BIOMEDICAL AND ENVIRONMENTAL SCIENCES 13, 97-104(2000)

# The Identification and Toxicity of the Microcystin in the Waterbloom Obtained From an Eutrophied Lake

XU JUN-KANG*, LIU YAN-FANG**,[1], ZHU HUI-GANG*, AND DING WEN-XING***

*Department of Environment Health, Shanghai Medical University, 200032, China;
** Weizmann Institute of Science, Rehovot, Israel;
*** National University of Singapore

The water of "J" lake has been seriously eutrophied; concentration of total nitrogen (TN), total phosphorus (TP) and chlorophyll a were all far above the 3rd level of the National Standard of Ground Water of China. The concentration of microcystin (MCYST) of the water at one site (M) was 1865 μg/l. There were 2.36μg MCYST-LR per mg dry waterbloom powder.

## INTRODUCTION

Environment is vital to human beings, and water is a vital part of the environment. Hence proper management of environment and protection of source water are important aspects of sustainable development. In 1960's, the Organization of Environmental Cooperation and Development initiated a systematic research on lake eutrophication. In 1980, international conferences of lake eutrophication were held in Japan and USA. The first Chinese conference of this kind was convened in 1984 in Beijing, where monitoring, mechanics and control policy of eutrophication were discussed.

Eutropication has become worldwide for decades (Elleman, Falconer, and Runnegar, 1978; Turner *et al*., 1990; Namikoshi, Sivonen, and Evans, 1992). It has been detected in China (Wang and Zhu, 1996). "J" lake is located in an "economic-develop-quickly" littoral city south of the Yangtze River, and it is the source of the tap water of a city nearby. Due to the pollutants in the domestic sewage, industrial waste and runoff, the water quality of J lake has become much worse, and waterbloom has often been found in summers. In August, 1996, we sampled at three sites at "J" lake: site E and X, are protected zones of source water; site M was where we found much waterbloom, water and waterbloom were collected there for study.

[1] Address for correspondence: Liu Yanfang, Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, 76100, Israel. Telephone: 00972-8-9342378, E-mail: lcliu@wiccmail.weizmann.ac.il

Abbreviations: TN: Total nitrogen; TP: Phosphorus; MCYST: Microcystin; WBE: Waterbloom extract; DMSO: Dimethyl sulfoxide; 3-MCA: 3-methylcholanthrene; TPA: Phorbol 12-myritate 13-acetate; ELISA: Enzyme-linked immunosorbent assay; HPLC: High performance liquid chromatography; MNNG: N-methyl-N′-nitro-N-nitrosoguaridine; GMA: Glycidyl methacrylate; RCE: Relative colony forming efficiency; TF: Transformation frequency

0895-3988/2000
CN 11-2816

## MATERIALS AND METHOD

Both the water and the waterbloom collectors were made in the Institute of Plankton, the Chinese Academy of Science (CAS). For the alga counting, the determination of total nitrogen (TN), total phosphorus (TP) and chlorophyll a, we collected 5 l of water at 0.5 m below the surface. Waterbloom was skimmed with a No.25 plankton net, placed in an ice kettle, and then lyophilized before analysis.

### *Eutrophied State*

Alga counting was done in accordance with the method recommended by the Institute of Plankton, CAS. Determination of TN was done by $K_2S_2O_8$ oxidation-ultraviolet spectrophotometry, TP by $SnCl_2$ reduction spectrophotometry and chlorophyll a by Lorenzen monochromatic spectrophotometry.

### *ELISA*

The Microcystin Elisa Kit was purchased from the Mitsubisi Kagaku Bio-Clinical Laboratories; the Microplate Autoreader was made by Bio-Tek Instruments Inc. (EL309); the Multihole feeder was made by IWAKI GLASS, Finland (50-300$\mu$l).

### *HPLC*

The methods used were those introduced by Harada and Suzuki *et al*. (Harada, Suzuki and Dahlem, 1988; Meriluoto and Eriksson, 1988) but with the following modifications: Lyophilized sample weighing 25mg was dissolved in 20ml distilled water, then vortexed in the middle of the ultrasonic bath for 5 min. The sample was then centrifuged (10 min, at 10 000 g) and the supernatant was retained. The sediment was extracted with a mixture of water-methanol-butanol (75:20:5, v/v/v, 20ml) and centrifuged. The supernatant was combined with the previous one. The combined supernatant was applied to Bond Elut C18 columns washed with 20% methanol, then eluted with methanol. After removal of methanol with $N_2$, the extract was dissolved in distilled water which was designated as WBE (Waterbloom Extract).

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-6A pump coupled to a SPD-6A set at 238nm and a CR-5A integrater. Separation was performed on a Nucleosil 5 C18 (140—4.6mm, GL Science, Tokyo, Japan) using the following mobile phase: acetonitrile-water-trifluoroacetic acid (TFA) (50:50:0.025, v/v/v). The flow rate was 1ml/min.

### *Transformation Assay*

Two-stage Transformation. The KMB-13 cells were grown in Dulbecco's modified eagle medium (Gibco. Ltd.) supplemented with 3.7g/l sodium bicarbonate, 10% fetal calf serum, 100U/ml penicillin, 100$\mu$g/ml streptomycin and 100$\mu$g/ml kanamycin in a humidified atmosphere of 5% $CO_2$ air at 37℃. Well growing cells were seeded at a density of $1 \times 10^4$ cells/5 ml culture medium in 20 $cm^3$ culture flasks (5 flasks for each treatment) for the transformation assay. After 24 h, the cells were treated with DMSO, 3-methylcholanthrene

(3-MCA) or different concentration of WBE, which were added to the medium for 72 h as initiating treatment. Then the medium was replaced with fresh one, and the cells were grown in the fresh medium for 72 h. Then DMSO, different concentrations of WBE and Phorbol 12-myritate 13-acetate (TPA) were added to the medium as promoting treatment. After 2 weeks of incubation, the medium was changed every 3 days. Finally, the cells were fixed with methanol and stained with Giemsa. Transformed foci were identified according to the criteria of Dunkle *et al*. (1991). The types of foci were recorded. The transformation frequency (TF) = Number of Transformed. Foci/(Number of cells seeded × Relative colony forming efficiency) × 100%. A response was considered positive when the transformation rate was twice the spontaneous transformation or the TF value exhibited a dose-response relationship.

*Cloning Efficiency*

Samples of $1 \times 10^4$ growing KMB-13 cells were seeded in 20 cm flasks in 5 complete medium for 72 h and grown in normal medium for 9 days. The culture was then fixed and stained, the number of colonies were counted. The results were expressed by the relative colony forming efficiency (RCE), which is the percentage of colony-forming cells in terms of the vehicle control.

## RESULT

*Eutrophied State*

The TN and TP of the three samples were much higher than the 3rd level of the National Standard of Ground Water (GB3838-88) which established the levels of TN at 1.0 mg/l and TP at 0.05 mg/l. Those from site X were the highest. The Chinese Environment Protection Bureau (CEPB) divided the concentration of chlorophyll a into three levels: malatrophied $< 4\mu g/l$, middle trophic 4-10$\mu g/l$ and eutrophied 10-15$\mu g/l$. As shown in Table 1, we believe that J lake has been seriously eutrophied.

Table 1 also shows the high concentration of algae, especially the blue-green algae which might exert severe adverse effects on people.

TABLE 1

The Main Eutrophied Indices of J Lake in Aug. 1996

| Sample Site | TN(mg/l) | TP(mg/l) | Chlorophyll a($\mu g/l$) | Alga(million/l) |
|---|---|---|---|---|
| E | 4.76 | 0.66 | 132.25 | 9.21 |
| M | 6.03 | 0.84 | 124.43 | 17.0(14.2[a]) |
| X | 6.22 | 0.87 | 32.08 | 9.41 |

[a] Blue-green algae.

The ELISA Tests revealed that the MCYST concentration of the water at site M was 1865ng/l (Table 2).

TABLE 2

The MCYST in Water Detected by Means of ELISA

| | Standard Series | | | | | Site M |
|---|---|---|---|---|---|---|
| Concentration(pg/ml) | 50 | 100 | 200 | 400 | 1600 | 1865 |
| OD | 1.288 | 0.888 | 0.707 | 0.656 | 0.260 | 0.188 |

*Note*. The standard equation: $y = 2.23 - 0.63x$, in which $y$ refers to the value of OD and $x$ refers to the common logarithm value of the concentration (pg/ml, or ng/l), $P < 0.05$.

*HPLC*

Observed under the light microscope, the waterbloom almost all consisted of *Microcystis aeruginosa* (Figs. 1 and 2). Using the most common subordinations of MCYST, i.e. MCYST-LR MCYST-RR and MCYST-YR as the standard samples, only MCYST-LR was detected in the waterbloom, with the mass of 58.94$\mu$g. Taking into account of the total mass of the lyophilized waterbloom powder, the mass was equivalent to 2.36$\mu$g MCYST-LR per mg dry waterbloom powder (Fig. 3).

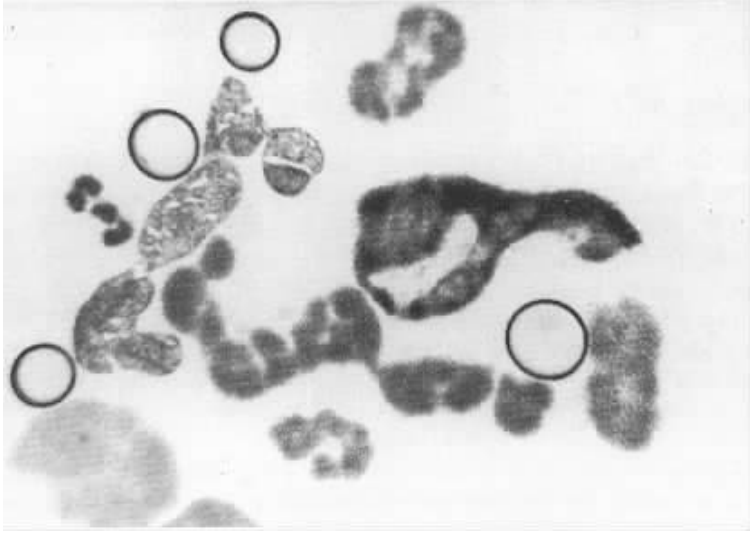

FIG. 1. The algae *Microcystis aeruginosa*, the main components of the waterbloom. ×80

*Transformation and Cloning Efficiency*

Initiated by MCA, WBE could result in the malignant transformation of KMB cells, and the numbers of transformation foci increased as the concentration of WBE did, which showed statistically significant dose-response relationship. On the other hand, no significantly higher transformation was observed when the cells were treated by WBE only or they are treated by WBE followed with the known promoter, TPA. Thus, the waterbloom extract, which contain MCYST-LR may be taken as promoter instead of initiator (Fig. 4 and Table 3).

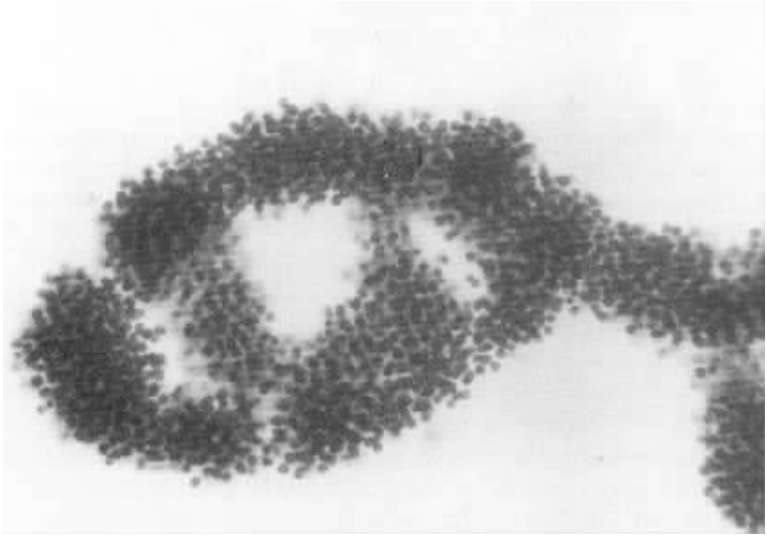

FIG. 2. *Microcystis aeruginosa*. ×320

TABLE 3

Result of Two Stage KMB Cells Transformation Assay

| Samples (Initiator Promoter) | Concentration ($\mu$g/ml) | CFE(%) | RCE(%) | No. of Transformation (foci/flasks) | TF(%) |
|---|---|---|---|---|---|
| DMSO DMSO | | 22.5 | 100 | 4/5 | 0.035 |
| TPA (0.25$\mu$g/ml) | | | | | |
| WBE | 0.5 | 21.80 | 96.9 | 4/5 | 0.037 |
| | 5.0 | 21.32 | 94.8 | 3/5 | 0.028 |
| | 10.0 | 21.06 | 93.6 | 8/5 | 0.076 |
| | 20.0 | 20.86 | 92.7 | 4/5 | 0.038 |
| | 40.0 | 20.62 | 91.6 | 10/5 | 0.097 |
| DMSO(0.2%) | | | | | |
| WBE | 0.5 | 21.14 | 94.0 | 3/5 | 0.028 |
| | 5.0 | 20.35 | 90.4 | 4/5 | 0.039 |
| | 10.0 | 19.42 | 86.3 | 4/5 | 0.041 |
| 3-MCA(0.5$\mu$g/ml) | | | | | |
| WBE | 0.5 | 19.87 | 88.3 | 9/5 | 0.090 |
| | 5.0 | 14.38 | 63.9 | 21/5[a] | 0.292 |
| | 10.0 | 9.43 | 41.9 | 32/5[a] | 0.679 |
| TPA | 0.5 | 7.25 | 32.2 | 73/5[a] | 2.013 |

[a] Significantly different ($P < 0.01$) from the group treated with DMSO alone.

The cytotoxicity of WBE is also shown is Table 3, which reflects that the relative colony-forming efficiency (RCE) was reduced when the dosage of WBE increased.

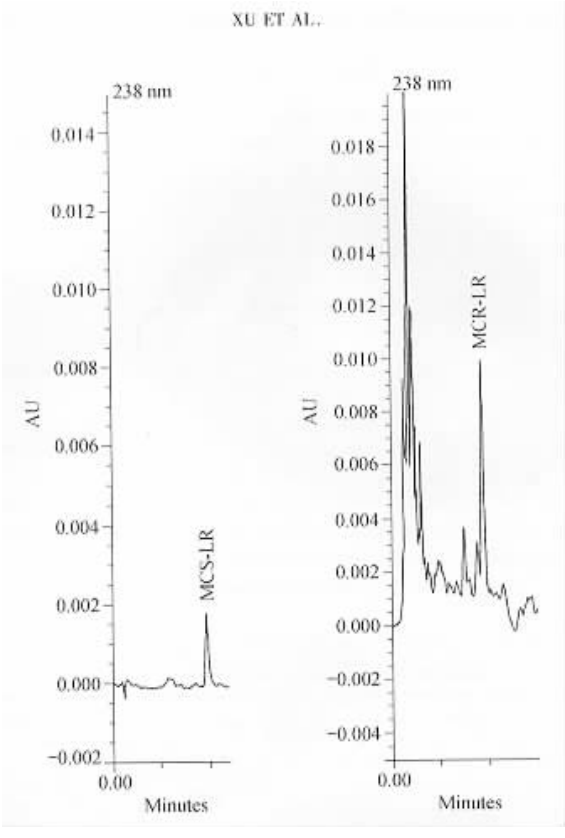


FIG. 3. The results of HPLC of standard microcystin-LR, 1.0 mg/ml (left curve), and extract from the waterbloom. The retention time of the microcystin peaks in both cases were 8.2 min.

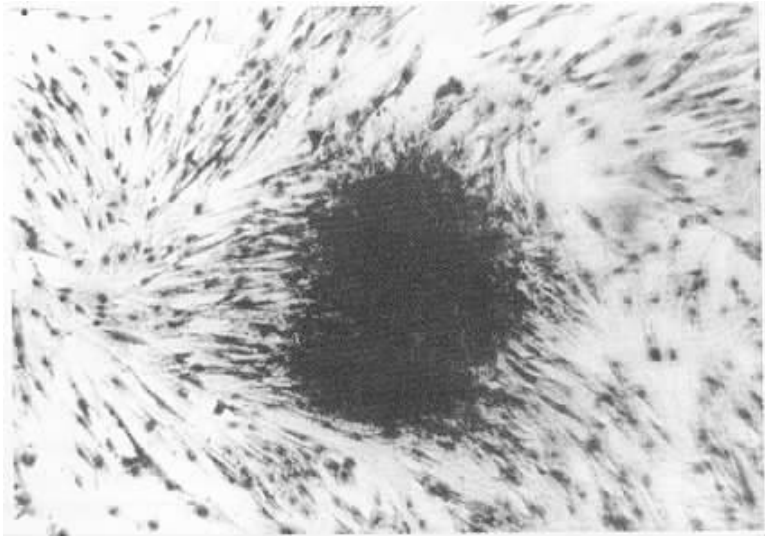

FIG. 4. A malignant tranformation focus of the KMB cells.

## DISCUSSION

Algal toxins are very stable, being resistant to heating and boiling (Rabin, 1989), with the acute and chronic toxicity of microcystins, the best policy is to prevent them from generating. Ressom (1994) recommend that water with a blue-green algae account of 500—2000 and abnormal smell should be the first-level warning, warranting successive monitoring twice a week. The eutrophication of "J" lake is very serious as shown by our studies. Furthermore, "J" lake is the source of the municipal water supply; consequently controlling the drainage and limiting the aquatic culture in the lake should be initiated.

Chu (1989) and Nagata *et al*. (1995) detected the MCYST in waterbody by means of ELISA. In this study, the concentration of 1865ng/l, which is much higher than the standard of concentration of MCYST in drinking water at 0.5μg/l recommended by WHO. There are contradictory reports on the removal of MCYST by chlorination. For example, Hoffman (1976) reported that chlorination is effective for the removal while Nicholson *et al*. (1994) reported on the contrary results. On the other hand, it has been widely documented that activated carbon and ozone are effective alternatives for the removal. However, these treatments on large scale are prohibitive.

Accroding to the latest studies on the algal toxins, they may be hepatotoxic, neurotoxic and cytotoxic (Xu, Wang and Zhu, 1996). Hepatotoxins do harm to liver, which are circular peptide in chemical character such as those produced by *Microcystis aeruginosa* and *Anabaeanflons-aqua*. MCYST is the kind researched much widely and deeply, which comprises of more than 40 subordination, among which MCYST-LR, MCYST-RR and MCYST-YR are reported most frequently. In Japan and China, the discovery of MCYST-RR has been reported, while MCYST-LR was found in some European and American countries (He, 1993). In the country where primary liver cancer was once prevalent, MCYST-LR was thought as predominant cause (Chen and Yu, 1995).

The phenotype of the transformed cells is very similar to that of tumor cells, and the injection of the transformed cells to homologous animals may bring about cancer. The transformation assay is generally taken as the best *in vitro* counterpart of the chemical carcinogenesis *in vivo* (Isfort and Lebeuf, 1996), and the enactivity of oncogen was reported to be in relation with the cell transformation (Ebert *et al*., 1990). Furthermore, chemical carcinogenesis was considered a multistage procession which may begin with DNA damage or gene mutagenesis, and tumors result from at least two additional stages, i.e. promoting and progressing stage. Treated with N-methyl-N′-nitro-N-nitrosoguaridine (MNNG) and glycidyl methacrylate (GMA) (Chang, Wang and Wang, 1987; Tang, Wang and Xie, 1997), KMB cells were found to be malignantly transformed. In this study, the promoting activity of the raw toxins extracted from the waterbloom were detected in KMB cells, which may have followed the activity of certain oncogen, such as C-myc or those of Ras gene family. We are undertaking other assays to identify the potential hazard of those microcystin-contained extract, and to elucidate the possible mechanism of action. Anyway, experimentats on intact animal and epidemiological studies in human population are necessary for the risk assessment of microcystins.

## ACKNOWLEDGEMENTS

The author express their sincere gratitude to Prof. Yu Shun-Zhang and Prof. Jin Xi-Peng for their valuable advice.

## REFERENCES

Chen Gang and Yu Shun-Zhang (1995). Investigation of Microcystin in various drinking water in the area with the prevalence of liver cancer. *Chinese Journal of Preventive Medicine* **30**, 6-9.

Chang Yi-Cang, Wang Zhi-Hua, and Wang Xiu-Qin (1987). The *in vitro* transformation of human diploid cells induced by MNNG. *Experimental Biology Acta* **20**, 135-137.

Chu, F. S., Huang, X., Weird, and Carmichael, W. W. (1989). Production and characterization of antibodies against microcystins. *Applied and Environmental Microbiology* **55**(8), 1928-1933.

Dunkel, V. C., Rogers C, Swierenga, S. H. H., Brillinger, R. L., Gilman, J. P. W., Nestmanner (1991). Recommended protocols based on a survey of current practice ingenotoxicity testing laboratories: cell-transformation in C3H/10T1/2 mouse embryo cell, BALB/C 3T3 mouse fibroblast and syrian-hamster embryo cell-cultures. *Mutation Research* **246**, 285-300.

Ebert, R., Barret, J. C., Wiseman, R. W., Pechan, R., Reiss, E., Rollich, G., and Schiffmann, D. (1990). Activiation of cellular oncogenes by chemical carinogens in Syrian hamster embro fibroblasts. *Environ. Health. Perspect* **88**, 175-178.

Elleman, Falconer, I. R., and Runnegar, M. T. (1978). Isolation characterization and pathology of the toxin from a *Microsystis aerugionsa* bloom. *Aust. J. Biol. Sci.* **31**, 209-218.

Harada, K. I., Suzuki, M., and Dahlem, A. (1988). Improved method for purification of toxic peptides produced by cyanobacteria. *Toxicon.* **26**, 433-439.

He Zhen-Rong (1993). The toxins of Microsystis aerugionsa strain M. 8641. *Journal of Biochemistry* **9**, 168-172.

Hoffman, J. R. H. (1976). Removal of microcystis toxins in water purification process. *Water SA* **2**, 58-60.

Isfort, R. J. and Lebeuf, R. A. (1996). Application of *in vitro* cells transformation assays o predict the carcinogenic pential of chemicals. *Mutat. Res.* **365**, 161-173.

Jussi, A. O. Meriluoto and John, E. Eriksson (1988). Rapid analysis of peptide toxins in cyanobacteria. *J. Chromatography* **438**, 93-99.

Nagata, S., Soutome, H., Tsutsumi, T., Hasegawa, A., Sekijima, M., Sugamata, M., Harada, K., Suganuma, M., and Ueno, Y. (1995). Novel monoclonal antibodies against microcystin and theirprotective activity for hepatotoxicity. *Nat. Toxins.* **3**(2), 78-86.

Namikoshi, M., Sivonen, K., and Evans, W. (1992). Structure of three new homotyrosine-containing microcystins and a new homophenylalanine variant from Anabaena sp. strain 66. *Chem. Res. Toxicol.* **5**, 661-666.

Nicholson, B. C., Rositano, J., and Burch M. D. (1994). Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. *Water Research* **28**, 1297-1303

Rabin, P. (1989). An improved extraction procedure for endotoxin from Microcystis aeruginosa NRC-1. *Biochem. SOC. Trans.* **3**, 428-430.

Ressom, R. (1994). Health effects of toxic cyanobaceria (blue-green alga). *Australia, National Health and Medical Research Council*, **74**.

Tang Min-Jia, Wang Li-E, and Xie Da-Yin (1997). Detection of P53 mutation in transformed cells by means of PCR-SSCP. *Health Research* **26**, 159-161.

Turner, P. C., Gammie, A. J., Hollinrake, K., and Codd, G. A. (1990). Pneumonia associated with cyanobacteria. *Br. Med. J* **300**, 1440-1441.

Wang Hong-Bing and Zhu Hui-Gang (1996). Promoting activity of Microsystins extracted from waterbloom in SHE cell transformation assay. *Biomedical and Envionmental Sciences* **9**, 46-51.

Xu Jun-Kang, Wang Hong-Bing, and Zhu Hui-Gang (1996). Progress of study on Cyanobacteria toxins and their methods of determination. *Shanghai Environmental Science* **15**, 38-41.

(*Received March* 30, 1999 *Accepted January* 28, 2000)