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**[Note]**

# The Analysis of Cyanobacterial Neurotoxins by High-Performance Liquid Chromatography-Mass Spectrometry

**Jong-Mun Jung[1,2]*, You-Jung Lee[1,2], Hong-Ki Park[2], Eun-Young Jung[2] and Gea-Jae Joo[1]**

[1]*Department of Biology, Pusan National University, Busan 609-735 and*
[2]*Busan Water Quality Institute, Busan Waterworks HQ, Kyoungnam 621-813, Korea*

Cyanobacteria were dominant from June to September in the Nakdong River and the Hoedong Reservoir. *Microcystis aeruginosa* was dominant from June to September; *Anabaena flos-aquae* from June to August and *Aphanizomenon flos-aquae* from July to August. Cyanobacterial neurotoxins, Anatoxin-a and saxitoxin were analyzed by electrospray ionization-mass spectrometry with strains of *Aphanizomenon flos-aquae* NIES-81 and *Anabaena flos-aquae* NIER-10002. Anatoxin-a was not detected from the cultured *Anabaena flos-aquae* nor from the wild samples. Low levels of saxitoxin were detected in the cultured *Aphanizomenon flos-aquae*; however, those of field samples were below the detection limit.

**Key Words:** anatoxin-a, *Aphanizomenon flos-aquae*, cyanobacterial toxins, LC-MS, saxitoxin

## INTRODUCTION

Recently annual cyanobacterial blooms occurred during summer and early fall in the Nakdong River-Reservoir system (Ha 1999). Cyanobacteria, especially members of the genera *Microcystis*, *Anabaena*, *Aphanizomenon* and *Oscillatoria* are common and potentially harmful in the freshwater environments (Falconer 1993). Some genera of cyanobacteria are known to produce three types of intracellular toxins: cyclic hepatotoxins (microcystins, nodularin), alkaloid neurotoxins (anatoxins, saxitoxin, neosaxitoxin) and alkaloid cytotoxin (cylindrospermopsin) (Carmichael 1992; Harada *et al.* 1994; Falconer 1999).

Anatoxin-a (ANTX-a) and saxitoxins (STXs) are neurotoxin alkaloids produced by a number of blue-green algae (cyanobacteria) including *Anabaena*, *Aphanizomenon* and *Oscillatoria*. ANTX-a is a potent agonist for nicotinic acetylcholine receptor, acts as a postsynaptic depolarizing neuromuscular blocking agent and has been associated with *Anabaena flos-aquae*, *Anabaena spiroides*, *Anabaena circinalis*, and *Oscillatoria*. *Anabaena circinalis* blooms in Australia have recently shown to produce paralytic shellfish poisons (Baker and Humpage 1994; Steffensen 1994).

STXs are carbamate alkaloids and more than 20 chemical analogues have been characterized (Oshima 1995) and 16 have been identified in freshwater cyanobacteria (Sivonen and Jones 1999). Especially, saxitoxin and neosaxitoxin (neoSTX) were shown to be the major neurotoxins present in *Aphanizomenon flos-aquae* (Mahmood and Carmichael 1986). The toxin was described as a "very fast death factor (VFDF)" since the toxin acts in a few minutes, with very few deaths occurring after 5 min (Alam *et al.* 1973). These toxins present acute hazards to human and animal health and are responsible for isolated and sporadic animal fatalities; in mammals, fish and birds. Monitoring the levels of these toxins is important in the management of reservoir and drinking waters (Namera *et al.* 2002).

Preparative separation of the ANTX-a and STX has been accomplished with a variety of techniques, including ion-exchange chromatography (Jaime *et al.* 2001), thin-layer chromatography (Ikawa *et al.* 1982), electrophoresis (Alam *et al.* 1973) and gas chromatography-mass spectrometry (Zotou and Jefferies 1993). This study was to applied to develop a sensitive, simple, reliable and highly selective method for the determination of optimized LC-MS method for anatoxin-a and saxitoxin of freshwater cyanobacterial toxins in the municipal water management.

*Corresponding author (jmjung4356@empal.com)

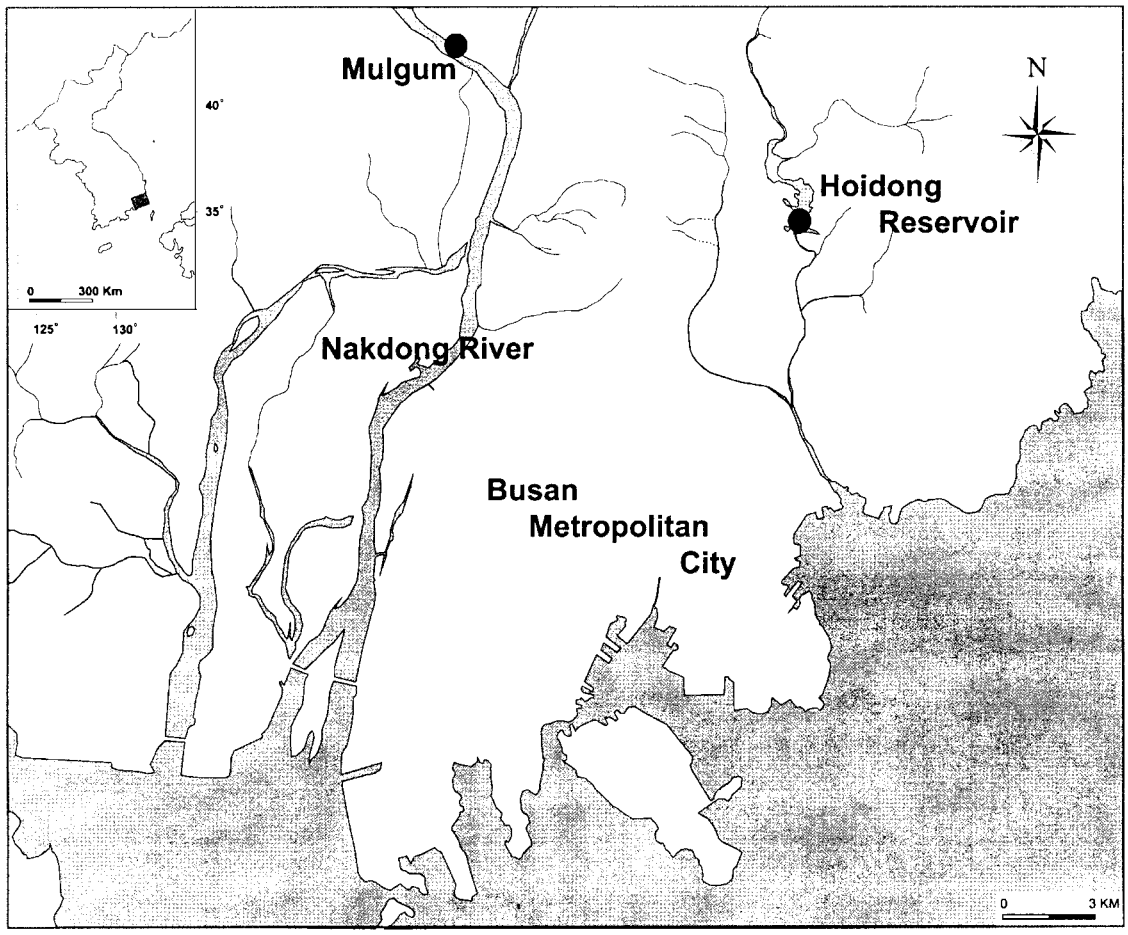


**Fig. 1.** Map and location of the study sites in Mulgum and Hoedong Reservoir.

## MATERIALS AND METHODS

### Collection of samples

Cyanobacterial samples for toxin analysis were collected by plankton net (20 $\mu$m mesh) at Mulgum in July 1999 and at Hoedong Reservoir in January 2000 (Fig. 1). The samples were stored in a cooler for transport to the laboratory, cleaned of debris, rinsed with distilled water, lyophilized (Samwon, SFDSF12, Korea), and stored at -20°C until screened for toxicity by mouse bioassay and analysis for toxin content by LC-MS. The concentrations of chlorophyll *a* as phytoplankton biomass were monitored spectrophotometrically (Wetzel and Likens 1991; Ha 1999).

### Cultivation of cyanobacterial samples

Strains of *Anabaena flos-aquae* NIER-10002 (National Institute for Environmental Research, Korea) and *Aphanizomenon flos-aquae* NIES-81 (National Institute for Environmental Studies, Japan) were used in this study. The culture was maintained in the CB medium buffered with TAPS (N-tris[Hydroxymethyl]-3-aminopropanesulfonic acid). The 0.5 m*l* of sample was inoculated to 100 m*l* CB medium in 250 m*l* glass Erlenmeyer flask and its initial cell density was about 105 cells $\cdot$ m$l^{-1}$. The cultures were maintained at 25 ± 1°C, under 1500 lux illumination and 12L:12D photoperiod. Cultures were harvested about 20 days after inoculation for analysis.

Cell densities were estimated by an inverted microscope (Axiovert 135M, Zeiss, Germany) with Sedgewick-Rafter Chamber. The ranges of densities were 1.0-2.0 $\times$ $10^7$ cells $\cdot$ m$l^{-1}$ for *Anabaena flos-aquae* NIER-10002 and 1.5-2.5 $\times$ $10^7$ cells $\cdot$ m$l^{-1}$ for *Aphanizomenon flos-aquae* NIES-81.

### Chemicals and standards

All solvents and chemicals were HPLC and analytical grade; acetonitrile (Merck, Germany) and heptafluorobutyric acid (analytical grade, Aldrich A16419-4. USA). Water was purified with a Milli-Q Ultra pure Water System (Millipore, USA). Standard for ANTX-a from ICN (No. 159873, USA) and STX from Sigma (S-1417, USA).

**Extraction of samples for ANTX-a**

Aliquiots of HPLC grade water (100 m*l*) were spiked at 1.0 $\mu g \cdot l^{-1}$ and 3.0 $\mu g \cdot l^{-1}$ with ANTX-a. Aliquiots of field sample water taken from Nakdong River (100 m*l*) were spiked at 1.0 $\mu g \cdot l^{-1}$ with ANTX-a. Solid-phase extraction cartridges (100 mg sorbent mass: OASIS Waters, UK) were preconditioned with methanol (10 m*l*) followed by HPLC grade water (20 m*l*). The samples were then applied to the cartridges and allowed to percolate through the sorbent bed gravity. The cartridges were dried under vacuum and the analyte eluted into a clean glass vial with methanol containing 0.1% v/v trifluoroacetic acid (2 m*l*). The extracts were evaporated at 40°C under nitrogen. The samples were reconstituted in 1 m*l* of 10% methanol, capped and stored prior to analysis (Fig. 2).

**Extraction of samples for STX analysis**

The lyophilized *Aphanizomenon flos-aquae* samples (1.0 g) were extracted with 5% acetic acid at 50 $ml \cdot g^{-1}$ of materials by sonicating (20KHz, 180W) for 30 seconds (Fig. 2). The mixture was stirred for 24 hour at room temperature and centrifuged at 20,000 × g for 30 min at 4°C. The suspensions were filtered through a filter (GF/C Millipore, USA) to remove cell debris. Filters were washed with 5% acetic acid, and samples were passed through a ODS cartridges (Sep-Pak $C_{18}$, Waters, USA) which had been washed and pre-equilibrated with 10 m*l* each of methanol and distilled water.

Toxins were eluted with 100% methanol (LiChrosolv UN 1230, HPLC grade Merck, Germany). The eluate was evaporated under reduced pressure and then the residue was dissolved in 1.0 m*l* of 5% methanol. Before HPLC analysis, each sample was filtered through a ultrafiltration membrane with 10,000 dalton cut-off (Ultrafree-MC, Millipore, USA) and centrifuged at 5,000 g for 15min. The concentrated extract was the transferred to a 300 $\mu l$ HPLC vial and stored prior to analysis (Fig. 2).

**Bioassay**

Mouse bioassays were performed according to the Association of Official Analytical Chemists method (AOAC 1995) for paralytic shellfish poisons (PSPs) with using male mice 16-21 g (Balb-C). Mice were injected intraperitoneally with 1.0 m*l* extracts obtained from the lyophilized material extracted with 0.1N HCl. Given sufficient amount of sample, tree mice were injected 0.25 mg, 0.5 mg and 1.0 g material, respectively. Symptoms of poisoning and survival times were recorded. Toxicities

| lyophilized cells 1.0 g (for ANTX-a ) | lyophilized cells 1.0 g (for STX) |
|---|---|
| ← extracted with 0.05M AcOH | ← extracted with 3% AcOH |
| ← tree times | ← tree times |
| ← centrifuged 10,000 rpm, 1 hr, 4℃ | ← centrifuged 20,000×g, 30min, 4℃ |
| supernatant | |
| ← pass $C_{18}$ cartridge | ← pass ODS $C_{18}$ cartridge |
| ← air dry (overnight) | ← air dry (overnight) |
| ← washed with water and 10% MeOH | ← elute 100% MeOH |
| ← elute 100% MeOH | ← evaporated dry |
| ← evaporated dry | ← elute 5% AcOH 1.0 m*l* |
| ← elute 5% AcOH 1.0 m*l* | ← 10,000 dalton cut-off |
| LC-MS injection (2.0 μ*l*) | |

**Fig. 2.** The analysis procedure of ANTX-a and STXs in cyanobacterial cells.

**Table 1.** HPLC gradient conditions for anatoxin-a

| time (min) | acetonitrile (0.1% TFA) | water (0.1% TFA) |
|---|---|---|
| 0.00 | 2 | 98 |
| 8.00 | 20 | 80 |
| 8.01 | 50 | 50 |
| 10.00 | 50 | 50 |
| 10.01 | 2 | 98 |

were expressed by mouse unit (MU), where one MU was defined as the amount of toxins required to kill a mouse of 20 g body weight in 15 min following i.p. injection. Survivals over 20 hours were determined to be non-toxic.

**LC-MS analysis of anatoxin-a**

LC-MS was used with electrospray ionization (ESI). The 150 × 2.1 mm XTera $C_{18}$ column (3.5 $\mu$m particle size, maintained at 30°C, Waters, USA) was used. The optimization of drying gas and nebulizer gas were done to introduce ANTX-a standard solution at 0.2 $ml \cdot min^{-1}$. The 2.0 $\mu l$ of sample was injected and a gradient elution was performed with using water and acetonitrile, both containing 0.1% trifluoroacetic acd (TFA) (Table 1).

**LC-MS analysis of saxitoxin**

The LC-MS system was consisted of a Waters ZQ micromass, Allience 2695 (Waters, USA) and a reversed-phase XTerra MS $C_{18}$ column (3.5 $\mu$m, 150 × 2.1 mm i.d., Waters, USA). Chromatographic conditions were similar

to those described by Lagos (1999) using heptafluorobutyric acid as an ion pair reagent. The mobile phases consisted of aqueous 10 mM heptafluorobutylic acid and acetonitrile in 80:20 for STX were used isocratically at a flow rate of 0.2 m$l \cdot min^{-1}$. A ZQ micromass spectrometer equipped with an atmospheric pressure ion source and an electrospray ionization (ESI) interface (Waters, USA) was employed for detection. ESI was effected by a spray voltage of +3.0 kV and heated capillary temperature was maintained at 250°C. High purity nitrogen served UHPLCMS nitrogen generater (NITROX, Korea) at an operating pressure of 80 psi. Full scan spectra were acquired in the positive ion peak continue mode over the mass ranges of *m/z* 200-500 for STX. Instrumental control, data acquisition and data processing were performed with an Empower soft program on COMPAQ (P720, USA) computer.

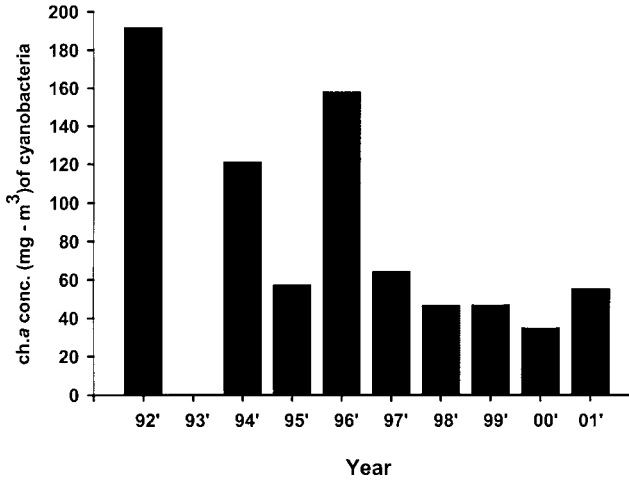


**Fig. 3.** Variations of average chlorophyll *a* concentratin in Mulgum during cyanobacteria water bloom from 1992 to 2001.

## RESULTS AND DISCUSSION

### Cyanobacterial bloom formation in the lower Nakdong River and Hoedong Reservoir

Cyanobacteria were dominant throughout from early summer to late autumn in the lower Nakdong River (Ha 1999). The *Microcystis aeruginosa* is most commonly reported species of the genus *Microcystis* in the Nakdong River. Six species of the genus *Microcystis*; *M. aeruginosa*, *M. viridis*, *M. wesenbergii*, *M. incerta*, *M. novacekii*, *M. ichthyoblabe* have been identified in Nakdong River system (Lee *et al.* 1997). The maximum chlorophyll (chl) a concentration was 194 m$g \cdot m^{-3}$ in 1992 and minimum was 43 m$g \cdot m^{-3}$ in 2000 during the cyanobacterial waterbloom season, except that the frequent rainfall prevented algal waterblooms in 1993 (Fig. 3). The *Microcystis aeruginosa* was dominant from 1992 to 1998, but *Aphanizomenon flos-aquae* was temporarily dominant in 1999. *Aphanizomenon flos-aquae* was appeared in late September 1998 in Mulgum and the average chl a concentration and the standing crops were 59.5 m$g \cdot m^{-3}$ and 150 cells $\cdot$ m$l^{-1}$, and were 202 m$g \cdot m^{-3}$ and $0.5 \times 10^3$ cells $\cdot$ m$l^{-1}$ in July 1999. Lee *et al.* (2000) reported that the standing crops were lower than $2.4 \times 10^4$ cells $\cdot$ m$l^{-1}$ in July 1999, in July 2000 and August 2002 at the same point. In this study, the mean chl. a concentration ranged between 32.5 m$g \cdot m^{-3}$ and 31.5 m$g \cdot m^{-3}$ and the standing crops were between $0.9 \times 10^3$ cells $\cdot$ m$l^{-1}$ and $1.3 \times 10^3$ cells $\cdot$ m$l^{-1}$.

In the Hoedong Reservoir *Aphanizomenon flos-aquae* occurred in summer and winter. Unusually, *Aphanizomenon* bloom occurred January 2000 and cell density was $2.2 \times 10^8$ cells $\cdot$ m$l^{-1}$. Though *Microcystis aeruginosa* occurred at the same time, over 95% was dominated by *Aphanizomenon flos-aquae* that had the highest standing crops.

### Mouse bioassay

A 1.0 m*l* of STX were injected to all mice at concentrations of 0.1 m$g \cdot l^{-1}$, 0.25 m$g \cdot l^{-1}$, 0.5 m$g \cdot l^{-1}$ and 5.0 m$g \cdot l^{-1}$. At 0.1 m$g \cdot l^{-1}$ of STX, mice showed the symptoms of gasping breathing, tail rigor (after 1 minute) and lachrymation (after 17 minutes), and then recovered gradually and survived over 20 hours. Therefore, these were not neurotoxic response. At 0.25 m$g \cdot l^{-1}$ of STX, mice had the symptoms of gasping breathing, salivation, diplegia (after 3 minutes), convulsion (after 9 minutes) and then died after 10 minutes. At 0.5 m$g \cdot l^{-1}$ of STX mice had the symptoms of sudden leaping movements, gasping breathing, mydriasis and convulsions and died after 3 minutes. At 5.0 m$g \cdot l^{-1}$ of STX, all mice died at once.

The mouse lethality of the cultured *Aphanizomenon flos-aquae*, evaluated by mouse bioassay, was 2.23 MU $\cdot$ mg dry cells$^{-1}$ (Pereira *et al.* 2000). Mouse bioassays were performed with extracts of *A. flos-aquae* samples collected in January 2000 and July 1999, but showed no signs of toxicity. Mice had no response at the results of the injection with 0.25 g freeze-dried *A. flos-aquae* material, had symptoms of diplegia at 0.5 g after 5 minute but recovered gradually, and recovered after gasping breathing and mydriasis in 2 minutes at 1.0 g. Therefore, *A. flos-aquae* materials were proved to be no neurotoxic response.

**LC-MS analysis of ANTX-a and STX**

Chromatographic separation was obtained on a reversed phase column at 30°C under gradient conditions with a mobile phase of acetonitrile-water containing 0.1% TFA and a flow rate of 0.2 $ml \cdot min^{-1}$. LC-MS analysis was implemented of *m/z* 166 for anatoxin-a concentration from 5.0 to 100 $\mu g \cdot l^{-1}$, 5.56 minutes of the retention time and correlation coefficient ($r^2$) higher than 0.999 as the result of analyzing ANTX-a standards.

There was no detection of ANTX-a toxin in cultured Anabaena flos-aquae NIER-10002 and cyanobacteria collected in Noksan (July 1997), Kangdong (1999) and Mulgum (1999), where cyanobacterial blooms were dominated by *Microcystis aeruginosa* and *Anabaena flos-aquae*. The distilled water and filtered raw water collected in the Nakdong River were spiked with 3.0 $\mu g \cdot l^{-1}$ ANTX-a, respectively. As this result, ANTX-a toxin was detected at 2.9 $\mu g \cdot l^{-1}$ and 2.4 $\mu g \cdot l^{-1}$ and the recoveries were 96.7 and 78.3%. This values were lower than 98.7-100% from Zotou and Jefferies (1999) but were similar to 80.5-97.6% from Powell (1997). Bumke-Vogt *et al.* (1999) reported that the anatoxin content was 13.1 $\mu g \cdot l^{-1}$ from cyanobacteria *Anabaena* sp. and *Aphanizomenon* sp. in freshwater, and Himberg (1989) detected anatoxin (5 $\mu g \cdot l^{-1}$) from *Anabaena flos-aquae*.

ANTX-a has occurred occasionally in North America and northern Europe and no occurrence has been reported in Japan so far (Codd *et al.* 1994). Choi *et al.* (2002) reported that during summer in may 2000, ANTX-a was not detected in the lower Nakdong River where *Anabaena flos-aquae* was reported to occur massively with *Microcystis aeruginosa*. On the other hand, Park *et al.* (1998) reported that analyzed ANTX-a toxins from Korean reservoirs were maximum of 1,444 $\mu g \cdot l^{-1}$ dw.

The presence of paralytic shellfish poisonings (PSPs) in cyanobacteria has been proven in *Aphanizomenon flos-aquae* (Alam *et al.* 1973; Mahmood and Carmichael 1986) and *Lyngbya wollei* from North America (Carmichael *et al.* 1997; Onodera *et al.* 1997), *Anabaena circinalis* from Australia (Humpage *et al.* 1994) and *Cylindrospermopsis raciborskii* from Brazil (Lagos *et al.* 1999). Under the analytical condition saxitoxin was detected at identical retention time of 5.80 minute by scanning respective protonated ions and similarity to the standard. STX toxins found in the cultured *Aphanizomenon flos-aquae* NIES-81 were further confirmed by LC-MS analysis. STX toxin from *Aphanizomenon flos-aquae* NIES-81 strain in the exponential growth phase (after culture 16 days) was detected for 0.45 $\mu g \cdot g^{-1}$ dw. According to Pereira *et al.* (2000), neoSTX (23.0 $nmol \cdot mg^{-1}$ dry cells), dcSTX (6.1 $nmol \cdot mg^{-1}$ dry cells) and STX (5.4 $nmol \cdot mg^{-1}$ dry cells) were detected from the cultured *Aphanizomenon flos-aquae* in Motargil reservoir. Kaas and Henriksen (2000) showed that neoSTX (0.9-6.5 $\mu g \cdot g^{-1}$ dw), dcSTX (0.8-7.0 $\mu g \cdot g^{-1}$ dw) and STX (14.7-218 $\mu g \cdot g^{-1}$ dw) was detected from the cyanobacterial samples in Danish Lake. This content was higher than our result. It was reported that the detectable saxitoxin content from cyanobacteria *Lyngbya wollei* was 9-20 $\mu g \cdot g^{-1}$ fw and was higher than the toxin content from *Anabaena flos-aquae* and *Aphanizomenon flos-aquae* (Steffensen *et al.* 1994; Carmichael *et al.* 1997). STX toxin from the cultured *Aphnizomenon flos-aquae* were not detected that were extracted from the natural materials in the Nakdong River (July 1999) and Hoedong Reservoir (January 2000). In addition, the neurotoxic reaction was not considered in the mouse bioassay.

On the other hand, the microcystins from *Aphanizomenon flos-aquae* were detected in the Nakdong River and Hoedong Reservoir. In that station, microcystin-RR and -LR were detected for 37.4 $\mu g \cdot g^{-1}$ dw and 16.3 $\mu g \cdot g^{-1}$ dw, respectively and in this station, for 24.9 $\mu g \cdot g^{-1}$ dw and 6.6 $\mu g \cdot g^{-1}$ dw (data not shown). Though STX was reported to be produced by *Aphnizomenon flos-aquae*, microcystins, one of the hepatotoxins were detected in it.

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