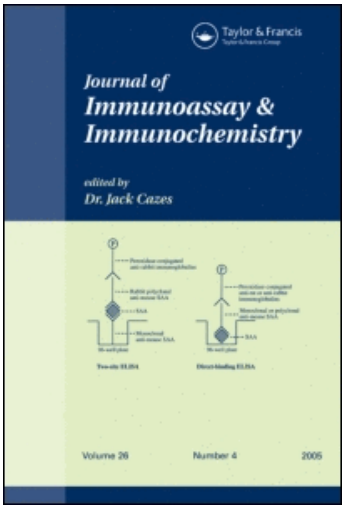

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Rapid Analytical Detection of Microcystins Using Gold Colloidal Immunochromatographic Strip

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Abstract: Routine monitoring of microcystin in natural waters is difficult because the concentration of the toxin is low and the detection method is usually complicated. We developed a rapid analytical detection method of microcystins gold colloidal immunochromatogeraphic strip. The sensitivity of the strip is about 1 ng/mL for microcystin LR; it is able to distinguish visually among different concentrations of microcystin solutions. The developed gold colloidal strip can detect microcystins within 15 min and does not require either a complicated extraction system, or trained or qualified experts.

Keywords: Microcystin, Detection, Gold colloid, Strip

INTRODUCTION

Cyanobacteria, also known as blue-green algae, are common members of the plankton of marine, brackish, and freshwaters throughout the world. *Microcystis aeruginosa* is the most common toxin-producing cyanobacteria found worldwide that produce microcystins. In the various reported incidents of poisoning in human and livestock caused by toxins of cyanobacteria, *Microcystis* is the most frequently cited organism. Microcystins are a family of potent toxic oligopeptides produced by freshwater cyanobacteria genera including *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc*.[1,2] The most

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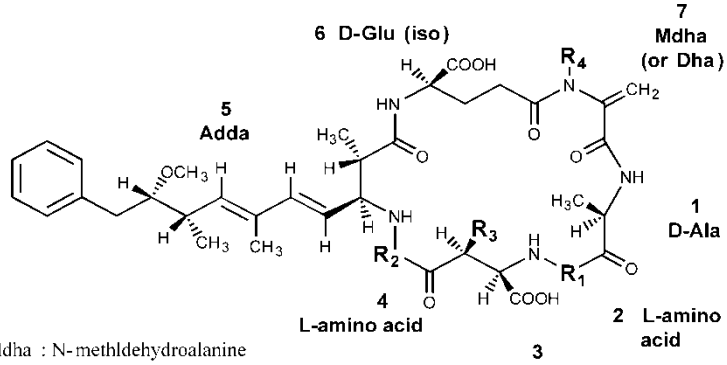
extensively studied form is microcystin-LR, which contains L-leucine and R-arginine in the two main variant positions. Nowadays, more than 60 of these compounds and their structural variants have been reported and characterized. They have become a great threat to the welfare of humans and animals. Natural resources are also in danger worldwide because cyanobacteria are capable of blooming by eutrophication of the water ecosystem and, thus, contaminating the drinking water. Some incidents have shown that acute exposure to these toxins could be fatal.[2,3] There has been a great demand for development of a fast and convenient analytical method to detect microcystins. One of the characteristic features of these monocyclic peptide toxins is sharing of the distinctive 20-carbon amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), as a common molecular component (Figure 1). The Adda component has been known to be a key player for the toxicity of the compounds.[4]

Many studies have revealed that the molecular mechanism of how these toxins affect tumor-promotion activity,[5,6] apoptosis,[7,8] and the onset of liver hemorrhage.[9] These very small oligopeptide (MW-1,000 Da) toxins accumulate in hepatocytes, their main target in the body, and bind irreversibly to



	R_1	R_2	R_3	R_4	MW
Microcystin - LR	Leu	Arg	CH_3	CH_3	994
Microcystin - YR	Tyr	Arg	CH_3	CH_3	1044
Microcystin - RR	Arg	Arg	CH_3	CH_3	1037

Figure 1. Structure of microcystins. The characteristic of microcystins and related cyanobacterial toxins is the hydrophobic amino acid, Adda, which contains two conjugated double bonds in position 5. Numbers represent the positions of the corresponding amino acid.

several serine/threonine phosphatases, such as phosphatase 1 (PP1) and/or phosphatases 2 (PP2A).[10,11] Since phosphorylation and dephosphorylation of protein is a key mechanism for the functional regulation of cells, the inhibition of enzyme activity may cause severe damage to cells. The binding of microcystins to the phosphatases initiates the formation of an intercellular reactive-oxygen species[12,13] which disturbs the fine organization of the cytoskeletal architecture.[12,14] It also causes typical morphological and biochemical changes of apoptosis in an affected cell, including plasma-membrane blabbing and DNA-fragmentation.[15]

Management of surface and drinking water is essential to protect human and animal health; it has become very important to develop fast, reliable, and accurate analytical methods to detect microcystins. Several methods have been developed through the years, including animal bioassay[16] enzyme phosphatease-inhibition assay,[17,18] and high-performance liquid chromatography (HPLC) after solid-phase extraction.[19] To detect microcystins, many researchers employ direct competition Enzyme-Linked Immunosorbent Assay (ELISA) using a monoclonal or polyclonal antibody, i.e., the prevailing method for detection of microcystins.[20] While the other detection methods have their own advantages, they are inconvenient due to the required time-consuming procedures, and special safety precautions needed when handling isotopes, expensive equipment, and need for qualified experts.

In this study, the rapid analytical detection method for microcystins, using a gold colloidal strip, was developed for the detection signal and the principle of traditional lateral-flow-type immunochromatographic assay. The method for purifying microcystins from cyanobacteria was developed by supercritical fluid extraction (SFE) using methanol as modifier.[21,22] The developed assay system consists of a nitrocellulose strip housed in a disposable cartridge. The samples can be analyzed at the test site using a real time base, within 15 min, without having to bring the samples back to the laboratory.

EXPERIMENTAL

Keyhole Limpet Hemocyanin (KLH), and 1-ethyl-3-(3-dimethylaminopropyl) carbadimide (EDAC) were purchased from Sigma (St. Louis, MO). Streptoavidin, Protein G, and 3,3′,5,5′,-tetramethylbenzidine (TMB) were purchased from Kem-En-Tec (Copenhagen, Denmak). Microcysin-L-leucine-R-arginine (MCLR) was extracted from *Microcystis aeruginosa* in the analytical chemistry laboratory, Kangwon National University. Cyanobacterial cells were collected from the Soyang Lake, which is the biggest water source in South Korea. Algal cells were freeze-dried for extraction; 100 mg of dried cells, pre-extracted with a ternary mixture (90% CO_2, 9.0% methanol, and 1.0% water) at 40°C and 250 atmosphere, was used.

Production and Purification of Microcystin

Supercritical fluid extraction of microcystins from cyanobacteria was performed using a JASCO (Tokyo, Japan) LC-900 SFE system. The system consisted of three sections; fluid delivery, extraction, and collection. The fluid delivery section included two pumps which delivered liquid carbon dioxide and a modifier solvent separately. In the extraction section, supercritical fluid extractions were performed with carbon dioxide modified aqueous methanol. The collection section included a back-pressure regulator, which kept the pressure of an extraction vessel at a desired value. The effluent flowing through the back-pressure regulator reduced its pressure to atmospheric pressure and, thereby, the solubilities of the solutes in the effluent were reduced to virtually zero. In this way, the solutes were deposited and collected in a collection vessel. The aqueous methanol modified CO_2 was used as an extracting solvent and the extracts were collected as a liquid solution in the collection vessel.

Production of Monocolonal Antibody Against Microcystin-LR

A good quality of mouse monoclonal antibody (mAb) against MCLR which was conjugated to Bovine Serum Albumin (BSA) or keyhole limpet hemocyanin (KLH), was produced in the presence of 1-ethyl-3,3′-dimethyl-aminopropyl-carbodiimide (EDAC). The immunization, cell fusion and screening of hybridoma cells producing anti-MCLR antibody were conducted according to the standard method.[23] Six- to eight-week-old BALB/c mice were immunized with 100 μg of MCLR-KLH conjugate which was mixed with complete Freund's adjuvant. The first injection was followed by three or four booster injections of the same amount of immunogen, which was mixed with incomplete Freund's adjuvant, at 3∼4 week intervals. Serum was taken from the tail of mouse and tested for antibody titer, usually after the third injection. For the fusion, spleen cells from immunized mouse were combined with SP2/0-Ag-14 myeloma cells, and 1 mL of 50% polyethylene glycol (PEG) 1500 in DME medium was added, drop by drop, during 60 sec. The fused cells were selected in Hypoxanthine-Aminopterin-Thymidine (HAT) medium for 2 week and culture supernatants of hybridoma cells were then collected; they were then screened with the BSA-conjugated MCLR as an antigen by ELISA assay. The positive clones were frozen first and screened further by two successive limiting dilutions after thawing. Ascitic fluids were generated for the large-scale production of the mAbs by injecting 1×10^7 hybridoma cells into the peritoneal cavity of a mouse.

The monoclonal antibody (mAb) was purified from the supernatant of hybridoma cells that were cultured in serum free DME medium through a series of purification steps: membrane ultra-filtration, ammonium sulfate

precipitation, and then protein G column chromatography. The eluted mAb from the column, with 100 mM glycine-HCl (pH 2.5), was neutralized with 0.1 vol. of 1 M Tris-Cl (pH 8.0), the concentration was measured, and it was stored at -70°C until use. Ascitic fluids were directly applied onto the protein G column after clearing by centrifugation. The antibodies were eluted with 100 mM glycine-HCl (pH 2.5), neutralized, and dialyzed against phosphate buffered saline solution (PBS).

Enzyme-Linked Immunosorbent Assay (ELISA)

For ELISA, 96-well microtiter plates (Greiner Labor-technik, Polystyrene) were coated with MCLR-mAb in 50 mM sodium-carbonate buffer at 2 μg mL^{-1} concentration and incubated at 37°C for 1 hour; they were then washed three times with 0.2% Tween 20/PBS. After washing, 100 μL of substrate solution (KPL, Bluephos phosphate solution A. B) was added and the plates were incubated for 30 min at room temperature. The reaction was stopped by the addition of 100 μL per well of 0.2 M EDTA (pH 8.0). The absorbance was measured at 630 nm by using an auto ELISA reader (Bio-rad model 550 reader).

Gold Colloidal Immunochromatographic Strip

The immunochromatographic strip consists of a nitrocellulose (NC) membrane, a sample pad, an absorption pad, and a backing card (Figure 2). These components are located on the adhesive side of the polystyrene card. The test line (detection zone) is located on the nitrocellulose membrane, and its bottom side was coated with a thin plastic film. Streptoavidin

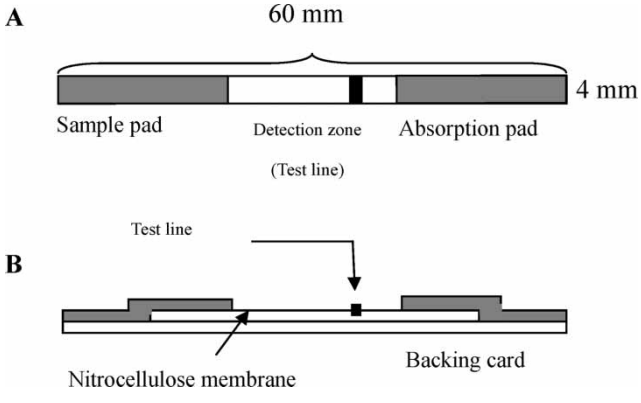


Figure 2. Schematic diagrams of developed gold colloidal immunochromatographic strip. (A) Upper view of the strip; (B) Side view of the strip.

($2.5\ mg\ mL^{-1}$) was dispensed on the control line of the detection zone as an internal standard. MCLR-BSA ($125\ mg\ mL^{-1}$) conjugates were dispensed on the test line to detect microcystins in the samples. When an automatic dispenser (Bio Dot, USA) was used, MCLR-BSA conjugates were dispensed onto the nitrocellulose membrane (0.8 mm width and $0.88\ \mu L\ cm^{-1}$ of volume). Before setting on NC, the sample pad was completely soaked in PBS containing 1% BSA and 0.05% Tween 20 and vacuum-dried at 50°C for 1 hour. The absorption pad was set up at the end of the nitrocellulose matrix.

Conjugation of Gold/MCLR-mAb and MCLR/BSA

In this study, the gold colloidal immunochromatographic strip was developed for rapid detection of microcystins. 30–40 nm gold particles were selected and purchased from BB International, Inc. The following procedures were carried out for the gold colloids conjugate to MCLR-mAb and MCLR-BSA.

MCLR-mAb ($1.2\ \mu g\ mL^{-1}$) and the colloidal gold were mixed, and the pH was adjusted to 9.0 by 40 mM Borax for dialysis against PBS. Then, 1% of the whole amount of BSA was added to the above mixture because of blocking of empty space which didn't adhere to colloidal gold surface. The mixture was centrifuged at 6,000 rpm for 40 min at 4°C; then it was filtered through a 2 μm filter.

For the MCLR conjugate to BSA, 1.2 mg of MCLR was dissolved in 1 mL of BSA solution, cross-linked by adding 11.5 mg of EDAC, and stirred overnight at 4°C. Then the mixture was purified by Sephadex G25 column chromatography.

RESULTS AND DISCUSSION

To produce a good quality of mouse monoclonal antibody, it is very important to prepare antigen, microcystin LR, in extremely pure form. The microcystins were extracted from cyanobacterium using supercritical carbon dioxide. The microcystin-LR was successfully extracted with a ternary mixture (90% carbon dioxide, 9.0% methanol, and 1.0% water). This supercritical fluid extraction method has several advantages over solid-phase extraction for the analysis of microcystins. Sample handling steps are minimized, thus reducing possible loss of analytes and saving analysis time. No organic solvent extractions are involved in this method and no clean-up steps are employed. The KLH-conjugated microcystin LR was used as an immunogen for the production of mouse monoclonal antibody. Immunization, cell fusion, and screening of hybridoma cells producing anti-microcystin LR antibody were conducted according to a standard method. Each mAb at an appropriate concentration, mixed with

indicated concentrations of MCLR which was added to MCLR-KLH coated wells of ELISA microtiter plates, followed by the addition of hybridomas producing (HRP) labeled anti-mouse IgG antibody. The binding interaction between anti-MCLR-mAb and MCLR-BSA conjugate was well inhibited by free MCLR in the water samples when seven different concentrations (1,600, 800, 400, 200, 100, 50, 0 pg mL^{-1}) of aqueous MCLR samples were tested. Then, the sensitivity (minimum detection limit) of the ELISA system was determined at low concentrations of aqueous MCLR samples on the above concentrations of water samples. The minimum detection level of ELISA system was found to be about 50 pg mL^{-1} of microcystin LR.

Fabrication of Gold Colloidal Immunochromatographic Strip

The gold colloidal immunochromatographic strip was developed for the rapid detection of microcystins. The components of the lateral strip are as follows: a nitrocellulose membrane, a sample pad, an absorption pad, and a backing card. These components are in direct contact with each other so that lateral flow of sample from the sample pad to the membrane and then to the absorption pad occurs continuously. Gold particles of any accurately defined size can be manufactured reproducibly under the appropriate manufacturing conditions. Since different sizes may be used for different applications, it is important to choose the optimum gold particle size. In this study, 30–40 nm gold particles were selected for conjugation with MCLR-mAb and MCLR/BSA. The structure of the lateral strip is shown in Figure 3. Instead of antibody, MCLR was immobilized on the test line in the form of MCLR-BSA conjugate in this system. The strip was fabricated into a plastic cartridge, completely sealed in a foil pouch with a desiccant, and stored at room temperature.

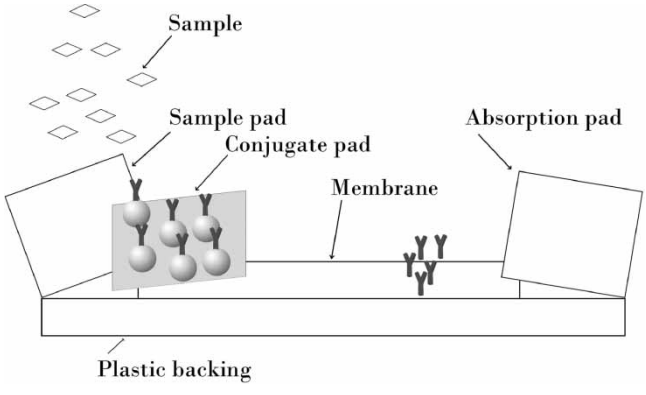


Figure 3. The structure of lateral strip for the rapid detection of microcystins.

The conjugate pad generally contains a detector reagent; the colloidal gold conjugate to an antibody specific for the analyte was of interest. The sample pad was pretreated with PBS buffer containing 1% BSA and 0.05% Tween 20 to prevent nonspecific binding and to ensure that the tests could be reproduced. In this study, a well containing Gold/MCLR-mAb with buffer was used instead of the conjugate pad. The defined distance from the junction between sample pad and membrane and the capture reagents (test line) were required to immobilize on the membrane. The developed gold colloidal strip for microcystin-LR is presented in Figure 4. The condition of the developed gold colloidal strip for detection of microcystins is presented in Table 1.

The test line indicates the presence of analyte contained in an antigen (MCLR-BSA). A provisional guideline value of 1 ng mL^{-1} has been adopted for microcystin-LR (WHO, 1998). Therefore, the developed gold colloidal strip for detection of microcystins is designed to fit into the WHO guideline value of 1 ng mL^{-1}. The volume of sample was controlled to flow laterally in the membrane and the absorption was well analyzed in this study. If the sample contains a large amount of microcytins, the test line shows a weak red color because of binding Gold/MCLR-mAb and MCLR. On the other hand, if the sample contains low levels of microcystins, the test line shows a strong red color.

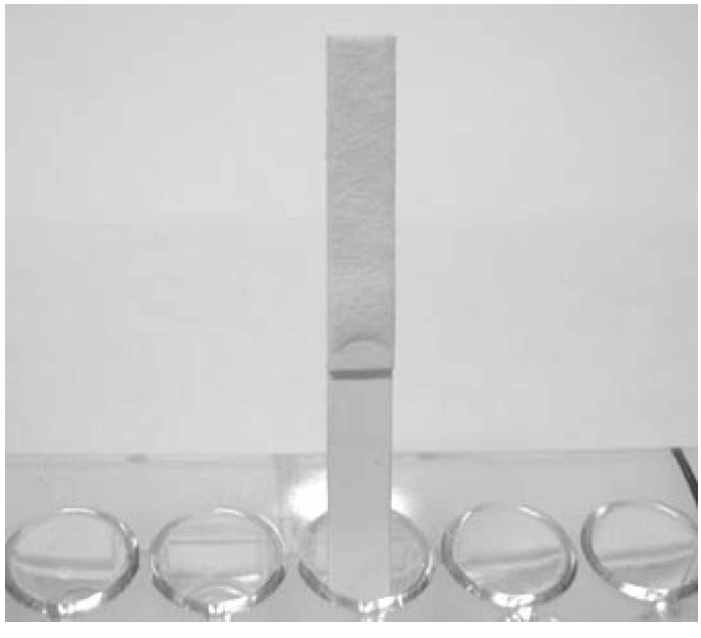


Figure 4. Gold colloidal immunochromatographic strip for the detection of microcystin-LR.

Table 1. Conditions of developed gold colloidal immunochromatographic strip for detection of microcystins

The immumochromatographic strip for microcystins		
Gold size and conjugation	NC matrix	Pad
Gold size: 30–40 nm MCLR mAb: 1.2μg/mL		Sample pad
Conjugated Gold/MCLR mAb: O.D = 1(520 nm)	Dispensed MCLR-BSA conjugate: 0.8 mm width and 0.88 μg/cm of volume	Absorption pad
Conjugated MCLR-BSA: 125μg/mL	Membrane: HF 180	Well (contains gold/MCLR mAb and PBS buffer)

Sensitivity and Stability of the Developed Gold Colloidal Strip for Algal Toxins

The performance of developed gold colloidal immunochromatographic strip was evaluated by its sensitivity and stability towards five different concentrations of microcystin-LR (0, 0.1, 1, 10, and 100 ng mL^{-1}). It was attempted five times during a two week interval to evaluate its stability. The sensitivity of developed assay for microcystin-LR is shown in Figure 5. According to Figure 5(c), 1 ng mL^{-1} MCLR is able to distinguish, visually, among the five different concentrations. Consequently, the developed gold colloidal immunochromatographic strip is successful. As a result, it shows the same color continually, which indicates good stability of the developed assay. Moreover, the developed gold colloidal strip can detect microcystins within 15 min and does not require either a complicated extraction system, nor trained or qualified experts. The stability of this assay is forecast at about 2 years.

Comparison of Gold Colloidal Strip and ELISA

The methods for comparison of gold colloidal strip and ELISA are time duration, manageability, sensitivity, quantitative analysis ability, and cost. The experimental results of these two methods are given in Table 2. According to the experimental results, the time duration of developed gold colloidal strip was 24 to 48 times faster than the ELISA detection method. In addition, the gold-colloidal strip can be used by everyone and not

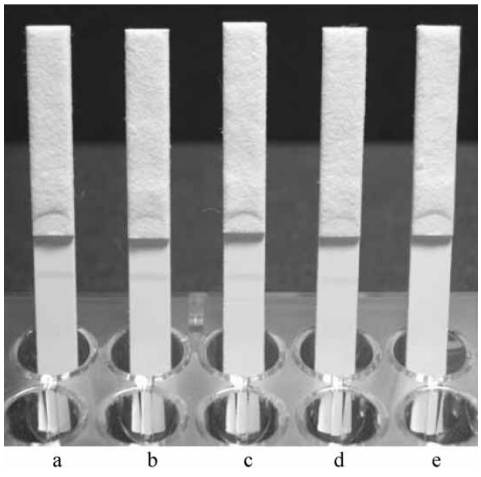


Figure 5. Sensitivity test of gold colloidal immunochromatographic strip using different concentrations of microcystin-LR solutions. a) 0 ng/mL; b) 0.1 ng/mL; c) 1 ng/mL; d) 10 ng/mL; e) 100 ng/mL.

necessarily qualified experts. Moreover, the cost of the gold colloidal strip per kit is 10 times less than ELISA detection. However, sensitivity and quantitative analysis show a remarkable weak point. The limit of detection was determined to be 50 pg mL^{-1} for the ELISA system and 1 ng mL^{-1} for the developed gold colloidal strip. Although the gold colloidal strip has lower sensitivity than ELISA, it appeared to be a better system. The reproducibility of the developed gold colloidal strip detection system was good through the entire range.

Apparently, the time duration, manageability, and cost are significantly strong points in the developed gold colloidal strip among these detection factors. Then, the gold colloidal strip does not require a substrate for signal generation, unlike ELISA. Thus, the developed gold colloidal immunochromatographic strip is a useful on-site detection tool for checking drinking water or surface water to detect microcystins.

Table 2. Comparison of gold colloidal immunochromatographic strip and ELISA

	Strip detection	ELISA detection
Analysis amount of time	About 15 minutes	About 6–12 hours
Manageability	Everybody	Only trained experts
Sensitivity	1 ng mL^{-1}	50 pg mL^{-1}
Quantitative analysis ability	impossibility	possibility
Cost	\$ 100/kit	\$ 1000/kit

CONCLUSION

Cyanobacteria produce a diverse range of small molecules (cyanobacterial toxins) which are hazardous to human and animal health. Consequently, reactive and proactive measurements are required to detect cyanobacterial toxin. Therefore, a rapid, sensitive immunoassay, based on a gold colloidal strip was developed for rapid detection of microcystins, i.e., cyanobacterial peptide toxins.

The aim of this study was to evaluate the performance of a newly developed gold colloidal strip, in terms of the sensitivity and stability of monoclonal antibodies with different concentrations of microcystin LR. This is the first study on the use of gold colloids in an immunochromatographic assay to detect microcystins. The successful development of the gold colloidal immunochromatographic strip emphasizes the fast, convenient, and reproducible method for detection of microcystins with rapidity and good sensitivity. It is relatively small, transportable, and easy to use. The samples can be analyzed at the test sites in the field, e.g., lakes, rivers, and water purification plants in a real time, in 15 min, without trained experts. Thus, the developed gold colloidal strip may replace many types of MCs-ELISA test kits that are commercially available.

We expect that many active researches can be initiated in the field of microcystins and this research can be utilized to control highly potent toxic microcystins in drinking water.

ACKNOWLEDGMENT

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