is concentrated in the liver as a result of active uptake by hepatocytes. Some microcystins, which are more hydrophobic than microcystin-LR may crosscell membranes by other mechanisms, including diffusion (cited from Chorus and Bartram [3]). Once in hepatocytes microcystin-LR is a potent inhibitor of eukaryotic protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A). PP1 and PP2A inhibition leads to hyperphosphorylation of cytoskeletal proteins resulting in deformation of hepatocytes. The liver swells to nearly double its size, due to massive intrahepatic centrilobular haemorrhaging, which is preceded by hepatocyte rounding and extensive dissociation as well as disruption of sinusoidal epithelium. Some chemicals have been used experimentally to prevent microcystin hepatotoxicity in laboratory animals. These include cyclosporin A, rifampin and silymarin. These antagonists have been most successful when given prior to or coadministered with toxin (cited from Rao *et al*. [54]).

Substances that inhibit PP enzymes are considered to be also nonphorbol ester-type tumour promoters. PPs serve an important regulatory role to maintain homeostasis in the cell. PP inhibition results in a shift in the balance towards higher phosphorylation of target proteins such as tumour suppresser proteins. This is a major post-transitional modification which can result in excessive signalling and may lead towards cell proliferation, cell transformation and tumour promotion. The inhibition of PP2A by microcystin-LR can be effectively reversed in the presence of polyclonal antibodies against microcystin-LR; such antibodies can also protect *in vivo* against microcystin-LR toxicity as shown with intraperitoneal (i.p.) coadministration studies in mice. The implications of PP inhibition in humans, due to low level chronic exposure to microcystins, are not known (cited from Chorus and Bartram [3]).

2.1.6.2 Pharmacokinetics

The liver appears to be the main target organ for both accumulation and excretion of microcystins. In tissue distribution studies in laboratory animals with i.p. and intravenous (i.v.) administration microcystin-LR, 50–70% was found in the liver, with another 7–10% in the intestine, 1–5% in the kidney and the remainder distributed throughout the body. It is likely that transport may occur in the kidney as well because the kidney has also a bile transport system similar to the transporter of the intestinal cell in rats. Microcystins are resistant to enzymatic hydrolysis and have no 'cleavage point for tryptic hydrolysis'. Therefore, microcystins are more resistant to degradation in tissues and may require excretion in bile in either its original form or after conjugation. There is evidence for biliary excretion of microcystins from the liver, indicating the potential for enterohepatic recirculation. It is suggested that microcystins may leave hepatocytes *via* the bile because they rapidly appear in the duodenum. It is conceivable that microcystin-LR may be excreted through both the liver and kidney routes. Plasma $t_{1/2}$ of microcystin-LR after i.v. administration was 0.8 and 6.9 min. for the α- and β-phases of elimination, respectively. The disappearance of microcystins in blood was found to follow two phases. The first phase had a $t_{1/2}$ of 2.1 min and the second phase 42 min. There is a species difference in clearance of microcystins (cited from Duy *et al*. [4]). After oral administration to mice less than 1% uptake in the liver was seen [52].

Based on the protective effect of microsomal enzyme inducers, it is evident that the liver plays a large role in the detoxification of microcystins. Three metabolic products have been identified, a glutathione conjugate, a cysteine conjugate and a conjugate with the oxidized ADDA diene (Chorus and Bartram [3]).

Table 1. Acute i.p. LD_{50} values in the mouse

LD_{50} values i.p. mouse	μg/kg bw	
Microcystin-LR	50–60	Generally accepted, see [3]
	25–150	Generally accepted, see [3]
	32.5–100	Cited from Wolf and Frank [11]
	56	Cited from Wolf and Frank [11]
Microcystin-LA, -YR and -YM	Similar to microcystin-LR	Cited from Wolf and Frank [11]
Microcystin-RR	500	Cited from Wolf and Frank [11]
(D-asp3,(E)-Dhb7)-microcystin-RR	250	Cited from Wolf and Frank [11]

2.1.6.3 Toxicity to laboratory animals

Acute toxicity – oral studies. The oral LD_{50} of microcystin-LR in mice is 5000 μg/kg bw in one strain while in another strain the oral LD_{50} is 10 900 μg/kg bw (cited from Chorus and Bartram [3] and Fawell *et al*. [55]). For rats the oral LD_{50} value is >5000 μg/kg bw [55]. The acute oral toxicity in mice was shown to be age dependent possibly because of disruption of surface cell structure of gastric mucosa and small intestine in older animals. This disruption may markedly influence gastrointestinal absorption of microcystin-LR (cited from Chorus and Bartram [3]) (for clinical symptoms see Section 2.1.6.1).

Acute toxicity – intraperitoneal studies. The i.p LD_{50} of microcystin-LR in the mouse (see Table 1) varies from 25 to 150 μg/kg bw (a value of 50–60 μg/kg bw is commonly accepted) (cited from Chorus and Bartram [3] and Fawell *et al*. [55]). The i.p. LD_{50}s of several of the commonly occurring microcystins (-LA, -YR, and -YM) are similar to that of microcystin-LR, but the i.p. LD_{50} for microcystin-RR is about ten times higher. However, because of the differences in lipophilicity and polarity between the different microcystins, i.p. LD_{50}s cannot predict toxicity after oral administration. After i.p. and i.v. administration of microcystin, severe liver damage is characterized by disruption of liver cell structure, 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 (cited from Chorus and Bartram[3]).

Wolf and Frank [11] calculated TEF values for other microcystins than microcystin-LR based on their acute i.p. LD_{50} value in the mouse. The TEF value for microcystin-LR was 1.0 by definition, for microcystin-LA, -YR and -YM the TEF value was also 1.0 and for (D-asp3,(E)-Dhb7)-microcystin-RR and microcystin-RR the TEF values were 0.2 and 0.1.

Acute toxicity – intravascular studies. In a study of swine groups six anaesthetized female animals were given intravascularly a lethal (72 μg/kg bw) or a toxic-sublethal (25 μg/kg bw) dose of microcystin-LR. This study indicated that death from acute microcystin-LR toxicosis is due to hypotensive, hypovolemic shock resulting from rapid and severe obstruction of blood flow through the liver and severe haemorrhage into, and destruction of, the hepatic parenchyma. The shock syndrome is complicated by a reduction in circulating platelets, a partially compensated (lactic) acidosis, reduced renal perfusion and terminal hyperkalemia as well as hypoglycaemia. An early and sensitive indicator of acute microcystin-LR toxicosis in swine is serum bile acid concentration. Increases in this parameter are consistent with decreased bile transport due to rapidly disrupted cytoskeletal form and function [56].

Irritation and sensitization studies. An intradermal reactivity test in rabbits, an ocular irritation test in rabbits and a sensitization test (maximization test) in guinea-pigs were performed with freeze-dried algal suspensions (from *Microcystis* and *Anabaena* blooms) in physiological saline. No to slight dermal irritation was seen and negative and positive results were found in studies for eye-irritation. In the sensitization study all algal samples were sensitizing (30–67% of the animals used, reacted). There was no correlation of the toxic effects seen in these studies, with the microcystin content of the algal samples. Pure microcystin-LR was only slightly sensitizing even at a high concentration (22% of the animals reacted at 1.5 mg/mL [57]).

Repeated dosing – oral studies. Repeated daily oral administration of 31.3 μg microcystin-LR/kg bw for 7 days caused a 75% increase in liver weight of mice (cited from Chorus and Bartram [3]).

In a 14-day range finding study groups of five male and five female mice received by gavage 0, 40, 200 or 1000 μg microcystin-LR/kg bw. The only findings were microscopic changes in the liver in four male and two female mice at 1000 μg/kg bw [55].

Groups of ten adult male rats (11-wk old) received 0, 50 or 150 μg microcystin-LR/kg bw *via* their drinking water (150 mL/day) for 28 days. Dose-dependent increases in liver weight and serum activities (SAP, ALAT, ASAT, lactate dehydrogenase (LDH)) were seen. Histopathology showed dose-dependent injuries in the liver (for type of effects see Section 2.1.6.1 [58]).

In a 44-day drinking water study in pigs (groups of five pigs) with an extract from *M. aeruginosa* (containing seven microcystin variants with microcystin-YR as the major component, doses 280–1312 μg total microcystins (as microcystin-LR eq)/kg bw based on i.p. mouse bioassay, 184–860 μg/kg bw based on HPLC; 88–441 μg/kg bw based on PPase inhibition assay) liver changes were also reported even at the lowest dose-level of 280 μg microcystin-LR eq/kg bw (for type of effects see Section 2.1.6.1) (cited from WHO [59] and Duy *et al*. [4]).

In a 13-wk gavage study in mice 40 μg microcystin-LR/kg bw was an NOAEL. This NOAEL was based on pathological changes in the liver accompanied by changes in enzymatic parameters and serum proteins at 200 and 1000 μg/kg bw. Additionally at the highest dose-level (1000 μg/kg bw), up to a 20% increase in food consumption was seen while bw was 7% lower than in controls [55] (cited from WHO [59]).

Three vervet monkeys received by gavage (3 times/week) for 46 wk microcystin-LA. The doses were gradually increased from 20 to 80 μg/kg bw over the duration of the study. No significant changes in clinical or haematological parameters or serym enzyme levels were seen. No histopathological changes in liver or other tissues were observed [52].

In a 1-year drinking water study in mice with an extract of *M. aeruginosa* (750–12 000 μg microcystin-YM/kg bw) no clear NOAEL was established. At the higher dose-levels (>500 μg/kg bw) increased death, increased bronchopneumonia (which was endemic) and chronic liver injury were seen (for type of effects see Section 2.1.6.1) (cited from Chorus and Bartram [3]).

An 18-month oral study in Balb/c mice was performed in which two groups of 100 (6-wk old) animals received 0 or 20 μg microcystin-LR *per* litre of drinking water. Mortality, clinical signs, body weights and food and water consumptions were recorded. Examinations on haematology, serum biochemistry, necropsy, organ weights and histopathology were performed at 3, 6, 12 and 18 months (10 animals/group). In addition immunohistochemical distribution of microcystin-LR was examined in the liver at these time points. Mean cumulative microcystin-LR intake after 18 months was estimated at 35.5 μg *per* mouse. No chronic toxicity nor accumulation of microcystin-LR in the liver was observed [60].

Repeated dosing – intraperitoneal studies. Female New Zealand rabbits (bw 3.5–4.0 kg) received i.p. 7.5 mg/kg bw of sterilized cyanobacterial lyophilizate containing microcystins (1 μg microcystin-LR/mg and 2.75 mg microcystin-RR/mg) every second day for 3 wk. Fatty infiltration and periportal fibrosis of the liver were seen (detected by MRI) [61].

Four groups of three rats (average bw 185 g) received *via* an intraperitoneal osmotic pump 0, 16, 32 or 48 μg microcystin-LR/kg bw in saline for 28 days. Dose-dependent hepatic inflammation was seen including infiltration of centrilobular regions by lymphocytes, macrophages and neutrophils, centrilobular fibrosis, apoptosis and steatosis. Analysis of lipid peroxidation products showed a dose-dependent increase in malondialdehyde levels with an approximate four-fold increase in the liver of the highest dosed rats. Livers from the exposed rats were more sensitive to the cytotoxic effects of the oxidizing agent *tert*-butylhydroperoxide (based on an MTT viability assay) than livers of control rats. These findings indicate that oxidative stress may play a significant role in the pathogenesis of chronic microcystin-LR toxicosis [62].

Groups of five male Wistar rats received i.p. every second day for 8 months 10 μg microcystin-LR/kg bw or 10 μg microcystin-YR/kg bw in a vehicle (0.8% ethanol and 0.2% methanol in physiological saline). A control group received vehicle only. Besides hepatotoxicity nephrotoxicity was observed. The kidneys were far more affected than the liver. The pathological changes in the kidney induced by microcystin-LR were more severe than those induced by microcystin-YR. At the cellular level, the mechanisms underlying the chronic nephrotoxicity were similar to the mechanisms of the acute hepatotoxicity of microcystins (for type of effects see Section 2.1.6.1) [63, 64].

***In vitro* toxicity studies.** In primary cultured rat hepatocytes 24 and 72 h LC_{50} values of 48 and 8 ng/mL, respectively, were reported for microcystin-LR [65, 66]. Exposure of rat hepatocytes to noncytotoxic concentrations of 2 or 10 ng/mL microcystin-LR for 3, 24 or 48 h provided evidence of the induction of oxidative stress as was demonstrated by an increase in reduced glutathione [65].

Primary hepatocyte cultures in the presence of picomolar and nanomolar concentrations of microcystin-LR showed selective cell toxicity and selective cell proliferation depending on the ploidy (chromosome copy number) of the cells (cited from Chorus and Bartram [3]).

Mankiewicz *et al*. [67] reported apoptotic effects in rat hepatocytes and human lymphocytes caused by extracts from cyanobacterial bloom samples from a Polish water reservoir (named Jeziorsko). The extracts contained predominantly microcystins (microcystin-LR concentration 250–1000 nM, total microcystin concentration 675–2700 nM microcystin-LR eq; determined by HPLC). The incubation time needed to observe the first morphological apoptotic changes in rat hepatocytes was approximately 30 min; however, the characteristic biochemical changes in DNA were not observed even after 120 min. The morphological changes characteristic for apoptosis in human lymphocytes were seen after 24 h of incubation and a 48-h incubation period was optimal for the analysis of internucleosomal DNA fragmentation.

Mutagenicity. No mutagenic activity was observed when a purified lyophilized extract of hepatotoxin (0.9 mg hepatotoxin/mL) was tested in the Ames *Salmonella* assay (strains TA 98, 100 and 102) with or without metabolic activation (doses 25–100 μL extract). The *Bacillus subtilis* multigene sporulation test was also negative using both the 168 and hcr-9 strains (doses undiluted extract and two- and four-fold dilutions) [68]. Another study with purified *Microcystis* extract was also negative in the Ames test [3].

Extracts from cyanobacterial bloom samples from Polish water reservoirs (Jeziorsko and Sulejòw, central Poland) containing predominantly microcystins, were tested for genotoxicity using the SOS chromotest with *Escherichia coli* PQ37 (without metabolic activation) and the comet assay with human leucocytes. The SOS chromotest revealed a higher toxicity and genotoxicity for extracts with higher amounts of microcystins including microcystin-LR. Standard microcystin-LR showed less toxicity and less genotoxicity in the SOS chromotest than the Polish extracts. This finding could be closely linked to the composition of the extracts which indicated the possibility of action of other variants of microcystins, whose presence in the extracts was determined as equivalents of microcystin-LR (by HPLC).

In the comet assay with human leucocytes, DNA damage was observed with the Polish extracts. The degree of DNA disturbance depended mainly on the extract concentration

and the time of exposure. A maximum was reached after 12 h incubation. In the comet assay standard microcystin-LR showed less genotoxicity than the Polish extracts [69].

Extracts from cyanobacterial bloom samples from Chinese water sources (Dianshan Lake, Shanghai) were studied for their genotoxic activity in the Ames test with *Salmonella typhimurium* strains TA 97, TA 98, TA 100 and TA 102 without and with metabolic activation, in the comet assay with primary rat hepatocytes (single cell electrophoresis) and in the *in vivo* micronucleus assay in the mouse. The extracts contained microcystin-LR as the main component, besides several other microcystin types. Strong mutagenicity was seen in the Ames test in all four *S. typhimurium* strains in the absence as well as the presence of a metabolic activation system. In the comet assay the extracts induced DNA damage in the primary rat hepatocytes. In addition, an increased number of micronucleated polychromatic erythrocytes in bone marrow of mice was seen in the micronucleus assay [70].

Zhan *et al*. [71] investigated the genotoxicity of microcystin-LR by means of an *in vitro* micronucleus assay and an *in vitro* thymidine kinase (TK) gene mutation assay using a human lymphoblastoid cell line TK6. In a standard 4 h treatment no cytotoxic effects were seen at concentrations up to 80 μg microcystin-LR/mL. However, at a 24 h treatment microcystin-LR-induced cytotoxic as well as mutagenic effects at concentrations >20 μg/mL up to 80 μg/mL. Microcystin-LR induced both micronuclei and gene-mutations at an exposure for 24 h. Maximum induction of micronuclei and gene-mutations was approximately five times the control value. In the gene-mutation assay microcystin-LR elevated not only the frequency of mutants but also the fraction of slow growing mutants, suggesting that predominantly gross structural changes such as large deletions, recombinations and rearrangements were induced. Molecular analysis of the mutants supported this hypothesis. These results indicated that microcystin-LR had a clastogenic effect.

In an assay with human lymphocytes *in vitro*, a purified lyophilized extract (0.9 mg hepatotoxin/mL) caused dose-related) increases in chromosomal breakages at the two highest dose-levels of 0.09 and 0.9 μg/mL. At the lowest dose-level of 0.009 μg/mL, no effect was seen [68].

Lankoff *et al*. [72] showed that treatment of human lymphocyte cells with microcystin-LR at concentrations of 1, 10 and 25 μg/mL in the comet assay caused a dose- and time-dependent induction of DNA-damage with a maximum at 18 h. Microcystin-LR affected the mitotic index but microcystin-LR had no effect on the frequency of chromosome aberrations in the lymphocytes. Microcystin-LR had an inhibiting effect on the repair of DNA damage induced in the lymphocytes by radiation. The results of the experiments indicated that the enhanced DNA damage in the comet assay might be related to the early stages of apoptosis due to cytotoxicity but not to genotoxicity.

In a two-stage cell-transformation assay using Syrian hamster embryo cells, extracts from *M. aeruginosa* blooms containing mainly microcystins (no further analytical data) from a lake in China, did not show initiating activity when followed by the tumour promotor 12-*O*-tetradecanoyl phorbol-13-acetate. With methylcholanthrene as an initiator (0.5 μg/mL), followed by the bloom extract, a dose-related (up to 7×) increase in transformation frequency was observed [3].

Microcystin-LR induced both ouabain-resistant mutations and K-*ras* codon 12 mutations in human RSa cells *in vitro* at the tested concentrations of 7.5–15 μg/mL [73].

Unpublished work showed the induction of DNA fragmentation by microcystin-LR in baby hamster kidney (BHK) cells and mouse embryo fibroblast cell cultures [74].

In the human hepatoma cell line HepG2 microcystin-LR-induced dose- and time-dependent DNA strand breaks at noncytotoxic doses (0.01–1 μg/mL). The DNA strand breaks were transient, reaching a maximum level at 4 h of exposure and declining with further exposure. In the presence of DNA repair inhibitors, DNA strand breaks accumulated after prolonged exposure. With the use of purified, oxidative DNA damage specific enzymes and a radical scavenger, it was shown that microcystin-LR-induced formation of reactive oxygen species that caused the DNA damage [75].

Microcystin-LR induced a dose (at 0.5, 1.0 and 2.0 LD_{50} intraperitoneal doses) and time-dependent DNA damage (DNA strand breaks measured by fluorimetric analysis of DNA unwinding) in the mouse liver *in vivo* [74].

Carcinogenicity. Microcystin-LR is a potent inhibitor of eukaryotic protein serine/threonine PP1 and PP2A both *in vitro* and *in vivo*. Substances that inhibit these enzymes are considered to be nonphorbol ester (TPA)-type tumour promoters. PPs serve an important regulatory role to maintain homeostasis in the cell. PP inhibition results in a shift in the balance towards higher phosphorylation of target proteins such as tumour suppresser proteins. This is a major post-transitional modification, which may result in an excessive signalling and may lead towards cell proliferation, cell transformation and tumour promotion. The implications of PP inhibition in humans, due to low level chronic exposure to microcystins, are not known (cited from Chorus and Bartram [3]). Regarding the possible role of microcystins in tumour promotion, further studies are especially needed to establish a dose-effect relationship for nodule induction with microcystin alone using various routes of administration [3].

No tumours in the liver were seen in a 1-year drinking water study in mice with an extract of *M. aeruginosa* (750–12 000 μg microcystin-YM/kg bw) (cited from Chorus and Bartram [3]) or in a 1-year drinking water study in Balb/c mice receiving 20 μg microcystin-LR/L [60]. In addition mice, which were given orally 100 times 80 μg microcys-

tin-LR/kg bw within 28 wk, did not develop liver injury or liver nodules (cited from Chorus and Bartram [3]). However, when mice received i.p. 20 μg microcystin-LR/kg bw, 100 times within 28 wk, all exposed animals developed liver nodules (up to 5 mm in diameter). When some mice were kept for a further 2 months after cessation of dosing, the liver nodules persisted (cited from Chorus and Bartram [3]).

Some evidence of tumour promotion by microcystin-LR was shown in a two-stage carcinogenesis mouse skin bioassay in female mice and a short-term two-stage carcinogenicity bioassay in male F344 rats with i.p. dosing. In a tumour initiation and promotion assay in C57 black mice aimed at evaluating possible tumour promoting effects in the upper small intestine, oral dosing of *Microcystis* extracts *via* drinking water after oral dosing of the initiator *N*-methyl-*N*-nitrosourea, no primary liver tumours were seen and no evidence of promotion of lymphoid or duodenal tumours by microcystin was observed (cited by Chorus and Bartram [3]). In another study, male C57Bl/6J mice received three i.p. injections with azoxymethane as an inducing agent (inducer of aberrant crypt foci in colon; 5 mg/kg bw at 7-day intervals) followed by the administration of drinking water containing *Mycrocystis* extract (~0, 382 and 693 μg microcystin-LR eq/kg bw at midpoint of trial) for 212 days starting from 19 days after last azoxymethane injection. A dose-dependent increase in the areas of aberrant crypt foci was observed. Number of crypts/colon was not increased. Two overt colonic tumours (~30 mm^3) were found in microcystin treated mice (one tumour at both the low and high dose group) and one in azomethane-alone treated mice. In the highest dose group signs of hepatic toxicity were observed [76].

Also *in vitro* studies, *viz.* a cell-transformation assay in Syrian hamster embryo cells and a study in primary mouse liver cells, showed the promoting activity of extracts from *M. aeruginosa* and microcystin-LR, respectively (cited from Chorus and Bartram [3]).

Reproduction and developmental – oral studies. In a teratogenicity study in pregnant mice receiving by gavage 0, 200, 600 or 2000 μg microcystin-LR/kg bw, the highest dose-level of 2000 μg/kg bw caused maternal toxicity and mortality. Seven out of 26 female mice died and two were sacrificed because of distress during the dosing period (day 6–15 of pregnancy). A number of surviving females at this dose-level had abnormal livers, and foetuses showed retardation of foetal weight and skeletal ossification. There was neither evidence of embryolethality or teratogenicity nor an effect on litter size, postimplantation loss or sex distribution of live foetuses at any dose-level. The NOAEL for any aspect of developmental toxicity was 600 μg/kg bw [55].

Male and female mice received an extract of *M. aeruginosa* (~750 μg microcystin/kg bw) *via* the drinking water since weaning throughout the mating period. Exposure was continued in female mice during pregnancy. No effects on fertility of the dams or on foetal weight or sex distribution were seen. There was some evidence of hippocampal injury and reduced brain size in 7/73 5-day-old young from exposed parents (cited from WHO [59]).

Reproduction and developmental – intraperitoneal studies. In a range-finding study pregnant CD-1 mice received i.p. 16–160 microcystin-LR/kg bw on two consecutive days during gestation days 7–12. Maternal death was observed at 64 and 96 μg microcystin-LR/kg bw. At 128 μg/kg bw 4/10 pregnant mice died and at 160 μg/kg bw 9/10 died. All deaths occurred within 1 day after the last injection. The estimated i.p. LD_{50} was 127 μg/kg bw. Significant hepatotoxicity was seen [77].

In a study in pregnant CD-1 mice, 2–8 animals/group were given daily i.p. injections with 0, 32, 64 or 128 μg microcystin-LR/kg bw or subcutaneous injections with 128 μg/kg bw during gestation days 7–8, 9–10 or 11–12. On day 17 of pregnancy the animals were killed and foetuses were weighed and examined for gross abnormalities and skeletal effects. No effect on litter size, viability, weight or the incidence of anomalies was seen [77]. In a second study pregnant mice were given daily i.p injections with 32–128 μg/kg bw on gestation days 7–8, 9–10 or 11–12 and were allowed to give birth. The growth and development of the pups were followed postnatally for 4 days. The microcystin-LR used in this study, was derived from a different lot that proved to be far more toxic than the microcystin-LR used in the foregoing studies. At 64 μg/kg bw 19/35 animals died. Number of survivors were four, three and five in gestation days 7–8, 9–10 and 11–12 groups, respectively. One single animal survived at 96 and 128 μg/kg bw dose-groups. No effect on the number of foetuses alive after 4 days, birthweight or postnatal growth during days 1–4 was observed in any of the surviving litters [77].

Reproduction and developmental – *in vitro* embryo (cultures) studies. Neurulation staged (gestation day 8) cultured CD-1 mouse embryos were exposed to 50–1000 nM microcystin-LR in whole culture for 24 h. No increase in abnormalities or developmental delays was seen [77].

Rabbit embryos and whole rabbit embryo cultures were exposed to microcystin-LR to study the possible effects on morphology and cytoskeletal elements. Growth, development and cytoskeleton organization of the embryos embedded in *zona pellicuda* are not affected by microcystin-LR at concentrations up to 100 μM, while whole rabbit embryo cell cultures are affected by the presence of microcystin-LR in the culture medium. High microcystin-LR concentrations (100 μM) caused cells to be detached and destroyed, while concentrations of 10–20 μM profoundly affect actin and microtubule organization [78].

Reproduction and developmental study in toads. A developmental study in toads (*Bufo arenarum*) was performed from stage 17 (tail bud) for 10 days at concentrations from 1 to 20 mg microcystin-LR/L. No effect on survival or morphological development was seen [77].

Immunotoxicity. Microcystin-LR and microcystin-YR (and also nodularin) inhibited the *in vitro* (spleen cells from virus-free female B6C3F1 mice) polyclonal antibody forming cell response and lymphoproliferation to LPS. The concanavalin A-induced T-lymphocyte proliferation was inhibited by microcystin-YR (and nodularin) but not by microcystin-LR [79].

Male and female BALB/c mice received daily for 14 days intraperitoneal injections with 16, 32 or 64 mg lyophilized algae cells (extract of a cyanobacterial bloom containing microcystins; 0.310 mg microcystins/g lyophilized algae cells)/kg bw. Body weights were decreased dose-dependently, liver/bw ratios were markedly increased and significant differences in spleen and thymus/bw ratios were observed. Uptaking capacity of peripheral phagocytes was decreased significantly at 32 and 64 mg/kg bw. Also, LPS-induced lymphoproliferation was inhibited and the numbers of antibody-forming cells after immunization by T-dependent antigen sheep red blood cells showed dose-dependent decreases. However, concanavalin A-induced T-lymphocyte proliferation was not affected [80].

2.1.6.4 Effects on humans

Humans can be exposed orally *via* drinking water or *via* consumption of algal health food tablets and dermally *via* recreational use of lakes and rivers (cited from Rao *et al*. [54]). A minor route is inhalation exposure from the use of showers [5].

Short-term effects in humans. The earliest case of gastroenteritis from cyanobacteria was in the population of a series of towns along the Ohio river, USA, in 1931. In Harare, Zimbabwe, during the years 1960–1965, children living in an area of the city supplied from a particular drinking water reservoir, developed gastroenteritis each year at the time when a natural bloom of *Microcystis* was decaying in the reservoir. Other children in the city with different water supplies were not affected and no infectious agent was identified [3].

In the summer of 1981, a dense bloom of *M. aeruginosa* was being monitored in the drinking water reservoir supplying Armidale, New South Wales, Australia. Drinking water consumers complained of bad taste and odour from the drinking water. Controlling water authorities terminated the bloom by copper sulphate addition to the reservoir. A retrospective study of the liver function in the residents (hospital patients) who were supplied with the drinking water from this reservoir, compared with that in residents of comparable areas with different water supplies, showed evidence of toxic liver damage (increase in the activity of the hepatic enzyme γ-glutamyl-transferase) coincident with the bloom and its termination [81, 54].

In a community consuming water of a reservoir contaminated by cyanobacterial blooms, a high human birth defect rate was associated with cyanobacterial toxins [4].

Eight towns in Australia along the lower Murray river, which was used for water supply, were monitored for gastroenteritis and allergic conditions among the population. Significantly, more gastroenteritis occurred in the population that drank chlorinated river water than among those drinking their own supply of unchlorinated rain water [81].

The two most lethal poisonings attributed to cyanobacteria in drinking water occurred in Brazil. A massive *Anabaena* and *Microcystis* bloom in Itaparica Dam were responsible for 2000 cases of gastroenteritis resulting in 88 deaths, mostly children (cited Rao *et al*. [54]). In 1996, an outbreak of acute liver failure at a haemodialysis centre in Caruaru occurred. At the clinic 116 out of 131 patients experienced visual disturbances, nausea and vomiting after routine haemodialysis treatment on 13–20 February 1996. Subsequently, 100 patients developed acute liver failure, and of these 76 died. Comparison of victim's symptoms and pathology using animal studies of the two suspected cyanotoxins (microcystins and cylindrospermopsin) led to the conclusion that the major contributing factor to death of the dialysis patients was intravenous exposure to microcystins, specifically microcystin-YR, -LR and -AR. From liver concentrations and exposure volumes, it was estimated that 19.5 μg microcystin/L was present in the water used for dialysis treatment [82, 83].

Oral exposure *via* the consumption of fish particularly the livers of fish, from waters containing cyanobacterial blooms, can also occur and may cause Haff disease (vomiting, brownish-black urine, muscular pain, respiratory distress mortality). The evidence of the involvement of cyanobacterial toxins in these cases is circumstantial, but accumulation of toxins in fish cannot be ruled out (cited from Rao *et al*. [54]).

Man can also be exposed orally *via* algal health food products. These products are potentially hazardous if they contain any of the toxigenic species or strains of cyanobacteria. Many of these products contain *Aphanizomenon flos-aquae*, a blue-green alga that is harvested from Upper Klammath Lake in southern Oregon, USA. Because *M. aeruginosa* coexists with *A. flos-aquae*, it can be collected inadvertently resulting in microcystin contamination of blue-green algae health products. The Oregon Health Division and the Oregon Department of Agriculture established a regulatory limit of 1 μg/g for microcystins in blue-green algae-containing products and tested blue-green algae products for the presence of microcystins. Microcystins were detected in 85 of 87 samples tested, with 63 samples (72%) containing levels >1 μg/g. HPLC and ELISA tentatively identified microcystin-LR [84].

Dermal exposure may occur during recreational use of water bodies and during showering and can cause blistering of lips and allergic reactions (contact dermatitis, asthma, hay fever and conjunctivitis) [54]. In the UK in 1989, 10 out of 20 army recruits developed vomiting, diarrhoea, central abdominal pain, blistering of the lips and sore throats after swimming and canoe training in water with a dense bloom of *Microcystis* spp. Two of the recruits developed pneumonia attributed to the aspiration of *Microcystis* toxin and needed hospitalization and intensive care. The severity of the illness appeared to be related to the swimming skills and the amount of water ingested [3]. Epidemiological evidence of adverse health effects after recreational water contact was established in a prospective study involving 852 participants. Results showed an elevated incidence of diarrhoea, vomiting, flusymptoms, skin rashes, mouth ulcers, fevers, eye or ear irritation within 7 days following exposure. Symptoms increased significantly with the duration of water contact and cell density of cyanobacteria [3].

Twenty-six cases with skin diseases and multiple systemic symptoms associated with exposure (some *via* drinking water) to river water or rain water were reported in Australia during 1991–1992. The water was stored in open tanks and contained *Anabaena* blooms [59].

Illness in humans associated with inhaling microcystins had been reported. The intranasal route appeared to be as toxic as the intraperitoneal route. Therefore, the risk posed by inhaling microcystins during showering should be of concern [4].

Between November 1995 and October 1996, 50 samples from five regions of Paraná, Brazil, were examined for the presence of *Microcystis* spp. and microcystins (by ELISA). *M. aeruginosa* was positive in all the samples. Heavy contamination with microcystins was observed in samples collected in May 1996 from two recreation (swimming-fishing sites 6.38 and 10 μg/L) and one human supply (6.627 μg/L) samples. No cases of human illness were reported [85].

Long-term effects in humans. The implications of low level chronic exposure to microcystins, are not known [3]. In China, studies were performed whether cyanobacterial toxins are part of risk factors in the development of human hepatocellular carcinoma (HCC). The incidencence of this disease is one of the highest in the world in China. The distribution of HCC varies geographically. Two proven risk factors are the intake of aflatoxin B_1 from food items such as maize, and the hepatitis B virus. The third significant element of association was the source of drinking water. On a village base, lower cancer mortality rates were seen when the water was drawn from deep wells compared with much higher rates when the water came from ponds and ditches. Cyanobacteria are abundant in surface waters in southeast China, where the incidence of HCC is the highest. Very low levels of cyanotoxins were found in one study with limited sampling of drinking water sources in China. All three risk factors are being reduced concurrently in China and HCC rates appear to be decreasing (cited from Chorus and Bartram [3]).

2.1.6.5 Effects on aquatic organisms

Exposure of *Ceratophylum demersum* (a common lake macrophyte) for 6 wk to microcystin-LR levels of 0.1–5.0 μg/L revealed at concentrations =1.0 μg/L a significant growth reduction after 6 and 3 wk, respectively. Significant inhibition of photosynthetic oxygen production and changes in pigment pattern (changing of ratio of chlorophyl *a* to chlorophyl *b*) were seen at microcystin-LR levels ≥0.5 μg/L. Photosynthesis of the aquatic macrophyte *Elodea canadensis*, the macroalgae *Cladophora* sp., the submergent macrophyte *Myriophyllum spicatum* and the emergent macrophyte *P. australis* was also inhibited significantly at exposure to 0.5 μg microcystin-LR/L [86].

A dose-dependent response in mortality of *A. salina* was seen for purified microcystin-LR and LC_{50} values decreased with time from 4.58 to 0.85 μg/mL between 24 and 72 h, respectively [87].

Exposure of adult *A. salina* to microcystin-LR caused an elevation of glutathione-*S*-transferase (GST) activity *in vivo*. Microcystin-LR was conjugated to glutathione *via* GST, which is an initial step of detoxication [88].

Duckweed (*Lemna minor*) plants were cultivated for 6 days in a nutrient solution with 0, 0.1, 0.5, 1.0, 3.0 or 5.0 mg microcystin-RR/L. At concentrations, ≤1.0 mg/L no effect on growth was seen. At 3.0 and 5.0 mg/L formation of fronds was already decreased significantly >25% and up to 37% at 3.0 and 5.0 mg/L, respectively) at 3 days of exposure. After 6 days of exposure most of the plants at 5.0 mg/L formed only dwarfish, partially malformed fronds, and some of these showed chlorosis compared to control plants. At 5.0 mg/L photosynthetic capacity was decreased (maximum electron transport rate was only 16% of control value) and contents of chlorophyll *a*, *b* and the total amount of carotenoids were significantly reduced by >50% after 6 days of exposure [89].

Microcystins can affect *Daphnia pulicania*, *D. hyalina*, *D. longispina* and *D. pulex* and are toxic to the brine shrimp, *A. salina*. Certain zooplankton such as rotifers, copepods and cladocerans are killed by microcystins [3, 41]. Montagnolli *et al*. [90] reported acute effects of *M. aeruginosa*, derived from the Patos Lagoon Estuary, Southern Brazil, on the microcrustacean *Kalliapseudes schubartii.*

Since 1996, the RIZA in the Netherlands has studied the ecological effects of cyanobacterial toxins in the lake called IJsselmeer. Preliminary results showed that toxic cyanobacteria are eaten by zooplankton and mussels. The measured microcystin levels in seston (*i. e.* organic and inorganic matter suspended in seawater), phytoplankton, zooplankton and mussels in the IJsselmeer are so high that intoxication of grazers and their predators can be expected. However,

the interpretation of the values is based on the toxicity of the very toxic microcystin-LR, and in the IJsselmeer other not yet identified microcystins occur, which at least for water fleas, are less toxic than microcystin-LR. Slow growth of zooplankton has been observed as well as liver degeneration in fish in the IJsselmeer, but a clear cause-effect relationship to cyanotoxins has not been established yet. Mussels seemed to be little affected by cyanotoxins. They obviously possess mechanisms that reduce intoxications by cyanotoxins. However, it cannot be excluded that cyanotoxins are being transformed and that they may therefore be missed in standard tests which determine microcystin levels. There are also indications that microcystin levels are being underestimated because covalent-bounded microcystins cannot be detected [91].

M. galloprovincialis fed on a toxic *M. aeruginosa* strain that showed 1% mortality within a 16-day exposure period [41].

Early larvae of axolotl (*Ambystoma mexicanum*) were exposed under laboratory conditions to 0.5, 5 or 50 μg/L of purified microcystin-LR, microcystin-YR or microcystin-R until the end of the embryonic period. After the embryonic period, larvae were reared in toxin-free water. No effects were seen during embryonic development. However, a delay in feeding (by 1 day) was seen at an exposure to 50 μg microcystin-LR or 50 μg microcystin-YR/L. Exposure to 5 and 0.5 μg microcystin-LR or microcystin-YR/L or to 50 μg microcystin-RR/L or lower did not cause a delay in feeding. After rearing to an age of 35 days, many larvae exposed to 50 μg microcystin-LR, microcystin-RR or microcystin-YR/L showed less developed forelimbs. No effect on survival rate was seen [92].

No effect on the embryonic development of larvae of the smooth newt (*Triturus vulgaris*) and Marsh frog (*Rana ridibunda*) was seen at exposure to 0.5, 5 or 50 μg purified microcystin-YR or microcystin-RR/L. However, at rearing in toxin-free water after embryonic development, a reduced weight of 26 and 18%, respectively, was seen after pre-exposure to 50 μg/L of microcystin-YR and microcystin-RR. No effect on survival rate was seen [92].

Acute exposure of zebrafish (*Danio rerio*) embryos during day 0–5 of age to 0.1, 1, 5 or 10 mg purified microcystin-LR, microcystin-YR, microcystin-RR or microcystin-LF/L did not result in visible effects. However, exposure at the highest concentration of microcystin-LR (10 mg/L) during day 4–5 of age (eleuthero embryos) resulted in 10% decrease in heart rate after 2 h exposure. One day later, the embryos had developed pectoral edema and showed an enlarged and opaque yolk. The malformations were reversible (in 95% of embryos 1 day later). The other microcystins tested or lower concentrations of microcystin-LR, did not induce these effects [92].

When zebrafish (*D. rerio*) larvae were exposed for 6 days to 0.5, 5 or 50 μg purified microcystin-LR or RR/L and reared thereafter in toxin-free water, survival rate and growth were adversely affected at 5 and 50 μg/L of both toxins. No significant effect was seen at 0.5 μg/L [92].

In rainbow trout (*Oncorhynchus mykiss*) eggs, no acute effects were seen during embryonic development at exposure to 0.5, 5 or 50 μg purified microcystin-LR, microcystin-YR or microcystin-RR/L. However, earlier hatching was seen at 50 μg microcystin-LR/L, at 5 and 50 μg microcystin-YR/L and at all the three concentrations of microcystin-RR; the delay in hatching was minor at 0.5 and 5 μg microcystin-LR/L and at 0.5 μg microcystin-YR/L. The effect was most pronounced with microcystin-RR and was dose-dependent [92].

When embryos of fish and amphibians (zebra fish (*D. rerio*), roach (*Rutilus rutilus*), axolotl (*A. mexicanum*), smooth newt (*T. vulgaris*)) were exposed from cleavage up to advanced stages of embryonic development to various aqueous crude extracts of *M. aeruginosa* from field samples and batch cultures, far more pronounced effects were seen as compared to the effects caused by purified microcystins (see studies above from Oberemm *et al*. [92]). Similar malformations combined with high mortalities and adverse effects on outer egg structures were seen concomitantly in all tested species. The most sensitive species was the smooth newt, and roach was more sensitive than zebra fish. HPLC data showed that the effects cannot be attributed to microcystins alone [92].

In 1992, a kill of approximately 2000 brown trout in Loch Leven and the River Leven, Scotland, was believed to have been caused by toxins of *Anabaena flos-aquae*. The killed fish had liver damage characterized by necrosis, cellular degeneration, condensation and disintegration of nuclei, edema and focal areas of congestion and gill damage (cited from Duy *et al*. [4]).

Microcystin-LR or a closely related microcystin has a link to netpen liver disease in Atlantic salmon. In an experiment on healthy salmon, this link to netpen liver disease was confirmed. Under laboratory conditions microcystins caused hepatocellular damage also in carp (*Cyprinus carpio*) and rainbow trout (*O. mykiss*). Rainbow trout injected with microcystin-LR showed a colour change from light silvery green to a dark olive-green and lost swimming coordination and buyancy control. Even though the fish appeared to be relatively tolerant to high microcystin-LR doses, deaths were seen at 1000 μg/kg bw supposed to be caused by general hepatic failure due to massive hepatocyte necrosis (cited from Duy *et al*. [4]).

Microcystins were found to inhibit enzymes of the gill microsomal fraction of carp involved in ion pumps and exchangers. Later on microcystin-LR and microcystin-LR-like toxin from *M. aeruginosa* were found to inhibit the reaction of the Na^+–K^+ pump of the gill of carp. This inhibition might block the gills function and so disrupt the ion homeostasis of the internal medium. In turn, the impairment of gill activity may result in fish death (cited from Duy *et al*. [4]).

In rainbow trout, given orally by gavage 5700 μg microcystin-LR/kg bw as lyophilized *M. aeruginosa*, no mortalities occurred during gavage or throughout the test duration (72 h). In the liver necrosis and apoptosis of hepatocytes as well as intrahepatic haemorrhage were seen. The sequence of hepatic cell death, with necrosis appearing very early and apoptosis rather late in the development of liver damage, suggested that the pathological events underlying microcystin liver damage is highly comparable in mammalian and fish species [93].

Crayfish (*P. clarkii*) larvae are resistant to *M. aeruginosa* cells and their toxins, surviving densities of 3.3×10^6 up to 3.3×10^7 cells/mL during acute exposures up to 72 h. Juvenile crayfish also survived feeding with toxic *M. aeruginosa* for 8 wk although a decrease in individual growth was seen [44].

Catfish in three rearing ponds in the USA died (>100 000 fish deaths during a 2-wk period) due to microcystin consumption as a consequence of an *M. aeruginosa* bloom. Microcystin levels in filter retained water samples were >100-fold higher than baseline values and microcystin was detected in livers of the catfish [94].

Dominance of the toxic cyanobacteria *Planktothrix* sp. since 1990 in Lake Ammersee in southern Germany (cell counts up to 80 000 cells/mL; microcystin levels in *Planktothrix* extracts up to 6.5 μg/mg dry weight, microcystin levels in water up to 0.08 μg/L), caused irregularities in the development and growth of coregonids (whitefish, *Coregonus lavaretus*). In winter 2000/2001, nearly 75% of whitefish eggs died before hatching in a Lake Ammersee hatchery. *Planktothrix* sp. produces the cyclic peptide toxin desmethyl microcystin-RR (microcystin dmRR) [95].

In a pond located in Japan, the mass death of spot-billed ducks was reported. The death of these birds was attributed to high concentrations of three types of microcystins in a bloom of *M. aeruginosa*. Histopathology of the liver of one of these birds showed a necrotic liver, severely jaundiced and dark green in colour [96].

2.1.6.6 Effects on wild and domestic animals

Microcystin are responsible for intermittent, but repeated cases of poisoning in wild and domestic animals. They were attributed as the cause of death of cattle, geese, sheep, pigs, horses, dogs, cats, squirrels, poultry, waterfowl and birds. In both wild and domestic animals, hepatoxicosis was seen. The signs of hepatoxicosis included weakness, reluctance to move about, anorexia, pallor of extremities and mucous membranes, and mental derangement. Death occurs within a few hours to a few days and is often preceded by coma, muscle tremors and general distress. Death is believed to be the result of intrahepatic haemorrhage and hypovolemic shock (cited from Duy *et al*. [4]).

Over the 1990s, episodic mass mortalities of Lesser Flamingos (*Phoeniconaias minor*) have occurred at Kenya's Rift Valley saline, alkaline lakes. Analyses of flamingo carcass livers and cyanobacterial samples from Lakes Bogoria and Nakuru demonstrated three cyanobacterial toxins in dead flamingo livers: microcystin-LR, microcystin-RR and anatoxin-a. The total extractable microcystin levels in the bird livers varied from 0.21 to 0.93 μg microcystin-LR eq/g fresh weight, and those of anatoxin-a from 1.06 to 5.82 μg/g fresh weight. These toxin concentrations may have been sufficient to have caused the bird's death. The presence of anatoxin-a was consistent with observations of staggering and convulsions in the flamingos before death and with opisthotonus postmortem [97]. Further research with materials from Lake Bogoria Lesser Flamingos has identified the microcystins-LR, -RR, -LF, and/or -YR) and anatoxin-a also in bird stomach contents, intestine contents and faecal pellets. Total microcystin level in stomach contents was 0.196 μg microcystin-LR eq/g fresh weight and the anatoxin-a level in stomach contents was 4.34 μg/g fresh weight [98].

In 2001, flamingo mass mortalities in southwest Spain have involved wild and captive birds; at least 579 of 943 greater flamingo (*Phoenicopterus ruber*) chicks died, together with a mixed population of other water birds at the Doñana National Park lagoon. Microcystins were identified as the likely cause of this death event in Spain, based on the presence of microcystin-producing cyanobacteria and microcystins in the water and crop contents, postmortem examination of livers and the elimination of alternatives [99].

An acute mortality of ten adult captive Chilean flamingos (*P. chilensis*) occurred at SeaWorld, Orlando, Florida, USA, in 2001. The *P. chilensis* deaths were also attributed to microcystin-LR and microcystin-LA, based on poisoning signs, postmortem examination of organs, toxin concentrations in gastrointestinal contents and pond water and elimination of alternatives [99].

Four Holstein–Friesian cows received daily for 21 days drinking water containing 1×10^5 *M. aeruginosa* cells/mL. Cattle consumed a total dose of microcystin-LR up to 15 mg corresponding to 1.21 μg/kg bw/day. No effects on the behaviour or liver function (by means of changes in ASAT, γ-GGT, GADH, bilirubin) were seen. Analysis of milk (ELISA) did not show any detectable amount of microcystin. This means that the concentrations of free microcystin-LR did not exceed the LOQ of the ELISA kit (3.2 pg microcystin-LR/well) which was corresponding to a maximum possible concentration in the milk of 2.0 ng/L. This is 500 times lower than the WHO guideline for drinking water of 1 μg/L (80% of TDI was assigned to drinking water). When 10% of the TDI was assigned to milk and a daily milk consumption of 280 mL is assumed, a threshold level for microcystin-LR in milk of 0.86 μg/L was calculated by the authors [100].

Two Holstein–Friesian cows received orally *per* gelatin capsule daily for 4 wk doses of microcystin-LR increasing weekly from 0 to a maximum of 13 μg/kg bw/day (as

freeze-dried cyanobacterial mass containing 0.26 wt.% microcystin-LR). Two cows were used as controls (did not receive empty gelatin capsules and/or biological mass without microcystin-LR). Twice daily behaviour (salivation, unsteadiness) was checked. Two to three times *per* week blood plasma was examined of for liver function parameters (γ-GGT, SAP, total bilirubin, albumin). Milk production of treated cows was measured and milk was examined for the presence of microcystin-LR (by PP-inhibition assay). Liver function parameters were not affected in the treated cows and milk production did not differ from control cows. microcystin-LR level in milk was below the detection limit of 0.2 μg/L (by PP inhibition assay) suggesting that either no microcystin-LR was present in the milk of the treated cows or microcystin-LR was sufficiently biotransformed to render it not active in the PP inhibition assay [101].

When Australian yearling beef cattle steers (four animals) received live, toxic, 1×10^5 *M. aeruginosa* cells/mL in their drinking water for 28 days, no measurable liver dysfunction could be detected. Four controls were used. No concentrations of microcystin in liver or blood plasma were found that would present an unacceptable risk to human health. The treated cattle consumed between 6.7 and 7.7 mg microcystin-LR each (average ingestion 1.42 μg/kg bw/day) [102].

2.1.6.7 Effects on crops/plants

The inhibition of photosynthesis was measured in *P. vulgaris* leaves after dipping once or repeatedly in microcystin-LR solutions. A single dipping of *P. vulgaris* leaves in microcystin-LR solutions did not cause inhibition of photosynthesis at a concentration of 1 mg/L; at 10 mg microcystin-LR/L the inhibition was transient (recovery after 5 days) and at 100 mg/L the net photosynthesis rate was still 42% of control value 8 days after dipping and necrosis of the leaves was observed. By repeatedly (once daily, for 9 days) dipping of *P. vulgaris* leaves in microcystin-LR solutions, the photosynthetic apparatus was inhibited already at relatively low concentrations (*ca*. 20 μg/L). Patchy leaf necrosis was observed at the end of the experiment, but it was not as marked as after a single application with 100 mg/L [103].

In a laboratory bioassay, microcystin-LR (1.12 μg/mL) appeared to have little effect on the growth of germinated runner bean (*P. vulgaris*) seeds (stem 20–30 cm) during the first 18 days. Stem length and leaf development were normal. However, after 25 days of exposure, necrosis was evident in leaves. This effect was followed by the desiccation and loss of older leaves in exposed plants. Furthermore, microcystin-LR impaired the development of the roots of exposed plants. After 3 days of exposure, the roots of exposed plants appeared discoloured, and exhibited poor growth which became more evident as the experiment progressed. After 18 days, the roots of the exposed plants were dark brown in colour, with very short, thick lateral roots and the uptake of growth medium by the roots was approximately 30% less [50].

Potato (*Solanum tuberosum*) shoot cultures (nine replicates/concentration) were exposed for 16 days to solutions containing 1–5000 μg microcystin-LR/L. Growth and chlorophyll content were determined. At 1 μg/L, no significant effect on growth was seen. At concentrations of 5, 10, 50, 100 and 500 μg/L significant, dose-dependent growth inhibition was seen, but no significant further inhibition was observed when the concentration increased to 5000 μg/L. Chlorophyll content was not decreased significantly at 1 μg/L. At 5 and 10 μg/L slight but no significant decreases were seen and significant, dose-dependent decreases of chlorophyll content were observed at concentrations of 50, 100, 500, 1000, 2500 and 5000 μg/L. In addition, shoots exhibited 50–100% necrosis in shoot and leaf tissue at concentrations from 50 to 5000 μg/L. At lower concentrations of 1–10 μg/L, 25% necrosis of shoot and leaf tissue was seen. Root development was evident in all cultures up to and including a concentration of 50 μg/L. However, as microcystin-LR concentration increased the number of roots was found to decrease. At 0, 1 and 5 μg/L, up to three roots were seen, decreasing to 1–2 at 10, 50 and 100 μg/L. The number of replicate shoots exhibiting no root development increased at 500 μg/L, and at 1000, 2500 and 5000 μg/L roots were absent in all replicates [50].

The growth of mustard seedlings (*Synapis alba*) was examined at exposure for 7 days to concentrations of 0, 100, 500, 1000, 2500, 5000, 10 000, 25 000 or 50 000 μg microcystin-LR, -RR or -LF/L (15 replicates/dose). Each microcystin caused significant dose-dependent growth inhibition. The GI_{50} (50% growth inhibition) for microcystin-LR, -RR and -LF was 1900, 1600 and 7700 μg/L, respectively. The lowest concentration causing significant growth inhibition was 500, 100 and 5000 μg/L for microcystin-LR, -RR and -LF, respectively [50].

Rape and rice seedlings were exposed to water containing 0, 0.024, 0.12, 0.6 and 3 μg microcystin-LR eq/mL. Inhibition of growth and development of both plant species was examined. Microcystins had a more powerful inhibitory effect on rape than rice in germination percentage of seeds and seedling height. Microcystins significantly inhibited elongation of primary roots of rape and rice seedlings. Determination of peroxidase and superoxide dismutase revealed that microcystin stress was manifested as oxidative stress [49].

Exposure of the emergent reed plant *P. australis* to 0.5 μg ^{14}C-labelled microcystin-LR/L for 3 days (in a 300 mL beaker) demonstrated a rapid uptake of the toxin (sampling times 0.5, 1, 2, 4, 6, 12, 24, 48 and 72 h). The main uptake route appeared to be in the stem and rizome, from which the toxin is transported into the higher parts of the plant to the leaves. Uptake directly through the leaves may occur by direct contact of small plants or by the lowest leaves of a

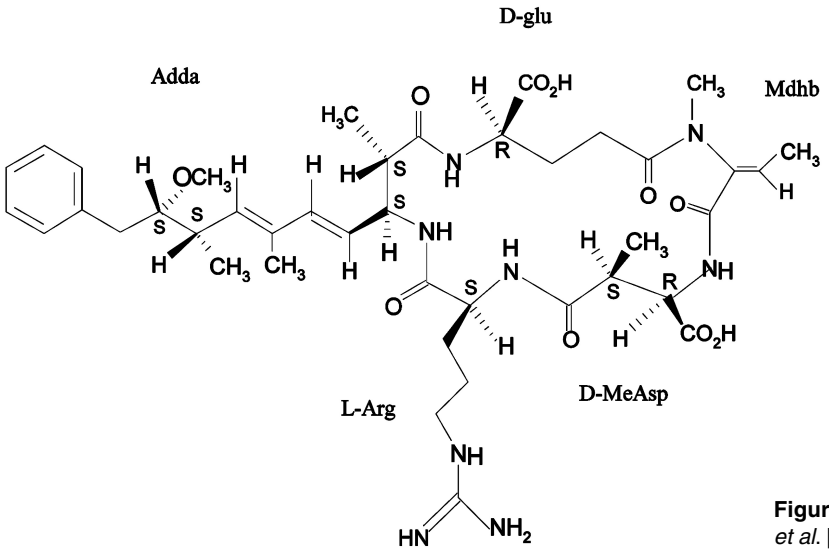


Figure 3. Chemical structure of nodularin (from Duy *et al.* [4]).

plant with surface water and with upper leaves by wave and spray contact [51].

2.1.6.8 Derivation of NOAEL and TDI

In 1998, the WHO derived a provisional guideline for drinking water for microcystin-LR [59]. The WHO concluded that for microcystins other than microcystin-LR there are insufficient data to derive a guideline. This conclusion is still valid. The drinking water guideline for microcystin-LR was derived from the NOAEL of 40 μg/kg bw in a 13-wk drinking water study in mice with microcystin-LR. The NOAEL was based on liver pathology. A TDI was calculated using an uncertainty factor of 1000 (100 for intra- and interspecies variation, 10 for limitations in the database). An allocation factor of 0.80 is used for the proportion of daily exposure arising from drinking water, because there is little exposure from any other source and route. The resulting guideline value for total microcystin-LR (free plus cell bound) is 1 μg/L (rounded figure) in drinking water [59]. The guideline value is supported by a 44-day study in which pigs were exposed, in their drinking water, to an extract from *M. aeruginosa* containing microcystin-LR.

When microcystins other than microcystin-LR are present, it may be necessary to use TEFs and to express the toxicity of the microcystin mixture in microcystin-LR equivalents [11] (see also Section 2.1.6.3).

2.1.7 Regulations

In 1998, WHO established a provisional guideline value for microcystin-LR in drinking water of 1 μg/L (cell-bound and extracellular) [59]. In Brazil, New Zealand and Great Britain, a guideline value of 1 μg/L is valid as well. In Canada [104] and Australia, guideline values in drinking water of 1.5 (for total microcystins (free and cell bound) expressed as microcystin-LR equivalents) and 1.3 μg/L (for total microcystins (free and cell bound) expressed as microcystin-LR equivalents), respectively, are established. In New Zealand, a health-based guideline value of 1.0 μg/L is valid but also a guideline value of 0.1 μg/L including a tumour-promotion factor of 10, is reported [105].

In Australia, a health alert value of 10 μg microcystin-LR eq/L drinking water (corresponding to 50 000 *M. aeruginosa* cells/mL) was proposed relating to potential acute or short-term health effects. Furthermore, a drinking-water action level of 2000 *M. aeruginosa* cells/mL and a recreational guideline of 20 000 *M. aeruginosa* cells/mL are valid in Australia [106]. In the USA, there is no guideline value for microcystins (or microcystin-LR) in drinking water. On 23 October 1997, the Oregon Department of Agriculture in the USA adopted 1 μg microcystin-LR/g as a regulatory standard for blue-green algae products for oral consumption [84].

2.2 Nodularins (NODLNs)

2.2.1 Chemical structures and properties

Nodularins are monocylic pentapeptides, with the two terminal amino acids of the linear peptide being condensed (joined) to form a cyclic compound (see Fig. 3). Nodularin itself has an M_r of 824 Da. The general structure of the nodularins is: cyclo-(D-MeAsp-L-Arg-Adda-D-glutamate-Mdhb) in which L-Arg is L-arginine, D-MeAsp is D-*erythro*-β-methylaspartic acid and Mdhb is 2-(methylamino)-2-dehydrobutyric acid. The amino acid Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is the most unusual structure in this group of cyclic peptide toxins. A few (seven) natural occurring variations of nodularin have been found; two demethylated variants, one with D-Asp instead of D-MeAsp, the other with DMAdda instead of Adda. A nontoxic natural occurring

variant of nodularin contains the 6Z-stereoisomer of Adda. In the marine sponge *Thenella swinhoei*, a nodularin analogue called motuporin has been found. In motuporin, instead of the polar L-arginine the hydrophobic L-valine is present. Motuporin might be also of cyanobacterial origin because the sponge is known to harbour cyanobacterial symbionts [3, 4]. Saito *et al*. [107] isolated and identified a new nodularin called nodularin-Har. This new nodularin-Har has homoarginine instead of arginine.

2.2.2 Methods of analysis

2.2.2.1 General

In many respects, nodularins have similar chemical properties as microcystins. Both are relatively polar molecules whereas amongst other parts the Adda residue gives a partially hydrophobic character to these toxins. Looking from this particular, chemically analytical angle, it may not be surprising that nodularins and microcystins are determined very often with the same methods and even within the same analytical run.

2.2.2.2 Extraction/concentration/clean-up procedures

See Section 2.1.2.2.

2.2.2.3 Determination/detection

PPIA. The biochemical assay PPIA is based on the fact that nodularins (as well as microcystins) inhibit enzymes responsible for the dephosphorylation of intracellular phosphoproteins, in particular PP1 and PP2. The first developed method [108] did make use of ^{32}P radiolabelled substrates (radiometric assay), which causes a huge problem: most routine laboratories are not sufficiently equipped to carry out radioactive determinations. To overcome this, other PPIAs, which do not require radiolabelled substrates, have been worked out. In colorimetric assays, the concentration in time of substrates such as *p*-nitrophenyl phosphate (*p*NPP) can be measured by the release of *p*-nitrophenol [14, 109]. This sensitive method achieves detection of nodularin concentrations in raw water below 1 μg/L. Later on, a fluorometric PPIA [110] was developed that has the ability of measuring microcystins (and consequently nodularins) in drinking water without clean-up and preconcentration procedures, with an LOD down to 0.1 μg/L; which is in fact comparable to ELISA. Neither the radiometric nor colorimetric PPIA could provide detailed information on the chemical identity of the inhibitors present in samples. Accordingly, PPIA may only be used as a screening method.

ELISA. Many studies have been performed to evaluate commercial kits [111, 112] for the detection of nodularins and microcystins. The conclusion of all those studies is that ELISA is a very sensitive method and simple in practice. But it has a potential for false positive reactions [113].

A major problem in ELISA is the crossreactivity of less toxic nodularin (and microcystin) variants. Cross-reactivity together with applicability and detection of analytes was profoundly discussed by Metcalf *et al.* [114]. Originally in many studies, an antibody was generated against a whole molecule (*e.g.* microcystin-LR). Interesting is a study [115] in which an mAb was generated against a common part of microcystins and nodularins, namely Adda. In this way an assay is obtained that measures all microcystins and nodularins equally and gives a sum concentration of them.

In combination with PPIA, immunoassay-based detection is a promising approach for nodularin detection [116]. A special technique (colorimetric immunoprotein phosphatase inhibition assay, CIPPIA) unites toxicity assessments and specificity for the analysis of nodularins (and microcystins). But also the (off-line) parallel combination of both PPIA and ELISA provides analysts a powerful procedure for determining microcystins and nodularins.

Bioassay. See Section 2.1.2.2.

HPLC. See Section 2.1.2.2.

2.2.2.4 Conclusion

See Section 2.1.2.2.

2.2.3 Source organism(s) and habitat/occurrence

2.2.3.1 Source organism(s)

Nodularin is produced only by the brackish water cyanobacterium *Nodularia spumigena*. The nodularin analogue motopurin is produced by the marine sponge *Theonella swinhoei* [3]. Saito *et al*. [107] isolated and identified a new nodularin called nodularin-Har from *Nodularia* PCC7804.

2.2.3.2 Habitat/occurrence

Nodularin has been found in brackish waters in Australia, New Zealand, Tasmania, Northern-Europe and the Baltic Sea [4, 54, 106]. Brackish water coastal lagoons and estuaries are frequently subject to summer blooms of *N. spumigena* [81]. The occurrence of *N. spumigena* blooms is determined by water temperature, light intensity and nutrient concentration; levels of nitrogen and phosphorus in particular are critical [117]. Moisander *et al.* [37] concluded that salt (NaCl concentrations tested 0–20 g/L) *per se* is not a primary controlling factor for bloom forming of *N. spumigena* and *N. sphaerocarpa* under laboratory conditions.

Intracellular nodularin concentrations increased with increasing temperatures, phosphate concentrations and irradiance. They decreased at low and high salinities and unnaturally high nitrogen concentrations [118]. In the Gulf of Gdansk, Poland, *N. spumigena* blooms were exceptional

during 2001 and 2002; in 2001 because of their high intensity and in 2002 because of their duration [117]. In South Australia frequent blooms of *N. spumigena* occur also in freshwater lakes of the lower River Murray. A survey in 1994–1995 indicated that the upper range for cell numbers was 50 000–80 000 cells/mL, while the upper range for nodularin levels was 1.0–1.7 μg/L (total extracellular and intracellular). Extracellular levels rarely exceeded the detection limit (HPLC) of 0.5 μg/L [106].

Hobson and Fallowfield [119] studied the influence of temperature, irradiance and salinity on growth, and nodularin production by *N. spumigena* isolated from Lake Alexandrina in the southeast of South Australia. The influence of salinity on total nodularin production rate was significant. However, the intracellular nodularin production rate was independent of salinity. Highest biomass production and nodularin production occurred at salinities of 360–13 200 mg TDS (= total dissolved solids)/L for cultures grown at both 30 and 80 μmol photons · $m^{-2}s^{-1}$. A significant combined influence of temperature and irradiance on the total nodularin production rate was observed. However, these variables did not affect the intracellular production rate. The highest nodularin production rates and yields, at a light intensity of 30 μmol photons · $m^{-2}s^{-1}$, were seen at 30°C, whereas at a light intensity of 80 μmol photons – m^{-2} s^{-1} the highest toxin production rates and yields were seen at 20 μmol photons · $m^{-2}s^{-1}$. The same irradiance/temperature interaction was also seen for biomass production rates and yields. A significant combined influence of temperature and irradiance on intracellular nodularin concentration *per* unit biomass was observed. However, in contrast to biomass and total nodularin production, *N. spumigena* grown at both 30 and 80 μmol photons · $m^{-2}s^{-1}$ produced the highest intracellular nodularin concentration *per* unit biomass at 10°C at 132 000 TDS/L. A higher intracellular nodularin concentration *per* unit biomass in cells grown at 320°C at 30 μmol photons · $m^{-2}s^{-1}$ compared to those grown at the same temperature but at 80 μmol photons · m^{-2} s^{-1}, suggested that photoinhibition at the higher irradiance level reduced nodularin production. Maximum nodularin production rates for all temperature, salinity and irradiance combinations were recorded at 20°C, light intensity of 80 μmol photons · $m^{-2}s^{-1}$ and salinity of 13 200 mg TDS/L for total nodularin production rate and 30°C, 80 μmol photons · $m^{-2}s^{-1}$ and 26 400 mg TDS/L for intracellular nodularin production rate.

2.2.4 Bioaccumulation in the environment

2.2.4.1 Accumulation in aquatic organisms

N. spumigena blooms occur regularly in the Baltic Sea during summer. The toxin produced by *N. spumigena*, *i.e.* nodularin, appeared to accumulate in mussels (*M. edulis* and *Dreissena polymorpha*) and clams (*Macoma balthica*) from the Baltic Sea and in some Baltic fish species, *e.g.* flounder (*Platichthus flesus*), Atlantic cod (*Gadus morhua*) [120–122] and three-spine stickleback (*Gasterosteus aculeatus*) [123].

Kozlowsky-Suzuki *et al*. [124] studied the feeding of *N. spumigena* and the accumulation of nodularin in the calanoid copepods *Acartia bifilosa* and *Eurytemora affinis* from the northern Baltic Sea. Both copepods fed actively on the cyanobacterium and survived. The authors concluded that, even if both copepods might act as a link transporting nodularin to higher trophic levels, a very small fraction of the esitmated ingested nodularin was found in the copepods. Therefore, the relative importance of this indirect pathway seemed limited.

The uptake and accumulation of nodularin *via* filtered seawater in the calanoid copepods *E. affinis* and *A. tonsa* and in the oligotrich ciliate *Strombidium sulcatum* was studied by means of tritiated-nodularin, ^{3}H-dihydronodularin. The minimum bioconcentration factor (BCF) was 12 for *A. tonsa* and 18 for *E. affinis*. For *S. sulcatum*, a maximum BCF of 22 was indicated [125].

Infaunal clams (*M. balthica*) from the Baltic Sea were exposed in experimental tanks for 96 h to *N. spumigena* (intracellular nodularin content 4 and 20 μg/L) or purified nodularin (10 and 50 μg/L). Tissue levels of nodularin-like compounds were measured by means of ELISA and HPLC. In the water 60–100% of nodularin in the pure toxin experiments and all the nodularin in the *N. spumigena* experiments appeared as an unidentified form with nodularin-like spectral characteristics. In the high level of the *N. spumigena* experiment (intracellular nodularin-like compounds in test water 20 μg/L) soft tissue levels (measured with ELISA) increased from the control level of 0.16 to 16.6 μg/g dry weight within 24 h of exposure, reaching 30.3 μg/g dry weight after 96 h of exposure. However, <5% of the toxin detected by ELISA was shown to be nodularin by HPLC. In the pure nodularin experiments, clams exposed to 50 μg/L accumulated one-third (0.32 μg/g dry weight) of the amount of nodularin-like compounds found in the highest exposure group with *N. spumigena* (0.96 μg/g dry weight) and twice the amount of nodularin-like compounds accumulated by clams treated with the lowest level of *N. spumigena* (0.16 μg/g dry weight) [126].

During an *N. spumigena* bloom in the Gippsland Lakes area of Southern Victoria, Australia, in the summer of 2001, seafood samples were collected and nodularin levels were determined by HPLC/MS/MS. In prawn flesh, nodularin levels varied from 5 to 22 μg/kg and in prawn viscera from 945 to 6400 μg/kg (the highest level was found while the amount of *Nodularia* cells was 29 000 cells/L). In mussels, the nodularin levels varied from 40 to 2500 μg/kg, in finfish flesh from 0.7 to 2.5 μg/kg and in finfish viscera from 0 to 152 μg/kg. Cooking of the prawn did not destroy the toxin, but simply redistributed it between viscera, flesh and water used for cooking. These results were used to restrict some seafood harvesting [127].

It has been suggested that pelagic planktivores may receive cyanotoxins indirectly, *i. e.* by preying on organisms that have ingested cyanobacteria. Engström-Öst *et al*. [128] studied the potential transfer and accumulation of nodularin in mysid shrimps (*Mysis relicta*) and three-spine stickleback (*G. aculeatus*) *via* cyanobacteria-fed copepods (*E. affinis*) in a 10-day laboratory experiment. The average ingested amount of nodularin/day varied from 0.0016 to 0.010 μg/kg stickleback/day and from 0.016 to 0.097 μg/kg mysid/day (detection method in copepods ELISA). Substantial concentrations of nodularin were detected in mysid shrimps and three-spined sticklebacks suggesting that nodularin may be transferred *via* copepods whereas accumulation of nodularin could not be shown with certainty. When measured with ELISA, nodularin levels were significantly higher in mysid shrimps (up to *ca.* 0.7 μg/g dry weight; levels increased with exposure time) than in three-spined stickleback (up to *ca.* 0.15 μg/g dry weight; levels did not increase with exposure time). When measured with the PPase inhibition assay, nodularin levels in both species did not differ (up to *ca.* 0.5 μg/g dry weight) and did not increase with time.

Kankaanpää *et al*. [123] reported nodularin levels in three-spine sticklebacks (*G. aculeatus*) varying from 35 to 170 μg/kg dry weight (expressed as microcystin-LR eq) during an *N. spumigena* bloom in the western Gulf of Finland, Baltic Sea, in August 1999.

In the summer of 1999 maximum nodularin levels of 2150 μg/kg dry weight were found in soft tissue of caged blue mussels from the western Gulf of Finland on 20th August coinciding with a peak of *Nodularia* blooms. The toxin level in caged mussels increased sharply between 8th July and 20th August (at least a 100-fold increase in about 1 month; detection method ELISA), after which the toxin level started to decrease. In wreck mussels, toxin levels continued to increase and reached a maximum on 24th September (363 μg/kg dry weight, 4–5 times more than on 4th August (79.8 μg/kg dry weight)) [120].

During summer 2000, blue mussels from the western Gulf of Finland contained a maximum of 1450 μg nodularin/kg dry weight on 23 August 2000. Blue mussels from other sites contained 40–130 μg nodularin/kg dry weight (detection method ELISA). LC-MS verified the presence of only nodularin. From August to December 2000 nodularin levels in blue mussels decreased and typical detoxication conjugates (GSH conjugates) were detected. In the summer of 2000 also another mussel species (*D. polymorpha*) and clams (*M. balthica*) from the Gulf of Finland (Russian waters) contained nodularin (100–130 μg/kg dry weight) [122].

Livers of flounders caught during August 1995 in the western Gulf of Finland and the Archipelago Sea contained after storage at −700C for several years, 82–637 μg of nodularin-R/kg wet weight (by LC-MS). Biotransformation products such as glutathione adducts, were not found [129]. In livers of flounders (*Platichthys flesus*) from the western Gulf of Finland caught in July 1996, September 1997 and September 1998 average nodularin levels varied from 34 to 140 μg/kg dry weight measured by ELISA and PP1 inhibition (recovery 30%). In the Gulf of Bothnia livers of flounders caught in August 1997 and September 1998 contained 44–60 μg nodularin/kg dry weight and no nodularin, respectively, and liver of flounders from Baltic Proper (September 1998) contained 25–71 μg nodularin/kg dry weight. Livers of flounders from the Atlantic Ocean (Tromsö, Norway) caught in May 1998, did not contain nodularin. In muscle tissues from flounders caught at the sites mentioned above, only the PPase inhibition assay showed inhibition, but this may be due to other compounds present in the muscle extracts rather than nodularin or due to matrix interference [121]. In July and August 1999, livers of flounders from the western Gulf of Finland contained 137 and 399 μg of nodularin eq/kg dry weight, respectively (by ELISA). During July and August 2000, livers of flounders from the western Gulf of Finland contained up to 400 μg nodularin/kg dry weight (detected by ELISA). In muscle tissue of flounders no nodularin was found [122].

Livers of cod (*G. morhua*) from the southern Baltic Sea caught in August 1998 showed nodularin levels of 53–56 μg/kg dry weight determined by ELISA and PPase inhibition [121].

Despite exceptionally heavy blooms of toxic *Nodularia* in the Baltic sea during the summer of 1997, liver of Baltic herring and muscle tissue of salmon contained very low quantities of nodularin (plus microcystins) (<0.7–6.5 and <1.1–4.9 μg/kg dry weight, respectively, expressed as microcystin-LR eq and determined by ELISA, not corrected for recovery (=30%)) [130].

Sea trout (*Salmo trutta*) were given an oral injection with a single dose of food containing nodularin (125 mg/kg wet weight (ww)) from *N. spumigena*. The exposure level was 210–620 μg nodularin/kg bw. An 8-day survey under laboratory conditions showed accumulation of nodularin-like compounds in the liver from 0.05% of the total nodularin dose on day 1 to 0.53% on day 8 (19 μg/kg on day 1; 1200 μg/kg on day 8) as measured by ELISA. In trout muscle, nodularin-like compounds were found in concentrations of 125–34 μg/kg as measured by ELISA. However, the ELISA test kit is also sensitive to metabolites of algal hepatotoxins. With HPLC no nodularin peak was detected after 24 h that also suggested nodularin absorbed in trout was metabolized or bound rapidly [131].

Eiders (*Somateria mollissima*) feed extensively on mussels and can be exposed to nodularin by contaminated mussels. Livers of common eiders from the Baltic Sea were analysed in August and September 2002 for nodularin (by ELISA and LC-MS). The analyses showed residues varying from 3 to 180 μg nodularin/kg dry weight [132].

2.2.4.2 Accumulation in crops/plants

'There are indicators that the scenario of exposure of crop plants to cyanobacterial toxins *via* irrigation is an area for concern, especially in countries with a high reliance on irrigation of high value crops, and a high incidence of eutrophication and blooms, like the Netherlands'. (G. A. Codd, Personal communication from a letter received in June 2004).

2.2.5 Biodegradation in the environment (n.d.)

2.2.6 Toxicity of nodularins

2.2.6.1 Mechanism of action

The mechanism of toxicity of nodularins is quite similar to that of microcystins. First, the toxins enter the blood from the ileum *via* the bile acid carriers that convey the toxins across the mucosa. Second, the toxins are transported preferentially into the hepatocytes *via* bile acid carriers. Third, the toxins induce changes in the actin microfilaments and elements of the cell cytoskeleton, and thus result in a dense aggregation of the microfilaments near the centre of the cell. The loss of cellular support may cause cells to roundup and in turn result in the destruction of the sinusoid endothelial cells. Eventually, destruction of the parenchymal cells and sinusoids of the liver cause lethal intrahepatic haemorrhage (within hours) or hepatic insufficiency (within days) [3, 4].

2.2.6.2 Pharmacokinetics (n.d.)

2.2.6.3 Toxicity to laboratory animals

Acute toxicity – oral studies. (n.d.)

Acute toxicity – intraperitoneal studies. Nodularins are specific liver poisons in mammals. Following acute exposure to high doses, they cause death from liver haemorrhage or liver failure [3, 4]. For nodularin the intraperitoneal LD_{50} in the mouse is 30–50 μg/kg bw [133]. For nodularin-Har the intraperitoneal LD_{50} in the mouse is 70 μg/kg bw [107].

When male Swiss mice were given i.p. nodularin (1, 5 or 10 μg/kg bw in 0.9% saline *per* 7 days), significantly increased liver/bw ratios were seen at 5 and 15 μg/kg bw and significantly decreased activities of superoxide dismutase, catalase and glutathione peroxidase at all dose-levels. These effects suggested oxidative damage might be involved in the toxicity of nodularin. Cotreatment and post-treatment with melatonin at i.p. doses of 10 or 15 mg/kg bw *per* 7 days might protect against the nodularin-induced oxidative stress as was demonstrated by reverse of the increased liver/body weight ratios and the decreased enzyme activities. Pretreatment with melatonin did not have a protective effect [134].

Irritation and sensitization studies. (n.d.)

Repeated dosing – intraperitoneal studies. Groups of 6–12 male F344 rats (age 7 wk) received, 2 wk after a single i.p. injection with saline, once a day for 2 days i.p. injections with 25 μg nodularin/kg bw. A control group received an i.p. injection with saline only (test was repeated twice). In serum and testes the testosterone levels were decreased accompanied by a higher (low-density lipoprotein) cholesterol level in the serum. The lower serum testosterone levels appeared to be caused by impaired biosynthesis in the testes. The potential causes of this phenomenon might have been apoptosis of Leydig cells induced by direct toxicity of nodularin on testes or hypothalamopituitary dysfunction [135].

***In vitro* toxicity studies.** The 24 h LC_{50} value for primary cultured rat hepatocytes is 62 ng/mL for nodularin. Exposure of the rat hepatocytes to noncytotoxic concentrations of 2 or 10 ng/mL nodularin for 3, 24 or 48 h provided evidence of the induction of oxidative stress as was demonstrated by an increase of reduced glutathione.

Mutagenicity. (n.d.)

Carcinogenicity. Nodularins may promote the growth of liver and other tumours following chronic exposure to low doses. In a two-stage liver carcinogenesis experiment in female F344 rats initiated with diethylnitrosamine and without partial hepatectomy, repeated i.p. administration of 10 μg nodularin/kg bw induced GST-P-positive foci (GST-P is the placental form of GST, a biomarker for preneoplastic changes in liver) more effectively than microcystin-LR. Nodularin alone also induced some foci [3, 4].

Reproduction and developmental studies. (n.d.)

Immunotoxicity. Nodularin inhibited the *in vitro* (spleen cells from virus-free female B6C3F1 mice) polyclonal antibody forming cell response and lymphoproliferation to LPS. The concanavalin A-induced T-lymphocyte proliferation was also inhibited by nodularin [79]. *In vivo* studies in B6C3F1 mice showed that intraperitoneal injection of nodularin decreased the humoral immune responses to sheep red blood cells, and the inhibitory effect became severe when hepatic uptake of nodularin was blocked by rifampicin [79].

2.2.6.4 Effects on humans

No human poisonings have been recorded as a result of ingestion of *N. spumigena* [81]. In 1991, at Lake Alexandrina and central Australia, five adults and three children showed skin rashes after contact with water containing toxins mainly from *Nodularia* and *Microcystis* [4].

2.2.6.5 Effects on aquatic organisms

Feeding and fecundity of two calanoid copepod species (*A. bifilosa* and *E. affinis*) were studied in a food assemblage dominated by cyanobacteria. Bloom conditions were simulated in a mesocosm by adding a high concentration of cultured hepatotoxic *N. spumigena* to 100 µm filtered seawater. This seston was fed to the copepods. Both copepods ingested large quantities of the cyanobacteria but switched to ciliates when those became more abundant. Egg production of *A. bifilosa* was seen irrespective of the high nodularin content in the mesocosm. The results demonstrated that *A. bifilosa* and *E. affinis*, two dominant copepod species of the Baltic Sea, are able to feed, survive and produce eggs in a plankton community dominated by *N. spumigena* [136]. Also Kozlowsky-Suzuki *et al*. [124] studied feeding and reproduction in the presence of *N. spumigena* from the northern Baltic Sea. The results suggested that *N. spumigena* was not directly harmful to these copepods if an alternative food source was available, even though reproduction was not sustained if the cyanobacterium was offered as a single diet.

Exposure of adult *A. salina* to nodularin caused an elevation of GST activity *in vivo*. Nodularin was conjugated to glutathione *via* GST, which is an initial step of detoxication [88].

In feeding experiments with mysid shrimps (*Mysis mixta*) both juvenile and adult mysid shrimps fed less active on the toxic *N. spumigena* than on the two nontoxic cyanobacterial strains *A. flos-aquae* and *N. sphaerocarpa* by reducing clearance rates, which can be interpreted as adaptive behaviour. No increased mortality of mysid shrimps (*M. mixta*) was seen at exposure to high concentrations of toxic *N. spumigena* in a 7-wk experiment with filtered seawater [137].

Herring larvae tend to avoid water layers with heavy *Nodularia* blooms, but furthermore little is known about possible toxic effects on the aquatic system (Kankaanpää, H., One kind of cyanobacteria (blue-green algae) frequently occurring in the Baltic, Undated, http//jolly.fimr.fr/boing/encyclopaedia. nsf).

In August 1999, during an *N. spumigena* bloom in western Gulf of Finland, Baltic Sea, threespine stickleback (*G. aculeatus*) were found floating on the surface. The sticklebacks contained approximately 35–170 µg of nodularin/kg dry weight (as microcystin eq) [123].

Sea trout (*S. trutta*) were given an oral injection with a single dose of food containing nodularin (125 mg/kg ww) from *N. spumigena*. The exposure level was 210–620 µg nodularin/kg bw. Histopatholgy showed rapid induction of severe but reversible liver damage. Apparently, nodularin accumulated from cyanobacteria in the intestine, but was detoxified rapidly [131].

2.2.6.6 Effects on wild and domestic animals

A water bloom of *N. spumigena* in Lake Alexandrina, Australia, which is a shallow lake at the termination of the River Murray, caused numerous livestock deaths in 1878 and was the first scientifically documented case of cyanobacterial intoxication (cited from Falconer [81]).

In 1963 in Rugen, Germany, 400 ducks were affected by toxins from *N. spumigena*; in 1974–1975, 34 sheep and 52 lambs in South Western and Western Australia were affected and in 1975 30 dogs became sick and 20 died at the Danish coast of the Baltic Sea. At the Swedish, German and Finnish coast of the Baltic Sea, nine dogs, 16 young cattle and one dog plus three puppies, respectively, were affected by toxins from *N. spumigena* in 1982, 1983 and 1984. In 1990 in Wilhelmshafen, Germany, two dogs became sick by toxins from *N. spumigena* and were sacrificed [4].

2.2.6.7 Effects on crops/plants (n.d.)

2.2.6.8 Derivation of NOAEL and TDI

No NOAEL can be derived for nodularin(s) because of the absence of suitable toxicological data. Because the same mechanism of action underlies the toxicity of both microcystin-LR and nodularin(s), the guideline value for microcystin-LR might also be used for nodularin(s).

2.2.7 Regulations

No guidelines have been set for nodularin in drinking water as this toxin is found only in brackish waters, although its presence should be regarded as a health risk [105]. It is reasonable to assume that nodularin represents at least the same human health risk as microcystin-LR. Therefore, Fitzgerald *et al*. [106] proposed a health alert for nodularin of 10 µg/L corresponding to 50 000 *N. spumigena* cells/mL relating to potential acute or short-term health effects.

3 Alkaloids

3.1 Anatoxin-a

3.1.1 Chemical structures and properties

Anatoxin-a is a low molecular weight tropane-related alkaloid (MW = 165), a bicyclic secondary amine, 2-acetyl-9-azabicyclo[4.2.1]non-2-ene and is a structural analogue of cocaine (see Fig. 4). It is synthesized in the cell from ornithine *via* putrescine with the participation of the enzyme ornithine decarboxylase. Anatoxin-a is not susceptible to enzymatic hydrolysis by cholinesterase because it does not possess an ester moiety. Anatoxin-a in the protonated form is more stable than the free base. It has a pK_a of 9.4 and at physiological pH it exists in the protonated form [4]. Homoanatoxin-a (MW = 179) is an anatoxin-a homologue (see Fig. 4). It has a propionyl group at C-2 instead of the acetyl group in anatoxin-a [3, 4].

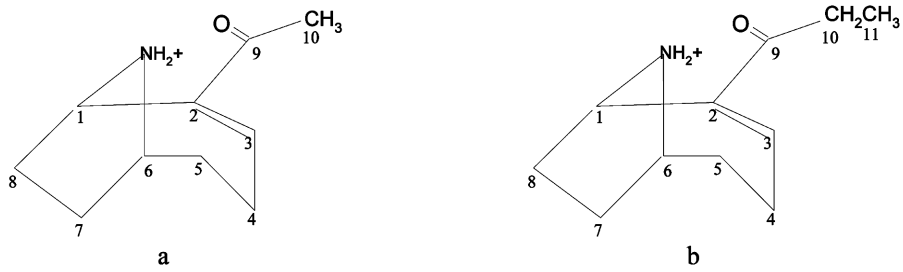


Figure 4. Chemical structures of anatoxin-a (a) and homoanatoxin-a (b) [3].

3.1.2 Methods of analysis

3.1.2.1 General

Cyanobacteria are microscopic photosynthetic organisms. Appropriate and careful handling of samples both prior to and during analysis is extremely important to ensure an accurate determination of toxin concentration. Anatoxin-a is relatively stable in the dark, but in pure solution in the absence of pigments it undergoes rapid photochemical degradation in sunlight. Breakdown is further accelerated by alkaline conditions [138]. Furthermore, toxins in water bodies at the time of a bloom will be present in both the water (free, dissolved or extracellular toxins) and the cyanobacterial cells (intracellular) [12].

3.1.2.2 Extraction/concentration/clean-up/ derivatization procedures

Effective extraction of anatoxin-a and its analogue homoanatoxin-a from cyanobacteria is difficult without disrupting or perforating the cell wall structure. Most procedures use freeze-drying for this purpose alternatively combined with oven drying [12] or ultrasonication prior to solvent extraction with methanol, acetic acid or Tyrode solution [139]. (Tyrode solution is an aqueous solution containing potassium, sodium, calcium and magnesium salts and dextrose with a pH = 7.4.)

Although minimal preliminary treatment of samples is possible – for example: the investigation of ‚health food supplements containing' tablets and capsules for the presence of neurotoxins related to anatoxin-a by means of only an extraction with methanol prior to micro-LC-MS-MS determination [140] – SPE is the most widely used technique in sample concentration and clean-up of cyanobacterial toxins. For anatoxin-a and analogues in particular, a weak cation exchange (WCX) cartridge [141] can be used because these toxins are highly water-soluble organic amines.

The ability to make fluorescent derivatives of anatoxin-a has resulted in the application of solid phase microextraction (SPME) methods. A fluorogenic agent, which reacts with anatoxin-a was placed on the SPME fibres. Then, after HPLC and fluorescence detection, anatoxin-a detection limits in water of 20 μg/L were reached [142]. But derivatization in general can have another advantage: altering the chemical characteristics of compounds. For anatoxin-a this means it can be converted from a polar to a nonpolar and more volatile compound (the same is true for analogues of anatoxin-a), which can be detected by GC [143].

3.1.2.3 Determination/detection

Chromatographic methods. A number of chromatographic methods are available for the analysis of anatoxin-a in cyanobacterial bloom material and these include HPLC and GC. Anatoxin-a has an absorbance maximum at 227 nm which is not really in the region that may provide specific conformational information.

To enhance selectivity and detection limit fluorescence is often used as detection tool. With NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) as fluorogenic agent on the surface of an SPME fibre and subsequently an RP separation and fluorescence detection, anatoxin-a in lake and drinking water was determined with a detection limit of 20 ng/L [144]. Still, this method is only suitable as screening method but it can be used for the analysis of highly contaminated water.

Derivatization with heptafluorobutyric acid anhydride was used prior to GC-ECD (electron capture detection) for the detection of anatoxin-a and homoanatoxin-a [139]. This technique was then compared with a GC-MS system (with negative ion chemical ionization: NICI and SIM). GC-ECD was able to detect 3 ng toxin in 1 L lake water sample. GC-MS had an LOD ten times lower.

In general, GC-MS is more sensitive than LC-MS. But a main advantage of LC-MS is that derivatization can be on-line coupled with this technique. ESI is suitable for the amines, and because ion-pairing reagents suppresses ion intensity (on-line), precolumn derivatization with a volatile ion-pair reagent is an alternative approach for analysing anatoxin-a and its analogues with LC-ESI-MS. Fluorenyl methyl chloroformate (Fmoc) is such a highly and rapidly reacting derivatization reagent for anatoxin-a. The LOD of anatoxin-a in drinking water with this method was calculated at 2 ng/L [145]. The power of LC-MS^n is that derivatization of anatoxin-a is not needed anymore. The detection limit for

anatoxin-a in lake water using LC-MS [13] was 0.6 μg/L [141]. Lowering the LOD should not be sacred. A main problem in analysing anatoxin-a and homoanatoxin-a is the occurrence of their epoxy- and dihydro-degradation products. Using RP micro-LC-MS-MS equipped with an IS (ionspray) interface, all the mentioned degradation products could be determined and identified in tablets and capsules [140].

Nonchromatographic methods. Beyond the area of chromatography not so many methods have been published for the analysis of anatoxin-a. ELISA was used for the determination of anatoxin-a in maize with an LOD down to 1 μg/kg [146]. And recently, a receptor radioligand-binding assay for determining (homo)anatoxin-a was used for high-throughput screening directly on cell extracts. The LOD was close to 1 μg/L [147].

Regarding (mouse) bioassays for anatoxin-a determination, the same analytical facts can be recited as for nodularins and microcystins (mouse) bioassays.

CZE and MEKC were applied to the simultaneous separation of anatoxin-a, microcystin-LR and cylindrospermopsin (CYN). The analytical performance data of both methods, optimized for the three toxins, were similar with a precision of migration times smaller than 0.8% RSD and a detection limit in the range of 1–4 μg/mL, using spectrophotometric detection at 230 nm. Both methods were applied to an analysis of cyanotoxins in water bloom samples and crude cyanobacterial extracts. The results obtained indicate that for complex matrices, the sequential application of CZE and MEKC is needed. It is recommended to use both CE techniques for the analysis of the same sample in order to confirm the results by an orthogonal approach [148].

3.1.2.4 Conclusion

In comparison with microcystins and nodularins less literature about the determination of anatoxin-a can be found. There are a few comparative studies but none of them gives a complete survey of all possible anatoxin-a determinations. An example of such a study is the determination of anatoxin-a, homoanatoxin and propylanatoxin with HPLC-UV, GC-MS and CE [149]. Although this study is somehow out of date (1994), it is in its simplicity very useful by discussing the basic characteristics of the above-mentioned three methods and in addition, by going into the possibility to determine anatoxin-a and its analogues in the presence of microcystin. In practical respect however, there is a very convenient, general methodology for the analysis of anatoxin-a (and other cyanobacterial toxins) with HPLC [14]. Aspects such as freeze-drying, filtering and extraction are put in a workflow easy for analysts to use. For screening purpose, HPLC-fluorescence appears to be a method of choice because of its selectivity, quickness and practicability. On the other hand, LC-MS is the better confirmative method and it will only take some time for this technique to sweep the analytical world of anatoxin-a determination. An example of the apparently endless number of applications of LC-MS, is the simultaneous determination of algal and cyanobacterial toxins in phytoplankton from lakes [30]. In one single chromatographic run, anatoxin-a, STX, nodularin, microcystins and other toxins were determined and quantified.

3.1.3 Source organism(s) and habitat/occurrence

3.1.3.1 Source organism(s)

Anatoxin-a is produced by *Anabaena* sp. (a.o. *A. planctonica*, *A. flos-aquae*, *A. spiroides* and *A. circinalis*), *Oscillatoria* (*Planktothrix*) sp., *Cylindrospermum* sp. and *Aphanizomenon* sp. It is produced in minor amounts by *Microcystis* sp. (in Japan) [3, 4]. Homoanatoxin-a was reported to be produced by *Planktothrix formosa* and by *O. formosum* (*Phormidium formosum*) [3]. Recently [150], homoanatoxin-a was found to be produced by some unidentified *Anabaena* sp. Watanabe *et al*. [151] isolated and identified homoanatoxin-a from *Raphidiopsis mediterranea*. Namikoshi *et al*. [152] reported the simultaneous production of anatoxin-a, homoanatoxin-a and the new nontoxic 4-hydroxyhomoanatoxin-a by *R. mediterranea*.

3.1.3.2 Habitat/occurrence

For anatoxin-a production, phosphorus levels have no effect as was seen for microcystin production [3].

Anatoxin-a was first isolated from Canadese strains of *A. flos-aquae* and later by Finnish strains of unidentified *Anabaena* strains and from individual species of *Oscillatoria*, *Aphanizomenon*, and *Cylindrospermopsis*, from benthic *Oscillatoria* from Scotland, and from *Anabaena* and *Oscillatoria* in Ireland. Anatoxin-a was present also in *A. planctonica* blooms in Sardinia, Italy, in *Anabaena* and *Aphanizomenon* blooms in Germany, and in minor amounts in some Japanese bloom samples [3, 4]. In a fishing pond in northern Italy anatoxin-a was isolated from a heavy bloom of *Planktothrix rubescens*. The bloom was sustained by the high N/P ratio [153].

Homoanatoxin-a was isolated from a Norwegian strain from *O. formosum* (*P. formosum*) [3] and from *Anabaena* sp. in Ireland [150]. In a lake in Japan the simultaneous production of, anatoxin-a, homoanatoxin-a and the new nontoxic 4-hydroxyhomoanatoxin-a by *R. mediterranea* was observed [152].

3.1.4 Bioaccumulation in the environment

3.1.4.1 Accumulation in aquatic organisms (n.d.)

3.1.4.2 Accumulation in crops/plants

See Section 2.2.4.2.

3.1.5 Biodegradation in the environment

Laboratory studies showed rapid decomposition of anatoxin-a in the presence of sunlight, particularly at elevated

pH. Although the nontoxic degradation products, including dihydroanatoxin-a and epoxyanatoxin-a, have been found in senescent cyanobacterial blooms, these compounds are seldom reported. This is probably due to the used detection method which is not capable of detecting these compounds (cited from Furey *et al*. [150]). Anatoxin-a in pure solution in the absence of pigments undergoes rapid photochemical degradation in sunlight. The half-life for photochemical breakdown is 1–2 h. Under normal day and night light conditions at pH 8 or 10, and at low initial concentrations (10 μg/L), the half-life was found to be approximately 14 days [3]. The expected degradation products of homoanatoxin-a, dihydrohomoanatoxin-a or epoxyhomoanatoxin-a have been synthesized, but these degradation products have not yet been found in natural samples (cited from Furey *et al*. [150]).

3.1.6 Toxicity of anatoxin-a

3.1.6.1 Mechanism of action

Anatoxin-a is a potent postsynaptic depolarizing neuromuscular blocking agent; a stereospecific nicotinic (cholinergic) agonist that binds to neuronal nicotinic acetylcholine receptors at the neuromuscular junction to cause persistent stimulation resulting in a block on further electrical transmission. As such transmission is necessary for activity in skeletal muscle, a sufficiently high dose can lead to muscular paralysis (in the case of respiratory muscles) leading to death by asphyxiation. Pharmacological screening studies confirmed that anatoxin-a is a potent nicotinic agonist which can produce neuromuscular blockade and death by respiratory arrest [5, 55, 154].

The homologue of anatoxin-a, homoanatoxin-a is a potent neuromuscular blocking agent. It enhances the flux of Ca^{2+} ions in the cholinergic nerve terminals [3].

3.1.6.2 Pharmacokinetics (n.d.)

3.1.6.3 Toxicity to laboratory animals

General. Anatoxin-a causes death within minutes to a few hours depending on the species, the amount of toxin ingested, and the amount of food in the stomach. Clinical signs of poisoning follow a progression of muscle fasciculations, decreased movement, abdominal breathing, cyanosis, convulsions and death. In smaller laboratory animals, death is often preceded by leaping movements, while in field cases, larger animals collapse and sudden death is observed. No known therapy exists, although respiratory support may allow sufficient time for detoxification to occur followed by recovery of respiratory control [5].

Acute toxicity – oral studies. The oral LD_{50} values for anatoxin-a in many species range from 1 to 10 mg/kg bw with a latent period after administration followed by symptoms of intoxication which may include twitching, gasping, convulsions and death [155].

Acute toxicity – intraperitoneal studies. For anatoxin-a the i.p. LDL_0 (lowest dose causing death) in mice is 250 μg/kg bw and the i.p. LD_{50} is 375 μg/kg bw [3]. Intraperitoneal LD_{50} values in the mice of <100 μg/kg bw and from 200 to 250 μg/kg bw are reported also in the literature (cited from Wolf and Frank [11]).

For homoanatoxin-a the i.p. LD_{50} in mice is 250 μg/kg bw [3]. Symptoms of poisoning are severe body paralysis, convulsions and death by respiratory arrest within 7–12 min [3].

A new homoanatoxin-a derivative was identified as 4-hydroxyhomoanatoxin-a and appeared to be not toxic to mice at i.p. injection up to 2 mg/kg bw [152].

Acute toxicity – intravenous studies. The i.v. LD_{50} in mice for anatoxin-a is <100 μg/kg bw [3]. At a single sublethal i.v. dose in mice, the animals recovered rapidly and completely [156].

Acute toxicity – intranasal studies. The intranasal LD_{50} in mice for anatoxin-a is 2000 μg/kg bw [3].

Irritation and sensitization. (n.d.)

Repeated dosing – oral studies. In a range-finding study, groups of two male and two female mice received oraly by gavage 1.5, 3, 7.5 or 15 mg of anatoxin-a hydrochloride/kg bw for 5 days. All mice at 15 mg/kg bw and one mouse at 7.5 mg/kg bw died within 5 min of dosing over the first 4 days of the study. No treatment related signs of clinical toxicity, or changes in body weight were observed. No treatment related changes were seen at necropsy [156].

Groups of ten male and ten female mice received orally by gavage 0, 120, 600 or 3000 μg anatoxin-a hydrochloride/kg bw (equal to 0, 98, 490 and 2460 μg anatoxin-a/kg bw) for 4 wk. All animals were examined daily for signs of clinical toxicity or illness. Body weights and food consumption were recorded weekly while opthalmoscopy was done at the beginning and end of treatment. During the final week, haematology and serum biochemistry were performed. Absolute and relative organ weights were determined. All tissues in 0 and 3000 μg/kg group were examined microscopically along with those from decedents. Attention was paid to any gross lesions seen at necropsy. One male at 600 μg/kg and one female at 3000 μg/kg died at day 10 and 14, respectively. No signs of clinical toxicity or histological abnormalities were observed and no cause of death could be found. One male on 120 μg/kg was killed for humane reasons after showing signs of having been attacked by its cage mates. Surviving animals displayed no significant treatment-related adverse effects. The NOAEL in this study was determined to be 120 μg anatoxin-a hydrochloride/kg bw/day (equal to 98 μg anatoxin-a/kg bw) [156].

Groups of 20 female rats received orally *via* the drinking water 0, 0.51 or 5.1 mg anatoxin-a/L (equivalent to 0, 51 or 510 μg/kg bw/day, respectively) for 54 days. No mortality was seen. No effects on body weight gain, haematology (Er, Leu) or clinical chemistry (SAP, SGPT, γ-GGT, whole blood and brain ChE) were observed. Macroscopy and microscopy (no further details) revealed no abnormalities [155].

Repeated dosing – intraperitoneal studies. Female rats received daily by i.p. injection 0 or 16 μg anatoxin-a/rat (equal to 0 or 80 μg/kg bw) for 21 days. No mortality was seen. No effects on body weight gain, haematology (Er, Leu) or clinical chemistry (SAP, SGPT, whole blood and brain ChE) were observed. Macroscopy and microscopy (no further details) revealed no abnormalities [155].

Mutagenicity. (n.d.)

Carcinogenicity. (n.d.)

Reproduction and developmental – oral studies. Two groups of pregnant female mice (number/group not given) received orally by gavage from day 6–15 of pregnancy, 0 or 3 mg anatoxin-a HCl/kg bw/day as a solution in sterile water (~2.46 mg anatoxin-a/kg bw). Maternal body weights and clinical signs were recorded. On day 18 of pregnancy the animals were killed. Necropsy was performed. The number of live and dead implantations were recorded, and the foetuses were weighed, sexed and examined for external abnormalities. No maternal deaths or treatment-related clinical signs were recorded. Maternal necropsy did not reveal abnormalities. Pregnancy incidence, mean number of implantations, live foetuses, mean postimplantation losses and foetal sex ratios were normal. No effect on foetal weight was seen and no foetal abnormalities were observed. The NOAEL in this study was 3 mg anatoxin-a HCl/kg bw equal to 2.46 mg anatoxin-a/kg bw [156].

Groups of pregnant female hamsters (number/group not given) received i.p. once daily on day 8–14 of pregnancy 0.20 mg anatoxin-a/kg bw, three times *per* day on day 8–11 of pregnancy 0.125 or 0.20 mg/kg bw or three times *per* day on day 12–14 of pregnancy 0.125 or 0.20 mg/kg bw. Concurrent control groups were used. The animals were killed on day 15 of pregnancy. No maternal toxicity was seen and the number of resorption sites did not show deviations. Foetal weights in many treated groups were lower than control weights. No external abnormalities and no skeletal malformations were observed. Examination of soft tissues showed hydrocephaly in all foetuses from one litter only containing ten embryos, from the group which received three times *per* day 0.125 mg/kg bw during day 12–14 of pregnancy [155].

***In vitro* studies.** Anatoxin-a-induced cytotoxicity and apoptosis in two non-neuronal type of cells, *viz.* cultured rat thymocytes and African green monkey cells (Vero). The cultured rat thymocytes showed all the typical morphological and biochemical features of apoptosis including DNA fragmentation, generation of reactive oxygen species and caspase activation, which are more pronounced than in Vero cells [157].

3.1.6.4 Effects on humans (n.d.)

3.1.6.5 Effects on aquatic animals

In Canada, a case of anatoxin-a poisoning of waterfowl showing neurotoxic effects, was reported [3]. Exposure of zebra fish (*D. rerio*) embryos to 400 μg of purified anatoxin-a/L under laboratory conditions caused an altered heart rate in different developmental stages. At the pec-fin stage (55 h), heart rate was decreased by 9% compared to control values, whereas at the protruding-mouth stage (80 h) an increase of 12% was measured. These effects were temporary. An extract of *A. flos-aquae* containing ≥200 μg anatoxin-a/L had no effect on developing embryos of zebra fish [92].

3.1.6.6 Effects on wild and domestic animals

Anatoxin-a causes death within minutes to a few hours depending on the species, the amount of toxin ingested, and the amount of food in the stomach. Clinical signs of poisoning follow a progression of muscle fasciculations, decreased movement, abdominal breathing, cyanosis, convulsions and death. In addition, opisthotonus (rigid 's'-shaped neck) is observed in avian species. In field cases, larger animals collapse and sudden death is observed [5].

Documented cases of animal fatalities due to the consumption of water with anatoxin-a included cattle in Finland and Canada, and dogs in the USA, New Zealand, Scotland and Ireland. The onset of the symptoms is very rapid [3, 4, 96, 158] (cited from Furey *et al*. [150]).

Over the 1990s, episodic mass mortalities of Lesser Flamingos (*P. minor*) have occurred at Kenya's Rift Valley saline, alkaline lakes. Analyses of flamingo carcass livers and cyanobacterial samples from Lakes Bogoria and Nakuru demonstrated three cyanobacterial toxins in dead flamingo livers: microcystin-LR and microcystin-RR and anatoxin-a. The total extractable microcystin concentrations in the bird livers varied from 0.21 to 0.93 μg microcystin-LR eq/g fresh weight. Anatoxin-a concentrations varied from 1.06 to 5.82 μg/g fresh weight. These toxin concentrations may have been sufficient alone to have caused the birds death, that of anatoxin-a being consistent with observations of staggering and convulsions in the flamingos before death and with opisthotonus postmortem [99]. Further research with materials from Lake Bogoria Lesser Flamingos has identified also the microcystins and anatoxin-a in bird stomach, intestine contents and faecal pellets (microcystin levels 0.196 μg/g fresh weight; anatoxin-a levels 4.34 μg/g fresh weight) [98].

3.1.6.7 Effects on crops/plants

The free-floating aquatic plant *L. minor* and the filamentous macroalga *Chladophora fracta* were exposed to anatoxin-a under laboratory conditions for 4–7 days. In both *L. minor* and *C. fracta* peroxidase activity was increased significantly after 4 days exposure at 25 μg anatoxin-a/L but not at 15 μg/L or lower. After 7 days exposure of *L. minor* significantly increased GST activity and significantly reduced photosynthetic oxygen production were observed at 20 and 5 μg/L but not at lower concentrations. In conclusion, anatoxin-a produced adverse effects on aquatic plants (1.0 and 0.1 μg/L) [142].

3.1.6.8 Derivation of NOAEL and TDI

Fawell *et al.* [156] derived an NOAEL of 98 μg/kg bw from their 28-day gavage study in mice. Using an uncertainty factor of 1000 (100 for intra- and interspecies variation and 10 for limitations in the database) a TDI of 0.1 μg/kg bw can be calculated.

3.1.7 Regulations

No official guidelines are known, but anatoxin-a has a suggested guideline limit of 3.0 μg/L [105].

3.2 Anatoxin-a(S)

3.2.1 Chemical structure and properties

The chemical structure of anatoxin-a(S) (see Fig. 5) is unrelated to anatoxin-a. It is a unique guanidinium methyl phosphate ester and has an M_r of 252 Da. Structural variants of anatoxin-a(S) have not been detected until now [3, 4]. Anatoxin-a(S) decomposes rapidly in basic solutions but is relatively stable under neutral and acidic conditions [3]. Anatoxin-a(S) becomes inactivated at elevated temperatures (>40°C) [5].

3.2.2 Methods of analysis

3.2.2.1 Survey

There is little literature available about the analysis of anatoxin-a(S). Anatoxin-a(S) lacks a chromophore and therefore HPLC-UV is not suitable for the detection of this compound [14]. Of course, LC-MS may be considered to become an important analytical tool in determining anatoxin-a(S) but yet alternatives have to be used, like methods based on the ability of anatoxin-a(S) to inhibit acetylcholinesterase (AChE). A major disadvantage of these so-called colorimetric assays is that all known organophosphorous pesticides and insecticides will also inhibit AChE.

The development of a specific biosensor indeed based on inhibition of AChE, but with the use of a set of mutant enzymes (two sensitive to anatoxin-a(S) and resistent to most insecticides and two reversed) resulted in a method to detect anatoxin-a(S) selectively in aqueous solution down to 0.1 μg/L [159]. However, it should be noted that this method still measures bioactivity instead of concentrations and therefore it can only be used as a screening tool.

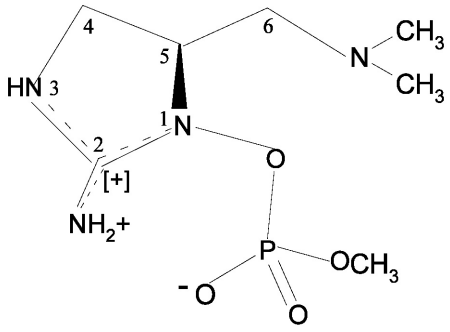


Figure 5. Chemical structure of anatoxin-a(S).

Regarding (mouse) bioassays for anatoxin-a(S) determination, the same analytical facts can be recited as for anatoxin-a, nodularins and microcystins (mouse) bioassays.

3.2.3 Source organism(s) and habitat/occurrence

3.2.3.1 Source organism(s)

Anatoxin-a (S) is produced by *Anabaena* species: *A. flos-aquae* and *A. lemmermannii* [3].

3.2.3.2 Habitat/occurrence

In Denmark anatoxin-a(S) has been detected in some lakes. *A. lemmermannii* was assumed to be the causative cyanobacterium. In Canada, USA and Scotland *A. flos-aquae* appeared to be the producing organism for anatoxin-a(S) [3].

3.2.4 Bioaccumulation in the environment

3.2.4.1 Accumulation in aquatic organisms (n.d.)

3.2.4.2 Accumulation in crops/plants

See Section 2.2.4.2.

3.2.5 Biodegradation in the environment (n.d.)

3.2.6 Toxicity of anatoxin-a(S)

3.2.6.1 Mechanism of action

Anatoxin-a(S) is a cholinesterase inhibitor with a mechanism similar to that of the organophosphorus insecticides. However, anatoxin-a(S) acts only in the periphery and thus brain and retinal cholinesterase activities remain normal even in lethally poisoned animals [96].

3.2.6.2 Pharmacokinetics (n.d.)

3.2.6.3 Toxicity to laboratory animals

Acute toxicity – intraperitoneal studies. The i.p. LD_{50} value of anatoxin-a(S) for mice and rats is 31 and 20 μg/kg

bw, respectively (cited from Monserrat *et al*. [160]). Wolf and Frank [11] cited an i.p. LD_{50} value in the mouse of 20 μg/kg bw. Whole cell aqueous extracts of *A. spiroides* gave an i.p. 24-h LD_{50} in mice of 369 mg/kg bw. Deaths at concentrations near and above the LD_{50} occurred within minutes [160]. Neurological effects in mice at acute toxic doses are muscle weakness, respiratory distress (dyspnea) and convulsions (effect on seizure threshold) preceding death. Death often occurs from respiratory arrest. In pigs and mice, anatoxin-a(S) can cause viscous mucoid hypersalivation [96].

Mutagenicity. No mutagenic activity has been observed when a purified lyophilized extract from anatoxin-a (S) (dose-levels 25–100 μL of a stock with 0.02 mg anatoxin-a(S)/mL) was tested in the Ames *Salmonella* assay (strains TA 98, 100 and 102) with or without metabolic activation [68].

In an assay with human lymphocytes *in vitro*, a purified lyophilized extract from anatoxin-a (S) (stock contained 0.02 mg anatoxin-(S)/mL) caused an increase in chromosomal breakages at the highest dose-level (0.8 μg/mL). At the two lower dose-levels (0.08 and 0.008 μg/mL) no effects were seen [68].

3.2.6.4 Effects on humans (n.d.)

3.2.6.5 Effects on aquatic organisms

The effect of aqueous extracts from *A. spiroides* on AChE from fish (*Odonthestes argentiniensis*), crab (*Callinectes sapidus*) and purified eel AChE was examined *in vitro.* Fifty percent inhibition of the enzyme activity (IC_{50}) was seen at 45.0, 17.2 and 23.0 mg/L of the lyophilized *A. spiroides* for fish, crab and eel AChE, respectively. It cannot be concluded from this study that the inhibitory effects on AChE activity were due solely to anatoxin-a(S), but the symptoms seen (see also Section 3.2.6.3. acute i.p. studies) were similar to those of organophosphorus pesticides and the chemical structure of anatoxin-a(S) fits with that of organophosphorus pesticides including a P=O bond [160]. Anatoxin-a(S) was responsible for the death of ducks in USA and waterfowl in Canada [96].

3.2.6.6 Effects on wild and domestic animals

Anatoxin-a(S) was responsible for the death of dogs, pigs and ducks in the USA [96]. Animal poisoning due to anatoxin-a(S) was seen in Denmark (cited from Kaas and Henriksen [161]).

3.2.6.7 Effects on crops/plants (n.d.)

3.2.7 Derivation of NOAEL and TDI

There are insufficient data to derive an NOAEL or LOAEL and calculate a TDI for anatoxin-a(S).

3.2.8 Regulations

There are no regulations known for anatoxin-a(S).

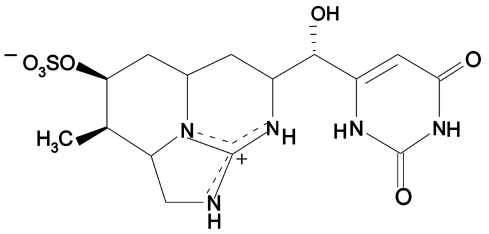


Figure 6. Chemical structure of cylindrospermopsin.

3.3 Cylindrospermopsin (CYN)

3.3.1 Chemical structure and properties

Cylindrospermopsin (CYN) is a tricyclic alkaloid, possessing a tricyclic guanidine moiety combined with hydroxymethyluracil (see Fig. 6). It has an M_r of 415 Da and is believed to be derived from a polyketide that uses an amino acid derived starter unit such as glycocyanamine or 4-guanodino-3-oxybutyric acid. New structural variants of cylindrospermopsin (a.o. demethoxy-cylindrospermopsin) have been isolated from an Australian strain of *Cylindrospermopsis raciborskii* [3, 4]. An analogue of cylindrospermopsin, deoxycylindrospermopsin (deoxy-cylindrospermopsin) was identified by Norris *et al*. [162]. Banker *et al*. [163] elucidated the chemical structure of 7-epicylindrospermopsin, a toxic minor metabolite of *Aphanizomenon ovalisporum*.

At treatment with chlorine-related water disinfectants 5-chlorocylindrospermopsin, a chlorine derivative of cylindrospermopsin, and cylindrospermic acid, an oxidated derivative of cylindrospermopsin, may be formed [164].

Under laboratory conditions cylindrospermopsin in aqueous solutions appeared to be reasonably stable in the dark or when exposed to a range of (artificial) light intensities, temperatures and pH. Water suspected of containing cylindrospermopsin cannot be cleared by boiling in 15 min, but such treatment promoted the decomposition of cylindrospermopsin to a structurally related compound. UV light and temperatures of 500C also similarly promoted the decomposition of cylindrospermopsin [165].

3.3.2 Methods of analysis

3.3.2.1 General

Cyanobacteria are microscopic photosynthetic organisms. Appropriate and careful handling of samples both prior to and during analysis is extremely important to ensure an accurate determination of toxin concentration. Cylindrospermopsin is stable in the dark in water. Furthermore, toxins in water bodies at the time of a bloom will be present in both the water (free, dissolved or extracellular toxins) and the cyanobacterial cells (intracellular) [10]. But, in contrast to blooms of *Microcystis*, a large proportion of cylindrospermopsin in environmental samples appears to be present as extracellular toxin [10]. It should be noted that standards

will soon be available from National Research Council Canada Canada (Quilliam, M. A., National Research Council Canada, Institute for Marine Biosciences, December 2005, http//www.imb-ibm.nrc-cnrc.gc.ca/crmp/index_e.php).

3.3.2.2 Extraction/concentration/clean-up procedures

Effective extraction of cylindrospermopsin from cyanobacteria is difficult without disrupting or perforating the cell wall structure. Some procedures use freeze-drying for this purpose, others utilize methanol. Other extractants for cylindrospermopsins in cyanobacterial material include water [10]. Due to its hydrophilic nature, cylindrospermopsin cannot be extracted and concentrated from water samples with SPE cartridges such as C18. SPE with graphitized carbon-based sorbents have been examined and with success [166].

Studies of cylindrospermopsin adsorption on materials and degradation have been reported. No adsorption by Pyrex glass was found, but cylindrospermopsin is strongly adsorbed by polyethylene [165].

3.3.2.3 Determination/detection

Chromatographic methods. Cylindrospermopsin can be determined by HPLC with UV-detection at 262 nm. But without the above-mentioned SPE, specificity of the method needs evaluation. One of the most common methods used involves HPLC-DAD [14], mainly because of the characteristic spectrum of cylindrospermopsin. The opportunities and limits of the analysis of cylindrospermopsin with HPLC-DAD have been studied [167]. By cleverly using an RP-C18 column – with a view to use it as a multimethod for other cyanobacterial toxins – and matching the mobile phase, cylindrospermopsin could easily be separated from deoxycylindrospermopsin. The main impediments of this method are the complex matrices when natural samples are analysed due to the difficulties of the water extraction used.

The problems to concentrate samples containing cylindrospermopsin were tackled shortly after by using graphitized carbon-based sorbents for SPE (instead of C18 sorbents) [166]. Cylindrospermopsin in lake water could then successfully be detected with HPLC-DAD at 1 μg/L.

Some years ago, a method for monitoring cylindrospermopsin using LC-MS/MS has been reported [168]. This method was sufficiently sensitive to be applicable directly to water samples without an extraction and preconcentration step. With detection limits lower than 1 μg/L in lake water, the results indicate the power of MS/MS as a detection method in chromatography.

Nonchromatographic methods. Initial studies involved the use of mouse bioassay (see microcystins, nodularins, anatoxin-a, antoxin-a(S)) but alternatives have been investigated. Animal invertebrate assays such as the Tamnotox kit and the brine shrimp *A. salina* provide toxicity assessments with LC_{50} values of 0.7–8.1 μg/mL [87]. The ability to inhibit the translation of mRNA into protein by cylindrospermopsin using the rabbit reticulocyte lycate assay system has resulted in the development of sensitive assays for this cyanobacterial toxin [14, 169].

CZE and MEKC were applied to the simultaneous separation of anatoxin-a, microcystin-LR and cylindrospermopsin. Analytical performance data of both methods, optimized for the three toxins, were similar with a precision of migration times smaller than 0.8% RSD and a detection limit in the range of 1–4 μg/mL, using spectrophotometric detection at 230 nm. Both methods were applied to an analysis of cyanotoxins in water bloom samples and crude cyanobacterial extracts. The results obtained indicate that, for complex matrices, the sequential application of CZE and MEKC is needed. It is recommended to use both CE techniques for the analysis of the same sample in order to confirm the results by an orthogonal approach [148].

3.3.2.4 Conclusion

Although there is an alternative to analyse cylindrospermopsin with bioassays, physicochemical methods are preferable to them. The opportunities of in particular LC, optical spectroscopy and MS are very promising and indicate that a combination of these techniques may be selected to be part of a standard method for the analysis of cylindrospermopsin in environmental samples. Another approach to determine cylindrospermopsin is the use of HILIC-MS (hydrophilic interaction LC-MS) [31]. The prospects of this method are quite good, certainly with a view on the need for multimethods.

The extraction of cylindrospermopsin in pure water provokes some problems due to a considerable matrix background. Efficient clean-up steps, like SPE based on graphitized carbon sorbent, should be developed.

3.3.3 Source organism(s) and habitat/occurrence

3.3.3.1 Source organism(s)

Cylindrospermopsin has been isolated and characterized from *C. raciborskii* and *Umezaka natans* [4]. An analogue of cylindrospermopsin, the nontoxic deoxycylindrospermopsin (deoxy-cylindrospermopsin) can be formed also by *C. raciborskii* [170, 162]. There is considerable variation in *C. raciborskii* and this is one of the reasons for its appearance recently in waters where it has been previously unrecorded [171]. In 1999, cylindrospermopsin was isolated also from *A. ovalisporum* [4]. A minor toxic metabolite of *A. ovalisporum*, an analogue of cylindrospermopsin, named epicylindrospermopsin, was also detected [162, 163].

Rhadiopsis curvata, isolated from a fish pond in Wuhan, P. R. China appeared to produce deoxy-cylindrospermopsin

and traces of cylindrospermopsin [172]. Furthermore, production of cylindrospermopsin by *A. bergii* was observed [173]. The coiled and the straight form of *C. raciborskii* produced different amounts of cylindrospermopsin with respect to cell concentrations in response to different growth conditions including the available form and the concentration of nitrogen. Different cylindrospermopsin production was also found between cultured and field populations from the lower Fitzroy River in Central Queensland, Australia. This indicates that cylindrospermopsin production might be influenced by a suite of genetic and environmental factors [174].

3.3.3.2 Habitat/occurrence

Cylindrospermopsis spp. can tolerate low light levels and therefore can compete more efficiently with other planktonic algae for available light, owing primarily to the presence of photosynthetic pigments. These pigments also allow photosynthesis in coloured waters [52].

Chonudomkul *et al*. [175] concluded that *C. raciborskii* is not only an ongoing invasive species but also a species with different physiological strains or ecotypes in temperature tolerance. No relationship of cylindrospermopsin synthesis to phylogenic or genetic clusters or geography exists. Synthesis of cylindrospermopsin is possibly a result of dynamic genetic and evolutionary processes, such as a horizontal transfer.

With respect to cylindrospermopsin content, cultures of cylindrospermopsin-producing isolates of *C. raciborskii* grown in the absence of fixed nitrogen, generally possess more toxin (on a cell dry-weight basis) than corresponding cultures grown in media with fixed nitrogen. Although cultures of *C. raciborskii* appeared to grow best at light intensities between 50 and 100 $\mu mol.m^{-2}s^{-1}$, the highest cellular content of cylindrospermopsin was obtained at light intensities outside this range.

There was a negative correlation between temperature and cellular toxin content in *C. raciborskii*. Maximum cylindrospermopsin contents were found in cultures grown at 20°C, a temperature well below the optimum for growth of *C. raciborskii*. Although all tested isolates of *C. raciborskii* grew well at 35°C, none produced any detectable amount of cylindrospermopsin. When these cultures were transferred to a lower temperature, the ability to produce cylindrospermopsin was fully restored. Cultures of *C. raciborskii* from different sources have shown great variation in their toxin content (as cited in Griffiths and Saker [176]).

Cylindrospermopsin-producing genera most commonly form toxic blooms in subtropical, tropical or arid zone water bodies [3]. *C. raciborskii* was found in Australia, *Umezakia natans* in Japan and *A. ovalisporum* in Israel and Australia [3, 4]. Data obtained from the Queensland Department of National Resources and the South East Queensland Water Board in Australia showed that the level of cylindrospermopsin ranged from 0.2 to 10.1 µg/L in storages in Queensland during 1997–1998 [4]. Blooms of *C. raciborskii* occur each year since 1991 in Lake Julius, a large man-made water impoundment in Australia's semiarid tropics with subsurface concentrations of >50 000 cells/mL. Low concentrations of cylindrospermopsin (~1–2 µg/L) were detected. An isolate of *C. raciborskii* from Lake Julius in 1995 which was grown in pure culture, produced no symptoms of poisoning when tested by the mouse bioassay [177]. Furthermore, increasing occurrences of *C. raciborskii* in Europe and North and South America are reported. Apparently *C. raciborskii* is highly tolerant of a range of salinity and nutrient concentrations and can grow in temperate climates under light and temperature regimes different from those of tropical environments [178]. Moisander *et al*. [37] reported an upper limit for salinity tolerance of 4 g NaCl/L for *Cylindrospermopsis* under laboratory conditions.

Beginning about 1995 *C. raciborskii* was identified in eutrophic Florida lakes, USA [5]. Around 1997, *C. raciborskii* began exponentially to increase and to dominate some Florida water bodies, for example Lake Jessup. Correlation analysis suggested that magnesium limitation, among other factors, may be an important factor influencing *C. raciborskii* growth in Lake Jessup, but appeared to be absent in Lake Apopka and Lake Griffin [179]. Invasive behaviour of *C. raciborskii* at mid-latitudes was observed. It was first reported in ponds in France in 1994 [178] and in 2002 in the river Seine in France [180], but was identified also in Germany [181, 182], Hungary [183], Brazil [184–186], Austria, Greece, Slovakia, Portugal [45, 187], Thailand, Mexico and Senegal [188]. The colonization of mid-latitudes by *C. raciborskii* may result from a combination of its ability to tolerate a rather wide range of climatic conditions and the global warming phenomenon, which provides this species with better environmental conditions for its growth [188]. However, blooms and strains of *C. raciborskii* from South America and Europe have so far not been found to contain cylindrospermopsin (cited from Fastner *et al*. [181]) (see also Section 3.3.6.3). In autumn 2000 *C. raciborskii* occurred for the first time in Lake Ouberia, Algeria [189].

In New Zealand, the presence of cylindrospermopsin was reported for the first time in autumn 1999 in water from a recreational lake (Waitawa) in the Wellington region. However, a rigorous identification of the causative organism was not carried out [190]. In autumn 2003, *C. raciborskii* was recorded in three shallow Waikato lakes and a hydroelectric dam on the Waikato River, New Zealand. It formed water blooms at densities >100 000 cells/mL in Lakes Waahi and Whangape. In Lake Waahi, cylindrospermopsin and deoxy-cylindrospermopsin were detected [171, 191].

3.3.4 Bioaccumulation in the environment

3.3.4.1 Bioaccumulation in aquatic organisms

Freshwater mussels *A. cygnea* were exposed to cylindrospermopsin-producing cultures of *C. raciborskii* for 16 days

followed by a depuration period up to day 32. Cylindrospermopsin in the culture medium (intra- plus extracellular) over the 16-day exposure period ranged from 14 to 90 μg/L. During the exposure period, maximum recorded cylindrospermopsin concentrations in the haemolymph, viscera and whole body of the mussels were 412, 1099 and 247 μg/kg wet weight, respectively. Directly after the exposure period cylindrospermopsin distribution in the mussels was as follows: haemolymph (68.1%), viscera (23.3%), foot and gonad (7.7%) and mantle (0.9%). No cylindrospermopsin was detected in the gills or the adductor muscle. After the first 2 days of depuration, the amount of cylindrospermopsin in the tissues was reduced by *ca.* 50% and then interestingly increased from days 22–28. At day 32 approximately 50% of the toxin remained in the tissues [192].

Redclaw crayfish (*Cherax quadricarinatus*) and rainbow fish (*Melanotaenia eachamensis*) were able to accumulate cylindrospermopsin from an aquaculture pond infested by *C. raciborskii*. Pond water contained 589 μg cylindrospermopsin/L (93% in cells, 7% in water). Exposure time was not given. The crayfish contained 4.3 mg cylindrospermopsin/kg freeze dried hepatopancreas tissue and 0.9 mg/kg freeze dried muscle tissue; the rainbow fish contained 1.2 μg/g freeze dried viscera tissue.

Under laboratory conditions the crayfish also accumulated cylindrospermopsin both at exposure to extracellular cylindrospermopsin (14-day exposure to an extract of harvested bloom material containing up to 568 μg (extracellular) cylindrospermopsin/L) and at exposure to a pure culture of *C. raciborskii* (111 μg cylindrospermopsin/L intracellular and 17 μg/L extracellular). However, accumulation occurred to a considerably lesser extent in the laboratory than in the culture pond [193].

3.3.4.2 Accumulation in crops/plants

See Section 2.2.4.2.

3.3.5 Biodegradation in the environment

In young blooms, a larger percentage of cylindrospermopsin was found in the cells, whereas in older blooms a larger percentage of cylindrospermopsin was dissolved in the water column. Cell numbers may be low in the decline of a bloom, but cylindrospermopsin levels in the water may persist, and it is common to find around 70–98% of the total toxin dissolved in the water column. When cells decompose in water blooms, cylindrospermopsin in the presence of plant pigments, enzymes and other material is exposed to sunlight, and this may promote natural rates of degradation [165]. Cylindrospermopsin in an algal extract solution (with high levels of plant pigmentation) decomposed rapidly ($t^{1/2}$ = 1.5–4 h) when exposed to sunlight in the laboratory. In natural waters in which plant pigment levels were low, $t^{1/2}$ was 11–15 days [165].

On treatment with chlorine-related water disinfectants, the nontoxic 5-chlorocylindrospermopsin (in mouse bioassay), a chlorine derivative of cylindrospermopsin, and nontoxic cylindrospermic acid (in mouse bioassay), an oxidated derivative of cylindrospermopsin, may be formed [164]. Destruction of the cylindrospermopsin molecule was most effective at pH levels above 6.0 (as cited from Griffiths and Saker [176]).

Effective degradation of cylindrospermopsin (in the form of cell-free extract from a cultured toxic strain of *C. raciborskii*) by UV in the presence of two brands of TiO_2 has been demonstrated. With the more effective form TiO_2, cylindrospermopsin was photodegraded from an initial concentration of 100 μg/L with a half-life of 0.7 min. An alternative form of the photocatalyst, yielded a half-life of 3.6 min under the same circumstances. This photocatalytic degradation of cylindrospermopsin was pH dependent, being most efficient at higher pH (9.00) (as cited from Griffiths and Saker[176]).

In systems in which water from the affected water body can be pumped into a holding reservoir prior to treatment and distribution, the seeding of buoyant species into the holding reservoir could perhaps be prevented by applying increased hydrostatic pressure in the connective pipe. Such a method has been reported to be effective in combating the buildup of populations of *A. ovalisporum* in Lake Kinneret, Israel (as cited from Griffiths and Saker [176]).

3.3.6 Toxicity of cylindrospermopsin

3.3.6.1 Mechanism of action

Cylindrospermopsin is a general cytotoxin that blocks protein synthesis, and the first clinical symptoms of poisoning are kidney and liver failure [3, 5]. By the oral route, cylindrospermopsin can cause gastroenteritis through injury to the gut lining, hepatitis from injury to liver cells, renal malfunction from cell injury to the kidneys and haemorrhage from blood vessel injury [4].

Primary rat hepatocytes were used to investigate the mechanism involved in cylindrospermopsin toxicity. Cylindrospermopsin caused significant dose-dependent cytotoxicity (52–82% cell death) at 1–5 μM. Protein synthesis inhibition was a sensitive, early indicator of cellular responses. After removal of cylindrospermopsin, the inhibition of protein synthesis could not be reversed. In contrast to the LDH leakage (measure for cell death), protein synthesis was inhibited maximally at 0.5 μM. No protein synthesis occurred over 4–18 h at or above 0.5 μM. This study confirms that cylindrospermopsin produces time- and concentration-dependent toxicity in freshly isolated rat hepatocytes. Although cylindrospermopsin caused rapid and irreversible inhibition of protein synthesis, this is unlikely to be the major toxic insult induced by cylindrospermopsin because cytochrome P-450 inhibitors that attenuated cylindrospermopsin toxicity, did not protect against the impairment of protein synthesis [194].

For the liver injury four consecutive phases of pathomorphological changes were described. In the initial phase, ribosomes detach from the membranes of the rough-surfaced ER and accumulate into the cytoplasm of hepatocytes. Usually, this process is accompanied by condensation and reduction in the size of nucleoli. The second phase often begins 24 h after administration and is correlated with membrane proliferation. In this phase the amount of total P-450 is considerably decreased in the toxin-treated hepatic microsomes. The authors believed that marked proliferation of agranular membranes is due to lipid peroxidation caused by P-450. The third phase is represented by an accumulation of fat droplets in the central portion of hepatic lobules, probably induced by free radicals generated in the injury. The last phase is characterized by severe liver necrosis [4].

Norris *et al*. [195] demonstrated that in male Quakenbush mice GSH depletion was unlikely to be the mechanism for cylindrospermopsin toxicity. Conversely, Quakenbush mice pretreated with piperonylbutoxide, a P-450 inhibitor, were protected against cylindrospermopsin toxicity suggesting activation of cytochrome P-450 by cylindrospermopsin. This is of primary importance in the mechanism of action.

3.3.6.2 Pharmacokinetics

Twelve male Quakenbush mice received i.p. 0.2 mg ^{14}C-labelled cylindrospermopsin/kg bw. Most of the radiolabel was excreted during the first 12 h (70.9%), predominantly in the urine (59.6% in mice with toxic effects, 70.5% in mice without effects), but in some individual mice significant excretion took place *via* the faeces (mean 7.2% in mice with toxic effects, 4.6% in mice without effects, range in all mice 0.7–19.6%). Total excretion in 24 h in urine plus faeces was 72.2 and 80.2% in mice with and without effects, respectively. Cylindrospermopsin accounted for 72.0% of the excreted urinary radioactivity at 12 h, which is equal to 47% of the administered dose. Accumulation of tissues occurred predominantly in the liver (20.6% of the dose at 6 h, 13.1% at 48 h and 2.1% at 5–7 days) and to a lesser extent in the kidneys (4.3% of the dose after 6 h, 0.15% after 5–7 days). All spleens contained <0.1% of the dose (5–7 days). There is at least one methanol-extractable and one nonmethanol-extractable metabolite in the liver. The methanol-extractable metabolite was not found in the kidney and is more hydrophilic than cylindrospermopsin itself on RP [196].

3.3.6.3 Toxicity in laboratory animals

Acute toxicity – oral studies. No data on oral toxicity of pure cylindrospermopsin are available but studies with aqueous extracts of freeze-dried *Cylindrospermopsis* provide a preliminary indication.

After single oral doses to male mice of 2.5 up to 8.3 mg cylindrospermopsin equivalent/kg bw, given *per* stomach tube as freeze-dried *C. raciborskii* (Woloszynska) culture containing 0.2% cylindrospermopsin, suspended in normal saline, a lowest lethal dose of 4.4 mg toxin equivalent/kg bw could be determined although one animal survived a dose of 6.7 and one of 6.9 mg/kg bw. Hundred percent mortality was observed in mice at 8 mg cylindrospermopsin eq/kg bw. Death occurred 2–6 days after dosing. Pathologic changes included marked fatty liver, often with periacinar coagulative necrosis, acute renal tubular necrosis, atrophy of the thymic cortex and the lymphoid follicles in the spleen, subepicardial and myocardial haemorrhages and multiple ulcerations of the esophageal part of the gastric mucosa [197].

After oral dosing of male mice with freeze-dried *C. raciborskii* (strain AWT 205) cells suspended in distilled water, also Falconer *et al*. [154] observed histological damage of liver and kidneys. Oral dosing of 1400 mg/kg bw of an extract containing 5.4 mg cylindrospermopsin/g (approximately 7.5 mg cylindrospermopsin/kg bw) did not cause mortality.

Shaw *et al*. [198, 199] reported an acute oral LD_{50} value of 6 mg/kg bw for male white Quakenbush mice. Cylindrospermopsin was given as a freeze-dried cellular suspension of *C. raciborskii*. Histopathology of the liver revealed lipid vacuolation in hepatocellular cytoplasm and in more severe cases extensive areas of hepatocyte necrosis were seen. Furthermore, thymic atrophy and necrosis in tubules of the kidney cortex were observed. A number of animals developed thrombohemorrhagic lesions in one or both eye orbits at 6 and 8 mg/kg bw.

Acute toxicity – intraperitoneal studies. Intraperitoneal injection of lysed *C. raciborskii* to mice resulted in wide-spread and progressive tissue injury, with cell necrosis in the liver, kidneys, adrenals, lung, heart, spleen and thymus. In mice, the i.p. LD_{50} at 24 h was 52 mg dry weight of cells/kg bw, equivalent to 300 μg cylindrospermopsin/kg bw, whereas the i.p. LD_{50} at 7 days was approximately 32 mg dry weight of cells/kg bw, equivalent to 180 μg toxin/kg bw. Administration of the pure toxin demonstrated this delayed toxicity more clearly, with the 24 h i.p. LD_{50} being 2100 μg/kg bw and the 5–6 day i.p. LD_{50} being 200 μg/kg bw. Major changes in mouse were seen in hepatocytes, with progressive proliferation of the smooth ER and accumulation of lipid over 5 days [3].

Deaths were observed in mice by i.p. administration with doses greater than 50 μg cylindrospermopsin/kg bw for both purified cylindrospermopsin and cylindrospermopsin as cell-free extract of *C. raciborskii*. Mice huddle and are anorexic and usually experience slight diarrhoea. Average hours until moribund were 70.5–9 h for i.p. doses of purified cylindrospermopsin of 0.1–0.8 mg/kg bw and 102–41 h for i.p. doses of 0.1–0.8 mg cylindrospermopsin/kg bw given as cell-free extract. Death is preceded by a slow gasping respiration and occasional limb paddling [4, 198].

By using the Spearman-Käber method the 7-day i.p. LD_{50} of cylindrospermopsin in mice was estimated to be 170 μg/kg bw [4].

After i.p dosing of freeze-dried *Cylindrospermopsis* (strain AWT 205) cells suspended in 0.9% saline, to male mice, 24 h LD_{50} values of 50–110 mg extract/kg bw (cylindrospermopsin contents of the batches were 5.4, 1.3, 2.0 and 3.2 mg/g extract) were observed, whereas the 7 days LD_{50} values ranged from 20 to 65m g extract/kg bw (cylindrospermopsin contents of the batches were 5.4, 1.3, 2.0 and 3.2 mg/g extract). Histological damage of liver and kidneys was observed. However, the severity of this damage caused by the different batches of *Cylindrospermopsis* with similar cylindrospermopsin content varied considerably, implying the presence of more than one toxin [154].

The i.p. toxicity in the mouse of extracts from a French isolate of *C. raciborskii* was compared to the toxicity of extracts from *C. raciborskii* isolates from Australia, Brazil, Mexico and Hungary. The toxicity of extracts from European and South American isolates of *C. raciborskii* appeared to be considerably lower than that of an extract from an Australian strain of *C. raciborskii* [200].

The i.p. LD_{50} of 7-epi-cylindrospermopsin, a natural derivative of cylindrospermopsin, was estimated by mouse bioassay and appeared to be 200 μg/kg mouse weight [164].

A single i.p. injection of 0.8 mg deoxy-cylindrospermopsin/kg bw in three (male white Quackenbush) mice did not cause any toxicity within 5 days [162].

At treatment with chlorine-related water disinfectants, 5-chlorocylindrospermopsin, a chlorine derivative of cylindrospermopsin, and cylindrospermic acid, an oxidated derivative of cylindrospermopsin, may be formed. After i.p. injection of 10 mg/kg bw in mice, these compounds did not cause any mortality and no physiological or pathological toxic symptoms were observed within 10 days after treatment [164].

Irritation and sensitization studies. In an intradermal skin irritation test in albino Californian rabbits, 0.2 mL of an extract of lyophilized *A. ovalisporum* was injected intradermally and skin reactions (erythema, oedema) were scored after 24, 48 and 72 h. Moderate skin irritation was seen [57].

A maximization test in guinea-pigs was performed with an extract of lyophilized *C. raciborskii* (0.015 mg cylindrospermopsin/g). Fifty percent of the animals appeared to be sensitized. The same test performed with a nontoxic *Aphanizomenon* strain resulted in sensitization of 91% of the animals [57].

Repeated dosing – oral studies. At repeated or dosing by gavage to male white Quakenbush mice, an NOAEL of 0.05 mg cylindrospermopsin/kg bw and an LOAEL of 0.15 mg/kg bw was observed based on fatty infiltration in the liver (both for cylindrospermopsin given as a cell-free extract of *C. raciborskii* as well as for cylindrospermopsin given purified). Lymphophagocytosis in the spleen was seen at dosing with the cell-free extract at 0.05 mg cylindrospermopsin/kg bw, whereas dosing with purified cylindrospermopsin did not cause lymphophagocytosis. One mouse dosed with the cell-free extract showed a retro-orbital haematoma in one eye at 0.15 mg cylindrospermopsin/kg bw [198].

Six mice given daily for 21 days drinking water from a dam containing 800 μg cylindrospermopsin/L did not show any observable pathological symptoms. The approximate dose based on water consumption was 0.2 mg/kg bw/day [198].

Groups of eight 4-wk-old male ICR mice received orally *via* the drinking water 0 or 0.6 mg cylindrospermopsin/L (~0 or 0.066 mg/kg bw) for 3 wk. After 21 days urinary excretion rate was decreased significantly and haematocrit value was increased significantly. Relative liver- and teste weights were increased significantly in the experimental group. Relative kidney weight was increased slightly and relative spleen weight did not show abnormalities. The typical metabolic disorder associated with the inhibition of uridine monophosphate (UMP) synthetase activity (as was seen in *in vitro* studies), known as ‚orotic aciduria' was not seen under these conditions, but other anomalous metabolic responses related to cholesterol metabolism were developed [201].

Male white Quakenbush mice given cylindrospermopsin in their drinking water *ad libitum* for 90 days did not show any gross morphological or histopathological effects at doses up to 0.15 mg/kg bw. Cylindrospermopsin was administered as a cell-free extract of *C. raciborskii* [198].

In a study with a crude aqueous extract of *C. raciborskii* (AWT205 strain), groups of 10–12 male Swiss albino mice (except at the highest dose-level; this group included five mice) received 0, 43, 85, 130 and 135 mg of the extract/kg bw *via* their drinking water (equal to 0, 216, 432, 657 and 687 μg cylindrospermopsin/kg bw) for 10 wk. Body and organ weights were determined, food and drinking water consumption were recorded, urine, serum and haematology analyses were performed and gross pathology and histopathology of tissues were carried out. At the highest dose-level of 687 μg/kg bw the animals reduced their liquid intake and became dehydrated. Therefore, the results at this dose-level were not accounted for. Body weights were decreased after 10 wk at all dose-levels and these decreases were significant at 432 and 657 μg cylindrospermopsin/kg bw. Significant increases in relative liver and kidney weights were seen at all dose levels. Clinical indicators for liver injury (ALAT, ASAT and SAP) did not show significant changes up to and including the dose level of 432 μg cylindrospermopsin/kg bw. A nonsignificant increase of ALAT was seen at 432 μg/kg bw and ASAT showed nonsignificant decreases at 216 and 432 μg/kg bw. Elevation of SAP was low compared to changes seen in the case of hepa-

tobiliary diseases. Serum albumin and bilirubin levels were increased significantly at all dose-levels with a dose-relationship, whereas serum bile acids and phosphate levels were decreased significantly. Urinalysis revealed decrease in urine protein/creatinine concentrations at all dose-levels and these decreases were significant at 432 and 657 μg cylindrospermopsin/kg bw. Histopathological changes in the liver were seen at all dose-levels. An NOAEL could not be derived in this study [202].

In a study with purified cylindrospermopsin, groups of ten male Swiss albino mice (except at the highest dose-level; this group included six mice) received by gavage 0, 30, 60, 120 and 240 μg cylindrospermopsin/kg bw for 11 wk. Body and organ weights were determined, food and drinking water consumption were recorded, urine, serum and haematology analyses were performed and gross pathology and histopathology of tissues were carried out. After 9 wk on test a clinical examination was conducted (physiological and behavioural signs of toxicity). Body weights were significantly increased at 30 and 60 μg/kg bw whereas nonsignificant increases were seen at 120 and 240 μg/kg bw. However, growth of control animals was reduced probably by physical discomfort due to gavage. Water intake was reduced in all groups. Relative liver weights were increased significantly at 240 μg/kg bw (nonsignificant increases at 60 and 120 μg/kg bw) and relative kidney weights were increased significantly at 60, 120 and 240 μg/kg bw (nonsignificant increases at 30 μg/kg bw). Absolute adrenal weights were increased significantly at 120 μg/kg bw and absolute testes weights at 60, 120 and 240 μg/kg bw. However, relative adrenal and testes weights did not show significant increases. Haematology revealed an increase in lymphocytes at 30 μg/kg bw only. However, no changes in bone marrow smears or spleen were observed. Indicators for liver injury (ALAT, ASAT and SAP) were not increased significantly at any dose-level as was expected after exposure to a hepatotoxin. Serum bilirubin levels were nonsignificantly increased and serum bile acids nonsignificantly decreased at 60, 120 and 240 μg/kg bw. At dose-levels ≥120 μg/kg bw urine protein/creatinine concentrations were depressed significantly. Urine specific gravity was decreased significantly at 240 μg/kg bw. At 120 and 240 μg/kg bw minor increases in histopathological damage to the liver was seen and at 240 μg/kg bw also damage to the proximal tubules of the kidney occurred. An NOAEL of 30 μg/kg bw was established in this study based on increased kidney weights. It has to be remarked that the magnitude of effect for some parameters (organ weights, serum bile acids, serum bilirubin, urine protein/creatinine levels) was greater in the afore-mentioned study at comparable levels of cylindrospermopsin. This may indicate that the extract used in the afore-mentioned study is more toxic than expected from its measured cylindrospermopsin content [202].

Two rats given daily for 21 days drinking water from a dam containing 800 μg cylindrospermopsin/L did not show any observable pathological symptoms. The approximate dose based on water consumption was 0.2 mg/kg bw/day [198].

Repeated dosing – intraperitoneal studies. In a study in male (white Quakenbush) mice given daily purified cylindrospermopsin by the intraperitoneal route for 14 days, an NOAEL of 1 μg cylindrospermopsin/kg bw was observed. When cylindrospermopsin was given as cell-free extract of *C. raciborskii* the NOAEL was <5 μg cylindrospermopsin/kg bw/day [198, 199].

Mutagenicity. An alkaline comet assay in Chinese hamster ovary (CHO) K1 cells *in vitro* did not reveal DNA damage after 24 h of treatment with cylindrospermopsin concentrations of 0.5 and 1 μg/mL. However inhibition of cell growth, cell blebbing and rounding were noticed. These morphological effects were linked to cytoskeletal reorganization and not to apoptosis. From this study it can be concluded that cylindrospermopsin does not obviously react directly with DNA in CHO K1 cells [203].

The cytokinesis-block micronucleus assay using the human lymphoblastoid cell-line WIL2-NS, was performed to test possible genotoxic activity of cylindrospermopsin. This assay can count aptotic and necrotic cells and can detect also centromeres in micronuclei to differentiate between aneugens causing whole chromosome loss, and clastogens which produce predominantly acentric fragments. The cell line was exposed for 24 h to 0, 1, 3, 6 or 10 μg cylindrospermopsin/mL. At 6 and 10 μg/mL a significant increase in the frequency of micronuclei in binucleated cells was seen. A significant increase of centromer-positive micronuclei was observed at all concentrations. The results suggested that cylindrospermopsin might induce cytogenetic damage *via* two mechanisms, one by induction of DNA strand breaks, the other by the loss of whole chromosomes at the level of kinetochore/spindle function (aneuploidy) [204].

In male white Quakenbush mice given i.p. cylindrospermopsin as a cell-free extract of *C. raciborskii*, adduct spots of liver DNA were seen in animals killed 24–96 h postdosing. A single adduct spot was seen in each case [198].

Male and female Balb/c mice were injected i.p. with 0.2 mg cylindrospermopsin/kg bw in 0.9% saline and were sacrificed after 6, 12, 24, 48 and 72 h. Significant DNA strand breakage was observed in the isolated liver [205].

***In vitro* studies.** Cylindrospermopsin has a significantly higher toxicity for primary rat hepatocytes than for ketone bodies (HeLa type) in cultures [198].

The 72 h-LD_{50} value of cylindrospermopsin for primary rat hepatocytes was 40 ng/mL (cell viability determined by MTT assay) (for comparison 72 h-LD_{50} value for microcystin-LR is 8 ng/mL). The involvement of bile acid carriers in the uptake of cylindrospermopsin into hepatocytes

appeared to be less than for the uptake of microcystin-LR. Since the molecular size of cylindrospermopsin was relatively small when compared with microcystin-LR, it is conceivable that a ‚second' transport system might be simple passive diffusion. This was supported by the fact that cylindrospermopsin showed cytotoxic effects at incubation with a permanent KB cell line which is devoid of a bile acid transport system [66].

The inhibitory effect of cylindrospermopsin on protein phosphatase 2A (PP2A) was measured using a colorimetric method. Cylindrospermopsin did not show a significant inhibitory effect on PP2A [66].

Carcinogenicity. The potential of a crude saline extract of freeze dried *C. raciborskii* (doses 500 or 1500 mg/kg bw) to initiate tumours in male Swiss Albino mice after one to three oral applications (time interval of 2 wk between applications) was investigated in a preliminary study. TPA was administered in some treatment groups until the end of the experiment (30 wk) with the aim of promoting the growth of initiated tumours. Totally five tumours among them two hepatic dysplastic foci and one frank HCC were found in 53 cylindrospermopsin treated animals compared to none in 27 control animals. No promoting activity of TPA was observed. Although the number of animals was too low to provide statistical significant evidence for tumour initiation, the range of the calculated relative risk suggests that it would be imprudent to reject this possibility [206].

3.3.6.4 Effects on humans

In Australia, a cyanobacterial bloom in the drinking water reservoir resulted in complaints from the water consumers of bad taste and odour from the drinking water. Controlling water authorities terminated the bloom by copper sulphate addition to the reservoir. Shortly after copper dosing of the reservoir, children were brought into the hospital with an unusual hepatoenteritis, initially showing acute tender liver enlargement, constipation, vomiting and headache. This was followed by bloody diarrhoea and loss of protein, electrolytes, glucose and ketones through the urine, with varying severity of dehydration. Severe cases were flown to the regional hospital, where they received intensive care with intravenous therapy. A total of 140 children and 10 adults received hospital treatment. The clinically most serious cases occurred among the Aboriginal population of Palm Island, off the Queensland coast of Australia in 1979. Cylindrospermopsin derived from *C. raciborskii*, was isolated. It showed similar toxicity in animal studies to that in the children reported above (cited from Falconer [81]).

3.3.6.5 Effects on aquatic organisms

Effects of a bloom of *C. raciborskii* on species composition and seasonal succession of some planktonic components were studied during 2 years (1997–1998) in Ingazeira reservoir, northeast Brazil. Cascading changes in the planktonic structure were induced by the *C. raciborskii* bloom. Data suggested the possibility of breakage of filaments by some zooplankton species (*e.g.* copepods) and, as a result of being broken, previously inedible cyanobacterial filaments may be efficiently grazed as small and edible particles by different zooplankton species (*e.g.* cladocerans). Changes in both the phytoplankton and zooplankton communities were also associated with shifts in bacterial community composition with the progressive occurrence of large grazing-resistant bacteria. Thus, the cyanobacterial bloom provided a pulse resource for herbivores, implying a significant change in the structure of other planktonic components, but that was not accompanied by a loss of biodiversity [207].

A dose-dependent response in *A. salina* mortality was seen for purified cylindrospermopsin and LC_{50} values decreased with time from 8.10 to 1.23 μg/mL between 24 and 72 h, respectively [87].

The large ciliate *Paramecium* cf. *caudatum* was found to be a successful grazer of cylindrospermopsin produced by *C. raciborskii* in the laboratory [174].

Natural *Daphnia* populations subjected to blooms of *C. raciborskii* may experience high mortality, reduced individual body growth and negative effects on fecundity because of the toxic metabolites present in *C. raciborskii* cells [208].

3.3.6.6 Effects on wild and domestic animals

In August 1997, a farm dam at McKinlay in northwest Queensland, Australia, contained an algal bloom which was identified as a monoculture of *C. raciborskii*. Cylindrospermopsin was detected in material harvested from the dam and in a pure culture of an isolate from the bloom. An extract of this material (freeze-dried) was lethal to mice 24 h after an i.p injection of 153 mg/kg bw. On a cattle property near the dam three cows and ten calves died. One animal showed signs of staggering and weakness before its death. Abdominal and thoracic haemorrhagic effusion, hyperemic mesenteries and pale and swollen liver were found at necropsy, with nothing abnormal observed in the brain, lungs, spleen or kidney. Histopathology of the liver from a calf carcass showed signs similar to the known toxicological effects of cylindrospermopsin in mice (extensive areas of hepatic degeneration and necrosis, with only isolated areas of intact hepatocytes remaining; deposits of fibrous tissue were common throughout the liver) [96, 209].

3.3.6.7 Effects on crops/plants

Cylindrospermopsin was shown to inhibit the germination of pollen (measured by Alcian Blue dye-binding) of the tobacco plant (*Nicotania tabacum*) at concentrations between 5 and 1000 μg/L. The amount of ^{14}C-(U)-leucine labelling in pollen tubes appeared to be significantly reduced. Implications of these effects for current spray-irrigation are discussed [210].

Table 2. Structures of the STXs

Name of toxin	Variable chemical groups in toxins				
	R1	R2	R3	R4	R5
STX	H	H	H	$CONH_2$	OH
GTX2	H	H	OSO_3^-	$CONH_2$	OH
GTX3	H	OSO_3^-	H	$CONH_2$	OH
GTX5 (B1)	H	H	H	$CONHSO_3^-$	OH
C1 (epiGTX8)	H	H	OSO_3^-	$CONHSO_3^-$	OH
C2 (GTX8)	H	OSO_3^-	H	$CONHSO_3^-$	OH
C3	OH	H	OSO_3^-	$CONHSO_3^-$	OH
C4	OH	OSO_3^-	H	$CONHSO_3^-$	OH
neoSTX	OH	H	H	$CONH_2$	OH
GTX1	OH	H	OSO_3^-	$CONH_2$	OH
GTX4	OH	OSO_3^-	H	$CONH_2$	OH
GTX6 (B2)	OH	H	H	$CONHSO_3^-$	OH
dcSTX	H	H	H	H	OH
dcneoSTX	OH	H	H	H	OH
dcGTX1	OH	H	OSO_3^-	H	OH
dcGTX2	H	H	OSO_3^-	H	OH
dcGTX3	H	OSO_3^-	H	H	OH
dcGTX4	OH	OSO_3^-	H	H	OH
LWTX1	H	OSO_3^-	H	$COCH_3$	H
LWTX2	H	OSO_3^-	H	$COCH_3$	OH
LWTX3	H	H	OSO_3^-	$COCH_3$	OH
LWTX4	H	H	H	H	H
LWTX5	H	H	H	$COCH_3$	OH
LWTX6	H	H	H	$COCH_3$	H

3.3.6.8 Derivation of NOAEL and TDI

Two studies may be used for derivation of an NOAEL/TDI. The first study is the 90-day drinking water assay in mice from Shaw *et al.* [199]. Based on the NOAEL of 150 μg/kg bw in this study a TDI of 0.3 μg cylindrospermopsin/kg bw/day can be calculated using an uncertainty factor of 500 (ten for interspecies variability, ten for intraspecies variability and five for less than lifetime exposure). When 100% of the exposure to cylindrospermopsin is assigned to drinking water, a drinking water consumption of 2 L/day is assumed, and the adult body weight is assumed to be 60 kg, a guideline value for drinking water of (0.3 μg × 60)/2 = 9 μg/L can be calculated (see Griffiths and Saker [176]).

A second study which may be used is an 11-wk gavage study in mice from Humpage and Falconer [202]. Based on the NOAEL of 30 μg/kg bw in this study a TDI of 0.06 μg cylindrospermopsin/kg bw/day can be calculated using an uncertainty factor of 500 (ten for interspecies variability, ten for intraspecies variability and five for less than lifetime exposure). When 100% of the exposure to cylindrospermopsin is assigned to drinking water, a drinking water consumption of 2 L/day is assumed, and the adult body weight is assumed to be 60 kg, a guideline value for drinking water of (0.06 μg × 60)/2 = 1.8 μg/L can be calculated. The possibility, that in addition to its major effect as a hepatotoxin, cylindrospermopsin may also be a tumour initiator, has been reported by Falconer and Humpage [206]. In addition some genotoxicity studies showed positive effects and DNA adducts in mouse liver were reported. Some authors recommend an additional safety factor of 10 for potentially genotoxic chemicals.

The potential risk from consumption of cylindrospermopsin-affected fish or shellfish is difficult to assess [176].

3.3.7 Regulations

No official guideline exists in the USA. In Australia no official guidelines were established but 1 and 15 μg/L (1.5 μg/L if genotoxic) are considered [5, 211]. A guideline value of 1.0 μg/L has been suggested for cylindrospermopsin in Canada based on suspected genotoxicity [105].

3.4 STXs

3.4.1 Chemical structures and properties

STX and neoSTX possess a unique tricyclic structure with hydropurine rings (see Fig. 7). STXs are a group of carbamate alkaloid toxins which are either nonsulphated (STXs), singly sulphated (gonyautoxins, GTXs) or doubly sulphated (C-toxins). In addition, decarbamoyl derivatives (dc) and several new toxins (*Lyngbya-wollei* toxins, LWTXs) have been identified in some cyanobacterial species (see Table 2 from Nicholson and Burch [10] and Chorus and Bartram [3]).

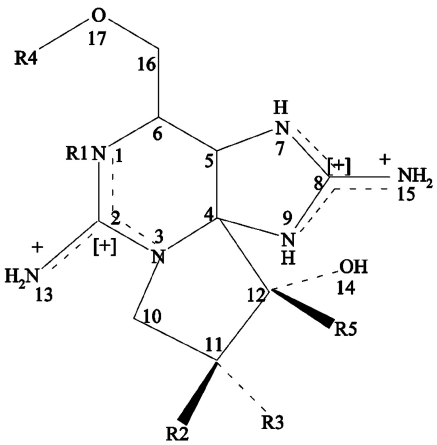


Figure 7. General structure of STXs.

3.4.2 Methods of analysis

3.4.2.1 General

Cyanobacteria are microscopic photosynthetic organisms. Appropriate and careful handling of samples both prior to and during analysis is extremely important to ensure an accurate determination of toxin concentration. STX is very stable in solution samples and could be adopted as a reference standard (Quilliam, M. A., National Research Council Canada, Institute for Marine Biosciences, December 2005, http//www.imb-ibm.nrc-cnrc.gc.ca/crmp/index_e.php).
This toxin can be kept in dilute acidic solutions for several months without loss of potency. However, neoSTX – for example – is unstable, possibly due to transformation to other toxins [212]. The lack of data concerning the stability of the different classes of STXs, confirm that preservation techniques for water samples containing STXs require investigation [10].

3.4.2.2 Extraction/concentration/clean-up procedures

The extraction of STX from intact cyanobacterial material using acetic acid was investigated. Recoveries were highest where freeze-thawing was used in conjunction with acetic acid. Ultrasonification was not necessary, provided the sample was freeze-thawed three times. Extractions with stronger acids (HCl) revealed substantial conversion of some classes of STX. Because of their hydrophilic nature, STXs cannot be extracted/or concentrated from water using organic solvents. Instead of C18-based sorbent SPE, graphitized carbon black cartridges have to be used [10].

3.4.2.3 Determination/detection

Chromatographic methods. The most commonly employed method is HPLC with fluorescence detection [14], using either precolumn [213] or postcolumn oxidation and derivatization of STXs [214]. More recently, considering the use of LC-MS techniques have become undispensible in analysing STXs. As discussed before, LC-ESI-MS-based method for the simultaneous determination of algal and cyanobacterial toxins in phytoplankton from marine waters and lakes is consequently suitable for the analysis of STX [30]. But also, evolving the high hydrofilicity of STXs, HILIC-MS (hydrophilic interaction LC-MS) [31] is well suited to detect STXs at sufficiently low levels.

Nonchromatographic methods. Some techniques for the determination of STXs look promising. Concerning ELISA, most important seems the development of suitable antibodies. A main problem of utilizing a specific antibody for STX is the poor crossreactivity to other STXs [112], whereas an advantage of ELISA by comparison with physicochemical methods, should be the wide variety of analogues that can be measured.

Regarding (mouse) bioassays for STXs determination, the same analytical facts can be recited as for anatoxin-a(S), anatoxin-a, nodularins and microcystins (mouse) bioassays.

3.4.2.4 Conclusion

A review of STXs research including analytical methods is present in the literature [215]. Much research on STXs – as paralytic shellfish poison – has been done in the past. Most of the methods used for the determination in marine waters and shellfish may be applied to the analysis of STXs in samples derived from blooms of blue-green algae. HPLC coupled with fluorescence is yet the method for the detection of STXs because of its low costs compared to LC-MS. Nevertheless, in future, LC-MS will play an increasing role in STXs determination, if only because of its prospects to be used as a multimethod for the detection of many different cyanobacterial toxins.

3.4.3 Source organism(s) and habitat/occurrence

3.4.3.1 Source organism(s)

STX and some of its analogues are produced by the cyanobacteria *A. circinalis* in Australian freshwaters (cited from Chorus and Bartram [3]). *A. circinalis* may not be the only *Anabaena* species in Australia producing STXs, as very low concentrations of GTX3 were detected in two other *Anabaena* species: *A. perturbata* var. *tumida* and *A. spiroides* [216]. In a few Danish lakes containing STXs, *A. lemmermanii* was the dominant cyanobacterium [161]. In addition the cyanobacteria *A. flos-aquae* and *Lyngbya wollei* from North America, *A. flos-aquae* from Portugal and *Planktothrix* sp. FP1 from Italy were reported to produce STX (analogues) [217]. In Brazil, the production of STXs (mostly neoSTX and smaller amounts of STX) was ascribed to the cyanobacterium *C. raciborskii* [3]. Dias *et al*. [218] reported the production of STXs by *A. isatschenkoi*.

3.4.3.2 Habitat/occurrence

In Australian populations of *A. circinalis* C1, C2, GTX2 and GTX3 showed to be the dominant components, whereas STX, GTX5, dcSTX, dcGTX2 and dcGTX3 were minor constituents. Variation in concentration and composition of STXs were seen in natural populations and cultured strains of *A. circinalis* reflecting possibly environmental conditions. Temporal variation in levels of STXs was seen during a persistent bloom of *A. circinalis* at Wongulla Lagoon, South Australia. An increase in toxin levels from 0 to 4423 μg STX/g dry weight was seen over a 3-month period. A unique toxin composition (exclusively STX and GTX5) was found in a geographically isolated strain from the southwest coast of Australia [216]. A linear relationship between cell density and concentration of STXs (intracellular + extracellular) was seen. Laboratory experiments showed that growth of *A. circinalis* was depressed by addition of ammonium (0.04 mg/L) and by high levels of nitrate (28 mg/L) and these treatments were associated with increased toxin release. Levels of extracellular STXs increased with the age of the cultures. Levels of STXs in *A. circinalis* appeared to be indirectly affected by the source and concentration of nitrogen through growth. In two other *Anabaena* species, vizually *A. perturbata* var. *tumida* and *A. spiroides*, very low concentrations of GTX3 were detected [219].

In 11 lakes in Denmark STXs were detected. In seven lakes STX (46–100% of the total toxin content) was the dominant toxin and in four lakes GTX4 (40–75%) was dominant. Most of the samples were totally dominated by cyanobacteria but unambiguous identification of the toxin producing species was not possible. *A. lemmermannii* was very common in the neurotoxic lakes and was distinctly dominant in two lakes with the highest total toxin content [161].

Ferreira *et al*. [220] reported STXs in cultures of *A. flos-aquae*, isolated from the Crestuma-Lever reservoir in northern Portugal (Douro river). GTX4, GTX1 and GTX3 as well as C-toxins were present in the *A. flos-aquae* cells.

Data from a bloom in the Tabocas reservoir in Caruaru, Brazil in 1997 and 1998 revealed the identification of several SXT analogues which were produced by an isolate (ITEP-018) of *C. raciborskii*. No cylindrospermopsin was detected (by HPLC). The compounds were STX itself, GTX6, dcSTX, neoSTX and dcneoSTX [217].

Castro *et al*. [221] demonstrated that the stability of the STXs produced by a *C. raciborskii* strain derived from a freshwater reservoir in Brazil was high enough to remain active in media after 30 days at 25°C or after 50 days at 19°C even at pH around 9. At both temperatures STX, GTX2 and GTX3 are produced. No significant differences were seen in the content of the STXs in either the cells or the extracellular media. Growth of *C. raciborskii* cells achieved at 25°C (measured by OD) was three times greater than at 19°C.

Lyngbya wollei from Alabama, USA was found to produce dcSTX, dcGTX2 and dcGTX3 and six Lyngbya-wollei-toxins (LWTX1-6) [3].

3.4.4 Bioaccumulation in the environment

3.4.4.1 Bioaccumulation in aquatic organisms

D. magna are able to accumulate STXs as was demonstrated in a laboratory study. In this study, *D. magna* were exposed to *A. issatschenkoi* cells and also to lyphilized material [222]. Accumulation of STXs was also seen in the freshwater bivalves *Elliptio camoplanatus* and *Corbicula fluminea* after exposure to *A. flos-aquae* [223]. Similarly STXs from *A. circinalis* are able to accumulate in the Australian freshwater mussel species *Alathyria condola*. Pereira *et al.* [224] demonstrated the ability of the freshwater mussel *A. cygnea* to accumulate STXs at exposure to high cell densities of the toxic cyanobacterium *A. issatschenkoi* in a 14-day experiment. The toxin levels decreased to traces or undetectable after a 14-day depuration period.

3.4.4.2 Accumulation in crops/plants

See Section 2.2.4.2.

3.4.5 Biodegradation in the environment

In the dark at room temperature, STXs undergo a series of slow chemical hydrolysis reactions. The C-toxins lose the *N*-sulphocarbamoyl group to form dc-GTXs; while the dc-GTXs, GTXs and STXs slowly degrade to, as yet unidentified, nontoxic products. The half-lives for the breakdown reactions are in the order of 1–10 wk, with more than 3 months often being required for greater than 90% breakdown. Because dc-GTXs are much more toxic than C-toxins (by a factor 10–100), a solution or water body containing a mixture of C-toxins and GTXs, will actually increase in toxicity over a period of up to 3 wk, before toxicity begins to abate during the succeeding 2–3 months [3].

3.4.6 Toxicity of STXs

Since the chemical structures of the STXs derived from marine organisms and freshwater organisms are identical, the FAO Food and Nutrition Paper 80 Marine Biotoxins, Food and Agriculture Organization of the United Nations, Rome 2004 (pp. 5–49 and p. 219) can be referred to for toxicity data on STXs.

3.4.6.1 Mechanism of action

All STXs act in the same way: nervous transmission is blocked when the toxin binds to site 1 of the sodium channels, and this induces muscle paralysis [96].

3.4.6.2 Pharmacokinetics

See FAO Food and Nutrition Paper 80 Marine Biotoxins, Food and Agriculture Organization of the United Nations, Rome 2004, pp. 5–49 and p. 219.

3.4.6.3 Toxicity to laboratory animals

General. Animals show typical neurological effects including nervousness, jumping, jerking, ataxia, convulsions and paralysis. The paralysis of respiratory muscles leads to death within a few minutes [96].

Acute toxicity – oral studies. An oral LD_{50} value for STX of 263 μg/kg bw is reported by Chorus and Bartram [3].

Acute toxicity – intraperitoneal studies. STX is the most toxic of all STXs (LD_{50} i.p is 10 μg/kg of mouse) and LTWX1, 4 and 6 can be more than 165 times less toxic [96]. For neoSTX Wolf and Frank [11] cited an i.p. LD_{50} value in the mouse of 65 μg/kg bw.

Mutagenicity. No mutagenic activity has been observed when a purified lyophilized extract from neoSTX (25–100 μL of a stock with unknown concentration) was tested in the Ames *Salmonella* assay (strains TA 98, 100 and 102) with or without metabolic activation [68].

In an assay with human lymphocytes *in vitro*, a purified lyophilized extract from neoSTX caused increases in chromosomal breakages at the two highest dose-levels (0.005 and 0.0005 mL of a stock with unknown concentration). At the lowest dose-level (0.00005 mL) no effects were seen [68].

3.4.6.4 Effects on humans

Till date no reports of human poisonings due to STXs in fresh water environments are known [3].

From the FAO Food and Nutrition Paper 80 Marine Biotoxins, Food And Agriculture Organization of the United Nations, Rome 2004 (p. 219) the following conclusion for toxicity in humans is adopted: Lowest doses causing mild symptoms of PSP in man vary between 120 and 304 μg/person and lowest doses associated with severe intoxications/fatalities vary between 456 and 576 μg STX/person.

3.4.6.5 Effects on aquatic organisms

The cyanobacterium *A. issatschenko* can affect both the fitness and growth potential of juvenile *D. magna* as was demonstrated in a laboratory study. In this study, *D. magna* were exposed to cells and also to lyophilized material [222].

Zebrafish (*D. rerio*) embryos and axolotl (*A. mexicanum*) were exposed to 10, 50, 100 or 500 μg STX/L. STX at 10 μg/L and above delayed hatching in zebrafish embryos and led to malformations and mortalities at 500 μg/L. Hatching was also delayed in axolotl at 500 μg/L [92].

3.4.6.6 Effects on wild and domestic animals (n.d.)

3.4.6.7 Effects on crops/plants (n.d.)

3.4.6.8 Derivation of NOAEL and TDI

From the FAO Food and Nutrition Paper 80 Marine Biotoxins, Food And Agriculture Organization of the United Nations, Rome 2004 (p. 219) the following conclusion is adopted: Lowest doses causing mild symptoms of PSP in man vary between 120 and 304 μg/person and lowest doses associated with severe intoxications/fatalities vary between 456 and 576 μg STX/person. In order to protect more susceptible persons (children, elderly, unhealthy), usually an uncertainty factor of 10 is applied for calculation of TDI values for contaminants, based on human data. However, for PSP the calculations are complicated by the following factors: at what levels should the effects be considered as 'adverse', and what level is the actual NOAEL and LOAEL? On the other hand, since the data on PSP represent many individuals, displaying large differences in susceptibility, an uncertainty factor of 10 may not be needed (see also Aune [225]).

3.4.7 Regulations

Most countries apply a tolerance level of 80 μg STX eq/100 g mussel meat. If the consumption of shellfish is estimated to be between 100 and 300 g/meal, a human being will ingest 80–240 μg STX eq/meal.

No official guidelines, but Australia is considering a 3 μg STX eq/L of drinking water be used based upon data from marine shellfish toxicity; this toxin level could be associated with cell counts of above 20 000 cells/mL [105].

3.5 Aplysiatoxins

3.5.1 Chemical structures and properties

Aplysiatoxin and debromoaplysiatoxin are phenolic bislactones (see Fig. 8) [54, 226].

3.5.2 Methods of analysis

3.5.2.1 Bioassays *in vivo*

Mouse bioassay. The mouse bioassay is used to detect the toxicity of suspected bloom material and detects all cyanotoxins together. The toxicity is tested by intraperitoneal injection to male Swiss Albino mice. About 0.1–1.0 mL of a lysate of cyanobacteria prepared either by sonication or by freeze-thawing of a cell suspension which has been sterilized by membrane-ultrafiltration. Samples can be suspended in water or physiological saline solution which is preferred if the volume to be injected is 0.5 mL or greater. Mice should be observed for 24 h and then killed. The observation period must be extended to 7 days where cylindrospermopsin is suspected. During the observation period clinical symptoms and mortality are recorded. The mice that have died are further examined for gross macroscopical changes in organs and tissues. The observed symptoms and the results of the postmortem examination are used to determine which type of cyanotoxin is present. When more than one type of cyanotoxin is present, the more rapid acting toxin may mask other symptoms (as cited in Chorus and Bartram [3]).

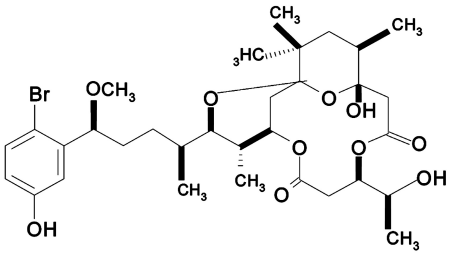


Figure 8. Chemical structure of aplysiatoxin.

Toxicity is expressed as LD_{50} mg cell dry weight *per* kg mouse body weight. Values are classified as follows:

>1000	Nontoxic
500–1000	Low toxicity
100–500	Medium toxicity
<100	High toxicity

This assay has been used primarily to determine the toxicity of bloom material, and, from toxic responses, the identity of the class of toxin can be inferred. It has generally been used in a qualitative manner to determine a bloom as 'toxic' or 'nontoxic'. In some countries nontoxic limits may be at least 2000 mg cell dry weight *per* kg mouse body weight.

The mouse bioassay plays an important role as screening tool and provides a measure of total toxicity (response), but the assay is not specific or sensitive. The peptide hepatotoxins (microcystins and nodularins) generally cause death within 4 h with symptoms of a liver engorged with blood. The neurotoxins (anatoxin-a, anatoxin-a(S) and STXs) can cause death more quickly (within 15 min) with neuromuscular symptoms and typically with no evidence of tissue damage on postmortem examination. This assay can potentially be calibrated against a specific toxin such as microcystin-LR, and produced a result in terms of microcystin-LR toxicity equivalents, but it does not have the detectability or precision to be applicable to water samples. Until now [3] no single method is available to replace the mouse bioassay for the detection of all cyanotoxins using a single assay. A license is needed to perform this test and in some countries the use of this test is not permitted [3, 10].

3.5.3 Source organism(s) and habitat/occurrence

3.5.3.1 Source organism(s)

Aplysiatoxins were first reported as toxins of the sea hare *Stylocheilus longicauda* and subsequently as the constituents of the benthic marine blue-green alga *Lyngbia majuscula*, a diet of the sea hare [227]. Debromoaplysiatoxin was also isolated from the blue-green algae *Schizotrix calcicola* and *O. nigroviridis* [3].

3.5.3.2 Habitat/occurrence (n.d.)

3.5.4 Bioaccumulation in the environment (n.d.)

3.5.5 Biodegradation in the environment (n.d.)

3.5.6 Toxicity of aplysiatoxins

3.5.6.1 Mechanism of action

Aplysiatoxin and debromoaplysiatoxin are potent tumour promoters and protein kinase C activators [3].

3.5.6.2 Pharmacokinetics (n.d.)

3.5.6.3 Toxicity to laboratory animals

Acute toxicity – oral studies. At oral administration to mice (three animals; age 3 wk) of 3000 μg aplysiatoxin/kg the same effects on the small intestine were seen as after i.p. administration of 57.1 μg/kg, but less severe [228] (and see also [228] under acute toxicity-intraperitoneal studies below).

Acute toxicity – intraperitoneal studies. For acute effects in the mouse after i.p. injection [229] of aplysiatoxin and debromoaplysiatoxin isolated from *Gracilaria coronopifolia* see Table 3.

At an i.p. dose of 107.1 μg/kg bw to six, 3-wk old, male mice, 3/6 animals died. In 5-wk-old mice 117.9 μg/kg bw caused death of one out of two animals and the other one recovered. Target site of a lethal dose was the whole small intestine where the toxin caused bleeding, from capillaries resulting in haemorrhagic shock [228]. Dilatation of lymphatic vessels in the villi and congestion of capillaries in the lamina propria were the initial changes which resulted in bleeding and death [230]. At 58.9 and 88.4 μg/kg bw of two male mice each (age 5 wk), no mortality was seen and the animals recovered within 24 h. With a sublethal dose of 57.1 μg/kg to ten male mice (age 3 wk), diarrhoea appeared within 4.5 h and continued for about 4 h intermittently. The site of diarrhoea was the large intestine, where the submucosa first accumulated fluid from edema. Then the fluid moved into the lamina propria, the surface epithelial cells were broken and the fluid flowed into the lumen. The cecum was the main target of the diarrhoea. In this study, the lowest dose of 35.7 μg/kg bw given i.p. to 3-wk-old male mice, caused already pathological changes in the cecum [228, 230].

Acute toxicity – intravenous studies. At i.v. doses of 100–200 μg aplysiatoxin/kg bw to mice, mortality was seen within 15 min, but lower doses of 50–75 μg/kg bw did not induce any changes. With a lethal dose, mice went into shock just after i.v. injection, then showed some sign of recovery, but finally lied down again after 10 min and died

Table 3. Acute i.p. toxicity in mice

Dose (μg/mouse)[a)]	Effects of aplysiatoxin	Effects of debromoaplysiatoxin
10		Died within 12 h
5	Died within 12 h	Diarrhoea; recovery within 12 h
1	Diarrhoea; recovery within 12 h	Diarrhoea; recovery within 5 h
0.2	Diarrhoea; recovery within 3 h	Diarrhoea; recovery within 3 h
0.04	Diarrhoea; reovery within 1.5 h	Diarrhoea; recovery within 3 h

a) Approximately 2–3 mice *per* dose.

gradually. Bleeding was observed only in the lungs, not in the small intestine, suggesting a different target organ on the i.v. route. The lung had bleeding around the distended pulmonary artery, accompanied by exudation of fibrin and fluid. The lumen of the pulmonary artery contained a lot of fibrin which flowed out from the cleavages in the arterial wall. Aplysiatoxin-stimulated fibrinogen resulted in inhibition of blood circulation by embolisms of fibrin deposition, and the toxin also weakened vessels by disturbances such as distension and cleavage [230].

Irritation and sensitization studies. Aplysiatoxin is known as strong skin irritant (as cited by Ito and Nagai [228]).

3.5.6.4 Effects on humans

In Hawaii, some algae including the red alga *Gracilaria coronopifolia* are often eaten by local residents. In September 1994, poisonings due to this red alga broke out. Two major toxins were isolated, namely aplysiatoxin and debromoaplysiatoxin. However, blue-green algal parasitism on the surface of the toxic *G. coronopifolia* was observed. Thus, parasitizing blue-green algae might be the true origin of the toxins. The characteristic symptoms of poisoning were diarrhoea and a burning sensation of mouth and throat [229].

Samples of *L. majuscula* from Okinawa, Japan, caused rashes and blistering of human skin. The extract of toxic compounds was later shown to be a mixture of debromoaplysiatoxin and aplysiatoxin (cited from Osborne *et al*. [226]).

3.5.6.5 Effects on aquatic organisms (n.d.)

3.5.6.6 Effects on wild and domestic animals (n.d.)

3.5.6.7 Effects on crops/plants (n.d.)

3.5.6.8 Derivation of NOAEL and TDI

There are insufficient data to derive an NOAEL or LOAEL and calculate a TDI for the aplysiatoxins.

3.5.7 Regulations

There are no regulations known for the aplysiatoxins.

3.6 Lyngbyatoxins

3.6.1 Chemical structures and properties

The structure of lyngbyatoxin-a (see Fig. 9) is identical to an isomer of teleocidin A found in the mycelia of several strains of *Streptomyces* [226].

3.6.2 Methods of analysis

3.6.2.1 Bioassays *in vivo*

Mouse bioassay. See Section 3.5.2.1, Mouse Bioassay

3.6.3 Source organism(s) and habitat/occurrence

3.6.3.1 Source organism(s)

Lyngbyatoxin-a, -b and -c were determined from a shallow-water variety of *Lyngbya majuscula*. *L. majuscula* is a benthic cyanobacterium that grows loosely attached to seagrass, sand and rocky outcrops. It grows in fine strands 10–30 cm in length. It usually resembles a clump of drab, olive-coloured hairy matted mass composed of filamentous threads, although colour can vary from red to white to brown [226].

3.6.3.2 Habitat/occurrence

L. majuscula has been found in estuarine and coastal waters in both tropical and subtropical regions. *L. majuscula* occurs in abundance from the intertidal zone to a depth of 30 m. The lyngbyatoxins were determined from a shallow-water variety of *L. majuscula* collected at Kahala beach, Oahu, HI (cited from Osborne *et al*. [226]). *L. majuscula* sometimes grows epiphytically on edible algae, such as *Acanthophora spicifera* which is eaten in Indonesia and in the Philippines [3]. Countries where *L. majuscula* has been recorded are: Australia, Curacao, Fiji, France, Granada, Guam, Hawai, Madagascar, Marshall Islands, Mozambique, Okinawa, Palau, Palmyra Island, Papua New Guinea,

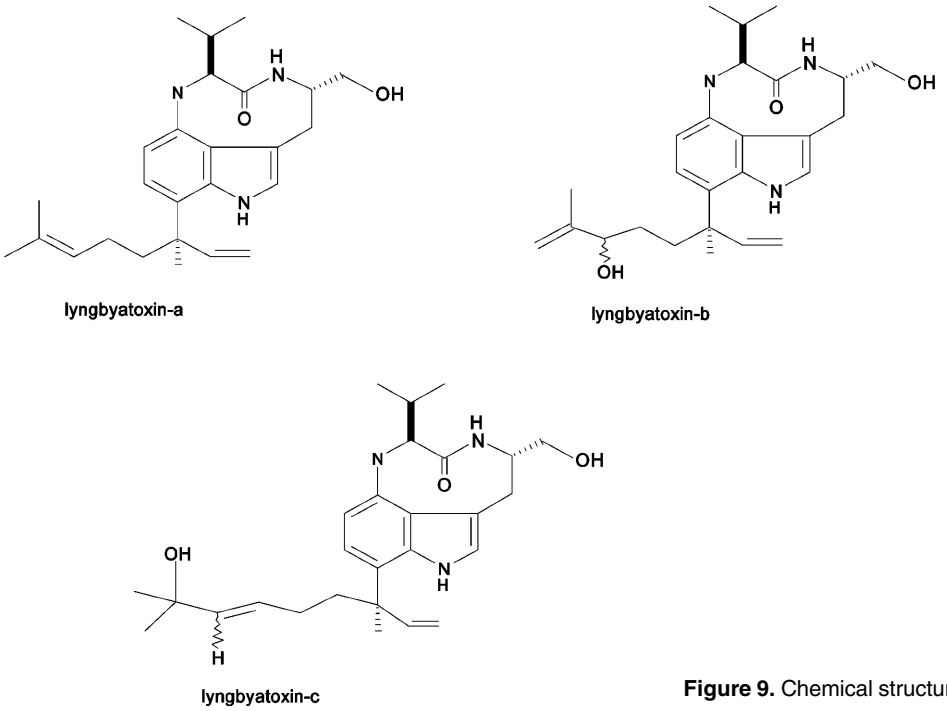


Figure 9. Chemical structures of lyngbyatoxin-a, -b and -c.

Philippines, Puerto Rico, Sri Lanka, Venezuela and Virgin Islands [226].

3.6.4 Bioaccumulation in the environment (n.d.)

3.6.5 Biodegradation in the environment (n.d.)

3.6.6 Toxicity of lyngbyatoxin

3.6.6.1 Mechanism of action (n.d.)

3.6.6.2 Pharmacokinetics

Dermal absorption. Lyngbyatoxin-a is slightly lipophilic and penetration through guinea pig and human skin was found to be 23 and 26% of the dose, respectively, over 1 h [226].

3.6.6.3 Toxicity to laboratory animals

As *L. majuscula* may produce several toxins [226], studies with extracts of *L. majuscula* which are not further identified, are not included.

Acute toxicity – oral studies. Mature mice received a single oral dose by gavage of 600 or 1000 μg lyngbyatoxin-a/kg bw and were observed up to 20 h. Young mice received a single oral dose of 1000 μg/kg bw and pathological changes were followed from 15 min to 5 wk after dosing. At 1000 μg/kg bw neither the young nor the mature mice appeared severely damaged. In mature mice at 600 μg/kg bw, enhanced mucus secretion and light erosion were seen at autopsy after 10 min both in stomach and small intestine. Autopsy 1 h after dosing revealed circular lined tears in the stomach. The small intestine was in the recovery process at 2 h and thereafter. Young mice at 1000 μg/kg bw did not move around for 20 min, they recovered without any changes of behaviour or body condition during the observation period of 5 wk. Pathology showed severe mucus secretion and injuries within 60 min in the intestine and within 24 h in the stomach followed by increased inflammatory cells. In the lungs edema was seen within 15 min followed by infiltration of inflammatory cells within 30 min. These injuries in lung, stomach and small intestine took a few weeks for recovery [231].

Acute toxicity – intraperitoneal studies. The i.p. lethal dose in immature mice (age 3 wk) for lyngbyatoxin-a is 250 μg/kg bw. Death occurred within 3.5 h. Most severely damaged capillaries of villi in the small intestine were observed. Immature mice were more sensitive than mature ones and died of bleeding from the small intestines. Mature mice given i.p. 300 μg/kg bw became inactive for 2 h but recovered thereafter. Autopsy of these mice 1, 2 or 3.5 h after injection showed dilation of the stomach and small bleeding in the lung. With sublethal doses of 100–150 μg/kg bw mature mice showed light diarrhoea at 2–4 h after injection. Light erosion in the stomach was observed at 5–8 h after injection and active mucus secretion continued from 1 to 24 h. The small intestine showed active mucus secretion after 2 h, but injuries were not observed [231].

Irritation and sensitization studies. A topical application of 0.011 nmol lyngbyatoxin-a at the ears of mice caused a reddening in 50% of the animals [226].

Carcinogenicity. Lyngbyatoxin-a has skin tumour promoting activity similar to the well-known tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [3]. Lyngbyatoxin-b and -c have different activities from lyngbyatoxin-a. The 50% inhibition for specific binding of TPA occurred at ED_{50} 2.2 and 0.2 μM for lyngbyatoxin-b and -c, respectively, corresponding to 1/200th and 1/20th the activity of lyngbyatoxin-a (cited from Osborne *et al*. [226]).

3.6.6.4 Effects on humans

Lyngbyatoxin-a found in a shallow water strain of *L. majuscula*, has caused dermatitis and severe oral and gastrointestinal inflammation in men [3].

Meat of the marine turtle *Chelonia mydas* was involved in a fatal intoxication. Lyngbyatoxin-a was identified as the causative toxin. As turtles feed on sea grass, contaminated by blue-green algae belonging to the genus *Lyngbya*, this blue-green algae were deduced to be the source of the toxin [232].

3.6.6.5 Effects on aquatic organisms (n.d.)

3.5.6.6 Effects on wild and domestic animals (n.d.)

3.6.6.7 Effects on crops/plants (n.d.)

3.6.6.8 Derivation of NOAEL and TDI

There are insufficient data to derive an NOAEL or LOAEL and calculate a TDI for lyngbyatoxins.

3.6.7 Regulations

No regulations are known for lyngbyatoxins.

This review was prepared within Project 310301 'Natural Toxins' of the National Institute for Public Health and the Environment, Bilthoven, the Netherlands. The work was carried out on behalf and for account of the Food and Consumer Product Safety Authority, the Netherlands.

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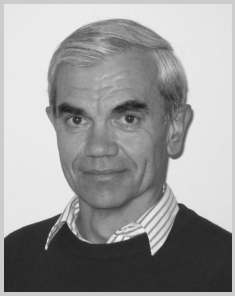

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