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Method for Simultaneous Determination of Anatoxin-a and Microcystins in Korean Water Systems by Using LC/MS/MS

Moon, Jeong-Suk*, Hwa-Bin Kim[1], Hae-Kyung Park[2], Jaejung Lee[3], Jong-Hwan Park[4] and Haejin Lee[5]

(Han River Water Environment Research Center, National Institute of Environmental Research, Gyeonggi 476-823, Korea
[1]Woongjin Coway Environmental Technology Research Center, Seoul 130-110, Korea
[2]Water Environment Research Department, Incheon 404-708, Korea
[3]Geum River Water Environment Research Center, Chungbuk 373-804, Korea
[4]Youngsan River Water Environment Research Center, Gwangju 500-483, Korea
[5]Nakdong River Water Environment Research Center, Gyeongbuk 717-870, Korea)

This study was purposed to develop an effective LC/MS/MS method for simultaneously determining five pre-treated cyanotoxins (anatoxin-a, microcystins-RR, -YR, -LR and -LA) of cyanobacteria blooms. Cyanobacterial bloom samples were collected from 11 major lakes and three downstream areas of river around Korea during 2005~2009. Cyanotoxins were identified in 38 samples from the lakes. The validity of the method was evaluated and the recovery rates were found ranging from 83~87%. The MDL turned out to be 0.046 μg L^{-1} for anatoxin-a and 0.066 μg L^{-1} for microcystins (RR, YR, LR and LA), which indicates that the method has high sensitivity and accuracy. The most dominant genus of the cyanobacterial blooms was *Microcystis*, which accounted for 71% of the analysed samples. *Microcystis* also contained the largest amount of microcystins (398.5 μg gDW^{-1}) among the analyzed cyanobacteria. The analysis of the five cyanotoxins showed that anatoxin-a ranged between 0~41.833 μg gDW^{-1} and microcystins ranged between 6.311~2,148.786 μg gDW^{-1}. Among the microcystins, micocystin-RR took up 58.3%, the largest portion. Anatoxin-a was found to account for 77.8% of the samples. This study has its significance in that it allowed the establishment of toxin criteria appropriate for the Korean water systems. Further studies may be necessary to conduct for improving water treatment methods.

Key words : simultaneous determination, LC/MS/MS, microcystin, anatoxin-a, cyanobacteria

INTRODUCTION

The massive algal growth in Korea, due to rising temperatures and higher nutrient concentrations, led to cyanobacterial blooming, raising concerns about the deterioration of water purification treatment and harmful toxic materials. The algal blooms following water eutrophication have often been seen in water systems, especially in eutrophic freshwaters such as lakes, rivers and reservoirs in a temperate or sub-trophic climate (Carmichael, 1993; Namikoshi and Rinehart, 1996; Codd, 2000).

* Corresponding author: Tel: 031) 770-7279, Fax: 031) 773-2268, E-mail: waterfa@korea.kr

Toxins from freshwater cyanobacteria are classified into three types of intracellular toxins: cyclic hepatotoxins (microcystines, nodularin), alkaloid neurotoxins (anatoxins, saxitoxin, neosaxitoxin) and alkaloid cytotoxin (cylindrospermopsin) (Carmichael, 1992; Harada *et al.*, 1994; Falconer, 1999). Cyanobactera, especially members of the genera *Mycrocystis, Anabaena, Aphanizomenon* and *Oscillatoria* are common and potentially harmful in the freshwater environments (Falconer, 1993). Microcystins (MCYST) as hepatoxins are produced by *Microsystis awruginosa* (Rinehart *et al.*, 1994), *M. viridis* (Kusumi *et al.*, 1987), *M. botrys* (Henriksen *et al.*, 1996b), *Anabaena flous-aquae* (Krishnamurthy *et al.*, 1989), *Osillatoria agardhii* (Meriluoto *et al.*, 1989), *Nostoc* sp. (Sivonen *et al.*, 1990a), etc. They are known to inhibit the activation of protein phosphatase in liver cells, leading to cell transformation, tumour promotion, etc. to pose health risk to humans and animals (Hilborn *et al.*, 2005; Dai *et al.*, 2008). These cyclic heptapeptides are composed of five common amino acids with a molecular weight of 900 ~ 1,100 as well as a pair of L-amino acid variations. A few apparently non-toxic microcystine congeners exhibit structural alternations in the Adda or Glu regions (Harada *et al.*, 1990; Scotts *et al.*, 1993; Rinehart *et al.*, 1994) Currently, 85 types are known around the world as having low polarity and high stability for heat (Sivonen, 2009). The toxicity of microcystins is usually LD_{50} (lethal dose resulting in 50 percent deaths) 466 μg kg^{-1}, but it depands on the type: microcystins-LR and -YR record 70 μg kg^{-1} while microcystin-RR represents 600 μg kg^{-1}. In Japan, the most common toxins are microcystins-RR, -YR and -LR (Kim *et al.*, 1995). With no criteria on cyanobacteria toxins in terms of water quality set in Korea, this study applied the WHO guidelines issued in 1997, which indicates that microcystins, the most common toxins, should be less than 1 μg L^{-1} microcystin-LR/L in drinking water (Chorus and Bartram, 1999).

Anatoxin-a (ANTX-a) as a neurotoxin is produced by *Anabaena flos-aque* (Carmichael *et al.*, 1975), *Anabaena planktonica* (Bruno *et al.*, 1994), *Osillatoria* sp. (Edwards *et al.*, 1992), *Planktothrix* sp. (Sivonen *et al.*, 1989a), *Aphanizomenon* sp. (Bumke-Vogt, 1998), *Cylindrospermum* sp. (Sivonen *et al.*, 1989a), etc. It is known to combine with the nicotinic acetylcholine receptor at the neuromuscular interface to cause death in animals apparently resulting from muscle contraction, respiratory paralysis, and convulsion (Masahiko *et al.*, 1999). Anatoxin-a has a high toxicity (LD_{50} i.p. mouse 200 ~ 250 μg kg^{-1}) with a low molecular weight (165), but easily degrades into nontoxic substances in the sunlight or at a high pH (Harada *et al.*, 1989; Botana, 2007; Kim *et al.*, 2009). In Korea, no criteria on cyanobacteria toxins in terms of water quality have been established, while Austrailia and New Zealand set guidlines for anatoxin-a in drinking water, each at 3 μg L^{-1} and 0.006 mg L^{-1} (Ministry of Environment, 2008). Most studies on toxic cyanobacteria have been focused on *Microcystis*, while studies carried out in Austrailia and Northern Europe have paid attention to *Anabaena* (Baker and Humpage, 1994; Codd, 2000).

As seen in Table 1, Hawkins *et al.* (2005) reported to compare feasibilities of detection limit, selectivity, cost, time and training in each methods for PPi, ELISA, PCR, Microscopy, HPLC, LC/MS and Mouse toxicity. LC/MS/MS analysis was needed high cost and training like LC/MS method but it was capable of quantitative analysis of high sensitivity. To analyze pre-treated microcystins and anatoxin-a, HPLC/PDA and HPLC/FLD have been employed respectively. So far, it has been impossible to determine both of them simultaneously because different pre-treatment methods and instrumental analysis conditions had to be applied. Technological advancement, however,

Table 1. Methods ranked on cost, selectivity, turnaround time and training needs. (ref: Hawkins *et al.*, 2005)

Method	Detection limit (μg L^{-1})	Detection limit (cells mL^{-1})	Selective (microscopy)	Cost (LCMS)	Time (mouse)	Training (LC/MS)
PPi	0.3	3,000	63%	19%	25%	20%
ELISA	0.2	2,000	50%	42%	25%	20%
PCR	0.001	10	56%	9%	50%	29%
Micoscopy	0.001	10	100%	3%	50%	42%
HPLC	0.5	5,000	38%	63%	50%	74%
LC/MS	0.5	5,000	13%	100%	75%	100%
Mouse	8	80,000	75%	13%	100%	42%

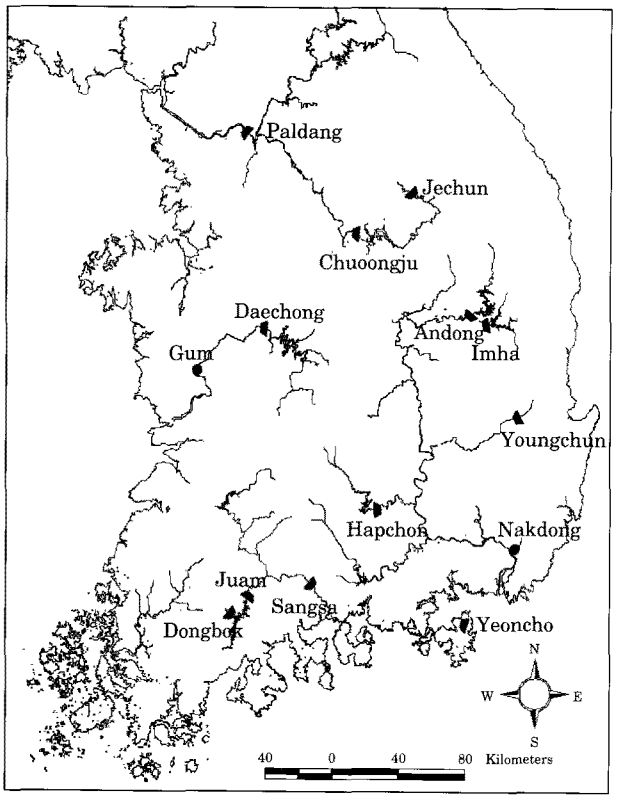

Fig. 1. Map of location of the study site.

allowed simultaneous determination by using LC/MS/MS, which is a significant progress given that there have been few data available on toxins in the Korean water systems.

The aim of this study was to suggest a new method for simultaneous determination of anatoxin-a and microcystins (microcystin-RR, YR, LR, LA) by applying LC/MS/MS to samples of cyanobacterial blooms collected from Korean water systems.

MATERIALS AND METHODS

1. Bloom samples

We used samples of cyanobacterial blooms from major Korean lakes and rivers (Fig. 1). The samples were examined under a microscope and the dominance value of the cyanobacteria was over 90%. The samples were lyophilized and preprocessed because of the large amount of cyanobacteria in them.

2. Standards, chemicals and instrumentation

We used anatoxin-a from Sigma (USA) and microcystins-RR, LR, YR, LA from from Alexis (USA).

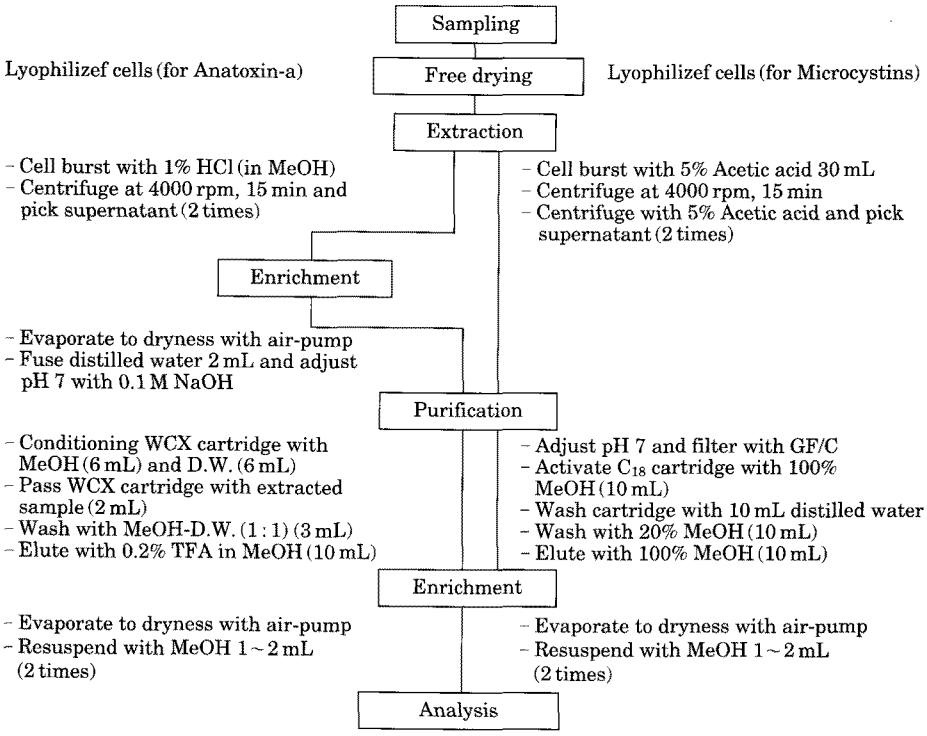

Fig. 2. Pretreatment for simultaneous determination of anatoxin-a, microcystins.

All Solvents and chemicals for pre-treatment and instrumental analysis were HPLC and analytical grade. As for cartridges, ODS Sep-Pak C_{18} and WCX from Waters (USA) were employed. Varian's 320 LC/MS/MS equipped with ZORBAX Eclipse XDB-C18 (Agilent) column was also used for this study.

3. Sample preparation

Before making chemical analyses, we lyophilized the cyanobacteria bloom samples and stored at −20°C to minimize changes in toxins. The lyophilized cells were fragmentized in order to analyze intracellular toxins in cyanobacteria. Pretreatment was carried out based on the method of Harada *et al.* (1988, 1989) in consideration of the characteristics of anatoxin-a and microcystins. As seen in Fig. 2, anatoxin-a and microcystins were pre-treated respectively through various processes such as lyophilization, extraction, enrichment and purification before they were mixed together for analysis.

Table 2. Mobile phase composition (%) of simultaneous determination.

Time (min)	0.1% formic acid	Acetonitrile
0	80	20
1	80	20
4	60	40
6	60	40
7	50	50
8	10	90
12	10	90
12.6	80	20
17	80	20

4. Simultaneous analysis of anatoxin-a and microcystins

LC/MS/MS was used with the Electrospray Ionization (ESI). To optimize the conditions of the mobile phase analysis, 0.1% formic acid and acetonitrile were employed. The flow velocity of the mobile phase was at 0.8 mL min^{-1}. Isocratic elution was made most effectively in the conditions shown in Table 2. Selective ion monitering (SIM) was performed to characterize each m z^{-1} ($[M+H]^+$ of anatoxin-a and microcystins-RR, YR, LR and LA). Multiple reaction monitering (MRM) enabled a simultaneous analysis which was not affected by other interfering substances (Fig. 3). As for the ESI, both of the positive and negative ion modes were applied. The analysis conditions of LC/MS/MS were as seen in Tables 3 and 4.

RESULTS AND DISCUSSION

1. Review of methods for simultaneous determination

After anatoxin-a and microcystins were pretreated respectively, the collected samples were mixed together. Then, 2 μg L^{-1} of the mixture was injected for simultaneous determination by LC/MS/MS. Calibration curves for the five toxins were made with 20~50 μg L^{-1} of the standard mixed liquid of anatoxin-a and microcystins. A quantitative analysis showed high correlation coefficients (R^2) and recovery rates as suggested in Table 5.

The standard mixed liquid was spiked 1 μg L^{-1} into distilled water to assess the recovery rate, relative standard deviation (RSD), accuracy and

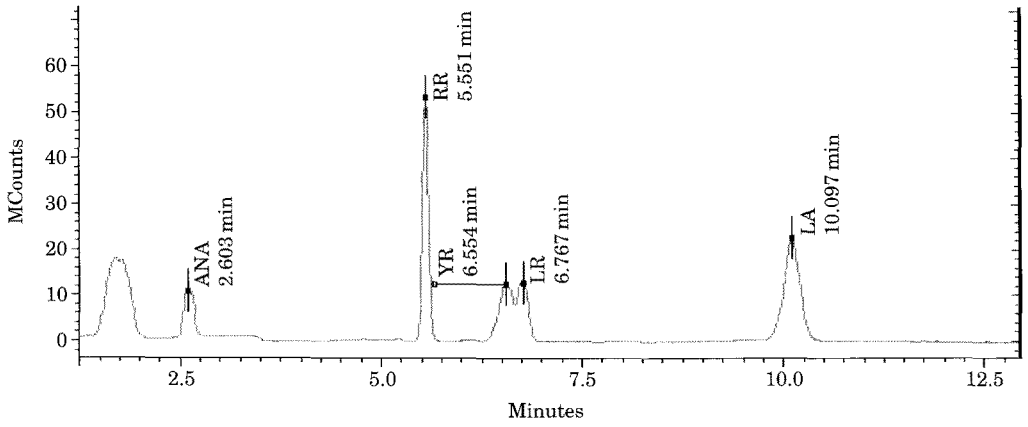

Fig. 3. Chromatogram for simultaneous determination of anatoxin-a, microcystins (RR, YR, LR, LA).

detection limit. After pre-treatment, the LC/MS/MS analysis were repeated three times to measure recovery rates. For the measurement of accuracies, relative standard deviations (RSD) and detection limits, it was repeated seven times.

Table 3. Summary of LC/MS/MS operating conditions used.

Mass	Conditions
MS	Varian 320-MS TQ Mass spectrometer
Flow	0.8 mL min^{-1}
Drying gas pressure °C	400°C
SIM width	0.5 amu total
Column	40°C
Inject volume	20 μL
Nebulizer gas pressure	55 psi
Drying gas pressure	35 psi
Housing °C	50°C
Ionization voltage	5.2 kV
CID gas pressure	1.8 mTorr

Table 4. Conditions of optimization for simultaneous determination by LC/MS/MS.

Toxin	Retention time (min)	Precusor ($m\ z^{-1}$)	Product ($m\ z^{-1}$)	Ion
Anatoxin-a	2.6	166.0	131.0	Positive
Microcystin-RR	5.5	520.0	520.0	Positive
Microcystin-YR	6.5	1043.5	1043.5	Negative
Microcystin-LR	6.7	993.5	993.5	Negative
Microcystin-LA	10.0	908.6	908.6	Negative

Table 5. Calibration and Recovery table of anatoxin-a, microcystins.

Toxin	R^2	Recovery (%)
Anatoxin-a	0.9995	87.0
Microcystin-RR	0.9996	84.6
Microcystin-YR	0.9992	83.2
Microcystin-LR	0.9995	86.2
Microcystin-LA	0.9995	83.3

Table 6. MDL, LOQ, Accuracy and RSD of anatoxin-a, microcystins.

Toxin	MDL (μg L^{-1})	LOQ (μg L^{-1})	Accuracy (%)	RSD (%)
Anatoxin-a	0.046	0.457	117.0	1.8
Microcystin-RR	0.045	0.451	117.6	1.8
Microcystin-YR	0.077	0.766	117.6	3.1
Microcystin-LR	0.082	0.815	118.8	3.2
Microcystin-LA	0.060	0.602	119.2	2.4

The recovery rates of anatoxin-a and microcystins were 87.0% and 84.3% (average) respectively. Their method detection limits (MDL) were 0.046 μg L^{-1} and 0.066 μg L^{-1} (average) each, which indicates that the method has a good sensitivity. The RSDs and accuracies were also high as suggested in Table 6. The limits of quantification (LOQ) of pre-treated anatoxin-a and microcystins

Table 7. Dominant genus from cyanobacteria blooms in water system of Korea (_: lake, _*: river, _**: stream).

Site	Date	Dominant genus
Paldang	'08. 07	*Mycrocystis*
	'08. 09	*Mycrocystis*
	'09. 07	*Anabaena*
	'09. 10	*Mycrocystis*
Sangsa	'06. 08	*Mycrocystis*
Chungju	'08. 08	*Mycrocystis*
	'09. 07	*Mycrocystis*
	'09. 07	*Mycrocystis*
	'09. 09	*Mycrocystis*
Daechung	'08. 06	*Aphanizomenon*
	'08. 08	*Mycrocystis*
	'08. 08	*Oscillatoria*
	'08. 10	*Aphanizomenon*
	'09. 06	*Aphanizomenon*
	'09. 07	*Mycrocystis*
	'09. 08	*Mycrocystis*
	'09. 08	*Mycrocystis*
	'09. 09	*Mycrocystis*
Jecheon*	'08. 09	*Mycrocystis*
	'09. 09	*Mycrocystis*
	'09. 09	*Mycrocystis*
	'09. 09	*Mycrocystis*
	'09. 10	*Mycrocystis*
Juam	'09. 08	*Anabaena*
	'09. 09	*Anabaena*
	'09. 09	*Anabaena*
	'09. 09	*Anabaena*
	'09. 09	*Anabaena*
	'09. 10	*Mycrocystis*
Geum*	'08. 10	*Mycrocystis*
	'09. 06	*Mycrocystis*
Andong	'08. 06	*Mycrocystis*
Youngchun	'08. 09	*Mycrocystis*
Nakdong*	'09. 06	*Mycrocystis*
Dongbok	'09. 06	*Anabaena*
Hapchon	'09. 09	*Mycrocystis*
Imha	'09. 09	*Mycrocystis*
Yeoncho	'09. 09	*Mycrocystis*

Table 8. Amounts of anatoxin-a, microcystins in cyanobacteria blooms from water system of Korea (_ : lake, _* : river).

Sites	Date	anatoxin-a (μg gDW^{-1})	Microcystins (μg gDW^{-1})				
			RR	YR	LR	LA	Total (μg gDW^{-1})
Paldang	'05. 08	–	5.638	0.811	4.667	N.D.	11.11
	'08. 07	–	88.590	8.360	39.257	2.986	139.19
	'08. 09	–	55.751	13.239	54.898	0.456	124.34
	'09. 07	1.760	76.700	10.510	88.575	1.191	176.98
	'09. 10	3.283	183.833	28.827	118.233	N.D.	330.89
Sangsa	'06. 08	–	9.197	1.463	3.991	N.D.	14.65
	'06. 11	–	4.210	1.212	5.362	0.104	10.89
Chungju	'06. 11	–	24.697	1.913	7.878	N.D.	34.49
	'08. 08	–	16.601	7.729	6.550	0.330	31.21
	'09. 07	1.839	63.350	4.399	29.825	1.953	99.53
	'09. 07	1.815	119.485	14.330	62.525	1.787	198.13
	'09. 09	N.D.	167.250	11.758	79.000	N.D.	258.01
Daechung	'08. 06	–	33.540	9.767	14.303	0.075	57.69
	'08. 08	–	167.381	61.707	75.733	0.217	305.04
	'08. 08	–	52.597	7.909	5.602	N.D.	66.11
	'08. 10	–	104.450	12.200	105.547	1.213	223.41
	'09. 06	2.426	30.381	5.802	19.168	0.699	56.05
	'09. 07	2.656	185.778	14.524	128.942	2.868	332.11
	'09. 08	0.740	139.367	23.350	135.833	N.D.	298.55
	'09. 08	1.108	33.550	3.943	42.893	N.D.	80.39
	'09. 09	1.031	33.213	1.341	16.187	N.D.	50.74
Jecheon*	'05. 09	–	424.144	61.876	177.529	N.D.	663.55
	'08. 09	–	168.660	26.133	44.480	N.D.	239.38
	'09. 09	1.360	22.307	4.268	30.367	N.D.	56.94
	'09. 09	2.784	518.467	72.253	280.200	N.D.	870.92
	'09. 09	N.D.	626.417	103.017	390.417	N.D.	1119.85
	'09. 10	N.D.	304.867	42.300	128.840	N.D.	476.01
Juam	'09. 08	3.480	4.029	N.D.	2.302	N.D.	6.33
	'09. 09	7.053	7.805	0.304	3.845	N.D.	11.95
	'09. 09	5.977	4.172	0.159	2.473	N.D.	6.80
	'09. 09	3.301	5.184	0.181	2.826	N.D.	8.19
	'09. 09	41.833	14.869	0.831	8.663	N.D.	24.36
	'09. 09	4.745	68.367	3.478	40.683	N.D.	112.53
	'09. 10	1.187	507.800	17.573	165.640	N.D.	691.01
Geum*	'08. 10	–	114.670	9.979	231.783	5.440	361.87
	'09. 06	1.171	387.013	30.685	255.813	1.251	674.76
Andong	'08. 06	–	29.443	2.620	24.993	13.770	70.83
Youngchun	'08. 09	–	141.413	23.837	214.250	3.123	382.62
Nakdong*	'09. 06	2.093	1420.533	61.980	663.967	2.306	2148.79
Dongbok	'09. 06	0.518	16.953	1.768	11.683	N.D.	30.41
Hapchon	'09. 09	N.D.	336.933	44.747	447.000	N.D.	828.68
Imha	'09. 09	N.D.	33.787	5.925	12.016	N.D.	51.73
Yeoncho	'09. 09	N.D.	440.067	3.111	79.267	N.D.	522.44

※ –: not determined, N.D.: toxins not detected.

were 0.457 μg L^{-1} and 0.659 μg L^{-1} (average). These results showed that it is possible to simultaneously determine the five toxic cyanobacteria by using LC/MS/MS in the summer.

2. Cyanobacteria toxin occurrence in Korea

Cyanobacteria bloom samples were collected from 11 lakes and three downstream areas of

rivers around Korea from 2005 to 2009. Cyanobacteria were found to be the dominant phytoplankton in these lakes and rivers (Table 7). The examination of cyanotoxins in 38 samples collected from the lakes showed that Microcystis was the most dominant (71%), followed by *Anabaena* (18%), *Aphanizomenon* (8%) and *Osillatoria* (3%) in order of frequency. *Microcystis* also contained 398.5 μg gDW^{-1} of microcystins on average, which was higher than *Anabaena* (39.1 μg gDW^{-1}), *Aphanizomenon* (112.4 μg gDW^{-1}) and *Osillatoria* (66.1 μg gDW^{-1}). This suggests that it is necessary to be more careful of *Microcystis* in controlling cyanobacteria occurrence during the summer.

3. Anatoxin-a and microcystins of cyanobacteria samples

We analyzed 43 cyanobacteria bloom samples in total: 16 of them for analyzing microcystins only and 27 for simultaneously determining both anatoxin-a and microcystins. The analyzed toxin contents were the dry weight of lyophilized bloom samples. It was difficult to estimate the total amount of toxins in the lakes and rivers because of the uneven distributions of cyanobacteria (Kim *et al.*, 1995). Microcystins were the major toxins in the samples. Microcystins-RR, LR and YR were detected from all samples while microcystin-LA was found in only 18 samples. Anatoxin-a was identified in 21 samples. Through analytical results in each sites, high amounts of microcystines were observed on June 2009 (Nakdong Mulkum) and those of anatoxin-a were observed on September 2009 (Juam). Environmental conditions needed by cyanobacterial blooms are related to eutrophic states (Rapala *et al.*, 1993). It is necessary to monitor the cyanotoxins due to rising temperatures and higher nutrient concentrations.

The contents of anatoxin-a ranged between 0 ~ 41.833 μg gDW^{-1}. The average was 3.41 μg gDW^{-1}. In an earlier study (Park *et al.*, 1997), anatoxin-a was detected from a few lakes including Chungju and Yeongrang. The simultaneous determination in this study, in contrast, found it from 77.8% of the samples. The amount of toxins produced by *Anabaena* depends on nutrient concentrations. Although a very small amount of it was traced in this study, it may be worth paying attention to its appearance (Sivonen *et al.*, 1989).

The contents of microcystins in the samples ranged between 6.331 ~ 2,148.786 μg gDW^{-1} and

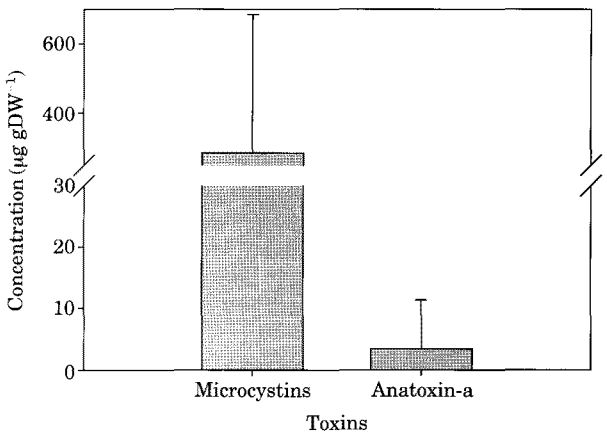

Fig. 4. Ranges from concentrations of Anatoxin-a, Microcystins.

the average was 285.10 μg gDW^{-1}, showing a wide variation (Fig. 4, Table 8). The study by Park *et al.* (1997) found that microcystin-YR took up 57.2%, the highest proportion, followed by microcystin-RR (25.6%) and microcystin-LR (17.1%). In this study, on the other hand, the relatively less toxic microcystin-RR accounted for 58.3%, followed by microcystin-LR (34.6%), microcystin-YR (6.3%) and microcystin-LA (0.8%), which indicates that microcystin-RR is the majority of cyanobacteria toxins in the Korean water systems.

Further studies are needed to develop better analysis methods that will enable simultaneous determination of various toxins from algae. It is also necessary to keep paying attention to the algal toxins and set toxin criteria appropriate for the algal occurrence patterns in Korea.

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