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Toxicity Assay of Cyanobacterial Strains Using *Artemia salina* in Comparison with the Mouse Bioassay

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ABSTRACT

Cyanobacterial samples were collected from aquaculture ponds and reservoirs in Taiwan in 1989-1994. Strains belonging to *Microcystis* and *Coelosphaerium* were isolated and maintained in laboratory cultures. Brine shrimp, *Artemia salina*, toxicity assay was applied and compared with the mouse bioassay for screening toxic strains among these cyanobacterial isolates in this study. We found that *M.*TY-1, *M.*TY-2, *M.*CY-1, *M.*TN-2, *M.*TN-3, and *M.*TN-4 strains of *Microcystis aeruginosa* were toxic in both assay methods. The same conclusion was also reached by HPLC analysis of known microcystins in the extract of different cyanobacterial strains. Also our data showed that the *Artemia* bioassay required more time than the mouse bioassay for detecting toxicity of microcystins, and the *Artemia* bioassay was less sensitive than the mouse bioassay at observation intervals shorter than 24 h. However, in view of its convenience and low expense, the *Artemia* bioassay still can be a reliable method for detecting toxicity of microcystins contained in toxic cyanobacterial strains.

Key words: *Artemia salina*, Mouse bioassay, Microcystin, Heptotoxin, Cyanobacteria

INTRODUCTION

Toxic cyanobacteria have been recognized as a worldwide hygienic problem in recent years (Gorham and Carmichael, 1988; Falconer, 1989). Some species of freshwater cyanobacteria produce hepatotoxic cyclic heptapeptides, the microcystins (abbreviated MCYST). So far, 52 microcystins have been identified from species of *Microcystis*, *Anabaena*, *Oscillatoria,* and *Nostoc* (Carmichael, 1997), and additional microcystins are still being reported.

The mouse bioassay, using intraperitoneal injection of cyanobacterial extracts or purified toxins, has long been applied for toxicity assessment of microcystins (Falconer, 1993; Hermansky *et al*., 1993; Lee *et al*., 1998). Among chromatographic analysis methods, HPLC has been widely used for the detection of microcystins after some of the microcystins standards became available (Lawton *et al*., 1994; Moollan *et al*., 1996). Immunoassays (Chu *et al*., 1990) and *in vitro* cytotoxicity assays (Henning *et al*., 1992) have also been developed for cyanobacterial toxin detection. Most of the above approaches, however, require special facilities and technical skills that are not usually available in most field-monitoring laboratories. Therefore, a convenient and reliable method for routine screening of cyanobacterial toxicity is still

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Table 1. Collection date and site of *Microcystis aeruginosa* and *Coelosphaerium küetzingianum* cultured in this experiment and results of mouse bioassays and *Artemia salina* bioassays.

Cyanobacterial strain	Sampling date	Sampling site	Threshold dosage on mice (mg dry cells/kg mouse)	Threshold conc. (24 h) on *Artemia* (mg dry cells/ml)
M. aeruginosa				
M. TY-1	Sept. 1992	Gongshi, Tauyuan	25	5
M. TY-2*	Nov. 1989	Neylih, Tauyuan	100	25
M. CY-1	Aug. 1993	Dongshyr, Chiayi	100	25
M. TN-1	Aug. 1992	Dahliao, Tainan	>1600	>100
M. TN-2	July 1993	Duujia, Tainan	25	10
M. TN-3	July 1993	Duujia, Tainan	100	25
M. TN-4	Sept. 1994	Duujia, Tainan	25	25
M. KS-1	Aug. 1989	Chengchin, Kaohsiung	>1600	>100
C. küetzingianum				
C. TN-1	Aug. 1992	Dahliao, Tainan	>1600	>100

*Strain was a gift from Dr. J. T. Wu, Institute of Botany, Academia Sinica, R.O.C.

needed. It has been reported that hepatotoxic bloom samples and laboratory isolates of cyanobacteria were toxic to brine shrimp larvae (Kiviranta *et al*., 1991; Campbell *et al*., 1994). A simple brine shrimp bioassay has been applied by Lahti *et al*. (1995) and Vezie *et al*. (1996) for the detection of toxicity of cyanobacteria.

The purpose of this study was to evaluate the suitability of the *Artemia* bioassay for detecting toxicity of microcystins contained in cyanobacterial strains. Also we compared the sensitivity and time requirements of the *Artemia* bioassay with those of the mouse bioassay. The results of both assay methods on cyanobacterial strains were confirmed by HPLC detection of known microcystins.

MATERIALS AND METHODS

Cyanobacterial strains

Eight strains of *M. aeruginosa* Kützing and one *C. küetzingianum* Naegeli strain were isolated from freshwater ponds and reservoirs at various localities in Taiwan. These species were collected from algal blooms and then cloned for laboratory cultures (Table 1). They were identified according to the species described in *Plankton Algae of Reservoirs in Taiwan* (Moriwaka and Chyi, 1996). All clones were cultured in modified Fitzgerald media (Hughes *et al*., 1958) at 23 ± 1°C and illuminated with fluorescent light of 26.4 μEin.$m^{-2}s^{-1}$ for 12 h/d. Cyanobacterial cells were collected in their late exponential phase of growth and concentrated by continuous centrifugation, followed by lyophilization and storage.

Artemia bioassay

Brine shrimp eggs were supplied by Ocean Star International Inc. (Snowville, USA). Larvae were used within 1 d of hatching.

Following extraction of lyophilized cell masses (50 mg) of each cyanobacterial strain with methanol, the extract was evaporated to dryness in a vacuum. The dried extract was dissolved in 250 μl filtered seawater to give a concentration as 200 mg/ml in terms of lyophilized cell masses, and further diluted with

seawater to give five concentrations of 100, 50, 20, 10, and 2 mg/ml. Assays were performed on a 96-well microtitration plate with 10~20 brine shrimp larvae in 50 µl of seawater per well. The brine shrimp larvae in each well were tested using 50 µl per concentration level of extract. They were observed for 24 h to calculate mortality (Meyer *et al*., 1982). The toxicity threshold concentration, expressed as dry weight of cyanobacterial mass per milliliter of seawater, was defined as the lowest concentration that kills all tested brine shrimp within 24 h. Each test was run in triplicate, and seawater was used as the control. Additionally, NMR-confirmed pure microcystins, MCYST-FR, -WR, -LR, -RR, -RA, and [Dha]MCYST-RR isolated from the *M*.TN-2 strain and wild populations (Lee and Chou, 1997) were also assayed with brine shrimp to observe their relative toxicity using the LC_{50} parameter. Each microcystin was diluted with seawater to give seven assay concentrations of 100, 50, 30, 25, 20, 10, and 5 µg/ml. Assays were performed on a 96-well microtitration plate with 20~30 brine shrimp larvae in 100 µl of each concentration level per well, and percentage of deaths was counted at 24 h. The LC_{50} determined by probit analysis (Finney, 1963) was defined as the concentration needed to cause half the tested brine shrimp to die within 24 h. The time needed for the death response of all tested larvae at each concentration level was also recorded in this experiment.

Mouse bioassay

Dried cell mass (150 mg) of each strain of cultured *Microcystis* and *Coelosphaerium* was extracted three times with 10 ml methanol and the combined extracts were dried in vacuum. Dried extract from each cyanobacterial strain was redissolved in 1 ml saline solution for mouse toxicity assay using three mice for each dose level. Swiss albino male mice at 20 g each, ICR strain, were injected intraperitoneally with cyanobacterial extracts and were observed for 4 h for lethal results (Aune and Berg, 1986). The toxicity threshold dosage, expressed as the dry weight of cyanobacterial mass per kilogram of mouse, is the smallest quantity of extract that kills all triplicates of the mice. Six dose levels of the extract equivalent at 1/32, 1/8, 1/2, 2, 8, and 32 mg dry cell mass of each strain were tested for toxicity threshold.

A wild population of blooming *Microcystis* in an eel pond was also studied for comparison with the cultured strains. One gram of the dried specimen was extracted three times with 30 ml methanol. The dried extract was then diluted with various amounts of saline solution to give five different doses equivalent to 1, 5, 10, 50, and 100 mg dried cells per ml. Six mice were used for each dose level as replications.

HPLC analysis of microcystins in each cyanobacterial strain

A lyophilized-cell mass (50 mg) of each cyanobacterial strain was extracted three times with 3 ml 5% aqueous acetic acid for 30 min while stirring. The combined extracts were centrifuged at 3000 rpm for 15 min, and the supernatant was collected and applied directly to a preconditioned C18 column for solid phase extraction. An Accubond C18 SPE column (6 ml, 1 g C18, Cosmosil, Japan) was preconditioned initially with 10 ml water:methanol (2:8, v/v), followed by 10 ml water. After loading the sample, the column was first eluted with 10 ml water and then with 10 ml water:methanol (2:8, v/v) to give the toxin fraction. The toxin fraction was evaporated to dryness and redissolved in 200 µl methanol using 10 µl for each HPLC detection. The HPLC system used a 4.6×250 mm Alltima C18 column (Alltech, USA) and an isocratic solution of 0.01 M ammonium acetate:acetonitrile (3:1, v/v) as the mobile phase, at a flow rate of 1 ml/min. All microcystin standards were purified and identified from one of the toxic strains, *M*.TN-2, and a field-collected sample of *Microcystis* from our previous study (Lee and Chou, 1997).

RESULTS

***Artemia* bioassay**

The threshold concentration was defined here as the minimal concentration of methanol

Table 2. Percentage of death of *Artemia* at 24 h by treatment with various concentrations of MCYST-FR, -WR, -LR, -RR, -RA, and [Dha]MCYST-RR, and LC_{50} values of these microcystins.

Microcystin	Percentage of death of *Artemia* at 24 h					LC_{50}*	95%*
	30μg/ml	25μg/ml	20μg/ml	10μg/ml	5μg/ml	μg/ml	Confidence interval
MCYST-FR	100	84	64	-	0	17.6	16.4 ~ 19.0
MCYST-WR	100	92	-	27	0	13.2	12.3 ~ 14.1
MCYST-LR	100	84	16	-	0	22.3	22.1 ~ 22.6
MCYST-RR	100	90	-	7	0	16.2	15.2 ~ 17.1
MCYST-RA	100	92	91	-	0	12.7	9.2 ~ 17.5
[Dha]MCYST-RR	100	89	54	-	0	19.6	19.1 ~ 20.2

*Data determined from 24-h counts using the probit analysis method.

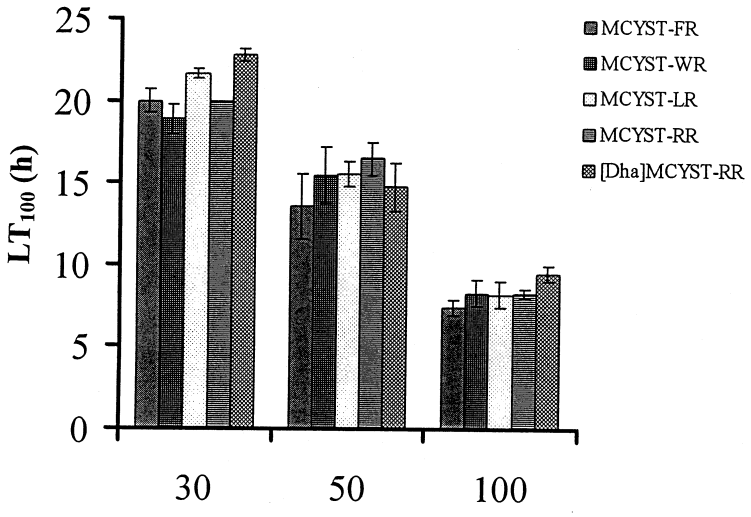

Figure 1. Relationship between microcystin concentration and LT_{100} (time needed to observe death response in all brine shrimp after treatment). Inserted bars represent standard errors.

extract of dried cells which killed all triplicates of the tested brine shrimp at 24 h. The threshold concentration of each strain of cyanobacteria is presented in Table 1. Among the assayed cyanobacteria, *M.*TY-1 strain had a threshold concentration lower than those of all other cyanobacterial strains, and thus seemed to be the most toxic, while *M.*TN-2 strain was ranked second. *M.*TY-2, *M.*CY-1, *M.*TN-3, and *M.*TN-4, with the same threshold, were less toxic than the *M.*TN-2 strain. *M.*TN-1, and *M.*KS-1 of *M. aeruginosa,* and *C.*TN-1 of *C. küetzingianum* were classified as non-toxic because few (< 10%) of the brine shrimp died even at the highest concentration level in this experiment. Microcystin-FR, -WR, -RR, -LR, -RA, and [Dha]MCYST-RR showed their LC_{50} to be within the range of 12.7~22.3 μg/ml at the observation interval of 24 h, and the potency of *Artemia* toxicity of these microcystins was found to be MCYST-RA > MCYST-WR > MCYST-RR > MCYST-FR > [Dha]MCYST-RR > MCYST-LR (Table 2). The quantity of microcystins which caused half of the brine shrimp to die within 24 h was between 1.27 and 2.33 μg per 100 μl seawater used in this experi-

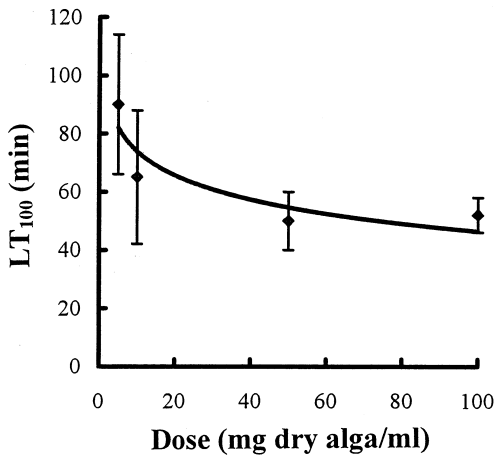

Figure 2. Relationship between injected dose and LT_{100} (time needed to observe death response after injection) of mice. Inserted bars represent standard errors.

ment. Also data have shown that all brine shrimp died before 24 h only at the assay concentration levels of 100, 50, and 30 μg/ml of these microcystins. The relationship between these three assay concentrations of each microcystin and death time of all the brine shrimp is shown in Fig. 1. It seemed that the assay concentrations of each microcystin correlated with death time of the brine shrimp, and approximately 8.5, 15, and 21 h were required for the death of all tested brine shrimp at concentrations of 100, 50, and 30 μg/ml of these toxins, respectively.

Mouse bioassay

The toxicity threshold doses of each cyanobacterial strain are presented in Table 1 to show the relative toxicity among these cultured strains. Among the cultured cyanobacteria, *M*.TY-1 was the most toxic, because a dose level (6.25 mg dry cell mass/kg mouse) lower than its threshold dosage (25 mg) showed lethal toxicity to 2/3 of the tested mice. *M*.TN-2 and *M*.TN-4 strains of *M. aeruginosa*, having the same threshold as *M*.TY-1 but without any toxicity at a lower dose level, were second. *M*.TN-1, *M*.KS-1, and *C*.TN-1 were classified as non-toxic because none of the injected mice died even at a dose level of 1.6 g dry cell mass/kg mouse, the highest dose level in this experiment. Totally, six of nine cyanobacterial strains were found to be toxic but variable in their toxicity.

The natural *Microcystis* population showed a lower toxicity threshold (50~250 mg dry alga/kg mouse) than the cultured strains, and from the result of this assay, a dose-response relationship was found to show a minimal reaction time. It can be observed in Fig. 2 that no significant difference was found between death time of the assayed mice and lethal doses of the toxic algal extract, especially for doses higher than 10 mg dry alga/ml, and at least 40 min was required to show the death response in treated mice.

HPLC analysis of microcystins in cyanobacterial strains

Toxin profiles of cultured cyanobacterial strains were studied by reverse-phase HPLC. Every component of each toxin fraction was identified by co-injecting the microcystin standards in the HPLC analysis. The results of HPLC analysis showed that each of six toxic *Microcystis* strains contained at least two different microcystins (Fig. 3), and no commonly known microcystin was found in the non-toxic strains of *M*.TN-1, *M*.KS-1, and *C*.TN-1.

DISCUSSION

When compared with the EC_{50} (concentration of [Dha]MCYST-RR that makes half of the *A. salina* move atypically at 24 h, at 5 μg/ml) given by Kiviranta *et al.* (1991), the LC_{50} (concentration of microcystin mixtures that causes half of the *A. salina* to die within 24 h, at 3~17 μg/ml) given by Lahti *et al.* (1995), and the LC_{50} (concentration of MCYST-LR that causes half of the *A. salina* to die within 48 h, at 5 ±0.2 μg/ml) reported by Vezie *et al.* (1996), the LC_{50} values of pure microcystins revealed in this study are quite reasonable (Table 2). Notably, the potency of *Artemia* toxicity of these pure

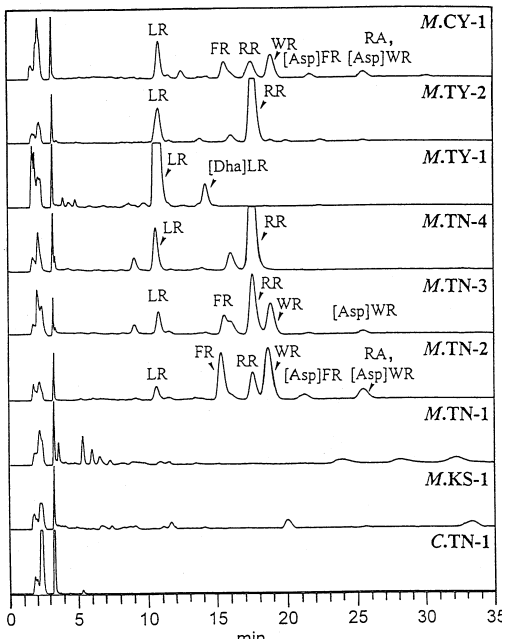

Figure 3. HPLC analysis of microcystins in eight strains of *Microcystin aeruginosa* and one *Coelosphaerium küetzingianum* strain, revealing MCYST-LR (LR), -FR (FR), -RR (RR), -WR (WR), -RA (RA), [Dha]MCYST-LR ([Dha]LR), [Asp]MCYST-FR ([Asp]FR), and [Asp]MCYST-WR ([Asp]WR) in the toxic strains. Column: Alltima C18, 4.6 x 250 mm. Mobile phase: 0.01 M ammonium acetate:acetonitrile (3:1, v/v), flow rate 1 ml/min. Detector: UV 238 nm.

microcystins was found to be opposite to the finding that MCYST-LR was the most toxic microcystin known to mice (Rinehart *et al.*, 1994). Also the relationship between microcystin concentration and death time in the *Artemia* bioassay (Fig. 1) is different from the dose-lethal time response of the mouse bioassay (Fig. 2) described in this report. In this study, the *Artemia* bioassay proceeded by toxin immersion while the mouse bioassay used intraperitoneal injection of the toxic algal extract. Thus we speculated that the mechanism of action of microcystins in *Artemia* is different to that in mice. Though the toxic action mode of microcystins in *A. salina* still has never been reported, our data show that it is a time-consuming process for microcystins to cause death of brine shrimp.

The result of relative toxicity of each cyanobacterial strain assessed using *Artemia* bioassay was almost the same as that of the mouse bioassay (Table 1). We conclude therefore, that the results of the *Artemia* bioassay coincide well with those of the mouse bioassay. Our data confirm the results of Vezie *et al.* (1996). Also the HPLC analyses of known microcystins in the extract of each cyanobacterial strain confirm that only the toxic strains produce microcystins (Fig. 3). These experiments show that brine shrimp is a reliable organism for detecting toxicity of microcystins contained in cyanobacterial strains. The sensitivity and time requirements of the *Artemia* bioassay were also compared with those of the mouse bioassay in this report. Many publications have shown that most microcystins display an LD_{50} value of 1~2 μg/mouse within 4 h (reviewed by Rinehart *et al.*, 1994). When compared to the quantity of microcystins used in this study on the *Artemia* bioassay with the above LD_{50} value in mice, it seems that the sensitivities of the two are similar. However, the *Artemia* bioassay will be less sensitive than the mouse bioassay at observation intervals shorter than 24 h.

Mouse bioassay with field-collected *Microcystis* showed a toxicity close to that (LD_{100} = 31 mg/kg mouse, intraperitoneally) obtained by Azevedo *et al.* (1994). Our data revealed that a minimum of 40 min was needed to observe the death effect in the mouse bioassay after intraperitoneal injection of algal extract at doses higher than the threshold. It has been reported that the time required for maximal toxin accumulation in liver varied from 1 min (Brooks and Codd, 1987) to 60 min (Robison *et al.*, 1989) after microcystin-LR administration to mice. A newly found inhibition activity of microcystins on protein phosphatase 1 and 2A was an assumed mechanism by which microcystins exert their hepatotoxity in mice (Nishiwaki-Matsushima *et al.*, 1992). Due to enzyme inhibition, hepatocytes shrink and cause liver damage, followed by internal hemorrhaging

(Runnegar and Falconer, 1986). The death of a 20 g mouse due to hemorrhage shock took at least 40 min in this case; nevertheless, it takes a much shorter time for the mouse bioassay to assess the toxicity of microcystins than does the *Artemia* bioassay.

Reliable HPLC analyses of microcystins require sophisticated and expensive instrumentation and a large battery of standards (Meriluoto *et al*., 1996). Also the toxicities of individual microcystins need to be determined in order to estimate the toxicity of each cyanobacterial strain. In this experiment, we show that although the *Artemia* bioassay is less sensitive and more time-consuming than the mouse bioassay, nevertheless, it is less constraining than the mouse bioassay because it requires less laboratory equipment, and is less problematical from an ethical point of view. Thus, we suggest that brine shrimp can be a convenient method for screening hepatotoxic cyanobacterial strains.

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