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High grazer toxicity of [D-Asp^3,(*E*)-Dhb^7]microcystin-RR of *Planktothrix rubescens* as compared to different microcystins

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Abstract

Planktothrix rubescens, the dominant cyanobacterium in Lake Zürich, is generally considered to be toxic to zooplankton. The major toxin was determined by NMR spectroscopy and chemical analysis to be [D-Asp^3,(*E*)-Dhb^7]microcystin-RR. The compound was isolated in high purity, and its 24-h acute grazer toxicity was compared with microcystin-LR, microcystin-RR, microcystin-YR, and nodularin using a *Thamnocephalus platyurus* bioassay. Based on LC_{50} values [D-Asp^3,(*E*)-Dhb^7]microcystin-RR was the most toxic microcystin tested. Nodularin was slightly more toxic under the conditions of the assay. The large number of individuals available for the grazer bioassay allowed the determination of dose-response curves of the different microcystins. These curves showed marked differences in their steepness. Microcystin-RR, which had nearly the same LC_{50} as microcystin-LR and microcystin-YR, exhibited a very flat dose-response curve. This flat curve indicates that, for some individuals, lower concentrations of this microcystin are much more toxic than are the other two microcystins. Mortality of 100% requires much higher concentrations of microcystin-RR, indicating the resistance of some animals to the toxin. The purified [D-Asp^3,(*E*)-Dhb^7]microcystin-RR exhibited a higher molar absorption coefficient determined by quantitative amino acid analysis than the coefficients generally used for other microcystins. This observation has consequences for the risk assessment for microcystins and makes a structural determination of microcystins an absolute requirement. The presence of the dehydrobutyrine residue may be the reason for the higher specific toxicity of [D-Asp^3,(*E*)-Dhb^7]microcystin-RR when compared to the N-methyldehydroalanine-containing microcystins. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Planktothrix rubescens; [D-Asp^3,(*E*)-Dhb^7]microcystin-RR; Molar absorption coefficient; *Thamnocephalus platyurus* bioassay

1. Introduction

Planktothrix rubescens is a filamentous cyanobacterium that is frequently found in natural prealpine lakes that are used extensively for drinking water supply. Throughout Europe, *P. rubescens* is distributed from the south (Sardinia, Italy, Bruno et al., 1992) to the north (Norway, Codd et al., 1984) and has been observed in many reservoirs. As with other toxic cyanobacteria, *P. rubescens* can accumulate to very high densities in these lakes. The collapse of such blooms liberates high amounts of toxins into the water. Assessing the public health risk caused by toxic cyanobacteria is complex and requires knowledge of specific toxicities of the individual compounds responsible for that risk. Monitoring water bodies and supply systems for cyanobacteria and cyanotoxins is not yet a common practice in all countries of the world. Provisional guideline values for microcystin-LR (MC–LR) were suggested by Bell and Codd (1994) at 1 $\mu g\, l^{-1}$; this value was later accepted by the WHO (WHO, 1998). However, Ueno et al. (1996) proposed a guideline value of 0.01 $\mu g\, l^{-1}$ based on the possible correlation between primary liver cancer and the presence of microcystins in ponds, rivers and shallow lakes in certain areas of China.

Beginning in the mid-20th century, intra peritoneal (i.p.) administration of samples to rabbits and mice was used to trace toxicity in natural cyanobacterial blooms and isolated fractions, and an i.p. mouse assay was established to quantify the toxicity (Bishop et al., 1959). However, ethical concerns restrict the application of this assay, and the

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number of mice used to determine toxicity is now generally minimized (usually three mice per dose). Alternative bioassays were introduced in response to these ethical concerns. The alternative bioassays include: *Artemia salina* (Kiviranta et al., 1991; Kiviranta and Abdel-Hameed, 1994; Feuillade et al., 1996), *Aedes aegypti* (Kiviranta and Abdel-Hameed, 1994), different species of *Daphnia* (DeMott et al., 1991; Rohrlack et al., 1999), *Tetrahymena pyriformis* (Ward and Codd, 1999), *Danio rerio* (Oberemm et al., 1997; Wiegand et al., 1999) and *Thamnocephalus platyurus* (Todorova and Jüttner, 1996; Kurmayer and Jüttner, 1999). The use of a large number of individuals in these bioassays allows robust statistical analysis. It is now believed that microcystins in natural ecosystems control grazer activity and act as a protection mechanism for cyanobacteria. This functional utility supports the validity of using aquatic invertebrates as sensitive bioassay organisms. *T. platyurus*, an anostracan crustacean, that occurs in ephimeric puddles and ditches, is particularly suitable as a test organism, because it has not developed resistance against microcystins, which are not found in these habitats. This contrasts with *Daphnia* and other grazers, which live in environments in which microcystin-producing cyanobacteria are frequently found and which therefore developed partial resistence against these cyclic peptides (Kurmayer and Jüttner, 1999). The aim of this study was to determine the specific toxicity of different, widely-distributed microcystins using the robust data of the 24-h acute toxicity assay of *T. platyurus* and to rank the specific toxicity of the major microcystin of *P. rubescens* among these microcystins.

2. Material and methods

2.1. Cyanobacterial biomass

The red-coloured filamentous cyanobacterium *P. rubescens* (BC 9307, Bristol Collection) isolated from Lake Zürich, Switzerland, in 1993 by A. E. Walsby was grown in Erlenmeyer flasks on a medium as described by Jüttner et al. (1983) at 20°C and 5 μmol quanta m^{-2} s^{-1} from a fluorescent tube. This strain was made axenic by extensive washings with 1–2 l of sterile 50% diluted cyanobacterial medium on membranes of cellulose acetate (8.0 μm pore size, Schleicher & Schuell, Dassel, Germany) and subsequent transfer to agarose dishes (6 cm diameter; 0.8% low melt agarose [No. A-9045, Sigma] in 50% cyanobacterial medium) which were incubated under the same environmental conditions as the liquid cultures. Naturally grown *Planktothrix* collected with a plankton net (95 μm mesh size) from Lake Zürich during blooms in the autumn 1997 and 1999 were nearly free of other phytoplankton organisms.

2.2. Purification of toxins

For the isolation and purification of microcystin from *P. rubescens*, the frozen cyanobacterial biomass was extracted with 60% MeOH for 20 h in the dark. After centrifugation for 15 min at 25,700 × *g*, the pellet was again extracted with 60% MeOH. The supernatants were combined and brought to dryness in a vacuum rotary evaporator at 40°C. The residue was extracted with 2 ml 60% MeOH and separated into four fractions on Bond-Elut C-18 cartridges (Varian, USA) by sequential elution with 30, 50, 80 and 100% aqueous MeOH. The 80% fraction contained the major amount of microcystins. HPLC purification was performed in accordance with Lawton et al. (1994), Lawton et al. (1995) and Krishnamurthy et al. (1986) on a Shimadzu 10AVP system with a photodiode array detector (PDA) and a C-18 Grom-Sil 120 ODS-4 HE reversed phase column (4.6 × 250 mm, 5 μm particle size, Stagroma, Germany). The flow rate was 1 ml/min. Two different solvent gradients were used. For identification of microcystins, solvent A was composed of UV-treated deionized H_2O and 0.05% trifluoroacetic acid (TFA; Fluka, Switzerland) and solvent B was acetonitrile and 0.05% TFA. To obtain purified microcystins that were free of isomerization products, solvent A and solvent B were used free of TFA. In both cases a linear increase in three steps was applied (solvent B from 30 to 35% in 10 min, 35 to 70% in 30 min, 70 to 100% in 2 min, isocratic 8 min).

2.3. Amino acid analysis and determination of the molar absorption coefficient

The amino acid composition of the microcystins was determined using a combined gas chromatograph-mass spectrometer (GC–MS; Fison Instruments, GC 8000 Top, MD 800). Separations were performed on a DB 1301 capillary column (30 m, 0.32 mm i.d., film thickness 0.25 μm; J & W Scientific).

The purified microcystin (100 μg) was hydrolyzed in 6 M HCl at 110°C for 48 h. For GC analysis the hydrolysate was esterified with 3 M HCl in methanol (Merck, Germany) and subsequently trifluoroacetylated with trifluoroacetic acid anhydride (TFAA; Fluka, Switzerland). Arginine was determined by GC–MS after conversion of the guanidino group into the dimethylpyrimidyl derivative by acetylacetone (Mori et al., 1978).

The enantiomers of the amino acids were determined by HPLC using Marfey's method (Harada et al., 1995). The hydrolyzed microcystin was derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (Fluka, Switzerland), and the enantiomers were separated on a C-18 Grom-Sil 120 ODS-4 HE reversed phase column (4.6 × 250 mm, 5 μm particle size, Stagroma, Germany). This method was also used for the determination of the molar absorption coefficient by adding a defined amount of L-alanine as an internal standard before hydrolysis.

As a second method to determine the molar absorption coefficient, a GC–MS separation was performed with a known amount of methyldeuterated L-alanine (L-alanine-3,3,3-d_3, 99 atom% D; Aldrich, WI, USA) added as an

internal standard. The hydrolysate of the microcystin was derivatised with N-*tert*-butyldimethylsilyl-N-methyl-trifluoroacetamide (MTBSTFA; Fluka, Switzerland) in a solution with tetrahydrofurane (1:1). The solution was acidified with TFA. For quantification of the deuterated L-alanine, the integral of the fragment ion *m/z* 263, and for quantification of D-alanine, the fragment ion *m/z* 260 were used.

2.4. NMR and mass spectrometry

The proton NMR spectra were recorded on a Bruker AVANCE 600 MHz spectrometer in d_4-MeOH–H_2O (2:1) at 300 K, with internal TMS reference. NMR assignments were made by standard methods using data from 2D DQF-COSY, TOCSY and NOESY spectra. The resonances within each spin system (amino acid) were identified from DQF-COSY and TOCSY spectra. ^{1}H NMR: 8.30 (d, HN, Ala^1), 4.50 (m, H–C(α), Ala^1), 1.35 (d, Me, Ala^1); 8.65 (d, HN, Arg^2), 7.36 (br. s, H–N(ϵ), Arg^2), 6.8 (br., HN = C–NH_2, Arg^2), 4.26 (m, H–C(α), Arg^2), 3.23 (m, H–C(δ), Arg^2), 2.01 (d, H–C(β), Arg^2), 1.79 and 1.68 (m, H–C(γ), Arg^2); 8.14 (d, HN, Asp^3), 4.69 (m, H–C(α), Asp^3), 2.99 and 2.43 (m, H–C(β), Asp^3); 8.61 (d, HN, Arg^4), 7.36 (br. s, H–N(ϵ), Arg^4), 6.8 (br., HN = C–NH_2, Arg^4), 4.37 (m, H–C(α), Arg^4), 3.16 (m, H–C(δ), Arg^4), 2.03 (d, H–C(β), Arg^4), 1.56 (m, H–C(γ), Arg^4); 7.92 (d, HN, Adda^5), 7.31 (t, H3,5–Ar, Adda^5), 7.23 (t, H4–Ar, Adda^5), 7.21 (d, H2,6–Ar, Adda^5), 6.26 (d, H–C(5), Adda^5), 5.52 (dd, H–C(4), Adda^5), 5.46 (d, H–C(7), Adda^5), 4.53 (m, H–C(3), Adda^5), 3.34 (m, H–C(9), Adda^5), 3.28 (s, OMe, Adda^5), 3.01 (m, H–C(2), Adda^5), 2.84 (m, H$''$–C(10), Adda^5), 2.72 (m, H$'$–C(10), Adda^5), 2.63 (m, H–C(8), Adda^5), 1.64 (s, Me–C(6), Adda^5), 1.05 (d, Me–C(2), Adda^5), 1.03 (d, Me–C(8), Adda^5); not detected (HN, Glu^6), 4.23 (m, H–C(α), Glu^6), 2.49 and 2.35 (m, H–C(γ), Glu^6), 2.10 and 1.96 (m, H–C(β), Glu^6); 9.87 (s, HN, Dhb^7), 5.84 (q, H–C(β), Dhb^7), 1.90 (d, Me–C(β), Dhb^7).

Mass spectra were recorded on a combined LC–MS (LCQ Duo mass spectrometer, Finnigan Thermoquest, USA) with an electrospray source (ES–MS).

2.5. 24-h Acute grazer toxicity bioassay

The toxicity of purified [D-Asp^3,(*E*)-Dhb^7]microcystin-RR ([D-Asp^3,(*E*)-Dhb^7]MC–RR), authentic microcystins, and nodularin (Calbiochem, San Diego, CA, USA) was tested in a 24-h acute toxicity assay performed with *T. platyurus* (Thamnotoxkit F; G. Persoone, State University of Ghent, Belgium). This freshwater crustacean occurs in temporary puddles and has been found to be sensitive to cyanobacterial toxins (Kurmayer and Jüttner, 1999). The 24-h bioassay was performed in a multiwell plate using instar II-III larvae. Nodularin and each microcystin were tested in at least seven different concentrations each in three independent replicates. Three to four parallel assays were conducted and combined.

3. Results

3.1. Bioassay-guided separations of toxicity

P. rubescens obtained from both an axenic culture and natural populations of Lake Zürich was used to determine the 24-h acute grazer toxicity. Extracts obtained with aqueous methanol contained the major part of the grazer toxicity of this cyanobacterium. These extracts were separated by HPLC into 14 fractions. Preliminary experiments have shown that the commonly used solvent gradient that contains TFA as an acidifying agent is not suitable for conducting bioassays because some toxicity is left in the fractions after removal of the solvent. Although the separation efficiency was less optimal in TFA-free solvents, such a system was used for the final purification step. The fractions of five runs were combined and tested for grazer toxicity in the *T. platyurus* 24-h acute grazer toxicity assay. Grazer toxicity was found at three different elution times: in the third fraction, which eluted after 6 min; in the fraction that eluted after 8 min and showed the typical absorption spectrum of microcystins; and in a late fraction at 18 min. The diode array absorption spectra of the first and last fractions gave no indication of the presence of microcystins. The bioassays clearly showed that the major toxicity was in the fraction containing the major microcystin. The separation profiles were identical for axenic *P. rubescens* and natural phytoplankton populations of Lake Zürich. The natural populations were enriched from a metalimnetic layer at a depth of 12 m in November 1997.

3.2. Purification and structural determinations of the major microcystin

Preparative HPLC was applied to obtain, in high purity, the major microcystin of *P. rubescens*. In front of the major microcystin a minor derivative with the mass *m/z* 1040.6 $[MH]^+$ (ES–MS) was observed; this minor derivative was not further investigated. ES–MS and retention time analysis were used to tentatively identify traces of two further microcystins as MC–LR and microcystin–YR (MC–YR). Another microcystin was present in low concentrations and showed a quasi molecular ion at *m/z* 981.6 $[MH]^+$ (ES–MS). Two additional microcystins were observed when the purified major microcystin was again chromatographed. However, these two additional microcystins turned out to be degradation products. They were formed during evaporation of the solvents of the purified fraction. However, when TFA-free solvents were applied in the final separation, this degradation was no longer observed.

The major toxin was identified as [D-Asp^3,(*E*)-Dhb^7]MC–RR (Fig. 1) on the basis of the following studies. The purified microcystin had an absorption maximum at

Fig. 1. Molecular structure of [D-Asp^3,(*E*)-Dhb^7] microcystin-RR isolated from *P. rubescens*.

239 nm in methanol and exhibited a quasi molecular ion (ES–MS) at *m/z* 1024.6 $[MH]^+$. The constitution of each amino acid residue could be confirmed from J-couplings in 2D ^{1}H COSY and TOCSY spectra. The Asp^3 residue showed no methyl group at the C(β) position, but rather two H–C(β) resonances. Also the connectivity and configuration of the 2-amino-2-butenoic acid (dehydrobutyrine, Dhb) residue^7 could be determined from TOCSY and NOESY spectra. The olefinic proton of Dhb showed an NOE connectivity to the HN of Dhb, whereas the methyl resonance showed an NOE to the HN of Ala^1. Therefore, the configuration is *E*. Finally, the connectivity between amino acid residues could be determined from inter-residue H-C(α) (i) to HN ($i+1$) NOEs in 2D NOESY spectra. This also allowed an assignment of the 1D ^{1}H NMR spectrum (see experimental part).

The stereochemistry of the amino acids of the hydrolysate was determined using Marfey's method for the separation of the enantiomers. Under the conditions applied, D-Asp and L-Asp exhibited retention times of 18.3 min and 17.4 min, respectively, D-Arg and L-Arg of 16.6 min and 18.6 min, D-Glu and L-Glu of 21.5 and 19.9 min and D-Ala and L-Ala of 29.9 and 24.3 min. In the hydrolysed microcystin only the enantiomers D-Asp and L-Arg, which both formed one unresolved peak at 18.7 min, D-Glu (21.9 min) and D-Ala (29.5 min) were observed. Adda (3-amino-9-methoxy-10-phenyl-2,3,8-trimethyldeca-4,6-dienoic acid) decayed upon hydrolysis and Dhb was converted to lactate. Lactate could be detected after derivatisation with MTBSTFA by its characteristic EI–MS spectrum (*m/z* 73 (100%), 147 (60%), 75 (30%), 189 (20%), 133 (16%), 233 (12%), 261 (11%), 57 (10%), 148 (9%) and 149 (7%), while the MTBSTFA derivative of the expected 2-oxobutyrate (*m/z* 73 (100%), 147 (81%), 189 (29%), 273 (25%), 57 (16%), 133 (15%), 75 (12%), 148 (11%), 59 (10%) and 41 (10%) was not observed.

3.3. Molar absorption coefficient

It is of primary importance to know the molar absorption coefficient for the determination of the specific toxicity of microcystins. Because the highly purified [D-Asp^3,(*E*)-Dhb^7]MC–RR was available only in μg amounts, gravimetric determinations were not acceptable. As a more reliable procedure, microcystins were hydrolysed and the obtained amino acids were determined quantitatively. L-Ala served as an internal standard for D-Ala in Marfey's separation on a HPLC column. By this method a molar absorption coefficient of $\epsilon = 50{,}500\ \mathrm{l\ mol^{-1}cm^{-1}}$ was obtained in methanolic solution for the maximum at 239 nm. A similar value ($\epsilon = 50{,}400\ \mathrm{l\ mol^{-1}\ cm^{-1}}$) was obtained by GC–MS analysis of the hydrolysate to which methyldeuterated L-Ala was added as an internal standard. Because traces of MC–YR were found in *P. rubescens*, and no molar absorption coefficient of MC–YR was available from the literature, the molar absorption coefficient of the commercially available compound was determined in the same way by GC–MS to be $\epsilon = 41{,}100\ \mathrm{l\ mol^{-1}\ cm^{-1}}$ (Table 1).

3.4. Acute grazer toxicity

Highly purified (>99% by HPLC at 239 nm) [D-Asp^3,(*E*)-Dhb^7]MC–RR, commercial microcystin-RR (MC–RR), MC–YR, MC–LR, and nodularin were assayed in a

Table 1
Molar absorption coefficients of different microcystins in methanol. [D-Asp^3,(*E*)-Dhb^7] MC–RR and MC–YR were determined by hydrolysis in this study, the others were taken from the literature (sh = shoulder)

Microcystin	Molecular mass	Absorption max. (nm) (solvent)	Molar absorption coefficient (l mol^{-1} cm^{-1})	Reference
[D-Asp^3,(*E*)-Dhb^7] Microcystin-RR	1023.6	239 (MeOH)	50,400	This study
Microcystin-RR	1037.6	238 (MeOH)	39,800	Harada et al. (1990)
Microcystin-YR	1043.5	231 (MeOH)	41,100	This study
		238 (MeOH), sh	38,100	
Microcystin-LR	994.5	238 (MeOH)	39,800	Harada et al. (1990)
Microcystin-LR	994.5	238 (MeOH)	36,500	Honkanen et al. (1990)
[D-Asp^3] Microcystin-LR	980.5	238 (MeOH)	31,600	Harada et al. (1991)
[Dha^7] Microcystin-LR	980.5	238 (MeOH)	46,800	Harada et al. (1991)
[D-Asp^3,(*E*)-Dhb^7] Microcystin-LR	980.5	239 (MeOH)	31,600	Sano and Kaya (1998)
[D-Asp^3,(*E*)-Dhb^7] Microcystin-HtyR	1044.5	239 (MeOH)	31,600	Sano and Kaya (1998)

24-h acute grazer toxicity assay against *T. platyurus*. It was essential to use [D-Asp^3,(*E*)-Dhb^7]MC–RR that was, in the final step, purified by a TFA-free solvent gradient. Traces of TFA gave false positive results. The obtained dose response curves for the different microcystins and nodularin showed typical sigmoidal shapes (Fig. 2). The non-linear-regression analysis [$y = y0 + a/(1 + \exp(-(x - x0)/b))$] of the percentage mortality exhibited correlation coefficients of all curves for which the R^2- values ranged between 0.983 and 0.999 ($P < 0.004$). A graph of different $LC_{(x)}$ values ($x = 10, 15, 50, 85, 90$) obtained from the regression curves is presented in Fig. 3, the values are given in Table 2. The obtained data show that *T. platyurus* was much more sensitive to nodularin than to all microcystins tested. The most toxic microcystin was [D-Asp^3,(*E*)-Dhb^7]MC–RR, followed by MC–LR and MC–YR. These three microcystins had dose response curves of the same shape unlike MC–RR, which exhibited a dose response curve that covers a wide range of concentrations. The MC–RR LC_{50} value was not markedly different from that of MC–LR and MC–YR but the flat curve extended the low percentage toxicity to much lower concentrations and demonstrated the resistance of some animals to higher concentrations.

4. Discussion

P. rubescens, the dominant organism of the phytoplankton community of Lake Zürich, contained several microcystins, as shown by PDA-HPLC and LC–MS. The microcystins were identical with those found in an axenic strain isolated from the lake. The major component of the microcystins exhibited a quasi molecular ion obtained by electrospray mass spectrometry at *m/z* 1024.6 $[MH]^+$ that was consistent with three previously isolated microcystins: [D-Asp^3]MC–RR (Meriluoto et al., 1989; Sivonen et al., 1992; Luukkainen et al., 1994), [Dha^7]MC–RR (Sivonen et al., 1992; Luukkainen et al., 1993) and [D-Asp^3,(*E*)-Dhb^7]MC–RR (Sano and Kaya, 1995). The isolation of a sufficient amount of the pure compound enabled extensive NMR spectrometric and chemical analyses to differentiate between these derivatives. Analysis of 1D and 2D ^{1}H NMR spectra provided evidence that the molecule is identical to that described by Sano and Kaya (1998), [D-Asp^3,(*E*)-Dhb^7]MC–RR, isolated from *Oscillatoria agardhii*. These analyses proved the constitution of the molecule and the configuration of the Dhb residue.

Marfey's analysis of the hydrolysed amino acids showed that their stereochemistry was identical with the general

Table 2
LC values (LC_{10}, LC_{15}, LC_{50}, LC_{85} and LC_{90}) of different microcystins and nodularin (μM) for *T. platyurus*

LC values	Toxins (μM)				
	[D-Asp^3,(*E*)-Dhb^7] MC–RR	MC–LR	MC–YR	MC–RR	Nodularin
LC_{10}	1.0	6.8	4.1	1.7	0.36
LC_{15}	1.3	7.2	4.5	2.5	0.65
LC_{50}	3.6	8.6	6.1	8.3	1.4
LC_{85}	6.7	10.3	7.7	18.6	1.7
LC_{90}	7.8	11.3	8.0	21.8	1.8

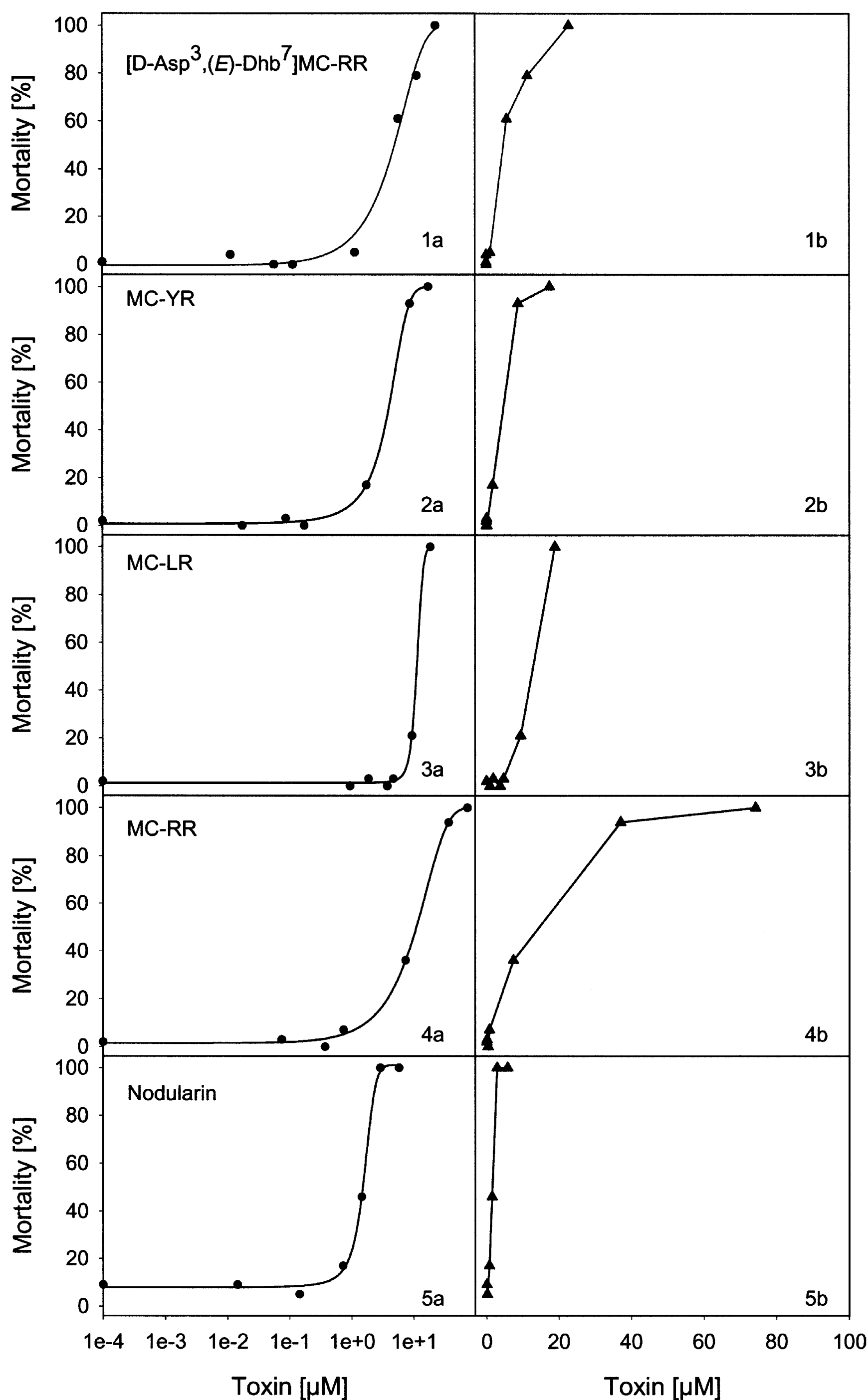

Fig. 2. Dose response curves for the 24 h-mortality (%) of *T. platyurus* of different microcystins and nodularin. The curves are presented in fitted logarithmic (a) and linear (b) mode. The data are calculated from seven to eight different doses with 30 animals per dose.

structure of microcystins (D-Ala, D-Glu, D-Asp and L-Arg). The structure was further supported by chemical degradation experiments. Lactate, rather than the expected 2-oxobutyrate, was obtained as a degradation product of Dhb that replaces N-methyldehydroalanine (Mdha) in the molecule. GC–MS analysis for lactate in the silylated hydrolysis products turned out to be an easy method to differentiate Dhb from Mdha-containing microcystins.

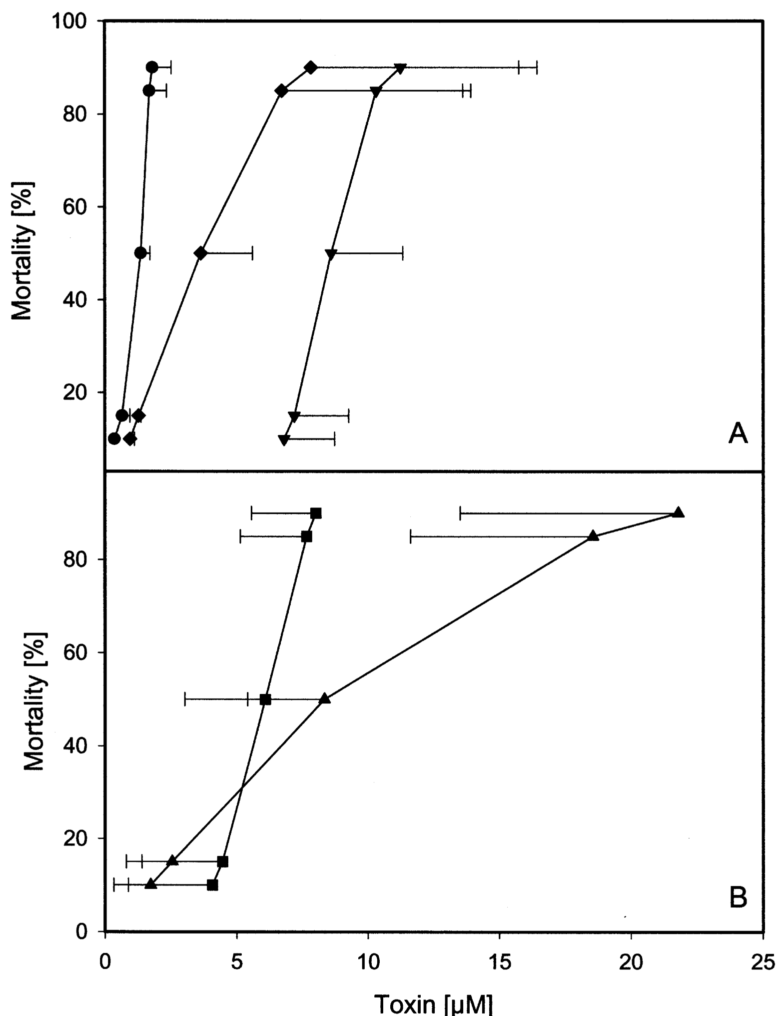

Fig. 3. Dose response curves for the mortality (%) of *T. platyurus* exposed to different concentrations (μM) of microcystins and nodularin. Data shown are means; horizontal lines represent standard deviation ($n = 3$); ◆ [D-Asp^3,(*E*)-Dhb^7] microcystin-RR; ● nodularin and ▼ microcystin-LR (A); ■ microcystin-YR and ▲ microcystin-RR (B).

The identification of [D-Asp^3,(*E*)-Dhb^7]MC–RR in an axenic strain of *P. rubescens* is consistent with the description of [D-Asp^3,Dhb^7]MC–RR in a natural bloom of *P. rubescens* on the basis of MALDI-TOF and partial NMR measurements (Fastner et al., 1999). The full NMR analysis and the determination of the amino acids in our investigation also allowed the identfication of the enantiomers of the amino acids in the molecule. Furthermore the configuration of the ethylidene group could be established to be (*E*).

To determine the specific toxicity of [D-Asp^3,(*E*)-Dhb^7]MC–RR, the molar absoprtion coefficient is an essential physical parameter. For the molar absorption coefficient it is normal to use $\epsilon = 36{,}500$ (l mol^{-1} cm^{-1}) and $\epsilon = 39{,}800$ (l mol^{-1} cm^{-1}) for MC–LR, and MC–RR and their derivates, respectively. Molar absorption coefficients between 46,800 (Harada et al., 1991) and 31,600 (Harada et al., 1991; Sano and Kaya, 1998) have also been reported (Table 1). Since the assessment of water quality is governed by this physical property, we determined the molar absorption coefficient of [D-Asp^3,(*E*)-Dhb^7]MC–RR by applying quantitative amino acids analysis after hydrolysis. We obtained an absorption coefficient that was larger than reported for other microcystins and we assume that the dehydrobutyrine moiety contributes to this increase in the absorption at 239 nm. The molar absorption coefficient of MC–YR was in the range of the coefficients reported for MC–LR and MC–RR. The observed difference of molar absorption coefficients for different microcystins has a major influence on the assessment of microcystin-caused toxicity of food and water. This factor has not been previously considered and necessitates the knowledge of individual structures of microcystins.

Unfortunately comparisons of the specific toxicity of different microcystins are rather scarce. The widely used toxicity assay by intraperitoneal injection of white mice gives only approximate values, because ethical constraints limit the number of mice which are used for the experiments. The large number of grazers used in the present toxicity bioassays makes possible robust statistical analysis and gradation of percentage lethal doses. Marked differences between different microcystins were found in the *Thamnocephalus* 24-h acute toxicity bioassay. On a molar basis [D-Asp^3,(*E*)-Dhb^7]MC–RR of *P. rubescens* was much more toxic than all the other microcystins tested. The relatively higher toxicity of [D-Asp^3,(*E*)-Dhb^7]MC–RR as compared to MC–RR was also observed with the i.p. mouse bioassays. The LD_{50} of [D-Asp^3,(*E*)-Dhb^7]MC–RR was reported to be 250 μg/kg mouse (Sano and Kaya, 1998) while the LD_{50} of MC–RR exhibited the much higher value of 500-800 μg/kg mouse (Painuly et al., 1988; Namikoshi et al., 1992). The data suggest that it may be possible to compare the mouse bioassay with the *Thamnocephalus* bioassay, but further investigations are necessary to establish invertebrate assays as reliable risk indicators for humans.

In the *Thamnocephalus* bioassay MC–LR, MC–YR and MC–RR exhibited LC_{50} values which were close to each other. However, nodularin, which was assayed as a similar compound of cyanobacterial origin, was even more toxic than [D-Asp^3,(*E*)-Dhb^7]MC–RR. The toxicological analysis showed that the evaluation of toxicity by LC_{50} values alone is insufficient to understand the full action of microcystins. Plots which also include the LC_{10} and LC_{90} values show that the slope of MC–LR and MC–YR dose response curves are much steeper than that of [D-Asp^3,(*E*)-Dhb^7]MC–RR. An entirely different toxic behaviour was found for MC–RR which exhibited a much lower slope. The consequences are that very low concentrations of MC–RR are toxic enough to kill some sensitive individuals while attaining a 90% mortality needs much higher concentrations. The reason for this pronounced difference in physiological response of *Thamnocephalus* against microcystins of different structures is not known.

In a structure-dependant toxicity study of microcystins, using *Tetrahymena pyriformis* as a bioassay organism, toxicity was correlated with the lipophilicity of different microcystins of the MC–LX (X = R, W, F and Y) series (Ward and Codd, 1999). Both growth and respiration rates were significantly reduced by equimolar concentrations of the more lipophilic MC–LF as compared to MC–LR. When the same procedure was applied to calculate the lipophilicity of [D-Asp^3,(*E*)-Dhb^7]MC–RR of *P. rubescens*, this component turned out to be more hydrophilic than MC–LR and, hence, should be less toxic. The much higher specific toxicity observed clearly contradicts the assumption and shows that other structural differences must be important for increased toxicity in this molecule.

The explanation for the abnormal behaviour of [D-Asp^3,(*E*)-Dhb^7]MC–RR may be found in the replacement of the methylene group of the Mdha of MC–LR by an ethylidene group of Dhb. The other changes of the molecular structure are not likely to contribute to the increased toxicity. Although both toxin types (nodularin that contains an ethylidene group, and the Mdha-containing microcystins) initially bind noncovalently to and inhibit the protein phosphatases type 1 and 2A, the Mdha-containing microcystins readily undergo a covalent interaction with the protein phosphatases (Bagu et al., 1995). The covalent binding of the Mdha residue is caused by the electrophilic addition of the cysteine sulfhydryl group of the binding site to the methylene group (MacKintosh et al., 1995). The importance of Dhb for the enhanced toxicity of [D-Asp^3,(*E*)-Dhb^7]MC–RR as compared to MC–LR is supported by the high specific toxicity of nodularin, which was tested under identical conditions. Nodularin contains the same structural elements that are believed to be essential for toxicity: ADDA, the free glutamyl carboxy group (Stotts et al., 1993) and the ethylidene group. The importance of the ethylidene group for toxicity was shown by Namikoshi et al. (1993) because the removal of the double bond of Mdhb by hydrogenation reduced the i.p. mice toxicity by more than a half. This contrasts to structural studies of Mdha-containing microcystins which were controversial. Hydrogenation of the Mdha of MC–LR showed no change of toxicity to mice (Namikoshi et al., 1993) while in two other studies (Meriluoto et al., 1990; MacKintosh et al., 1995) reduced toxicity was shown by changes of the Mdha moiety. The addition of ethane thiol to the Mdha residue of MC–YR, that prevents the formation of a covalent bond between this molecule and the enzyme, reduced the inhibitory property of MC–YR against protein phosophatase 1 (MacKintosh et al., 1995). Reduced lethal potency was reported by Meriluoto et al. (1990) also for the epimers of the hydrogenated MC–LR.

Although protein phosphatases are targets of the microcystins, the toxicity against live grazers is further modulated by processes which include transport into, and export from, cells, degradative and detoxifying enzymes, and, possibly, the presence of additional target molecules.

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