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Protein Phosphatase Inhibition Assay for Detection of Microcystins in Lake Water and *Microcystis* Cultures

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The protein phosphatase inhibition assay was used to quantify low levels of microcystin in lake water polluted by cyanobacterial blooms of *Microcystis*, and toxin concentrations in cultures of 15 strains of *Microcystis*. This method was highly sensitive permitting measurement of microcystin in concentrations of the order of $\mu g\,l^{-1}$. The concentrations of microcystin in the surface water of Lake Kasumigaura in the summer of 1995 were in the range of 0.16 to 2.7 $\mu g\,l^{-1}$ microcystin-LR equivalents.

On the other hand, markedly high levels of microcystin (160 to 1,429 $\mu g\,l^{-1}$) were measured in the culture fluids of *M. aeruginosa* TAC 192–2, *M. ichthyoblabe* TAC 69, *M. ichthyoblabe* TAC 113–1, *M. viridis* TAC 45–1, *M. viridis* TAC 64 and *M. viridis* TAC 92. There were strains which did not produce microcystin in the culture fluid within the same species of *Microcystis*. Therefore, the toxin productivity in the culture fluid is not a useful criterion for discrimination of the species of *Microcystis*.

Introduction

Microcystis is one of the dominant genera of cyanobacteria forming waterblooms in eutrophicated lakes and reservoirs. Outbreaks of cyanobacterial blooms bring about numerous problems such as deoxygenation of the lake water, high mortality of cultivated carp in net cages, formation of a musty odor and production of cyanotoxins. Therefore, waterblooms of toxic cyanobacteria have become a wide world problem. Microcystins produced by *Microcystis, Anabena* and *Oscillatoria* have been reported to be cyclic heptapeptides[9,28,30] which show high hepatotoxicity causing mortality of many kinds of animals including domestic animals[20].

Increasing concern about microcystin toxicity in eutrophicated lakes and reservoirs has led to a demand for a rapid, sensitive and reliable method of measuring microcystins even at low concentrations for the management of lake water quality, especially for the assurance of safety of drinking water. For the detection of microcystins in water samples, high-performance liquid chromatography (HPLC), liquid chromatography / mass spectrometry (LC/MS), enzyme-

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linked immunosorbent assay (ELISA) and protein phosphatase inhibition assay (PPI) have been developed. HPLC and LC/MS analyses are important methods for determination and identification of microcystin variants[4,12]. However, sophisticated pretreatment of water samples is necessary for both methods. In addition, the sensitivity of HPLC is not sufficient to detect microcystins in concentrations lower than 0.1 $\mu g\ l^{-1}$, although it is now the most widely used method.

Recently, sensitive immunoassay techniques for detection of microcystins in water were developed[1,2,15,16]. The cross-reactivity of a rabbit anti-microcystin-LR polyclonal antibody with 18 microcystin variants showed that 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4(E),6(E)–dienoic acid (Adda) was essential for these toxins to express antibody specificity[1]. Ueno et al. developed a highly sensitive ELISA with a detection limit of 50 $pg\ ml^{-1}$. Using this method, they extensively analyzed natural water samples[17,24,25].

Microcystins were found to inhibit protein phosphatase 1 and 2 A and to promote tumor growth in the rat liver[18,32]. Among these, microcystin-LR inhibited the enzymes with Ki values below 0.1 nM, showing inhibitory potency over 10 times as strong as that of okadaic acid[5,14]. A sensitive assay for microcystin based on inhibition of protein phosphatase was developed[8,10,11,21]. This method was available to measure total concentration of microcystins in water samples since protein phosphatase inhibition was related to the mechanism of microcystin toxicity[1].

In this study, the protein phosphatase inhibition assay was used to quantify low levels of microcystins in water samples from Lake Kasumigaura as well as high levels of extracellular microcystin in the culture fluids produced by several species of *Microcystis*.

Materials and methods

Protein phosphatase inhibition assay

The Gibco protein phosphatase assay system (Life Tech. Inc., Gaitherburg, MD., U.S.A.) was used for the assay. It consists of purified phosphorylase kinase, purified phosphorylase *b* and additional reagents required for this assay. The assay mixture consisted of 20 μl of diluted crude protein phosphatase prepared as described below, 20 μl of sample and 20 μl of ^{32}P-labeled phosphorylase *a* solution in a total volume of 60 μl. The mixtures were incubated at 30°C for 20 min. Incubation was terminated by adding 180 μl of 20% trichloroacetic acid (TCA) and the precipitated protein was removed by centrifugation at $12,000 \times g$ for 5 min. The radioactivity of ^{32}P-orthophosphate in 200 μl of supernatant obtained by centrifugation was assayed with a Beckman LS 5000 TD liquid scintillation counter (Beckman, Palo Alto, Calif., U.S.A.) using Clear-sol 1 scintillation cocktail (Nacalai Tesque, Inc., Kyoto, Japan).

Preparation of crude protein phosphatase and ^{32}P-labeled phosphorylase a

Crude protein phosphatase was prepared from rat brain homogenate[7]. Samples of 3 g of frozen rat brain were homogenized in 30 ml of 50 mM Tris-HCl buffer containing 0.1 mM EDTA, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, 25 $\mu g\ ml^{-1}$ leupeptin and 25 $\mu g\ ml^{-1}$ aprotinin, pH 7.0. The homogenate was centrifuged at $10,000 \times g$ at 4°C for 10 min. The extract stored at −20°C was thawed on the day of analysis and diluted 10-fold with protein phosphatase assay buffer containing 20 mM imidazole-HCl, 0.1% (v/v) β-mercaptoethanol, 0.1 mM EDTA and 1 mg/l BSA, pH 7.63.

The substrate for protein phosphatase (^{32}P-labeled glycogen phosphorylase *a*) was prepared by reacting [γ-^{32}P] ATP with purified phosphorylase *b* in the presence of purified phosphorylase kinase according to the instructions provided with the assay kit.

Calculation of microcystin concentration by protein phosphatase inhibition assay

A standard inhibition curve was plotted using microcystin-LR in the concentration range of 0 to 10 $\mu g\ l^{-1}$ in each experiment, and was used for calculation of microcystin concentration of samples and for 50% inhibition of the enzymes (IC_{50}). The concentration of microcystins determined by this method was expressed as microcystin-LR equivalents (eq.).

HPLC analysis of microcystins in lake water

A liter of lake water was applied to a C_{18} silica gel cartridge (Sep-Pak C_{18}, Water Assoc.). It was rinsed with 20 ml of water followed by 20 ml of 10% methanol in water. Microcystins were finally eluted from the C_{18} cartridge with 20 ml of methanol. The eluate was evaporated and dissolved in 0.5 ml of methanol[3]. Determination and identification of microcystin molecules was carried out by HPLC with a reversed phased TSKgel ODS-80 TS (250×4.6 mm, Tosoh Co., Tokyo, Japan) using methanol-0.05 M phosphate buffer (pH 3.0, 6:4) as the mobile phase[3]. Concentrations of microcystin-LR, RR and YR were determined by peak areas at the absorbance of 238 nm of standard microcystin-LR, RR and YR, respectively.

Collection of lake water

Surface water was collected from Tsuchiura harbor of Lake Kasumigaura, Ibaraki, Japan, in the summer of 1995. Waterbloom proliferated from the end of July to the end of September that year. Water samples were filtered through a glass fiber filter (1.2 μm mean pore size, GF/C filter, Whatmann, U.K.) and were stored at 4°C for further analysis. Cells on the filters were dried at 105°C for 6 hours and weighed.

Strains and cultural conditions of Microcystis

Strains used in this study are listed in Table 2. They had been isolated from lakes and reservoirs in Japan, and classified in the Tsukuba Algal Collection (TAC), National Science Museum, Tsukuba, Japan[27]. All strains were purified to monoclonal cultures using the capillary pipette washing method and kept in TAC. As shown in Table 2, 10 strains of 15 cultures were axenic strains, whereas *M. ichthyoblabe* TAC 69, TAC 76, TAC 113-1, TAC 128 and *M. viridis* TAC 64 were monoalgal strains.

They were cultivated in 200 ml of CT medium under 13.5 $\mu E\ m^{-2}\ s^{-1}$ of continuous fluorescent light at 30°C for 25 days and stored under 3.37 $\mu E\ m^{-2}\ s^{-1}$ of light at 20°C for 22 days. Cell growth was estimated by determination of chlorophyll-*a* (chl-*a*) concentration in the cultures[22]. The cultures were centrifuged at 10,000×*g* at 4°C for 15 min and the supernatant obtained was filtrated through a membrane (0.45 μm mean pore size, Millipore) and stored at −20°C until analysis of microcystin.

Intracellular toxins of the cell pellet were extracted three times with 200 μl of 5% aqueous acetic acid by stirring at 4°C for 30 min. The crude extracts were centrifuged at 10,000×*g* at 4°C for 15 min and supernatants were applied to C_{18} cartridges as described above. The intracellular concentration was expressed as the total amount of microcystins in cyanobacterial cells contained in a liter of culture ($\mu g\ l^{-1}$), and was also expressed as the concentration in dried cells ($\mu g\ g^{-1}$)

Chemicals

Microcystin-LR, RR and YR which were used for standard curves were purchased from Calbiochem, La Jolla, Calif., U.S.A. [γ-^{32}P] ATP (222 TBq m mol^{-1}, 370 MBq ml^{-1}; aqueous solution) was purchased from DuPont NEN™, Boston, Mass., U.S.A.

Results

Protein phosphatase inhibition assay of microcystin-LR, YR and RR

The effects of microcystin-LR, YR and RR on protein phosphatase activities are shown in Fig. 1. Microcystin-LR, YR and RR inhibited the activity of protein phosphatase in a concentration-dependent manner as shown on semi-logarithmic plots. Marked differences were observed in the extent of the protein phosphatase inhibition by these three microcystin variants. Microcystin-LR and YR showed 10-fold stronger inhibition of protein phosphatase activity than microcystin-RR. Concentrations required for 50% inhibition (IC_{50}) with microcystin-LR, YR and RR were 0.4, 0.9 and 11.0 $\mu g\ l^{-1}$, respectively. The linear portions of protein phosphatase inhibition curves were used to calculate concentrations of microcystins present in samples (0.1 to 10 $\mu g\ l^{-1}$ with microcystin-LR and YR and 1 to 100 $\mu g\ l^{-1}$ with RR).

Figure 2 shows calibration curves of microcystin-LR, YR and RR by HPLC. The standard microcystin-LR, YR and RR were eluted at retention times of 7.0, 7.9 and 9.6 min, respectively, and linear

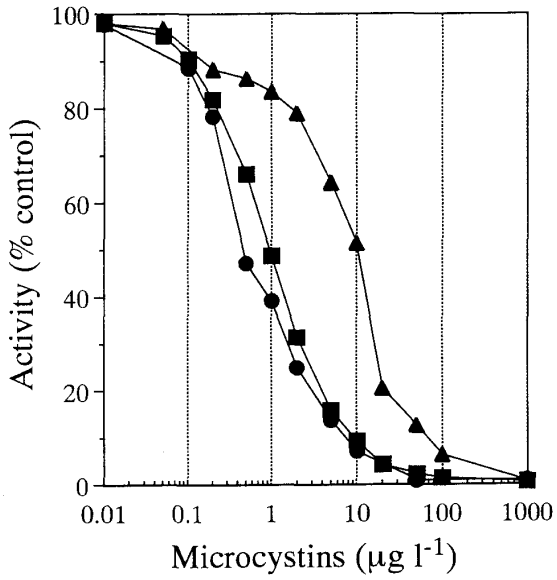

Fig. 1. Calibration curves of microcystins by protein phosphatase inhibition assay.
Microcystin-LR (●), YR (■) and RR (▲).

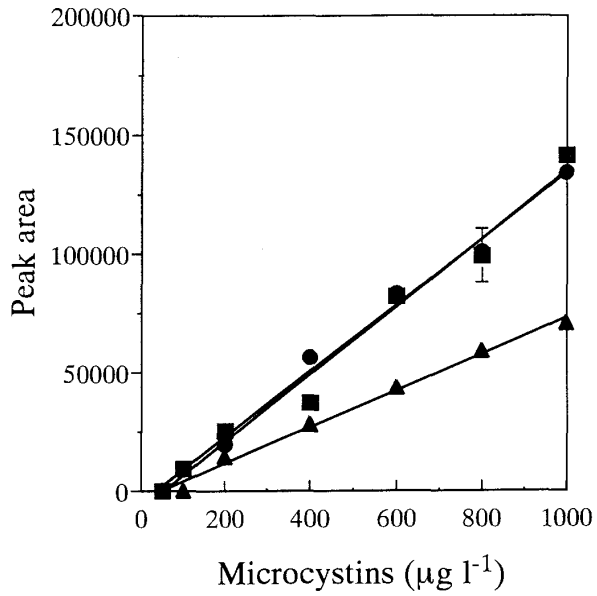

Fig. 2. Calibration curves of microcystins by HPLC analysis.
Microcystin-LR (●), YR (■) and RR (▲).

calibrations were obtained in a range of 100 $\mu g\ l^{-1}$ to 1,000 $\mu g\ l^{-1}$. The lower limit of HPLC detection for toxins was determined to be 100 $\mu g\ l^{-1}$ with microcystin-LR and YR and 200 $\mu g\ l^{-1}$ with RR. In comparison with HPLC analysis, protein phosphatase inhibition assay was highly sensitive to detect microcystin at concentrations of less than 100 $\mu g\ l^{-1}$.

Table 1. Concentration of microcystin in the coastal area of Lake Kasumigaura

Date	Microcystin in lake water	Dry cell weight
	$\mu g\ l^{-1}$	$mg\ l^{-1}$
June 30	0.16	25
August 22	1.3	2,200
August 24	1.6	4,000
August 29	2.7	1,750
September 5	2.0	2,000
September 6	1.1	1,860
September 13	2.4	4,000
September 14	2.6	2,000
September 20	1.0	1,800

Water samples were collected and immediately filtered through a glass fiber filter. Microcystin in filtered samples was assayed by protein phosphatase inhibition assay and expressed in microcystin-LR eq.

Quantification of microcystins in lake water by protein phosphatase inhibition assay

The concentrations of microcystins in the surface water of Lake Kasumigaura (Tsuchiura harbor) were determined by the protein phosphatase inhibition assay. Water samples were collected in June, August and September of 1995. During this period, the cyanobacteria in water samples collected from the lake mainly consisted of *Microcystis* and *Oscillatoria* species, and the former was usually the dominant species since the cyanobacterial colonies were usually accumulated in the coastal areas in these seasons.

For the measurement of microcystins, no pretreatment of water samples such as concentration or purification of microcystin was necessary because of the high sensitivity of the assay. Usually, more than 1,000-fold dilution of water samples is required to enable quantification. As shown in Table 1, the concentration of microcystins in the water sample collected in June 30 was 0.16 μg microcystin-LR eq. l^{-1}. This microcystin level was below the limit of detection of HPLC analysis in our study. Microcystin concentrations determined by the protein phosphatase inhibition assay of lake water samples collected in August and September were between 1.0 and 2.7 μg microcystin-LR eq. l^{-1}. HPLC analysis showed that

6.45 μg microcystin-LR l^{-1} and 12.18 μg microcystin-RR l^{-1} were present in the sample collected on August 29. However, HPLC was not applied for the determination of microcystin-YR because of interference by contamination with a large number of impurities which appeared at the same retention time as microcystin-YR. The average concentration of microcystins measured by this assay from eight water samples collected from Tsuchiura harbor of Lake Kasumigaura in August and September was 1.8 μg microcystin-LR eq. l^{-1}.

When water samples containing cyanobacterial cells were stored at 25°C, the concentration of released microcystin of lake water samples increased during the first 24 hours and dropped within the next 24 hours (Fig. 3). On the other hand, when the samples were stored at 4°C, microcystin was released into lake water and reached 25 μg l^{-1} in 8 days. Since dark blue pigment was released into the surrounding water, cyanobacterial cells might be decomposed and intracellular microcystins might be released during storage.

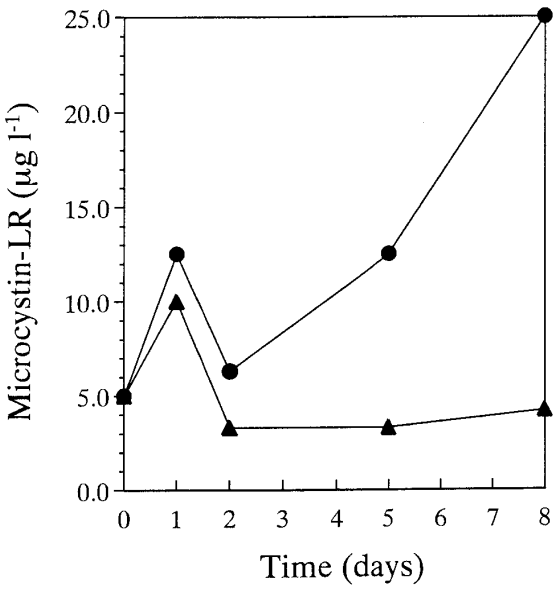

Fig. 3. Change of released microcystin concentration of lake water samples during storage.
Lake water samples were stored at 4°C (●) and 25°C (▲) for 8 days. Water samples were filtered and the concentrations of released microcystin were determined by protein phosphatase inhibition assay.

Productivity of microcystins of various strains of Microcystis cultures

Productivity of microcystins by various strains of *Microcystis* in culture was examined by the protein phosphatase inhibition assay. Since dark blue pigment was found in some culture fluids after storage under 3.37 μE m^{-2} s^{-1} of light at 20°C for 22 days, cultured cells might start to decompose and intracellular microcystins might be released. For determination of extracellular microcystin concentrations of the culture fluids of toxic strains, 100 to 10,000-fold dilution was necessary.

As shown in Table 2, 8 strains of 15 cultures were determined to be toxin-releasing strains. Among them *M. ichthyoblabe* TAC 113–1 and *M. viridis* TAC 45–1 showed potent productivity of the toxin, more than 1,000 μg l^{-1} of microcystin-LR eq., whereas it was not found in the culture fluids of *M. ichthyoblabe* TAC 48–1, TAC 48–3, TAC 76, TAC 110–1, TAC 128, TAC 136–1 and *M. wesenbergii* TAC 57–1. It is also noted that *M. ichthyoblabe* TAC 48 released small amount of microcystin into the culture fluid, although it was reported to be non-toxic[30].

The intracellular toxin concentrations of *M. aeruginosa* TAC 192–2, *M. ichthyoblabe* TAC 48–3, TAC 69, *M. viridis* TAC 92 and *M. wesenbergii* TAC 57–1 were examined by this method (Table 3). *M. aeruginosa* TAC 192–2, *M. viridis* TAC 92 and *M. ichthyoblabe* TAC 69 contained the toxin within the cells at concentrations of 557.8, 363.9 and 157.0 μg microcystin-LR eq. l^{-1}, respectively. On the other hand, *M. ichthyoblabe* TAC 48–3 and *M. wesenbergii* TAC 57–1 did not have intracellular toxins. These results indicated that there was a good correlation between extracellular and intracellular amounts of the toxins under our culture conditions.

Microcystin production during the growth of M. viridis TAC 45–1

Since *M. viridis* TAC 45–1 had the highest concentration of microcystin among the 15 strains examined (Table 2), toxin production during the growth of the organism was examined. Figure 4 shows the changes in concentration of microcystins in the culture fluid

Table 2. Concentration of microcystin released into culture fluid of *Microcystis* strains

Strains	Extracellular microcystin	Toxicity
	$\mu g\ l^{-1}$	
M. aeruginosa TAC 192–2*	588.0	N.T.[a]
M. ichthyoblabe TAC 48*	2.2	non-toxic[30]
M. ichthyoblabe TAC 48–1*	<0.5	N.T.[a]
M. ichthyoblabe TAC 48–3*	<0.5	N.T.[a]
M. ichthyoblabe TAC 69**	160.0	toxic[30]
M. ichthyoblabe TAC 76**	<0.5	N.T.[a]
M. ichthyoblabe TAC 110–1*	<0.5	non-toxic[30]
M. ichthyoblabe TAC 113–1**	1205.0	toxic[30]
M. ichthyoblabe TAC 128**	<0.5	N.T.[a]
M. ichthyoblabe TAC 136–1*	<0.5	N.T.[a]
M. viridis TAC 45–1*	1429.0	toxic[27]
M. viridis TAC 64**	280.0	toxic[28]
M. viridis TAC 78*	1.2	toxic[28]
M. viridis TAC 92*	312.5	toxic[28]
M. wesenbergii TAC 57–1*	<0.5	N.T.[a]

* Axenic strain
** Monoalgal strain
[a] Not tested previously
Released microcystin in filtrated culture fluids was measured by protein phosphatase inhibition assay and expressed in microcystin-LR eq. Intracellular toxicity was determined by mouse assays or HPLC analysis as reported previously[27,28,30].

Table 3. Concentration of microcystin in the culture fluid and cells of *Microcystis* strains determined by protein phosphatase inhibition assay

Strains	Extracellular	Intracellular	
	$\mu g\ l^{-1}$	$\mu g\ l^{-1}$	$\mu g\ g^{-1}$
M. aeruginosa TAC 192–2	588.0	557.8	170.0
M. ichthyoblabe TAC 48–3	<0.5	<4.5	<0.5
M. ichthyoblabe TAC 69	160.0	157.0	9.8
M. viridis TAC 92	312.5	363.9	17.9
M. wesenbergii TAC 57–1	<0.5	<4.5	<0.5

during growth of *M. viridis* TAC 45–1 estimated by chlorophyll-*a* (chl-*a*) concentration in the culture.

The cell density had reached a maximum of around 3.6 mg chl-*a* l^{-1} after 15 days of cultivation, and decreased gradually to 2.8 mg chl-*a* l^{-1} in 25 days. During the first 10 days, the concentration of microcystin increased gradually with increases in cell density. The concentration of microcystin in the culture fluid was associated with growth of *M. viridis* TAC 45–1 during the exponential phase, and still gradually increased

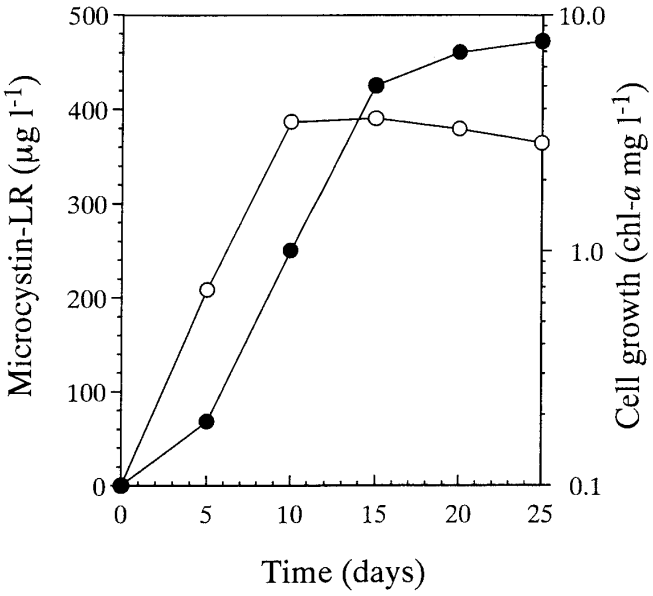

Fig. 4. Changes in the concentration of released microcystin during cell growth of *M. viridis* TAC 45–1.
The concentration of microcystin (●) was measured by protein phosphatase inhibition assay and the cell growth (○) was estimated by determination of chlorophyll-*a* concentration.

during the stationary phase reaching around 480 μg microcystin-LR eq. l^{-1}.

Discussion

For quantification of low levels of microcystin in lake water, the protein phosphatase inhibition assay was carried out. Our results confirmed that the protein phosphatase inhibition assay is useful for estimation of total concentration of microcystins in both lake water and *Microcystis* culture samples.

The sensitivity of this method is high enough to determine the concentrations of microcystin in the range of 0.1 to 10 μg microcystin-LR eq. l^{-1} without any treatment. Since the concentrations of microcystin in the environment are usually less than 1 $\mu g\, l^{-1}$, this method can be applied to measure natural water samples without any dilution or concentration. It is also possible to handle numerous samples at the same time using this method. Therefore, it might be a suitable method for monitoring microcystin in lakes and reservoirs.

Microcystin is a potent inhibitor of protein phosphatase 1 and 2 A similarly to okadaic acid and nodularin[32]. IC_{50} values of microcystin-LR, YR and RR calculated by this method were 0.4, 0.9 and 11.0 $\mu g\, l^{-1}$, respectively (Fig. 1), which correlated well with LD_{50} estimated by mouse assays[6,13,23,29]. Therefore, the protein phosphatase inhibition assay could be used to estimate toxicity of microcystin of natural water samples in place of the mouse bioassay. This assay needs a radioactively labeled protein substrate which is not useful for many laboratories or for field tests. Therefore, an alternative assay using a non-radioactive substrate, para-nitrophenylphophate (pNPP), was developed[1].

The major drawback of the protein phosphatase inhibition assay is its possible reaction with nonspecific phosphatase in the sample or with endogenous protein phosphatase that will lead to underestimation of microcystin[21]. Our results showed a good correlation between the results obtained by protein phosphatase inhibition assay and HPLC, although the concentrations of microcystin measured by protein phosphatase inhibition assay were 2 to 3-fold lower than those determined by HPLC analysis.

It is necessary to filtrate water samples as soon as possible to remove cyanobacterial cells to prevent the release of microcystins during storage. The concentration of released microcystins increased from 0.4% to approximately 50% of total toxin during storage of *Microcystis* cells in the dark at 20°C for 22 days (data not shown). Watanabe measured the released microcystin LR and YR from naturally decomposing cells of *M. aeruginosa* in non-axenic culture[31]. Our results using axenic strain *M. viridis* TAC 45–1 showed that microcystin might be excreted by the cells during the course of cell growth, since the concentration of microcystin in the culture fluid was associated with growth of the organism as shown in Fig. 4. For 5 cultures of monoalgal strains, the possibility still remains that associated bacteria might decompose the algal cells or that they might stimulate production of the toxin.

As shown in Table 2, a strain of *M. aeruginosa* and 4 strains of *M. viridis* were toxic, which was consistent with the results reported by Watanabe et al.[30]. However, *M. viridis* 78 which had been reported to be a toxic strain[28] did not have significant amounts of microcystin in our study. For *M. wesenbergii*, several studies have documented this species as both toxic[26] and non-toxic[19,28]. We also observed that there are both toxic and non-toxic strains in *M. wesenbergii* species by this method since *M. wesenbergii* TAC 38 released 18.0 μg microcystin-LR eq. l^{-1} into the culture fluid. From the above results, it was concluded that there are toxic and non-toxic strains within the same species of *Microcystis*. The productivity of the toxins is not admissible as a criterion for the discrimination of species within the *Microcystis* genus.

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