Annex to the SKLM opinion "Microcystins in Algae Products Used as a Food Supplement"

Overview and evaluation of the literature on microcystins

Author: Barbara Engeli*

** Swiss Federal Office of Public Health, Food Toxicology Section, Zürich*

The following Annex provides background information for the toxicological evaluation of microcystins given in the SKLM opinion "*Microcystins in Algae Products Used as a Food Supplement*" from 28 September 2005 (German version, English version was agreed on 1st June 2007). This Annex contains data on the metabolism and toxicology of microcystin-LR, a discussion of the analytical and biochemical aspects, an exposure assessment and an extensive bibliography. The collected data is based on the WHO report published in 1999 and on relevant publications from 1997-2004. Studies carried out with *Microcystis* extracts are difficult to evaluate owing to their differing compositions and the resulting ambiguities with respect to consumed quantities, which are only specified in exceptional cases.

Contents

1 BIOCHEMICAL ASPECTS

1.1 STRUCTURAL FEATURES OF MICROCYSTINS

Microcystins are cyclic heptapeptides that are synthesised by *Microcystis aeruginosa*, a blue-green algae (cyanobacterium). More than 60 microcystin congeners are now known. Their general structure is cyclo-(D-Ala[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-Glu[6]-Mdha[7]), see Fig. 1. Microcystins contain the unusual amino acid ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), where MeAsp stands for *erythro*-β-methyl aspartate, Mdha for *N*-methyl-dehydroalanine, X and Z are variable amino acids, see Tab. 1. The molecular weight of microcystins is between 800 and 1100 Da. Most congeners are hydrophilic (WHO, 1999).

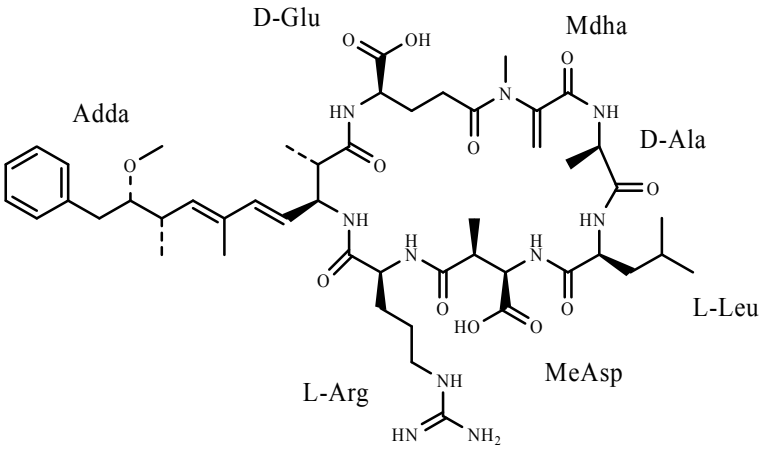

Fig. 1: Structure of microcystins

Tab. 1. Microcystins

Microcystin congener	Amino acid X[2]	Amino acid Z[4]	CAS Number	Molecular weight [Da]
-LA	L-Leu	L-Ala		
-LR	L-Leu	L-Arg	101043-37-2	995.17
-RR	L-Arg	L-Arg		
-YA	L-Tyr	L-Ala		
-YM	L-Tyr	L-Met		
-YR	L-Tyr	L-Arg		

1.2 ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION

Studies on the oral administration in mice showed that microcystin-LR is primarily absorbed in the small bowel and is systemically distributed (Ito *et al*, 2000). Microcystin-LR is taken up by bile acid transporters (multispecific organic ion transport system) that are present in the small bowel and in hepatocytes in animals and humans (WHO, 1999, Fischer *et al*, 1999). It is also possible that there are systems that transport microcystins across the blood-brain barrier (Fischer *et al*, 2005).

After a single i.v. dose of 0.035 mg ^{3}H-microcystin-LR/kg body weight (bw) to mice, the substance was eliminated from the plasma in two phases with a first half-life of 0.8 min and a second of 6.9 min. One hour to 6 days after administration, approx. 70% of the dose was localised in the liver. Within 6 days after administration, 9.2% of the dose had been excreted via urine and 14.5% via faeces. Six to twelve hours after administration, 60% of the radioactivity in the urine and faeces was attributed to non-metabolised microcystins. Metabolites were detected in urine, faeces and in liver cytosol; however, they were not identified more accurately (Robinson *et al*, 1991). After i.p. administration of radioactively marked microcystins in mice and rats, approx. 70% of the toxin was localised in the liver (WHO, 1999, Brooks & Codd, 1987).

After a single i.p. injection of microcystin-LR (0.02-0.1 mg/kg bw) into mice, it was detected in liver extracts only in the bound form (adducts of protein phosphatase 1 and 2A) and not in the free form (detection limit of the HPLC method: 0.15 µg) (Yoshida *et al*, 1998).

In a rat study, animals were given 2.5 or 5 mg microcystin-LR/kg bw administered directly into the lumen of an *in situ* isolated segment of the ileum or the jejunum. They were sacrificed after 6 hours and then examined for relative enlargement of the liver. In all animals treated with microcystin, the relative weight of the liver was higher, the liver was discoloured to a dark red and there were microscopic lesions. Doses administered into the ileum led to dose-dependent effects that were significantly more pronounced than those resulting from administration into the jejunum. The authors concluded that microcystin-LR is primarily absorbed in the ileum in rats (Dahlem *et al*, 1989).

According to the WHO, no pharmacokinetic studies have been carried out with orally administered microcystin (WHO, 1999). No data is available, even in more recent literature, and there is a lack of information on oral bioavailability, in particular. However, there are indications that oral doses of microcystin-LR are absorbed to a lesser degree. A comparison

of the LD_{50} values shows that orally administered microcystin-LR is approx. 100 times less toxic than when administered parenterally (see Chapter 2.1 Acute toxicity). Furthermore, mice only exhibited hepatic neoplasia after i.p. administration of microcystin-LR, but not after oral administration of a four-fold dose (see Chapter 2.3 Chronic toxicity/carcinogenicity, Ito *et al*, 1997b).

1.3 BIOTRANSFORMATION

After i.p. administration of 0.01-0.02 mg microcystin-RR/mouse or 0.004 mg microcystin-LR/rat, several metabolites were found in the liver. In both animal species, the Mdha moiety was conjugated with glutathione or cysteine, and one of the glutathione conjugates also had a sulphate group inserted on the ADDA amino acid (WHO, 1999 and Kondo *et al*, 1996).

1.4 EFFECTS ON ENZYMES AND OTHER CELL COMPONENTS

Effects on enzymes

In liver cytosol, the N-methyl-dehydroalanine (Mdha) group of the microcystin binds covalently to Cys-273 or Cys-266 of the Ser/Thr protein phosphatases 1 and 2A (WHO, 1999, Matsushima *et al*, 1990, Runnegar *et al*, 1995, MacKintosh *et al*, 1990, Yoshida *et al*, 1998, Gehringer 2004, MacKintosh *et al*, 1995). Binding to and inhibition of the phosphatases is considered to be the basic mechanism to explain the toxicity of microcystins (Hitzfeld *et al*, 2000). Inhibition of protein phosphatases 1 and 2A has been found *in vitro* (Honkanen *et al*, 1994, MacKintosh *et al*, 1990) and *in vivo* in mice (Runnegar *et al*, 1993, Runnegar *et al*, 1995) and rats (Solter *et al*, 1998). The IC_{50} value of phosphatase inhibition by microcystin-LR was 0.1-1.0 nM (Runnegar *et al*, 1995) or 0.05 nM (Tsuji *et al*, 1997). Microcystin-LR and microcystin-LA also showed *in vitro* inhibition of Ser/Thr protein phosphatase 3 isolated from cattle brain (Honkanen *et al*, 1994). Synthetic microcystin analogues consisting of the ADDA side chain and 1-3 further amino acids, also showed *in vitro* inhibition of protein phosphatases 1 and 2A; however, to a significantly lower degree than microcystin (Gulledge *et al*, 2003a Gulledge *et al*, 2003b).

After i.p. administration of a single lethal dose (0.1 mg/kg bw) or sublethal dose (0.012, 0.023 and 0.045 mg/kg bw) to Balb/c mice, microcystin-LR was found to be localised to equal parts in the cytosol and in hepatocyte nuclei. Protein phosphatases 1 and 2A only exhibited inhibition in the extract of hepatocyte nuclei at the lethal dose. (*Comment:* no information is given on protein phosphatase inhibition in the cytoplasm). The administration of sub-lethal

doses led to hyperphosphorylation of the tumour suppressor protein p53 in hepatocytes. This protein plays an important role in controlling apoptosis and cell proliferation caused by oxidative stress and DNA damage (Guzman *et al*, 2003).

Three protein-microcystin adducts were identified in cell lysates from Mel-7 (human melanoma cells) and J3 cells (interspecies hybridoma from chipmunk hepatocytes and rat hepatoma H4TG) that had been treated with microcystin-LR. Microcystin-LR was bound to the catalytic subunits of protein phosphatases 1 and 2A and to the beta-subunit of ATP synthase (Mikhailov *et al*, 2003).

2 TOXICOLOGY

2.1 ACUTE TOXICITY

The LD_{50} value of microcystin-LR in mice was 5-10.9 mg/kg bw for oral administration and 0.025-0.15 mg/kg bw for i.p. administration. The i.p. LD_{50} values of other microcystins (microcystin-LA, -YR, -YM) were of the same order of magnitude or, in the case of microcystin-RR, slightly higher than those for microcystin-LR (WHO 1999), see Tab. 2.

The oral LD_{50} value for rats was greater than 5 mg microcystin-LR/kg bw (mortality rate: 1/5 males for 5 mg/kg bw) (Fawell *et al*, 1999).

Tab. 2. Acute toxicity of microcystins

Microcystin congener	**Animal species**	**Administration route**	**LD_{50}** [mg/kg bw]	**References**
-LR	mouse	oral	5-10.9	WHO, 1999
-LR	mouse	i.p.	0.025-0.15	WHO, 1999
-LR	mouse	i.p.	0.043	Gupta *et al*, 2003
-LR	mouse	i.p.	0.05	Tsuji *et al*, 1995
-LR	mouse (male)	i.v.	0.04	Kondo *et al*, 1992
-LR	rat	oral	>5	Fawell *et al*, 1999
-LR	rat (male)	i.p.	0.05	Towner *et al*, 2002
-LR	rat (fed)	i.p.	0.122	Miura *et al*, 1991
-LR	rat (starved)	i.p.	0.072	Miura *et al*, 1991
-RR	mouse	i.p.	0.235	Gupta *et al*, 2003
-YR	mouse	i.p.	0.11	Gupta *et al*, 2003
-YR	mouse (male)	i.v.	0.09	Kondo *et al*, 1992
9 congeners	mouse	i.p.	0.1-0.75	Stotts *et al*, 1993

(not –LR)				
Purified toxin from *Microcystis aeruginosa*	mouse	i.p.	0.125	Brooks & Codd, 1987
Toxins from *Microcystis aeruginosa* extract	mouse	i.p.	0.098	Falconer et. al., 1988, 1991 and 1994

Rodents exhibited primary acute hepatotoxic effects after i.p. and i.v. administration. The liver was seriously injured due to damaged hepatocytes and sinusoids; the weight of the liver had increased as a result of internal bleeding. Microcystin also affected the kidneys, lungs and bowel (for more information, please see the studies described below). Microcystin-LR was more toxic than microcystin-YR and -RR (WHO, 1999, Gupta *et al*, 2003).

A single i.p. dose of 0.058-0.101 mg microcystin-LR/kg bw was lethal in BALB/c mice. However, this paper does not provide information on the mortalities in the individual dosage groups (Yoshida *et al*, 1998).

A single oral dose of 0.5 mg microcystin-LR/kg bw was given to 5- and 32-week-old mice. Microscopically visible changes of the liver were found in 62% of the older mice after 2-19 hours. None of the younger mice showed these effects. The authors attributed this age-dependent toxicity to the degeneration of the intestinal mucosa cells and the small intestine epithelium, which increases with age. In the older animals, this degeneration could led to increased uptake of microcystin in the bowel and to a correspondingly enhanced liver toxicity (Ito *et al*, 1997a).

Intranasal administration of microcystin-LR in mice led to necrosis of the nasal epithelium. The intranasal LD_{50} value was of the same magnitude as that of i.p. administration (WHO, 1999).

The i.p. LD_{50} value of *Microcystis aeruginosa* extract in rats was 15.8 mg of lyophilised algae cells/kg bw (Rao *et al*, 1995). The animals died within 2-6 hours after administration of the single or double LD_{50} dose. Before exitus, they showed signs of sedation, movement disorders, spasms, spreading of the rear extremities and severe cramps. The liver was enlarged and filled with blood (Rao *et al*, 1995, Rao *et al*, 1998).

2.2 SUBACUTE AND SUBCHRONIC TOXICITY

Mice

Groups of 15 male and 15 female mice were administered doses of 0, 0.04, 0.2 or 1 mg microcystin-LR/kg bw/day by gavage for 13 weeks, and the animals were examined with respect to their body weight, food intake, as well as effects on the eyes and haematology. At the end of the treatment, the liver, kidney and lungs were examined histopathologically.

The most sensitive end point was found to be histopathological changes in the liver at dosages from 0.2 mg/kg bw/day. All animals given the highest dosages of 1 mg/kg bw/day and some of the animals given dosages of 0.2 mg/kg bw/day exhibited chronic inflammation of the liver, focal degeneration of hepatocytes in the hepatic lobuli and haemosiderin deposits in the liver. The body weight increase in male mice given 0.2 mg/kg bw/day was significantly reduced and at 0.04 and 1 mg/kg bw/day tendentiously reduced. In female mice, it was significantly increased for dosages of 0.2 mg/kg bw/day compared to the control group. Male mice given dosages from 0.04 mg/kg bw/day and female mice at the highest dosage exhibited significantly increased serum activities of alanine and aspartate aminotransferase. At the highest dosage, the alkaline phosphatase activity was increased in both genders and the levels of albumin and total protein in the serum of male animals was slightly, but significantly, reduced. The authors are uncertain of the relevance of the blood chemistry results; however, they do state that there was a clear NOAEL of 0.04 mg microcystin-LR/kg bw/day with respect to hepatic changes (Fawell *et al*, 1999).

Mice (a total of 430 animals) were given *Microcystis aeruginosa* extract at a dilution ratio of 0, 1/16, 1/8, or 1/4 for up to 1 year in their drinking water. Seven interim kills were carried out (5 animals/gender/dosage group). Animals who died prematurely were replaced by animals from a correspondingly dosed reserve pool (110 animals) (main study). Owing to the low mortality rate, a 10-week study was carried out with a control group and two dosage groups (1/2 diluted and undiluted extract) with 2 interim kills (a total of 90 animals). To determine the toxin content in the extract, Falconer *et al.* experimentally determined the i.p. LD_{50} of the extract to be 1.7 mg extract/kg bw, which equates to a toxin content of 56.6 µg toxin/ml extract (i.p. LD_{50}: 98 µg toxin/kg bw, see Falconer et al., 1994).

Results of the 1-year study (0, 1/16, 1/8, 1/4 dilution, assumed to be equivalent to 0, 0.75, 1.5, 3 mg toxin/kg bw/day*): The mortality rate was significantly increased in males at 1/4 dilution and in females at 1/16 and 1/4. No neoplasia or histopathological changes were found in the liver.

Results of the 10-week study (0, 1/2, undiluted extract, assumed to be equivalent to 0, 6, 12 mg toxin/kg bw/day*): There was a dose-dependent increase in the mortality rate from 1/2 dilution, and the animals exhibited histopathological changes in the liver (hepatocyte damage, necrosis, leucocyte infiltration, fibrosis) (Falconer *et al*, 1988).

In a review published by the WHO (1999), this study is summarised as follows: A group of mice were given 5 doses of *Microcystis aeruginosa* extract in concentrations equivalent to 0.75-12 mg microcystin-YM/kg bw/day in their drinking water for a period of up to one year. At the higher concentrations there was increased death, increased bronchopneumonia (which was endemic in the colony), and chronic liver injury. There was no evidence for an increased incidence of hepatic tumours. It was not possible to derive an unambiguous NOAEL (WHO 1999, original literature Falconer *et al*, 1988).

**Comment on dosage data:* It must be assumed that the highest microcystin-YM dose stated in the WHO article of 12 mg/kg bw/day refers to undiluted extract (own calculations based on a toxin concentration in the extract of 56.6 µg toxin/ml extract, bw mouse 20 g, 5 ml fluid/day → 283 µg toxin/animal day→ ~14 mg microcystin-YM/kg bw) and thus to the highest dosage of the 10-week study (not the 1-year study).

Rats

An i.p. dosage of 0, 0.016, 0.032 or 0.048 mg microcystin-LR/kg bw/day was administered over a period of 28 days to groups of 5 male Sprague-Dawley rats. The microcystin-LR concentrations in the liver cytosol exhibited dose-dependent increases from a dosage of 0.016 mg/kg bw/day. Immunohistological staining showed that the microcystin-LR is mainly localised in zone 3 hepatocytes. Histopathological investigations of the liver indicated apoptosis and cytosolic vacuolisation of zone 3 hepatocytes from 0.032 mg/kg bw/day. Furthermore, at this dosage and higher, the serum activities of sorbitol dehydrogenase and alkaline phosphatase were dose-dependent and significantly increased, the concentration of serum albumin significantly reduced and the activities of protein phosphatases 1 and 2A in the liver were dose-dependent and significantly reduced. The activity of protein phosphatase 2A was reduced to a greater degree than that of protein phosphatase 1. At 0.048 mg/kg bw/day, the increase in body weight was significantly reduced and there were significant increases in the serum activities of aspartate aminotransferase and gamma-glutamyl

transferase and in the bile acid concentration. The mitotic index remained unaffected in all animals (Solter *et al*, 1998).

An i.p. dosage of 0, 0.016, 0.032 or 0.048 mg microcystin-LR/kg bw/day was administered over a period of 28 days to groups of 4 male Sprague-Dawley rats. The activity, concentration and mRNA concentration of alanine aminotransferase (ALT) in the liver exhibited a dose-dependent decrease from 0.016 mg/kg bw/day. The effects were already significantly at the lowest dosage, except for the mRNA concentration, which was significantly reduced only at the highest dosage. The serum activity of ALT was tendentiously but not significantly increased for the highest dosage group. The authors concluded that measurement of the ALT activity in the serum is not a very sensitive marker for liver damage (Solter *et al*, 2000).

An i.p. dosage of 0, 0.016, 0.032 or 0.048 mg microcystin-LR/kg bw/day was administered over a period of 28 days to groups of 3 male Sprague-Dawley rats. At dosages of 0.032 mg/kg bw and 0.048 mg/kg bw/day, dose-dependent inflammation of the liver with infiltration of lymphocytes, macrophages and neutrophiles, centrilobular fibrosis, apoptosis and adipose liver were observed. The ALT activity in the liver was significantly lowered from the middle dosage. The malondialdehyde concentration (marker for lipid peroxidation) in the liver showed significant and dose-dependent increases at dosages from 0.032 mg/kg bw/day. In the highest dosage group, malondialdehyde concentration was increased by approximately four times compared to the control group. Liver sections from rats treated with microcystin-LR were more sensitive to the cytotoxic effect of *tert*-butyl hydroperoxide (ROS-generator). The authors concluded from the histological and biochemical results that oxidative stress may play a significant role in the chronic toxicity of microcystin-LR (Guzman & Solter, 1999).

The effects of microcystin-LR and -YR on the kidneys was studied in detail. Groups of 5 male Wistar rates were given i.p. doses of 0.01 mg microcystin-LR/kg bw or 0.01 mg microcystin-YR/kg bw every second day over a period of 8 months. There were histopathological changes in the kidneys that were more pronounced for animals treated with microcystin-LR than with microcystin-YR. The kidneys showed collapsed glomeruli with thickened basal membranes, enlarged tubules with eosinophilic deposits and accumulations of cytoplasm and actine filaments in epithelial tubule cells (Milutinovic *et al*, 2003).

Pigs

Groups of 5 male pigs were given *Microcystis aeruginosa* extract in their drinking water over a period of 44 days. Doses of 0.28, 0.8 and 1.31 mg microcystin/kg bw/day were estimated from a mouse i.p. bioassay. The extract contained at least 9 different microcystins, with microcystin-YR as the probable major constituent. Animals treated with at least 0.8 mg/kg bw/day exhibited dose-dependent histopathological changes of the liver (cytoplasmatic degeneration, necrosis of individual hepatocytes, periacinous degeneration, blocked bile ducts and Kupffer cell proliferation); only one animal was affected at the lowest dose. The albumin concentration in the serum was decreased from 0.8 mg/kg bw/day; the activities of gamma-glutamyl transpeptidase and alkaline phosphatase were increased and the bilirubin concentration was elevated. A LOAEL of 0.28 mg microcystin/kg bw/day was established by the WHO. On the basis of the authors' estimates, this corresponds to a LOAEL of 0.1 mg microcystin-LR equivalents/kg bw/day. (The authors determined the toxicity of their extract by means of a mouse i.p. bioassay, HPLC and phosphatase inhibition; an i.p. LD_{50} of 0.1 mg/kg bw was used in the calculations) (WHO 1999 and Falconer *et al*, 1994).

Cows

Four yearling beef cows were given *Microcystis aeruginosa* (10^8 cells/L) in their drinking water for 28 days. No effects were found with respect to drinking water, feed consumption and blood plasma parameters (gamma-glutamyl transferase, glyceraldehyde dehydrogenase, aspartate aminotransferase, bilirubin). No free microcystin was found in the plasma (HPLC, ELISA), and there was no free or bound microcystin in the liver (HPLC and GC-MS). In contrast, an ELISA detected 0.92 mg microcystin-LR/kg liver. The authors are sceptical about this result because HPLC and GC-MS did not detect any microcystin (the detection limit is more than 1000 times lower than the ELISA results). The value appears to be very high because, even supposing that all the administered microcystin is absorbed and accumulated in the liver, the authors expect this to lead to maximum value of approx. 3 mg microcystin/kg liver (Orr *et al*, 2003).

2.3 CHRONIC TOXICITY/CARCINOGENICITY

No data are available from long-term studies on the chronic toxicity or carcinogenicity.

The IARC does not intend to evaluate microcystin-LR because there is insufficient animal data (internet IARC website).

Mice

A group of 13 ICR mice given a total of 100 i.p. doses of 0.02 mg microcystin-LR/kg bw over a period of 28 weeks (5 times per week) all showed neoplastic nodules of up to 5 mm diameter in the liver (*Comment:* the number of doses is not clear: 5x28=140, not 100). The neoplasias occurred without previous administration of a tumour initiator and were still present 2 months after the last dose (5 animals were examined directly after the end of the treatment period ended and 8 animals 2 months later). Microcystin-LR was primarily found as a cysteine conjugate in the liver. After administration of a total of 100 doses of 0.08 mg microcystin-LR/kg bw over a period of 28 weeks via a gavage, the mice did not exhibit any nodules or any liver damage (10, 5 and 7 animals were killed after 80 doses, 100 doses and 2 months after the last dose, respectively). (Ito *et al*, 1997b). *Comment:* There are no data on the tumour incidence in the control group (3 animals). This experiment indicates that microcystin may be only poorly absorbed by the gastrointestinal tract.

Groups of 30 male C3H/HeN mouse were given i.p. doses of 0, 0.0125 and 0.025 mg microcystin-LR/kg bw once a week over a period of 14 months without previous administration of a tumour initiator. The incidence of liver adenomas and adenocarcinomas showed a dose-dependent increase (2+1 of 25, 5+5 of 22, 2+15 of 22 animals*). Treatment with [D-Asp^3-(E)-Dhb^7]microcystin-LR (Dh-LR) (same experimental set-up) did not increase the incidence of liver tumours. Two and 14 months after the start of the trial, the 8-hydroxy-2-deoxy-guanosine (8-OHdG) level was determined in the liver and plasma. According to the authors, the synthesis of 8-OHdG agreed with the tumour incidence 2 months after the start of treatment for both substances (Sano *et al*, 2004). **Comment*: this study has only a limited meaningfulness because there is no information given on the significance of the effects and the cause of death of 5 control animals and 8 animals from each of the two microcystin-LR dosage groups who died before the end of the 14-month treatment period. It appears that these animals were not taken into account in the tumour incidence data.

A group of 20 female Swiss albino mice were given a single 0.5 mg dermal dose of 7,12-dimethylbenzanthracene (DMBA) followed a week later by a dose of *Microcystis aeruginosa* extract (according to WHO, 80 mg microcystin-YM per litre, which corresponds to 50 mg microcystin-LR equivalents/L) in their drinking water. Water and croton oil (tumour

promotor) were used as negative and positive controls, respectively. Fifty-two days after administration of DMBA, animals who had been given *Microcystis* extract exhibited a 1.6 times higher incidence and a 7 times higher average weight of cutaneous papillomas (benign tumours) (Falconer, 1991). WHO explained that the interpretation of this study is difficult because serious liver damage occurred, the active mechanism of which is unclear because very little microcystin (-LR, -YR, -RR) is able to penetrate the epidermis cells of mice (WHO 1999 and Matsushima *et al*, 1990).

Male C57Bl/6J mice (20-60 animals per dosage group) were given three i.p. doses of 5 mg azoxymethane/kg bw in 7-day intervals to induce aberrant crypt foci. Nineteen days later, the animals were given *Microcystis* extract (0, 0.382 and 0.693 mg microcystin-LR equivalents/kg bw/day) in their drinking water for 30 weeks. The microcystin congeners could not be identified; however, according to HPLC and capillary electrophoresis, they were not microcystin-LR, -YR or -RR. (*Comment:* The calculation of the microcystin-LR equivalents is unclear). The mice were killed, and their blood cells, serum enzymes and organs were examined. In the highest dosage group, the area of the focis were an average of 20% larger than those in the lower dosage group; however, the number of azoxymethane-induced foci per colon remained about the same. Colon tumours were found in one animal of each of the lower and higher microcystin dosage groups, as well as in one control animal that had only been given azoxymethane. The livers from the higher dosage group exhibited 5–10 times more foci from white blood cells compared to those of the lower dosage group. Administration of microcystin led to a dose-dependent decrease in the serum albumin concentration and to a significant dose-dependent increase in the serum activity of alkaline phosphatase. The other serum parameters (total amount of protein, globulin, alanine and aspartate aminotransferase, gamma-glutamyl transferase and total bilirubin) as well as the increase in body weight were not affected (Humpage *et al*, 2000).

Black C57 mice (total of 155 animals) were given two 40 mg doses of the tumour initiator *N*-methyl-*N*-nitrosourea/kg bw with an interval of one week, followed by approx. 20 weeks of treatment with 0, 10 and 40 mg *Microcystis* toxin/L (corresponds to 0, 1.2, 4.2 mg toxin/kg bw/day according to the authors) in their drinking water. The mortality rate in all initiated animals (with and without administration of *Microcystis* toxin) was greatly increased from week 10 (10-40% survivors in week 18). Adenomas and adenocarcinomas were found in the duodenum and lymphoid tumours in the liver, thymus and spleen. Administration of *Microcystis* extract did not promote tumours. Both dosage groups showed a significant

increase in the serum activity of sorbitol dehydrogenase and alanine aminotransferase (Falconer & Humpage, 1996).

Rats

A group of 18 male Fischer 344 rats were given i.p. doses of 0.025 mg microcystin-LR/kg bw twice a week for 10 weeks. Only animals that had previously been given diethylnitrosamine (DEN, liver tumour initiator) showed increases in the surface areas and in number of liver cell foci (glutathione S-transferase placental-form positive liver cell foci) (Ohta *et al*, 1994).

Male Fischer 344 rats were given a single i.p. dose of 0.5 mg aflatoxin B1/kg bw or 200 mg diethylnitrosamine/kg bw (liver tumour initiators), followed 2 weeks later by an i.p. dose of 0.001 or 0.01 mg microcystin-LR/kg bw twice a week for 6 weeks. The administration of microcystin-LR without the initiator did not increase the number of liver cell foci (glutathione S-transferase placental-form positive liver cell foci). Previous administration of aflatoxin B1 led to a significantly higher number and larger areas of liver cell foci for both microcystin-LR doses or for the higher dose in the case of diethylnitrosamine (Sekijima *et al*, 1999).

Fischer F344 rats were initiated with an i.p. dose of 0.2 mg diethylnitrosamine/kg bw, then given two i.p. doses of 0.001 or 0.01 mg microcystin-LR/kg bw and partially hepatectomized (increases the sensitivity of the test by stimulating cell division). Subsequently, in two separate experiments, i.p. doses of 0.001 and 0.01 or 0.01, 0.025 and 0.05 mg microcystin-LR/kg bw were given twice a week over a period of 8 weeks. From a dose of 0.01 mg/kg bw, the animals exhibited a dose-dependent 1 to 3-fold increase in the number and a 1 to 11-fold enlargement of the surface of glutathion S-transferase placental-form positive liver cell foci (GST-P). No liver cell foci were induced in animals treated only with microcystin-LR, without diethylnitrosamine. The NOEL was 0.001 mg/kg bw. Visible macroscopic neoplastic nodules were observed after administration of 0.01 mg microcystin-LR/kg bw before or 0.05 mg/kg bw after partial hepatectomy (Nishiwaki-Matsushima *et al*, 1992).

A DNA microarray test showed an increased transcription of the glutathione-S-transferase gene in the liver of male Wistar rats that had been given a single i.p. dose of 0.05 mg microcystin-LR/kg bw. No histopathological changes were found in the hepatocytes and there were no elevated liver enzyme activities in the serum (Bulera *et al*, 2001).

Sprague-Dawley rats (no information given on the number of animals) were given a single i.p. dose of DEN followed two weeks later by doses of microcystin-LR over a period of 7 weeks

(0.01, 0.04 and 0.08 mg/kg bw/day by gavage or 0.01 mg/kg bw i.p. 3 times a week.). The animals were partially hepatectomized 3 weeks after starting microcystin treatment. At the end of the treatment, the levels of GST-P-positive foci were increased for 0.08 mg/kg bw oral and 0.01 mg/kg bw i.p. (Charbonneau et al., 2004 unpublished data; cited in Dietrich & Hoeger, 2005). *Comment:* This study has limited meaningfulness owing to the lack of detailed information on the size of the dosage groups and on the effects.

2.4 GENOTOXICITY

Tables 3 and 4 give an overview of the genotoxicity test carried out with microcystin-LR and microcystin-containing cyanobacteria extracts.

An *in vivo* study of mice showed time- and dosage-dependent DNA strand breaks in the liver of animals given a single high i.p. dose of microcystin-LR (21.5-86 µg/kg bw = 0.5–2 times LD_{50}) (Rao & Bhattacharya, 1996). In a study of rats given a single i.p. dose of 50 µg microcystin-LR/kg bw (≈40% of the LD_{50}), significantly higher levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine was formed in the liver, which indicates oxidative DNA damage (Maatouk *et al*, 2004).

Microcystin-LR was not mutagenic *in vitro* in the Ames test (+/- S9 mix) with *S. typhimurium* (Ding *et al*, 1999, Tsuji *et a.,* 1997, Tsuji *et al*,1995); however, it did stimulate DNA repair in *E. coli* (Mankiewicz *et al*, 2002). Extracts of *Microcystis aeruginosa* and other cyanobacteria gave a positive Ames Test in one study (Ding *et al*, 1999), and a negative result in two other studies (Repavich *et al*, 1990, Runnegar & Falconer, 1982). In genotoxicity tests with mammalian cells (mice hepatocytes, mice embryo fibroblasts, rat hepatocytes, hamster ovary and kidney cells, human lymphocytes and human hepatoma cells), microcystin-LR and cyanobacteria extracts showed mainly positive results (see Tab. 3 and Tab. 4). Most of the tests were carried out with high dosages at which apoptosis and/or cytotoxicity has been observed. However, the latest genotoxicity tests with rat hepatocytes and human hepatoma cells also showed DNA damage at low concentrations, cited as non-cytotoxic, and which indicate a relationship with oxidative stress (Zegura *et al*, 2003, Zegura *et al*, 2004, Maatouk *et al*, 2004).

Tab. 3. Genotoxicity studies with microcystin-LR

Test system	Dose or concentration	Result	References
In vitro			
- bacteria:			
S. typhimurium TA-97, TA-98, TA-100, TA-102 ; with and without S9	2.5 µg/ml	Negative	Ding *et al*, 1999
S. typhimurium TA-98, TA-100; with and without S9	up to 20 or 50 µg. (No indication of whether per ml or per agar plate)	Negative	Tsuji *et al*, 1997; Tsuji *et al*, 1995
E. coli PQ37, SOS chromotest	1-10 µM (corresponds to 1-10 µg/ml)	Positive. Stimulation of the SOS repair system at 8-10 µM. Toxicity: (unclear. Suppressed phosphatase activity, increased beta-galactose activity)	Mankiewicz *et al*, 2002
- Mouse:			
Male. C3H/HeJ mouse, primary hepatocytes. (Studied: cell proliferation, DNA synthesis, DNA content/cell, apoptosis)	Single dose of 1-10 nM (corresponds to 0.001-0.01 µg/ml) or 3 "doses" of 0.01-100 nM (i.e. new medium after 0, 22 and 46 hours), measurements after 65 hours.	Positive *Single dose:* significantly increased no. of cells and total DNA content (but not DNA/cell). 50% dead cells at 10 nM. *Triple dose:* significantly increased no. of cells at 0.01-0.1 nM. In contrast to a single dose of 10 nM, the mortality rate was not increased for 3 x 10 nM (no information on when the cytotoxic effect starts)	Humpage & Falconer, 1999
Mouse embryonic fibroblast cell cultures (MEF)	1 µg/ml, 1 or 3 hours exposure	Positive. Significantly increased DNA fragmentation (50-60% versus 18% in the controls) Cytotoxicity: positive (60 or 40% surviving cells after 1 respectively 3 hours of exposure versus 85% in the control)	Rao *et al*, 1998
- Rat:			
Primary rat hepatocytes (no information on which rat strain), comet assay (single-cell gel electrophoresis)	1 µg/ml	Positive. DNA damage in approx. 40% of the cells. Toxicity: morphological cell changes (irregular and rough surface of the cell nucleus, shrunk cells)	Ding *et al*, 1999
Primary rat hepatocytes (Sprague-Dawley), oxidative DNA damage	0.002 and 0.01 µg/ml	Positive. Dose- and time-dependent formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine Toxicity: No apparent morphological evidence of cytotoxicity (however, no further tests carried out)	Maatouk *et al*, 2004
Primary rat hepatocytes (Sprague-Dawley), DNA replication	0.001-10 µg/ml	Negative. No inhibition of DNA replication. Toxicity: 75% intact cells on incubation with 1 µg/ml. According to calculations by the authors, an intracellular concentration of 1 µM (= 1 µg/ml) led to apoptosis in 50% of the cells.	Mellgren *et al*, 1993

- Hamster:			
Chinese hamster ovary cells (CHO-K1) (investigated: cell cycle, anaphase, chromosome segregation by mitotic spindle, apoptosis)	25-100 µg/ml for 14, 18 or 22 hours	Dose- and time-dependent increase in the mitotic index, increased abnormal G(2)/M ratio with hypercondensed chromosomes, abnormal anaphase with incorrect chromosome distribution and polyploid cells. Most mitotic cells exhibited monopolar and multipolar spindles (multipolar asters). Furthermore, the microtubuli bundles were present in interphase cells. Toxicity: The number of dead cells correlated positively with the number of polyploid cells. According to the authors, these results indicate that microcystin-LR induces dose- and time-dependent apoptosis and necrosis.	Lankoff *et al*, 2003
BHK-21 (baby hamster kidney) cell cultures	1 µg/ml, 1- and 3-hour exposure	Positive. DNA fragmentation (50-70% versus 20% in the controls) Cytotoxicity: positive (40 or 2% surviving cells after 1 respectively 3 hours of exposure versus 80% in the controls)	Rao *et al*, 1998
- Humans:			
human lymphocytes, alkaline comet assay	0.25-4 µg/ml	Positive. DNA damage DNA strand breakes were time-dependent with a maximum at 12-18 hours' exposure; no information on the significance. Toxicity: No information	Mankiewicz *et al*, 2002
Human lymphocyte (peripheral blood), alkaline comet assay, chromosome aberrations	1, 10, 25 µg/ml for 6, 12, 18 and 24 hours	- Positive comet assay: Time-dependent DNA damage. - Negative with respect to chromosome aberration Toxicity: Dose- and time-dependent increase in the apoptotic cells from the lowest dose and shortest incubation time (measured with the TUNEL method and a comet assay) The results indicate that DNA damage is more likely to be associated with cytotoxicity rather than with genotoxicity.	Lankoff *et al*, 2004
Human lymphocytes (peripheral blood), DNA repair	1 µg/ml for 4 hours then irradiation with 2 Gy (only microcystin-LR concentration and time tested)	Positive. Microcystin-LR retarded/inhibited the repair of DNA damaged by irradiation. (Controls: DNA repair was induced by irradiation (without microcystin-LR). No information on DNA repair on incubation with microcystin-LR (without irradiation)). Toxicity: no information for this test. In the same publication (see the line above, comet assay), a dose-dependent increase in apoptotic cells is described from 1 µg/ml)	Lankoff *et al*, 2004
Human hepatoma cell line HepG2	0.01-1 µg/ml	Positive. Time- and dose-dependent DNA strand breaks. Transient effects with a maximum after 4 h exposure. Toxicity: not cytotoxic at the tested concentrations (determination of the optical density after	Zegura *et al*, 2003

		treatment with MTT)	
Human hepatoma cell line HepG2, comet assay	0.01, 0.1 and 1 µg/ml for 4, 8 and 12 h	Positive. Dose-dependent DNA strand breaks already at lowest concentration after 4 h exposure. For 8 and 12 h exposure, there was a significant increase in DNA damage compared to the control only if DNA repair inhibitors AraC/HU (=beta-D-arabinofuranoside hydrochloride) and HU (hydroxyurea) had been added. This indicates that DNA damage caused by microcystin-LR has been efficiently repaired. Time- and dose-dependent increase in intracellular reactive oxygen species (ROS). Toxicity: Probably not determined. The authors cite Zegura et al. 2003.	Zegura *et al*, 2004
Human lymphoblastoid cell line TK6	20-80 µg/ml, 4 or 24 h treatment	Positive after 24 h treatment: clastogenic, LOH (loss of heterozygosity), i.e. more extensive genetic changes than point mutations. Negative after 4 h standard treatment. Cytotoxicity: 2 parameters measured: RS (relative plating efficiency directly after exposure) and RSG (rel. cell growth 3 days after exposure). - 4 h treatment: non-significant cytotoxicity up to 80 µg/ml - 24 h treatment: dose-dependent cytotoxicity from 20 µg/ml. RSG significant, RS tendentious (non-significant). Genotoxic effects only observed at cytotoxic concentrations	Zhan *et al*, 2004
Human RSa cell line (human embryo fibroblasts infected with Simian Virus 40 and Rous sarcoma virus)	7.5-15 µg/ml	Positive from 7.5 µg/ml: Ouabain resistance mutations and base substitution mutations on K-ras codon 12. Toxicity: 80% or 35% surviving cells for 10 or 15 µg/ml, respectively.	Suzuki *et al*, 1998
In vivo			
Male Swiss albino mouse, hepatocytes (no data on the group size)	21.5, 43, 86 µg/kg bw i.p. (=0.5, 1 and 2-fold LD_{50})	Positive. Time- and dose-dependent DNA strand breaks already at lowest dose. Toxicity: Animals generally died within 120-150 min after administration. Animals were killed after 60 min (dosage-dependence study) or after 0, 15, 30, 60, 90, 120 min (time-dependence study)	Rao & Bhattacharya, 1996
Male. Sprague-Dawley rats (n=4), oxidative DNA damage	50 µg/kg bw i.p. (= approx. 40% of the LD_{50})	Positive. Significant increase in 8-oxo-7,8-dihydro-2'-deoxyguanosine in liver DNA, 24 and 48 h after administration (maximum after 24 h)	Maatouk *et al*, 2004

Tab. 4. Genotoxicity studies with cyanobacteria extracts

Extract/Toxin	Test system	Dose or concentration	Result	Reference
In vitro				
- Bacteria				
Microcystis extract (mainly microcystin-LR)	*S. typhimurium* TA-97, TA-98, TA-100, TA-102 (with and without S9)	1.25-125 µg lyophil. algae/ml	Positive from 12.5 µg/ml (dose-dependent)	Ding *et al*, 1999
Cyanobacteria extracts (*Microcystis, Anabaena, Gloetrichia*) and purified	*S. typhimurium* TA-98, TA-100, TA-102 (with and without S9)	0.9 mg hepatotoxin/ml (positive in mouse	Negative	Repavich *et al*, 1990

hepatotoxin*		i.p. bioassay)		
Purified hepatotoxin (peptide) from *Microcystis*	*S. typhimurium* TA-98 and TA-100 (with and without S9)	0.2-20 µg toxin/plate	Negative	Runnegar & Falconer, 1982
Cyanobacteria extracts (*Microcystis, Anabaena, Gloetrichia*) and purified hepatotoxin*	*Bacillus subtilis* multigene sporulation test; strains 168 and hcr-9	0.9 mg hepatotoxin/ml (positive in mouse i.p. bioassay)	Negative	Repavich *et al*, 1990
Cyanobacteria extract (mainly *Microcystis aeruginosa*) and microcystin-LR as standard	SOS chromotest with *E. coli* PQ37	2-100 mg cyanobacteria/ml	Stimulation of the SOS DNA repair system (difficult to give concentrations)	Mankiewicz *et al*, 2002
- Mouse				
Microcystis aeruginosa extract	MEF (Mouse Embryo Fibroblast) cells	100 µg toxin/ml	DNA fragmentation if cytotoxic	Rao *et al*, 1998
- Rat				
Microcystis extract (mainly microcystin-LR)	Primary rat hepatocytes. Comet assay (single-cell gel electrophoresis)	1.25-125 µg lyophil. algae/ml	Positive. Dose-dependent DNA damage in approx. 40% of the cells.	Ding *et al*, 1999
Cyanobacteria extract (microcystin-LR, -RR, -YR, -WR)	Primary rat hepatocytes	0.05-1 µM MC-LR, (corresponds to 0.1-2.7 µM MC-LR equivalents if all congeners considered) 0.5-2 h incubation	DNA fragmentation, condensation of chromatin as a typical sign of apoptosis. *Comment:* no negative control.	Mankiewicz *et al*, 2001
- Hamster				
Microcystis aeruginosa extract	2-Stage transformation assay with Syrian Hamster Embryo (SHE) cells (initiator methylcholanthrene, promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA))	No information	Negative with respect to initiation Positive with respect to promotion	Wang & Zhu, 1996
Microcystis aeruginosa extract	BHK-21 (Baby Hamster Kidney)	100 µg toxin/ml	DNA fragmentation with slight cytotoxicity	Rao *et al*, 1998
- Human				
Purified hepatotoxin*	Human lymphocytes	0.009-0.9 µg toxin/ml	Positive (dose-dependent chromosome breaks)	Repavich *et al*, 1990
Cyanobacteria extract (mainly *Microcystis aeruginosa*)	Human lymphocytes, comet assay	Extracts with 0.25-1 µM microcystin	DNA breaks (time-dependent) Apopototic lymphocytes with severe DNA fragmentation after 24-h incubation	Mankiewicz *et al*, 2002
Cyanobacteria extract (microcystin-LR, -RR, -YR, -WR)	Human lymphocytes (peripheral blood)	0.25-1 µM MC-LR, (corresponds to 0.7-2.7 µM MC-LR equivalents if all	DNA fragmentation and condensation of chromatin as a typical sign of apoptosis,	Mankiewicz *et al*, 2001

		congeners considered) 24 or 48 h incubation	according to the authors, from 0.75 µM MC-LR, 48 h, (*Comment:* no negative control)	
In vivo				
Microcystis extract (mainly microcystin-LR)	Mouse micronucleus test	1-100 mg lyophil. algae/kg bw	Positive. Dose-dependent chromosome damage already at lowest dosage	Ding *et al*, 1999
Microcystis aeruginosa extract (UTEX 2385)	Mouse liver (male Swiss albino mouse)	32.7, 65.4, 130.8 mg toxin/kg bw i.p. (=0.5, 1 and 2-fold LD_{50})	DNA fragmentation	Rao *et al*, 1998

*The publication does not clearly state whether it refers to microcystin.

2.5 REPRODUCTION TOXICITY

No data on multigeneration studies of rodents are available.

Mice

Groups of 26 female mice were given oral (gavage) dosages of 0, 0.2, 0.6 or 2 mg/kg bw/day of microcystin-LR from gestation day 6 to 15. The mothers were killed on gestation day 18 and examined. In the group given the highest dosage, the mortality rate of the mothers was increased, the weight of the fetuses decreased and ossification of the skeleton delayed. Microcystin-LR did not influence the fetus mortality rate, gender ratio, number of implantations, post-implantation losses or visceral and skeletal abnormalities. The NOAEL for the maternotoxicity and fetotoxicity was 0.6 mg/kg bw/day (WHO 1999 and Fawell *et al*, 1999).

A group of 8 female and 2 male mice were given 1/4-diluted *Microcystis aeruginosa* extract in their drinking water (probably equivalent to 3 mg microcystin/kg bw/day (see the comment on Falconer *et al.* 1988 in Chapter 2.2 Subchronic toxicity) from weaning (3rd week of life) until mating in the 20th week of life. The fertility, the gender ratio and the weight of the newborn mice was not affected. Seven of the 73 newborn mice had a smaller brain and exhibited changes in the hippocampus compared to 0 of 67 control animals (Falconer *et al*, 1988).

Pregnant mice were given i.p. doses of 0.002-0.128 mg microcystin-LR/kg bw on two consecutive days on gestation day 7-8, 9-10 or 11-12. The mothers were killed on gestation

day 17, and the fetuses were examined for abnormalities. Some of the mothers in dosage groups receiving 0.032 mg/kg bw or more were not killed and their offspring were examined. Microcystin-LR was not teratogenic and there was no evidence for any apparent reprotoxic effects (Chernoff *et al*, 2002).

In vitro experiments

Mouse embryo cultures in the neurulation stage were treated *in vitro* with 50-1000 nM microcystin-LR for 24 h. This led to a greater number of abnormalities (non-significant); development was not retarded (Chernoff *et al*, 2002).

Embryos and embryo cell cultures from rabbits were treated *in vitro* with 10-100 µM microcystin-LR. No effects were found with respect to size, development and cytoskeleton organisation of the embryo embedded in the zona pellucida. In embryo cell cultures, 10-20 µM led to changes in the actin and microtubuli organisation, 100 µM to detachment and destruction of cells (Frangez *et al*, 2003).

2.6 IMMUNOTOXICITY

Immunosuppression in BALB/c mice was observed after i.p. administration of *Microcystis* extract in sublethal dosages of 16, 32 or 64 mg lyophilised algae cells/kg bw (0.005, 0.010, 0.020 mg microcystin equivalents/kg bw, calculated based on LD_{50} of the extract) over a period of 14 days. The relative weight of the spleen was significantly increased at the middle dosage, and significantly lower at the highest dosage compared to control animals. The relative weight of the thymus was significantly decreased at the highest dosage. The lipopolysaccharide-induced proliferation of B lymphocytes was already reduced at the lowest dosage; the concanavalin-induced proliferation of T-lymphocytes was not affected. The general condition of the treated animals was adversely affected: they were inactive, their body weight gain was reduced, the liver was enlarged, and there was liver bleeding. In mice immunised with sheep erythrocytes (sRBC), the middle dosage and higher had a significant adverse effect on the transformation of B-cells into antibody-forming plasma cells (Shen *et al*, 2003).

3 TOXICOLOGY OF METABOLITES

Metabolites occurring in rats and mice (liver):

Microcystin-LR and -YR administered i.v. to male mice was acutely toxic, namely, approx. 2–20 times more toxic than the corresponding synthetic conjugates with glutathione and cysteine. The i.v. LD_{50} values in mice for microcystin-LR and its glutathione and cysteine conjugates were 0.04, 0.63 and 0.27 mg/kg bw, respectively. The i.v. LD_{50} values in mice for microcystin-YR and its glutathione and cysteine conjugates were 0.09, 0.30 and 0.22 mg/kg bw, respectively (Kondo *et al*, 1992).

Metabolites produced during treatment of drinking water by ozonation and chlorination:

Microcystin-LR was rapidly converted into many reaction products by chlorination with sodium hypochlorite. This included the addition of two chlorine atoms on the conjugated double bonds in the ADDA amino acid and subsequent hydrolysis to form dihydroxymicrocystin. Tsuji *et al.* (1997) suggest that the other metabolites may be its stereoisomers and/or regioisomers. They investigated the reaction products with respect to acute toxicity, mutagenicity and inhibition of protein phosphatase. The acute toxicity in mice given i.p. doses of the reaction products present after 3 or 24-h chlorination was reduced (LD_{50} i.p mouse 0.05, >1 and >3 mg/kg bw for microcystin-LR, metabolites after 3 h or after 24 h chlorination; no information is given on dihydroxymicrocystin). The inhibition of protein phosphatase 2A was also reduced (IC_{50} 8 µg/L for dihydroxymicrocystin or 700 µg/L for another unidentified metabolite compared to 0.05 µg/L for microcystin-LR). The reaction products were not mutagenic in the Ames test with *S. typhimurium* TA98 and TA100 without S9 up to 20 µg. (*Comment:* There is no information as to whether the 20 µg is per plate or per ml) (Tsuji *et al*, 1997).

In a laboratory test, microcystin-LR (no concentrations given) was treated with 0.5 mg/L ozone until ozone was no longer detectable. In a protein phosphatase inhibition assay, one HPLC fraction with a similar retention time as that of the starting compound, also showed an inhibiting effect on protein phosphatases 1 and 2A. (Hoeger *et al*, 2000). *Comment*: This substance has not yet been isolated in a pure form (personal communication, D. Dietrich, 2004).

When 10 µg/L microcystin-LR was treated with 0.5, 1 or 1.5 mg/L ozone, directly or 60 min after ozonation, there was practically no longer a protein phosphatase-inhibiting effect (90-100% activity versus 60-70% prior to ozonation). In an analogous experiment carried out with *Microcystis aeruginosa* extract (50000 cells/ml), the protein phosphatase activities were

approx. 0.5, 40-80 and 80-90% before, immediately after, and 60 min, respectively, after ozonation (Hoeger *et al*, 2002). *Comment:* The article does not mention whether the inhibiting effect after ozonation can be attributed to a residual amount of starting compound or to ozonolysis products.

According to Hitzfeld *et al.* (2000) and Höger *et al.* (2002), the structure as well as the toxicity of the ozonolysis products have not yet been investigated.

Microcystins were rapidly degraded by sunlight in the presence of cyanobacteria pigments to afford 6(Z)-Adda-microcystin-LR and further isomers. Two of the metabolites, 6(Z)-Adda-microcystin and 4(Z)-Adda-microcystin were less acutely toxic compared to the starting compound (LD_{50} i.p. mouse >1.5 mg/kg bw for both metabolites, 0.05 mg/kg bw for microcystin-LR) and they also inhibited protein phosphatase 2A to a lesser degree (IC_{50} 5000 µg/L versus 0.05 µg/L). The starting compound and the two investigated photometabolites did not exhibit mutagenicity in the Ames test with *S. typhimurium* TA98 and TA100, with and without metabolic activation, at dosages up to 50 µg (no information whether this is 50 µg per ml or per agar plate) (Tsuji *et al*, 1995).

4 MECHANISTIC STUDIES: EFFECTS ON THE CYTOSKELETON, MITOTIC CELL DIVISION, APOPTOSIS AND OXIDATIVE STRESS

The mechanism of microcystin-LR-induced hepatotoxicity and tumour promotion is not yet fully understood. A number of publications, summarised in review articles by Gehringer (2004) and Ding & Nam (2003), deal with the effects of microcystin-LR on the cytoskeleton, mitotic cell division, apoptosis and oxidative stress.

Effects on the cytoskeleton

Rat hepatocytes, kidney cells and skin fibroblasts were incubated for a maximum time of one hour with 13.3 µM microcystin-LR, and the effects on the cytoskeleton were studied. All cell types exhibited condensation and collapse of the intermediary filaments (vimentin and cytokeratin), microfilaments and microtubuli (Khan *et al*, 1996).

Rat renal epithelial cells and fibroblasts were incubated for 6-18 h with 100 or 200 µM microcystin-LR, and rat hepatocytes were incubated for a maximum of one hour with 1 or 10 µM microcystin-LR. Fibroblasts and some hepatocytes exhibited changes in the cytoskeleton

that were initiated by disorganisation of the intermediary filaments and microtubuli followed by rapid breakdown of cytoskeleton constituents around the cell nucleus. Subsequently, the microfilaments also changed due to aggregation, condensation and collapse of actin. The authors concluded on the basis of similar effects in various types of cells that there is a common active mechanism and postulated that probably at least two different phosphorylation sites are affected because the kinetics of disaggregation of the intermediary filaments and the microtubuli are different (Wickstrom *et al*, 1995).

Desmosomes, which form the connections between adjoining cells, are connected to keratin filaments in epithial cells via desmoplakin I and II. Exposure of primary rat hepatocytes to 1 µM microcystin-LR for 12 min resulted in damage of desmoplakin I and II, and thus to disaggregation of desmosomes followed by reorganisation of micro- and intermediary filaments, whereby the latter were concentrated around a core of actin. It was shown that microcystin-LR leads to hyperphosphorylation of keratins 8 and 18 (as heterodimers they form the framework of intermediary keratin filaments) and of desmoplakin I and II. A possible explanation for this is inhibition of protein phosphatases 1 and 2A (Toivola *et al*, 1997).

Keratins 8 and 18 in primary rat hepatocytes were hyperphosphorylated with 1 µM microcystin-LR (Ohta *et al*, 1994).

Rat hepatocytes exposed to 0.05-0.5 µM microcystin exhibited dose-dependent inhibition of the frequency, speed and path length of microtubuli-dependent vesicle movements and the activities of protein phosphatases 1 and 2A. In contrast, microtubuli organisation was unaffected, even at the highest microcystin concentration (Hamm-Alvarez *et al*, 1996).

Effects on mitotic cell division

It is postulated that microcystins are able to intervene in the regulation of proteins via inhibition of protein phosphatases 1 and 2A, which thus affects the formation of the mitotic spindle and cell division. For example, proteins that are responsible for tubulin polymerisation and the stability of microtubuli are regulated by de-/phosphorylation. The tumour supressor protein p53 is also a substrate of protein phosphatases 1 and 2A (Lankoff *et al*, 2003).

Chinese hamster ovary cells (CHO-K1) were incubated with 0, 25, 50 and 100 µM microcystin-LR and with cyanobacteria extract for 14, 18 or 22 hours, and the effects on the

cell cycle, start of the anaphase, chromosome segregation by the mitotic spindle and apoptosis were studied. The results showed a dose- and time-dependent increase in the mitotic index, a higher abnormal G(2)/M ratio with hypercondensed chromosomes, an abnormal anaphase with incorrect chromosome distribution and polyploid cells. Most of the mitotic cells exhibited mono- and multipolar spindles (multiple asters). Furthermore, there were microtubuli bundles in the interphase cells. The number of dead cells correlated positively with the number of polyploid cells. According to the authors, these results indicate that microcystin-LR induces dose- and time-dependent apoptosis and necrosis (Lankoff *et al*, 2003).

During incubation of Syrian Hamster Embryo cells (SHE) with *Microcystis* extract, the transcription factors c-fos and c-jun were overexpressed during the 6-hour test period and up to 6 hours afterwards. As a result, the transition from the resting phase G(0)/G(1) into the S phase was induced in 45% of the cells. The authors postulate that the upregulation of the c-fos and c-jun transcription factors (proto-oncogenes) leads to abnormal cell proliferation, possibly leading to cancer (Zhao & Zhu, 2003, Original article in Chinese).

Apoptosis

High microcystin-LR doses, as described in detail below, induced apoptosis *in vivo* in mice (Yoshida *et al.* 1998 Yoshida *et al*, 1998) and rats (Guzman & Solter, 1999) and *in vitro* in rat hepatocytes, human lymphocytes and other mammalian cells within a few minutes (Fladmark *et al*, 1999, Fladmark *et al*, 2002, Boe *et al*, 1991, McDermott *et al*, 1998, Mankiewicz *et al*, 2001 and review article [Gehringer, 2004, Ding & Nam, 2003]).

in vivo

Immunohistochemical methods showed that a single i.p. dose of 0.02-0.1 mg/kg bw microcystin-LR given to mice led to the formation of adducts with protein phosphatases 1 and 2A in the centrilobular regions of the liver. These regions also exhibited haemorrhages and apoptotic cells, some of the latter were immunopositive (monoclonal antibodies against microcystin-LR). In the midlobular region of the liver, where chiefly necrosis occurred, all the cells were immunonegative and no microcystin adducts were detected (Yoshida *et al*, 1998).

After administration of i.p. doses of 0.048 mg/kg bw/day microcystin-LR for 28 days to rats (3 animals/dose), apoptotic cells (apoptosis bodies with and without chromatin) were

observed in the pericentral (zone 3) region of the liver. Doses of 0.016 or 0.032 mg/kg bw/day gave less pronounced effects (no further information) (Guzman & Solter, 1999).

in vitro

Microcystin-LR-induced *in vitro* apoptosis in various types of mammalian cells. Primary rat hepatocytes exposed to 0.8 µM microcystin-LR for less than 15 min exhibited typical apoptotic effects (budding of the cell surface, cell shrinkage, organelle redistribution, condensation of chromatin in the cell nucleus and partial fragmentation of the cell nucleus, intact mitochondria in apoptotic vesicles or on one pole of the cell). In the tested non-hepatocytes (human fibroblasts, endothelial cells and promyelocytes), 100-times higher concentrations were necessary (100 µM) and the effects started only after 6-8 h (McDermott *et al*, 1998). *Comment:* The article does not state how long microcystin-LR was administered and which route was used for application. The cells were probably incubated and not micro-injected. The fact that a higher concentration is required to incubate the cells is plausible because non-hepatocytes do not have a microcystin uptake mechanism.

Apoptosis had been induced in 50% of the incubated primary rat hepatocytes 2 min after addition of 16 µM microcystin-LR. Four minutes after addition, the cytoplasm and nucleus volume had decreased by 20% and intensive budding of the cell surface with detachment of apoptotic bodies (containing mitochondria) commenced. After fifteen minutes, most of the cells no longer contained any microvilli and no glycogen. After two hours, the chromatin in the cell nucleus condensed. Protein phosphatases 1 as well as 2A must be inhibited to induce rapid cell death. In another experiment, microinjection (because only hepatocytes exhibit good uptake) of microcystin-LR (no dosage information) led within a few min to intensive budding and rounding of the cells of Swiss 3T3 fibroblasts, human HEK 293 cells and normal rat kidney cells (NRK) (Fladmark *et al*, 1999).

Microinjection of 100-200 µM microcystin-LR induced apoptosis in rat hepatocytes. In another experiment with rat hepatocytes, incubation with 1 µM microcystin-LR led to increased protein phosphorylation. If an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) was also added, apoptosis was prevented and protein phosphorylation was not increased. The authors concluded from these results that microcystin-LR inhibits protein

phosphatases 1 and 2A in a CaMKII-dependent process, which leads to increased phosphorylation of proteins and thus to apoptosis (Fladmark *et al*, 2002).

Incubation of rat hepatocytes with 0.8 µM microcystin-LR caused cell fragmentation, condensation of chromatin in the cell nucleus and aggregation of internal membranes on one pole of the cell (Boe *et al*, 1991).

Incubation with 0.0125-0.05 µM microcystin-LR led to morphological changes in primary human hepatocytes and in rat hepatocytes. The actin network collapsed and aggregated in the middle of the cell, chromatin in the cell nucleus condensed, and, in addition, the cells detached from the cell cluster and disintegrated to form vesicles, fragments and apoptotic bodies. The estimated EC_{50} value (effective concentration) after 6-hour exposure was 0.025 µM for primary human hepatocytes and 0.05 µM for rat hepatocytes (Batista *et al*, 2003).

Effects on oxidative stress and lipid peroxidation

in vivo

Groups of 3 rats were given i.p. doses of 0, 0.016, 0.032 and 0.048 mg microcystin-LR/kg bw/day for 28 days. The malondialdehyde concentration in the liver showed significant and dose-dependent increases at a dosage of 0.032 and 0.048 mg/kg bw/day. In the highest dosage group, it was increased by approximately four times compared to the control group. Liver sections from rats treated with microcystin were more sensitive to the cytotoxic effect of *tert*-butyl hydroperoxide (ROS-generator). The authors concluded from the histological and biochemical results that oxidative stress plays a significant role in the chronic toxicity of microcystin (Guzman & Solter, 1999).

Eight male rats were given a single i.p. dose of 0.05 mg/kg bw microcystin-LR (=LD_{50}), and their oxidative lipid metabolism was investigated spectroscopically up to 3 h after administration. The liver of the treated animals contained significantly more lipid radicals; the glutamine/glutamate and lactate concentrations were significantly lowered. Significantly increased enzyme activities of alanine aminotransferase and aspartate aminotransferase in the serum indicate liver damage. The authors concluded from these results that oxidative stress plays an important role in hepatotoxicity induced by microcystin-LR (Towner *et al*, 2002).

in vitro

Five-hour incubation with 0.01, 0.1 and 1 mg microcystin-LR/ml (0.01, 0.1, 1 mM) induced intracellular reactive oxygen species (ROS) in human hepatoma HepG2 cells. *Comment:* The

authors state that the tested concentrations were non-cytotoxic; however, they refer to Zegura et al. 2003 (Zegura *et al*, 2004).

Exposure to 0.01-1 µg/ml microcystin-LR (non-cytotoxic concentrations) induced time- and dose-dependent DNA strand breaks in human hepatoma cell line HepG2. The effects were transitory, with a maximum after 4 hours of exposure. The DNA strand breaks accumulated when DNA repair inhibitors (cytosine, arabinoside and hydroxyurea) were added. In another experiment, DNA was treated with endonuclease III and formamidopyrimidine DNA glycosylase (FPG), which can specifically generate additional strand breaks at oxidatively modified DNA bases. Cells treated with microcystin-LR showed more DNA strand breaks compared to untreated cells. DNA damage in cells treated with microcystin-LR was significantly decreased by the addition of DMSO, a hydroxyl radical scavenger. The authors concluded that microcystin-LR induces the formation of reactive oxygen species that damage DNA (Zegura *et al*, 2003).

The incubation of primary rat hepatocytes with 0.002 and 0.01 µg microcystin-LR/ml (0.002 and 0.01 µM) led to significantly elevated intracellular glutathione (reduced form GSH) concentrations (transient increase after 2 h, baseline values after 24 and 48 h), along with the formation of reactive oxygen species and lowering of the malondialdehyde concentration. The tested concentrations were not cytotoxic (MTT test). The LC_{50} was 0.048 µg microcystin-LR/ml for 24 h incubation (Bouaicha & Maatouk, 2004).

Influence of other substances on microcystin-LR-induced toxicity

Female mice were given a single i.p. dose of 0.1 mg microcystin-LR/kg bw (lethal dose) together with biologically active substances to study their influence on the microcystin-LR-induced toxicity by means of the mortality rate, the time of death and the lactate dehydrogenase activity in the serum. Membrane-active antioxidants, such as vitamin E, silymarin, glutathione and the monoethyl ester of glutathione, significantly protected the mice against the lethal dose. The lethal effect of microcystin-LR was completely suppressed by penicillin G as well as by rifampicin and ciclosporin-A, two membrane-active substances with an immunosuppressive effect and which also inhibit bile acid transport in hepatocytes. Glucose significantly delayed the time of death. Calcium channel blockers (verapamil, nifepidine), free radical scavengers (allopurinol, mannitol, glycerol, ethanol), and further substances, such as vitamin C and dioxins, did not have a significant influence on the investigated parameters (Hermansky *et al*, 1991).

5 MICROCYSTIN CONCENTRATIONS IN ALGAE PRODUCTS, DRINKING WATER AND FOODS

Microcystin concentrations in algae products

USA and Canada

In a measuring campaign of the Oregon Health Division, microcystin-LR was detected in 85 of 87 AFA algae products from Klamath Lake; the concentration in 63 of these samples (72%) was greater than 1 µg/g. The algae samples studied in 1996-1999 were obtained from retail outlets or directly from the four largest manufacturers. Individual values lay between 0.04 and 18.4 µg microcystin/g, average values between 0.4 and 10.9 µg microcystin/g (values averaged per year and manufacturer). The separation of two samples using HPLC and subsequent quantification with ELISA showed that more than 90% of the microcystins in the samples was microcystin-LR. According to the authors, this agrees with the results from other authors (cited in the paper as a "personal communication"). The average microcystin-LR concentrations in 15 spirulina products collected by the Oregon Health Division in 1998 and 1999 were 0.15 and 0.52 µg/g, respectively. One sample contained 2.12 µg/g (Gilroy *et al*, 2000).

In a Canadian study, the microcystin concentrations in 117 algae products (AFA, spirulina, unidentified species) were determined by means of ELISA, protein phosphatase inhibition assay (PPA), LC-MS and LC-MS-MS. The microcystin concentrations in the 50 products, given in a table, were 0.1-10 µg/g, and one sample contained more than 30 µg/g. Analysis of the latter sample gave a microcystin concentration of 25-35 µg/g by LC-MS, LC-MS-MS and ELISA and 49 µg/g microcystin by PPA (each used 1-3 measured values, average of all measured values = 33 µg/g microcystin-LR and -LA). The authors did not comment on these noticeably elevated values. All positive samples were shown to contain microcystin-LR along with microcystin-LA as the only other congener. An interlaboratory study (4 laboratories), carried out for Health Canada, demonstrated a good agreement in the measured values between the different laboratories and concentrations of between 0.5-35 µg/g. The publication, which mainly focused on the analytical aspects, gives no information regarding

which algae products (AFA, spirulina, other species) the microcystin concentrations refer to and which concentrations were measured in the products not listed in the table (Lawrence *et al*, 2001).

In 1984, AFA algae were harvested from Upper Klamath Lake for a feeding study in mice. The algae were washed and lyophilised and stored at room temperature for approx. 13 years. The microcystin-LR content was analysed in six algae samples by means of HPLC, ELISA and PPA. Values of 11-24.7 µg microcystin-LR/g AFA algae were measured, with an average value of 20.0 ± 5.0 µg/g (Schaeffer *et al*, 1999).

Since the introduction of an acceptable maximum concentration of 1 µg microcystin-LR/g AFA algae product by the Oregon Department of Agriculture in 1997, manufacturers have been carrying out batch tests of freshly harvested AFA algae, and only batches containing less than 1 µg/g are used to manufacture AFA algae products. In a comprehensive investigation of algae samples from Upper Klamath Lake (1994-1998), the majority of the microcystin concentrations were significantly greater than 1 µg/g. Most of the values lay between 0.2 and 10 µg/g with a maximum value of 25 µg/g. The samples were measured by means of ELISA and PPA, whereby the ELISA values were all significantly higher (Carmichael *et al*, 2000). *Comment:* The publication does not mention how many samples were analysed. There is also no information on how representative the random samples were.

In an extensive measuring campaign carried out by Health Canada in Canada, no microcystin was found in any of the spirulina samples, in contrast to the other algae products (*Comment:* no information is given on how many samples were analysed, with which method and with which detection limit) (Health Canada online, 2001).

Germany and Switzerland

AFA algae in food supplements retailed in Germany and Switzerland also originate from Upper Klamath Lake, which is why comparable microcystin contents to those found in Canada and the USA are to be expected.

Switzerland: The microcystin-LR content in 7 AFA, 3 chlorella and one spirulina algae product was determined by means of ADDA-ELISA (triple determination) and colorimetric PPA; 5 random samples were analysed per product. The algae samples were obtained on the Swiss retail market or were submitted to the Bundesamt für Gesundheit (BAG, Swiss Federal Office of Public Health) by applicants. Concentrations of 0.4-1.4 µg microcystin-LR equivalents/g dry weight were found in 6 AFA algae products; one sample contained 5.3 µg

microcystin-LR equivalents/g dry weight. The results of the two methods of investigation were in good agreement. Chlorella and spirulina algae products were found to contain 0.01-0.02 µg (ELISA) and 0.1-0.3 µg (PPA) microcystin-LR equivalents/g dry weight (*Comment:* no information is given on the detection limits) (BAG, 2003, internal report).

Table 5 summarises the microcystin contents of AFA algae products.

Germany: No data are available.

Tab. 5. Microcystin content in AFA algae products from Upper Klamath Lake (USA)

Microcystin concentration [µg/g]	**Number of samples**	**Microcystin congener**	**Comments**	**References**
0.04-18.4	87	90% as -LR	98% samples positive 72% >1 µg/g	Gilroy *et al,*. 2000
0.1-10, 33	>100* (1 sample)	-LR and -LA	*not only AFA	Lawrence *et al,* 2001
11-24.7	6	-LR		Schäffer *et al,* 1999
0.4-1.4, 5.3	7 (1 sample)	-LR		BAG, 2003, internal report

Microcystin concentrations in drinking water

The WHO guideline value for the drinking water concentration is 1 µg microcystin-LR /L (WHO 1999). This value appears to be complied with in Germany and Switzerland (SKLM, 2003, Hoeger, 2003, Chorus, 2001). A compilation of microcystin concentrations measured around the globe showed that the values in drinking water were 0.01-0.4 µg/L, although there were a few exceptions. However, noticeably higher values were also given, e.g. 1.9 µg/L in China, 7.8 µg/L in the Czech Republic and 90 µg/L in the USA (Hoeger, 2003, Hoeger *et al*, 2005).

Microcystins are efficiently eliminated from raw water by ozonation during drinking water treatment. If insufficient ozone is used, e.g. if the cell density of an algal bloom is too high, the cyanobacteria cells undergo lysis thus liberating the toxins that are then only partially degraded (Hoeger *et al*, 2002).

Microcystin concentrations in foods of animal origin

Studies have been carried out on microcystin concentrations in fish, mussels and shellfish from Portugal (Vasconcelos, 1999), Brazil (Magalhaes *et al*, 2003, Mohamed *et al*, 2003) and the USA (Hathaway, 2001); however, they do not allow any representative statements to be made because the number of samples was usually too small and very little detail is given on the sampling parameters. Depending on the study, maximum values of 0.5 mg microcystin-LR equivalents/kg were found in fish (Mohamed *et al*, 2003), up to 2.7 mg microcystin-LR/kg in lobsters (Vasconcelos, 1999) and a maximum value of 0.1 mg microcystin-LR equivalents/kg in prawns (Magalhaes *et al*, 2003). In one publication, values of up to 16 mg microcystin-LR/kg were measured in mussels (Vasconcelos, 1999); however, in another publication, only max. 0.11 mg microcystin/kg was found in mussel livers (Hathaway, 2001).

Data is also available from studies of the passage of microcystin into cow's milk. Four lactating cows (Holstein Friesian dairy cattle) were given *Microcystis aeruginosa* extract (10^8 cells/L water, strain MASH01-A19) in their drinking water of a period of 21 days. (*Comment:* values greater than 10^6 cells/L are regarded as an algal bloom). The cows' total intake was 15 mg microcystin-LR or an average of 1.21 µg/kg bw/day. Neither HPLC nor ELISA detected any microcystin-LR in the milk. The detection limit of the ELISA method was 0.002 µg/L, that of the less sensitive HPLC method was not given (Orr *et al*, 2001).

Microcystin concentrations in bathing waters

Cyanobacteria were detected in 55% of the 155 bodies of water investigated in Germany (325 samples). The average measured value was 45 µg microcystin/L and the maximum was 566 µg/L (Wolf & Frank, 2002).

6 EXPOSURE ASSESSMENT

According to the WHO (1999), the main source of cyanobacterial toxins in humans is drinking water. The consumption of algae products as a food supplement is an additional source. Further sources include swimming in bathing waters and the consumption of fish, mussels and vegetables from irrigated fields (WHO, 1999, Höger, Dissertation 2003).

Microcystin-LR intake from the consumption of AFA algae products

The daily dose recommended by the manufacturers is 1 to 3 g AFA algae. If it is assumed that an adult (60 kg) has a daily consumption of 2 g AFA algae that contains 1 µg microcystin-LR/g (Oregon guideline value), the PTDI recommended by the WHO of 0.04 µg/kg bw/day is reached. Other exposure paths (e.g. drinking water) are regarded as negligible. Some AFA algae products have been found to contain significantly higher microcystin concentrations of up to 33 µg/g (see Tab. 5), and the dose recommended by some manufacturers is more than 2 g/day, which means that it is possible to significantly exceed the provisionally tolerable daily intake (PTDI). Because food supplements may be consumed every day and over a long period, adverse effects on the health cannot be excluded. Children are at particular risk owing to the higher intake per kg body weight.

Microcystin-LR intake from drinking water

At present, it is not possible to give a reliable estimate of the exposure arising from drinking water on the basis of the available data. The drinking water guideline value recommend by the WHO of 1 µg microcystin-LR/L appears to be complied with in Germany and Switzerland, where the concentrations are generally below 0.4 µg/L. However, the literature data does not indicate how representative the measured values are (no information as to whether the data refers to peak values after algal blooms or average values) (Hoeger, 2003, Chorus, 2001). Adults (60 kg) who consume 2 L of drinking water per day that contains 0.4 µg microcystin-LR/L have an intake of 0.013 µg/kg bw/day, which is already 33% of the PTDI recommended by the WHO. This means that, under certain circumstances, drinking water is not an insignificant source of exposure.

Other routes of exposure, such as swimming in bathing waters, consumption of fish, seafood and vegetables from irrigated fields probably do not contribute to a noteworthy chronic microcystin intake.

7 ANALYSIS OF MICROCYSTIN-LR AND MICROCYSTINS

Microcystins can be detected by liquid chromatography methods, enzyme-linked immunosorbent assays (ELISA) and protein phosphatase inhibition assays (PPA) (Weller, 2002).

An ISO method (ISO/DIS 20179: 2004) has been developed to determine the microcystin-LR, -RR and -YR concentrations in drinking water. This method could be used to analyse algae and algae products after appropriate work-up. Methods for the detection of microcystins in algae products used as a food supplement have been described by e.g. Lawrence *et al*. (2001), Gilroy *et al.* (2000) and Codd *et al.* (2001; review). Lawrence *et al.* (2001) found good agreement between the results from immunochemical and chromatographic methods for different algae products (AFA, spirulina and non-identified cyanobacteria species) in the range of 0.5-35 µg microcystin/g algae product.

Chromatographic methods

Liquid chromatography methods coupled with UV/VIS and/or mass spectrometric detection have been developed and are in use. However, at present, reference substances are only available for a few microcystins, including microcystin-LR (Hummert *et al.*, 2000, Dahlmann *et al*., 2003). Useful methods for structural determination include nuclear magnetic resonance spectrometry, tandem mass spectrometry and determination of amino acids after acid hydrolysis (Weller, 2002).

Immunoassays

The advantages of immunochemical methods include high sensitivity, selectivity, speed and low costs. The ELISA test with the corresponding antibodies can be used to detect microcystin-LR (Chu *et al*., 1990, Gilroy, 2000), other microcystins (Yu *et al*., 2002) and, on account of the shared rare amino acid ADDA, the sum of all microcystins and nodularins (Fischer *et al*., 2001, An & Carmichael, 1994, Lawrence *et al*., 2001).

Inhibition test

PPA can be used as a general method to detect substances that inhibit the activities of protein phosphatase 1 (An & Carmichael, 1994) or 2A (Heresztyn & Nicholson, 2001). However, because not only microcystin-LR has an inhibitive effect, further microcystin congeners, nodularins and any other substances that the purified extracts may contain will also be included in the value.

8 ASSESSMENTS OF INTERNATIONAL COMMITTEES

8.1 DERIVATION OF THE TDI AND THE WHO DRINKING WATER GUIDELINE VALUE (WHO, 1998 UND 1999)

The WHO derived a provisionally tolerable daily intake (PTDI) value of 0.04 µg/kg bw/day for microcystin-LR, based on a NOAEL of 40 µg/kg bw/day obtained from a 13-week sub-chronic oral dosing study in mice (study by Fawell *et al.*, 1999). For this, the WHO used an uncertainty factor of 1000, composed of a factor of 10 for animal/human extrapolation, a factor of 10 for differences in sensitivities within the human population and a factor of 10 for the inadequate database on the toxicology of microcystin-LR (lack of chronic studies and carcinogenicity studies). At this point, there were only a few studies on the genotoxicity of microcystin-LR.

The WHO derived a further PTDI based on a LOAEL of 0.1 mg microcystin-LR equivalents/kg bw/day from a 44-day drinking water study of pigs (study by Falconer *et al.*, 1994). For this, the WHO used a safety factor of 1500, i.e. a factor of 3 for animal/human extrapolation (pigs are closer to humans physiologically than rodents), a factor of 10 for differences in sensitivities within the human population, a factor of 5 for the extrapolation from LOAEL to NOAEL (10 appeared to be unjustified owing to the lower incidence of the effects and the course of the dosage-response relationship) and a factor of 10 to account for the short study period. This gave a PTDI of 0.067 µg/kg bw/day.

The two PTDIs have the same order of magnitude. The lower value of 0.04 µg/kg bw/day was used to derive a provisional drinking water guideline value.

Drinking water guideline value (WHO, 1998 und 1999)

Based on a PTDI of 0.04 µg/kg bw/day, a daily drinking water intake of 2 L per person (60 kg bw) and the supposition that 80% of the microcystin uptake is from drinking water, the WHO recommends a drinking water guideline value for microcystin-LR of 1 µg/L (free and cell-bound forms).

Drinking water guideline value = (0.04 µg/kg bw x 60 kg x 0.8)/2L = 0.96 µg/L (rounded to: 1 µg/L)

There was insufficient data available for all other microcystins so that no drinking water guideline values could be derived for these substances.

8.2 FURTHER RISK ASSESSMENTS AND GUIDELINE VALUES

TDI

The risk assessments by Kuiper-Goodman (Kuiper-Goodman, 1997) (Health Canada, Food Directorate) and Duy *et al.* (Duy *et al*, 2000) for microcystin-LR were based, like those of the WHO, on a NOAEL of 40 µg/kg bw/day obtained from oral 13-week studies in mice. Kuiper-Goodman und Duy *et al.* propose a PTDI of 0.013 µg/kg bw/day on the basis of an uncertainty factor of 3000. This uncertainty factor is composed of a factor of 100 for intra- and interspecies variations, a factor of 10 to account for the short study period and an additional factor of 3 to account for the tumour-promoting effect and the weak evidence for a possible carcinogenic effect in humans.

The legal maximum value for microcystin in food supplements based on algae in Oregon

In 1997, the Oregon Health Division and the Oregon Department of Agriculture introduced a statutory maximum concentration for adults of 1 µg microcystin/g in blue-green algae (BGA) products. This applies to products sold in Oregon and is based on a PTDI of 0.04 µg/kg bw/day of the WHO, a daily consumption of 2 g algae products and a body weight of 60 kg (Gilroy *et al*, 2000).

9 REFERENCES

An, J., and Carmichael, W.W (1994) Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. *Toxicon.* 32 (12):1495-1507.

Batista,T., de Sousa,G., Suput,J.S., Rahmani,R. & Suput,D. (2003) Microcystin-LR causes the collapse of actin filaments in primary human hepatocytes. *Aquat.Toxicol*, **65**, 85-91.

BgVV (2001) AFA Algen und AFA Algenprodukte, Opinion from 23.09.2001.

BgVV (2002) BGVV und BfArM warnen: Nahrungsergänzungsmittel aus AFA-Algen können keine medizinische Therapie ersetzen. Joint press release, 21.03.2002.

Boe,R., Gjertsen,B.T., Vintermyr,O.K., Houge,G., Lanotte,M. & Doskeland,S.O. (1991) The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. *Exp.Cell Res*, **195**, 237-246.

Bouaicha,N. & Maatouk,I. (2004) Microcystin-LR and nodularin induce intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation in primary cultured rat hepatocytes. *Toxicol Lett.*, **148**, 53-63.

Brooks,W.P. & Codd,G.A. (1987) Distribution of Microcystis aeruginosa peptide toxin and interactions with hepatic microsomes in mice. *Pharmacol.Toxicol*, **60**, 187-191.

Bulera,S.J., Eddy,S.M., Ferguson,E., Jatkoe,T.A., Reindel,J.F., Bleavins,M.R. & De La Iglesia,F.A. (2001) RNA expression in the early characterization of hepatotoxicants in Wistar rats by high-density DNA microarrays. *Hepatology*, **33**, 1239-1258.

Bundesamt für Gesundheit (BAG) (2003), internal report

Carmichael,W.W., Drapeau,C. & Anderson,D.M. (2000) Harvesting of Aphanizomenon flos-quae Ralfs ex Born. & Flah. var. flos-aquae (Cyanobacteria) from Klamath Lake for human dietary use. *J.Appl.Phycol.*, **12**, 585-595.

Chernoff,N., Hunter,E.S., III, Hall,L.L., Rosen,M.B., Brownie,C.F., Malarkey,D., Marr,M. & Herkovits,J. (2002) Lack of teratogenicity of microcystin-LR in the mouse and toad. *J.Appl.Toxicol.*, **22**, 13-17.

Chorus, I. ed. (2001) Cyanotoxins. Occurrence, Causes and Consequences. Springer, Berlin.

Chu, F.S., Huang, X., and Wei R.D. (1990) Enzyme-linked immunosorbent assay for microcystins in blue-green algal blooms. *J Assoc.Off Anal.Chem.* 73 (3):451-456.

Codd, G.A., Metcalf, J.S., Ward, C.J.,Beattie, K.A., and Bell, S.G. (2001) Analysis of Cyanobacterial Toxins by Physicochemical and Biochemical Methods. J Assoc Off Anal Chem. 84 (5):1626-1635.

Dahlem,A.M., Hassan,A.S., Swanson,S.P., Carmichael,W.W. & Beasley,V.R. (1989) A model system for studying the bioavailability of intestinally administered microcystin-LR, a hepatotoxic peptide from the cyanobacterium Microcystis aeruginosa. *Pharmacol.Toxicol*, **64**, 177-181.

Dahlmann J., Budakowski W.R, and Luckas B. (2003) Liquid chromatography-electrospray ionization-mass spectrometry based method for the simultaneous determination of algal and cyanobacterial toxins in phytoplankton from marine waters and lakes followed by tentative structural elucidation of microcystins. J Chromatography A. 994: 45-57.

Dietrich,D. & Hoeger,S. (2005) Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? *Toxicol Appl.Pharmacol*, **203**, 273-289.

Dietrich, D. (2004) University of Konstanz, Environmental Toxicology Workgroup, Germany. Personal communications.

Ding,W.X. & Nam,O.C. (2003) Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol.Lett.*, **220**, 1-7.

Ding,W.X., Shen,H.M., Zhu,H.G., Lee,B.L. & Ong,C.N. (1999) Genotoxicity of microcystic cyanobacteria extract of a water source in China. *Mutat.Res*, **442**, 69-77.

Duy,T.N., Lam,P.K., Shaw,G.R. & Connell,D.W. (2000) Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. *Rev.Environ Contam Toxicol*, **163:113-85.**, 113-185.

Falconer,I.R. (1991) Tumor promotion and liver injury caused by oral consumption of cyanobacteria. *Environ Toxicol Water*, **6**, 177-184.

Falconer,I.R., Burch,M.D., Steffensen,D.A., Choice,M. & Coverdale,O.R. (1994) Toxicity of the blue-green alga (cyanobacterium) Microcystis aeruginosa in drinking water to growing pigs, as an animal model for human injury and risk assessment. *J.Environ.Toxicol.Water Qual.*, **9**, 131-139.

Falconer,I.R. & Humpage,A.R. (1996) Tumour promotion by cyanobacterial toxins. *Phycologia*, **35**, 74-79.

Falconer,I.R., Smith,J.V., Jackson,A.R., Jones,A. & Runnegar,M.T. (1988) Oral toxicity of a bloom of the Cyanobacterium microcystis Aeruginosa administered to mice over periods up to 1 year. *J.Toxicol.Environ.Health*, **24**, 291-305.

Fawell,J.K., Mitchell,R.E., Everett,D.J. & Hill,R.E. (1999) The toxicity of cyanobacterial toxins in the mouse: I microcystin-LR. *Hum.Exp.Toxicol.*, **18**, 162-167.

Fischer,W.J., Altheimer,S., Cattori,V., Meier,P.J., Dietrich,D.R. & Hagenbuch,B. (2005) Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. *Toxicol Appl.Pharmacol*, **203**, 257-263.

Fischer,W.J., Hagenbuch,B., Cattori,V., Meier,P.J. & Dietrich,D.R. (1999) The cyclic heptapeptide microcystin, a cyanobacterial toxin, is transported by the human OATP. *Hepatology*, **30**, 465A

Fischer, W.J., Garthwaite, I., Miles, C.O., Ross, K.M., Aggen, J.B., Chamberlin, A.R., Towers, N.R., and Dietrich, D.R. (2001) Congener-independent immunoassay for microcystins and nodularins. *Environ Sci Technol.* 35 (24):4849-4856.

Fladmark,K.E., Brustugun,O.T., Hovland,R., Boe,R., Gjertsen,B.T., Zhivotovsky,B. & Doskeland,S.O. (1999) Ultrarapid caspase-3 dependent apoptosis induction by serine/threonine phosphatase inhibitors. *Cell Death.Differ.*, **6**, 1099-1108.

Fladmark,K.E., Brustugun,O.T., Mellgren,G., Krakstad,C., Boe,R., Vintermyr,O.K., Schulman,H. & Doskeland,S.O. (2002) Ca2+/calmodulin-dependent protein kinase II is required for microcystin-induced apoptosis. *J.Biol.Chem.*, **277**, 2804-2811.

Frangez,R., Zuzek,M.C., Mrkun,J., Suput,D., Sedmak,B. & Kosec,M. (2003) Microcystin-LR affects cytoskeleton and morphology of rabbit primary whole embryo cultured cells in vitro. *Toxicon*, **41**, 999-1005.

Gehringer,M.M. (2004) Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. *FEBS Lett.*, **557**, 1-8.

Gilroy,D.J., Kauffman,K.W., Hall,R.A., Huang,X. & Chu,F.S. (2000) Assessing potential health risks from microcystin toxins in blue-green algae dietary supplements. *Environ Health Perspect*, **108**, 435-439.

Gulledge,B.M., Aggen,J.B. & Chamberlin,A.R. (2003a) Linearized and truncated microcystin analogues as inhibitors of protein phosphatases 1 and 2A. *Bioorg.Med.Chem.Lett.*, **13**, 2903-2906.

Gulledge,B.M., Aggen,J.B., Eng,H., Sweimeh,K. & Chamberlin,A.R. (2003b) Microcystin analogues comprised only of Adda and a single additional amino acid retain moderate activity as PP1/PP2A inhibitors. *Bioorg.Med.Chem.Lett.*, **13**, 2907-2911.

Gupta,N., Pant,S.C., Vijayaraghavan,R. & Rao,P.V. (2003) Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. *Toxicology*, **188**, 285-296.

Guzman,R.E. & Solter,P.F. (1999) Hepatic oxidative stress following prolonged sublethal microcystin LR exposure. *Toxicol Pathol.*, **27**, 582-588.

Guzman,R.E., Solter,P.F. & Runnegar,M.T. (2003) Inhibition of nuclear protein phosphatase activity in mouse hepatocytes by the cyanobacterial toxin microcystin-LR. *Toxicon*, **41**, 773-781.

Hamm-Alvarez,S.F., Wei,X., Berndt,N. & Runnegar,M. (1996) Protein phosphatases independently regulate vesicle movement and microtubule subpopulations in hepatocytes. *Am.J.Physiol*, **271**, C929-C943

Hathaway, R. A. (2001) Mussels as biomonitors of lake water microcystin: A final report for the summer 2000 microcystin. Monitoring study.

Health Canada online (14-9-2001) Blue-green algae (cyanobacteria) and their toxins. Internet .

Heresztyn, T. and Nicholson, B.C. (2001) Determination of cyanobacterial hepatotoxins directly in water using a protein phosphatase inhibition assay. *Water Res.* 35 (13):3049-3056.

Hermansky,S.J., Stohs,S.J., Eldeen,Z.M., Roche,V.F. & Mereish,K.A. (1991) Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. *J.Appl.Toxicol*, **11**, 65-73.

Hitzfeld,B.C., Hoger,S.J. & Dietrich,D.R. (2000) Cyanobacterial toxins: removal during drinking water treatment, and human risk assessment. *Environ Health Perspect*, **108 Suppl 1:113-22.**, 113-122.

Hoeger, S. J. (2003) Problems during drinking water treatment of cyanobacterial-loaded surface waters: Consequences for human health. Dissertation, University of Konstanz, Department of Biology.

Hoeger, S. J., Dietrich, D. R., and Hitzfeld, B. C. (2000) Microcystin-LR Ozonation by-products: Chemical and Toxicological Characterization. 39th annual meeting of the Society of Toxicology, Philadelphia, PA, USA, March 2000.

Hoeger,S.J., Dietrich,D.R. & Hitzfeld,B.C. (2002) Effect of ozonation on the removal of cyanobacterial toxins during drinking water treatment. *Environ Health Perspect*, **110**, 1127-1132.

Hoeger,S.J., Hitzfeld,B.C. & Dietrich,D.R. (2005) Occurrence and elimination of cyanobacterial toxins in drinking water treatment plants. *Toxicol Appl.Pharmacol*, **203**, 231-242.

Honkanen,R.E., Codispoti,B.A., Tse,K., Boynton,A.L. & Honkanan,R.E. (1994) Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. *Toxicon*, **32**, 339-350.

Hummert C., Reichelt M., and Luckas B. (2000) New strategy for the determination of microcystins and diarrhetic shellfish poisoning (DSP) toxins, two potent phosphatase 1 and 2A inhibitors and tumor promoters. Fresenius J Anal Chem. 366: 508-513.

Humpage,A.R. & Falconer,I.R. (1999) Microcystin-LR and liver tumor promotion: Effects on cytokinesis, ploidy, and apoptosis in cultured hepatocytes. *Environ Toxicol*, **14**, 61-75.

Humpage,A.R., Hardy,S.J., Moore,E.J., Froscio,S.M. & Falconer,I.R. (2000) Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. *J.Toxicol.Environ.Health A*, **61**, 155-165.

ISO/DIS 20179: 2004, Water quality - Determination of microcystins - Method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

Ito,E., Kondo,F. & Harada,K. (1997a) Hepatic necrosis in aged mice by oral administration of microcystin-LR. *Toxicon*, **35**, 231-239.

Ito,E., Kondo,F. & Harada,K. (2000) First report on the distribution of orally administered microcystin-LR in mouse tissue using an immunostaining method. *Toxicon*, **38**, 37-48.

Ito,E., Kondo,F., Terao,K. & Harada,K. (1997b) Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. *Toxicon*, **35**, 1453-1457.

Khan,S.A., Wickstrom,M.L., Haschek,W.M., Schaeffer,D.J., Ghosh,S. & Beasley,V.R. (1996) Microcystin-LR and kinetics of cytoskeletal reorganization in hepatocytes, kidney cells, and fibroblasts. *Natural Toxins*, **4**, 206-214.

Kondo,F., Matsumoto,H., Yamada,S., Ishikawa,N., Ito,E., Nagata,S., Ueno,Y., Suzuki,M. & Harada,K. (1996) Detection and identification of metabolites of microcystins formed in vivo in mouse and rat livers. *Chem.Res.Toxicol.*, **9**, 1355-1359.

Kondo,F., Oka,H., Okumura M., Ishikawa,N., Harada,K., Matsuura,K., Murata,H. & Suzuki,M. (1992) Formation, characterization, and toxicity of the glutathione and cysteine conjugates of toxic heptapeptide microcystins. *Chem.Res.Toxicol.*, **5**, 591-596.

Kuiper-Goodman,T. (1997) Risk assessment of microcystins in Canada. *WaBoLu-Hefte*, **4**, 9-12.

Lankoff,A., Banasik,A., Obe,G., Deperas,M., Kuzminski,K., Tarczynska,M., Jurczak,T. & Wojcik,A. (2003) Effect of microcystin-LR and cyanobacterial extract from Polish reservoir of drinking water on cell cycle progression, mitotic spindle, and apoptosis in CHO-K1 cells. *Toxicol Appl.Pharmacol.*, **189**, 204-213.

Lankoff,A., Krzowski,L., Glab,J., Banasik,A., Lisowska,H., Kuszewski,T., Gozdz,S. & Wojcik,A. (2004) DNA damage and repair in human peripheral blood lymphocytes following treatment with microcystin-LR. *Mutat.Res*, **559**, 131-142.

Lawrence,J.F., Niedzwiadek,B., Menard,C., Lau,B.P., Lewis,D., Kuper-Goodman,T., Carbone,S. & Holmes,C. (2001) Comparison of liquid chromatography/mass spectrometry, ELISA, and phosphatase assay for the determination of microcystins in blue-green algae products. *J.AOAC Int.*, **84**, 1035-1044.

Maatouk,I., Bouaicha,N., Plessis,M.J. & Perin,F. (2004) Detection by (32)P-postlabelling of 8-oxo-7,8-dihydro-2'-deoxyguanosine in DNA as biomarker of microcystin-LR- and nodularin-induced DNA damage in vitro in primary cultured rat hepatocytes and in vivo in rat liver. *Mutat.Res*, **564**, 9-20.

MacKintosh,C., Beattie,K.A., Klumpp,S., Cohen,P. & Codd,G.A. (1990) Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.*, **264**, 187-192.

MacKintosh,R.W., Dalby,K.N., Campbell,D.G., Cohen,P.T., Cohen,P. & MacKintosh,C. (1995) The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. *FEBS Lett.*, **371**, 236-240.

Magalhaes,V.F., Marinho,M.M., Domingos,P., Oliveira,A.C., Costa,S.M., Azevedo,L.O. & Azevedo,S.M. (2003) Microcystins (cyanobacteria hepatotoxins) bioaccumulation in fish and crustaceans from Sepetiba Bay (Brasil, RJ). *Toxicon*, **42**, 289-295.

Mankiewicz,J., Tarczynska,M., Fladmark,K.E., Doskeland,S.O., Walter,Z. & Zalewski,M. (2001) Apoptotic effect of cyanobacterial extract on rat hepatocytes and human lymphocytes. *Environ Toxicol*, **16**, 225-233.

Mankiewicz,J., Walter,Z., Tarczynska,M., Palyvoda,O., Wojtysiak-Staniaszczyk,M. & Zalewski,M. (2002) Genotoxicity of cyanobacterial extracts containing microcystins from Polish water reservoirs as determined by SOS chromotest and comet assay. *Environ Toxicol*, **17**, 341-350.

Matsushima,R., Yoshizawa,S., Watanabe,M.F., Harada,K., Furusawa,M., Carmichael,W.W. & Fujiki,H. (1990) In vitro and in vivo effects of protein phosphatase inhibitors,

microcystins and nodularin, on mouse skin and fibroblasts. *Biochem.Biophys.Res.Commun.*, **171**, 867-874.

McDermott,C.M., Nho,C.W., Howard,W. & Holton,B. (1998) The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. *Toxicon*, **36**, 1981-1996.

Mellgren,G., Vintermyr,O.K., Boe,R. & Doskeland,S.O. (1993) Hepatocyte DNA replication is abolished by inhibitors selecting protein phosphatase 2A rather than phosphatase 1. *Exp.Cell Res*, **205**, 293-301.

Mikhailov,A., Harmala-Brasken,A.S., Hellman,J., Meriluoto,J. & Eriksson,J.E. (2003) Identification of ATP-synthase as a novel intracellular target for microcystin-LR. *Chem.Biol.Interact.*, **142**, 223-237.

Milutinovic,A., Zivin,M., Zorc-Pleskovic,R., Sedmak,B. & Suput,D. (2003) Nephrotoxic effects of chronic administration of microcystins -LR and -YR. *Toxicon*, **42**, 281-288.

Miura,G.A., Robinson,N.A., Lawrence,W.B. & Pace,J.G. (1991) Hepatotoxicity of microcystin-LR in fed and fasted rats. *Toxicon*, **29**, 337-346.

Mohamed,Z.A., Carmichael,W.W. & Hussein,A.A. (2003) Estimation of microcystins in the freshwater fish Oreochromis niloticus in an Egyptian fish farm containing a Microcystis bloom. *Environ Toxicol*, **18**, 137-141.

Nishiwaki-Matsushima,R., Ohta,T., Nishiwaki,S., Suganuma,M., Kohyama,K., Ishikawa,T., Carmichael,W.W. & Fujiki,H. (1992) Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *J.Cancer Res.Clin.Oncol.*, **118**, 420-424.

Ohta,T., Sueoka,E., Iida,N., Komori,A., Suganuma,M., Nishiwaki,R., Tatematsu,M., Kim,S.J., Carmichael,W.W. & Fujiki,H. (1994) Nodularin, a potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver. *Cancer Res.*, **54**, 6402-6406.

Orr,P.T., Jones,G.J., Hunter,R.A. & Berger,K. (2003) Exposure of beef cattle to sub-clinical doses of Microcystis aeruginosa: toxin bioaccumulation, physiological effects and human health risk assessment. *Toxicon*, **41**, 613-620.

Orr,P.T., Jones,G.J., Hunter,R.A., Berger,K., De Paoli,D.A. & Orr,C.L. (2001) Ingestion of toxic Microcystis aeruginosa by dairy cattle and the implications for microcystin contamination of milk. *Toxicon*, **39**, 1847-1854.

Pouria,S., de Andrade,A., Barbosa,J., Cavalcanti,R.L., Barreto,V.T., Ward,C.J., Preiser,W., Poon,G.K., Neild,G.H. & Codd,G.A. (1998) Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet*, **352**, 21-26.

Rao,P.V. & Bhattacharya,R. (1996) The cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver in vivo. *Toxicology*, **114**, 29-36.

Rao,P.V., Bhattacharya,R., Pant,S.C. & Bhaskar,A.S. (1995) Toxicity evaluation of in vitro cultures of freshwater cyanobacterium Microcystis aeruginosa: I. Hepatotoxic and histopathological effects in rats. *Biomed.Environ Sci.*, **8**, 254-264.

Rao,P.V., Bhattacharya,R., Parida,M.M., Jana,A.M. & Bhaskar,A.S. (1998) Freshwater cyanobacterium *Microcystis aeruginosa* (UTEX 2385) induced DNA damage *in vivo* and *in vitro*. *Environmental Toxicology and Pharmacology*, **5**, 1-6.

Repavich,W.M., Sonzogni,W.C., Standridge,J.H., Wedepohl,R.E. & Meisner,L.F. (1990) Cyanobacteria (blue-green algae) in Wisconsin (USA) waters: Acute and chronic toxicity. *WATER RES*, **24**, 225-232.

Robinson,N.A., Pace,J.G., Matson,C.F., Miura,G.A. & Lawrence,W.B. (1991) Tissue distribution, excretion and hepatic biotransformation of microcystin-LR in mice. *J.Pharmacol.Exp.Ther.*, **256**, 176-182.

Runnegar,M., Berndt,N., Kong,S.M., Lee,E.Y. & Zhang,L. (1995) In vivo and in vitro binding of microcystin to protein phosphatases 1 and 2A. *Biochem.Biophys.Res Commun.*, **216**, 162-169.

Runnegar,M. & Falconer,I.R. (1982) The *in vivo* and *in vitro* Biological Effects of the Peptide Hepatotoxin from the Blue-green Alga *Microcystis aeruginosa*. *S Afr J Sci*, **78**, 363-366.

Runnegar,M.T., Kong,S. & Berndt,N. (1993) Protein phosphatase inhibition and in vivo hepatotoxicity of microcystins. *Am.J.Physiol*, **265**, G224-G230

Sano,T., Takagi,H., Sadakane,K., Ichinose,T., Kawazato,H. & Kaya,K. (2004) Carcinogenic effects of microcystin-LR and Dhb-microcystin-LR on mice liver. *Sixth International Conference on Toxic Cyanobacteria, Bergen, Norway, pp.59.Abstract*,

Schaeffer,D.J., Malpas,P.B. & Barton,L.L. (1999) Risk assessment of microcystin in dietary Aphanizomenon flos-aquae. *Ecotoxicol.Environ.Saf*, **44**, 73-80.

Sekijima,M., Tsutsumi,T., Yoshida,T., Harada,T., Tashiro,F., Chen,G., Yu,S.Z. & Ueno,Y. (1999) Enhancement of glutathione S-transferase placental-form positive liver cell foci development by microcystin-LR in aflatoxin B1-initiated rats. *Carcinogenesis*, **20**, 161-165.

Shen,P.P., Zhao,S.W., Zheng,W.J., Hua,Z.C., Shi,Q. & Liu,Z.T. (2003) Effects of cyanobacteria bloom extract on some parameters of immune function in mice. *Toxicol Lett.*, **143**, 27-36.

SKLM (2003) Opinion of the DFG Senate Commission on Food Safety (SKLM) on Algal Toxins.

Solter,P., Liu,Z. & Guzman,R. (2000) Decreased hepatic ALT synthesis is an outcome of subchronic microcystin-LR toxicity. *Toxicol Appl.Pharmacol.*, **164**, 216-220.

Solter,P.F., Wollenberg,G.K., Huang,X., Chu,F.S. & Runnegar,M.T. (1998) Prolonged sublethal exposure to the protein phosphatase inhibitor microcystin-LR results in multiple dose-dependent hepatotoxic effects. *Toxicol Sci.*, **44**, 87-96.

Stotts,R.R., Namikoshi,M., Haschek,W.M., Rinehart,K.L., Carmichael,W.W., Dahlem,A.M. & Beasley,V.R. (1993) Structural modifications imparting reduced toxicity in microcystins from Microcystis spp. *Toxicon*, **31**, 783-789.

Suzuki,H., Watanabe,M.F., Wu,Y., Sugita,T., Kita,K., Sato,T., Wang,X., Tanzawa,H., Sekiya,S. & Suzuki,N. (1998) Mutagenicity of microcystin-LR in human RSa cells. *Int.J.Mol.Med.*, **2**, 109-112.

Toivola,D.M., Goldman,R.D., Garrod,D.R. & Eriksson,J.E. (1997) Protein phosphatases maintain the organization and structural interactions of hepatic keratin intermediate filaments. *J.Cell Sci.*, **110**, 23-33.

Towner,R.A., Sturgeon,S.A. & Hore,K.E. (2002) Assessment of in vivo oxidative lipid metabolism following acute microcystin-LR-induced hepatotoxicity in rats. *Free Radic.Res*, **36**, 63-71.

Tsuji,K., Watanuki,T., Kondo,F., Watanabe,M.F., Nakazawa,H., Suzuki,M., Uchida,H. & Harada,K. (1997) Stability of microcystins from cyanobacteria--IV. Effect of chlorination on decomposition. *Toxicon*, **35**, 1033-1041.

Tsuji,K., Watanuki,T., Kondo,F., Watanabe,M.F., Suzuki,S., Nakazawa,H., Suzuki,M., Uchida,H. & Harada,K.I. (1995) Stability of microcystins from cyanobacteria--II. Effect of UV light on decomposition and isomerization. *Toxicon*, **33**, 1619-1631.

Vasconcelos,V.M. (1999) Cyanobacterial toxins in Portugal: effects on aquatic animals and risk for human health. *Braz.J.Med.Biol.Res*, **32**, 249-254.

Wang,H.B. & Zhu,H.G. (1996) Promoting activity of microcystins extracted from waterblooms in SHE cell transformation assay. *Biomed.Environ Sci.*, **9**, 46-51.

Weller,G (2002): Algengifte im Wasser, *Nachrichten aus der Chemie*, 50, June 2002.

WHO (1998) Guidelines for Drinking-Water Quality. Second edition, Addendum to Volume 2, Health Criteria and Other Supporting Information. World Health Organization, Geneva.

WHO (1999) Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management.

Wickstrom,M.L., Khan,S.A., Haschek,W.M., Wyman,J.F., Eriksson,J.E., Schaeffer,D.J. & Beasley,V.R. (1995) Alterations in microtubules, intermediate filaments, and microfilaments induced by microcystin-LR in cultured cells. *Toxicol Pathol.*, **23**, 326-337.

Wolf, H. U. and Frank, C. (2002) Häufigkeit, Ursachen, toxikologische Relevanz sowie Massnahmen zur Abwehr und Reduzierung von Massenentwicklungen toxikologischer Cyanobakterien (Blaualgen) in Badegewässern zum Schutz von Badenden. Research report FZKA-BWPLUS, University Clinic of Ulm (Germany), Pharmacology and Toxicology Department.

Yoshida,T., Makita,Y., Tsutsumi,T., Nagata,S., Tashiro,F., Yoshida,F., Sekijima,M., Tamura,S., Harada,T., Maita,K. & Ueno,Y. (1998) Immunohistochemical localization of microcystin-LR in the liver of mice: a study on the pathogenesis of microcystin-LR-induced hepatotoxicity. *Toxicol Pathol.*, **26**, 411-418.

Yu, F.Y., Liu, B.H., Chou, H.N. and Chu, F.S. (2002) Development of a sensitive ELISA for the determination of microcystins in algae. *J Agric.Food Chem.* 50 (15):4176-4182.

Yu,S.Z., Huang,X.E., Koide,T., Cheng,G., Chen,G.C., Harada,K., Ueno,Y., Sueoka,E., Oda,H., Tashiro,F., Mizokami,M., Ohno,T., Xiang,J. & Tokudome,S. (2002) Hepatitis B and C viruses infection, lifestyle and genetic polymorphisms as risk factors for hepatocellular carcinoma in Haimen, China. *Jpn.J.Cancer Res*, **93**, 1287-1292.

Zegura,B., Lah,T.T. & Filipic,M. (2004) The role of reactive oxygen species in microcystin-LR-induced DNA damage. *Toxicology*, **200**, 59-68.

Zegura,B., Sedmak,B. & Filipic,M. (2003) Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. *Toxicon*, **41**, 41-48.

Zhan,L., Sakamoto,H., Sakuraba,M., Wu,d.S., Zhang,L.S., Suzuki,T., Hayashi,M. & Honma,M. (2004) Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells. *Mutat.Res*, **557**, 1-6.

Zhao,J.M. & Zhu,H.G. (2003) [Effects of microcystins on cell cycle and expressions of c-fos and c-jun]. *Zhonghua Yu Fang Yi.Xue.Za Zhi.*, **37**, 23-25.

Zhou,L., Yu,H. & Chen,K. (2002) Relationship between microcystin in drinking water and colorectal cancer. *Biomed.Environ Sci.*, **15**, 166-171.

Abbreviations:

ADDA	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ADI	acceptable daily intake
AFA	Aphanizomenon flos aquae
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
DEN	diethylnitrosamine
ELISA	enzyme-linked immunosorbent assay
GST-P	glutathione S-transferase placental-form positive liver cell foci
HPLC	high-pressure liquid chromatography
i.p.	intraperitoneal
bw	body weight
LD_{50}	lethal dose
LOAEL	lowest observed adverse effect level
NOAEL	no observed adverse effect level
PPA	protein phosphatase inhibition assay
PTDI	provisionally tolerable daily intake
ROS	reactive oxygen species
WHO	World Health Organization