Cyanobacteria cell damage and cyanotoxin release in response to alum treatment
Jisun Han, Bong-seok Jeon and Ho-Dong Park

ABSTRACT
Alum (aluminium sulfate) has been evaluated as a less-hazardous chemical for cyanobacteria that does not cause physiological toxicity and causes little or no release of cyanotoxins. In this study, the effect of alum treatment on toxic Microcystis cells was evaluated on weekly and monthly scales using a microcosm experiment designed to simulate common lakes or reservoirs. Alum treatment caused cell damage and subsequent release of large amounts of microcystin-LR (MC-LR). Moreover, MC-LR and Microcystis cells were degraded by microbes that existed in the lake sediment. This is the first study to report a serious release of microcystin by alum treatment. For this reason, care about release of a large amount of hepatotoxic microcystin should be taken when adding alum for cyanobacterial removal in lakes and reservoirs.

INTRODUCTION
Cyanobacteria frequently dominate the freshwater phytoplankton community in eutrophic waters. Cyanotoxins produced by freshwater cyanobacteria are very common worldwide, and have been reported to cause adverse effects and even death in wild and domestic animals (Dawson 1998; Carmichael 2001). These compounds have also been found to pose a significant hazard to human health (Yu 1995; Ueno et al. 1996; Pouria et al. 1998). Several species of commonly occurring cyanobacteria such as Microcystis, Anabaena and Oscillatoria produce microcystins, which are a type of cyanotoxin (Carmichael 2001; Holst et al. 2003). Microcystin-LR (MC-LR), which is the most commonly reported hepatotoxin, is regarded as one of the most toxic microcystins. Indeed, it has been reported that the LD₅₀ of MC-LR is approximately 50 μg per kg body weight (Sivonen & Jones 1999), and the World Health Organization (WHO) published a guideline value for MC-LR of 1 μg L⁻¹.

Due to the health concerns associated with toxic cyanobacterial blooms in drinking water sources, cell removal and toxin release are considered during the water treatment processes. Many studies of the removal of cyanobacteria using alum treatment have investigated the effect of coagulation/flocculation with alum on cyanobacterial cells and cyanotoxin release during common water treatment. For example, Chow et al. (1999) evaluated the effect of water treatment processes using alum on the integrity of toxic Microcystis cells using both jar tests and pilot plant studies. Peterson et al. (1995) also investigated the physiological toxicity and release of cellular dissolved organic carbon in response to treatment with several chemicals that are commonly used in water treatment processes. Finally, Drikas et al. (2001) and Jurczak et al. (2005) evaluated the integrity of cyanobacterial cells and additional toxin release under normal operating conditions during the treatment of drinking water.

However, the release of toxins by alum treatment has not been reported in any studies that have been conducted to date. Chow et al. (1999) reported that alum treatment did not cause cell damage leading to the release of MC-LR. Drikas et al. (2001) also reported that alum treatment had no significant effect on cell viability, and that cells remained intact in all experiments. Furthermore, Peterson et al. (1995) suggested that the chemicals fall into three categories based on their potential hazards; specifically, Type 1 chemicals caused no physiological toxicity and little or
no release of cellular components, while Type 2 and 3 chemicals caused physiological toxicity at or below concentrations typically used in water treatment operations. Alum was considered a Type 1 chemical.

To remove toxic cyanobacterial blooms in natural lakes, reservoirs and recreational ponds using alum, an approach from a different angle than typical water treatment processes is required. During the treatment of drinking water, the contact time is much shorter between raw water and alum because the supernatant is separated from the precipitate after the sedimentation process. Conversely, in natural aquatic ecosystems, the precipitate is present in the sediment due to settling, which could have a continuous effect on the water column being subject to treatment.

This study was conducted to examine the effect of alum treatment on MC-LR release on a monthly scale and to evaluate Microcystis cell damage and MC-LR release on a weekly scale in a microcosm experiment designed to simulate the conditions in lakes and reservoirs.

### MATERIALS AND METHODS

#### Microcystis suspension preparation

A unialgal culture of the *Microcystis ichthyoblabe* strain, TAC95 (Tsukuba Algae Collection, National science museum), was grown in 10 L of MA medium (Table 1) at 23 °C under illumination at ca. 16 μmol m⁻² s⁻¹ and a 12:12 h light:dark cycle. The composition of MA medium is described in Table 1. Cultures were harvested during the late exponential growth phase or the early stationary growth phase. *M. ichthyoblabe* strain TAC95 is known to produce only MC-LR (Yokoyama & Park 2003).

A cultivated *Microcystis* suspension was centrifuged at 3,500 rpm for 15 min. The supernatant was then discarded and the *Microcystis* cells were washed with tap water that had been allowed to dechlorinate by aeration overnight. All processes were conducted in triplicate.

#### Preparation of chemicals

Aluminium sulfate (anhydrous), which is a Cica Reagent produced by KANTO Chemical Co., Inc. in Tokyo, Japan (>92% purity, Al₂(SO₄)₃, MW 342.15 g), was used as a coagulant. Distilled water was used for the preparation of alum stock solution.

#### Jar test

In the jar test, alum solution was added to a beaker containing 100 mL of rinsed *Microcystis* suspension with stirring. The pH value was recorded after adding 100 μL of alum, which was expected to result in a pH of 6 (Cooke et al. 2005).

#### The effect of added alum on the release of microcystin (the flask experiment)

First of all, an experiment at flask scale was conducted in order to examine *Microcystis* cell damage and MC-LR release by alum treatment. The experiment was conducted over 30 days because aluminium-floc could exist and effect continuously in case of alum treatment in natural aquatic systems. Three 800 mL suspensions of rinsed *Microcystis* were added to individual 1,000 mL flasks. The maximum dose of alum (14 mg L⁻¹ as Al) was added to one flask, while half of the maximum dose (7 mg L⁻¹ as Al) was added to another flask and the remaining flask was left

### Table 1 | The composition of MA medium for culture of the *Microcystis ichthyoblabe* strain, TAC95

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Conc. (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>50</td>
</tr>
<tr>
<td>KNO₃</td>
<td>100</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>40</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>50</td>
</tr>
<tr>
<td>MgCl·6H₂O</td>
<td>5</td>
</tr>
<tr>
<td>β-Na₂ glycerophosphate</td>
<td>100</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>5</td>
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<tr>
<td>FeCl₃·6H₂O</td>
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</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>5</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.5</td>
</tr>
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<td>CoCl₂·6H₂O</td>
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<tr>
<td>NaMoO₄·2H₂O</td>
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</tr>
<tr>
<td>H₃BO₃</td>
<td>20</td>
</tr>
<tr>
<td>Bicine</td>
<td>500</td>
</tr>
</tbody>
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untreated as a control. Rapid mixing (300 rpm) for 1 min was followed by 5 min of slow mixing (50 rpm) with a magnetic stirrer. The experiment was conducted in a controlled chamber at 24±1 °C under illumination with fluorescent light at 40 μmol m⁻² s⁻¹ in a 16:8 h light:dark cycle. The optical density at 405 nm (OD₄₀₅ nm) was measured every day, and samples (20 mL) were taken for analysis of the intra and extracellular MC-LR before adding alum (day 0), and at days 1, 2, 3, 5, 7, 10, 15, 20 and 30. All samples were taken from the supernatant.

Comparison of microcystin release by alum treatment with and without sediment (the microcosm experiment)

This microcosm experiment was conducted in order to examine Microcystis cell damage and MC-LR release by alum treatment in common lake environments, which exist in the sediment. A microcosm experiment was conducted using three acryl chambers of size 320×180×150 mm (length × width × height). To make the microcosms similar to common lakes or reservoirs, sediment taken from a small eutrophic pond (Chikato-ike in Nagano, Japan) was added to two of the microcosms to give a sediment bed of 320×20 mm (length × width × height). One of the chambers did not receive sediment. Next, 6 L of rinsed Microcystis suspension was carefully added to each chamber. The microcosms were then allowed to stand overnight. Alum solution was subsequently added to each chamber. The microcosms were then homogenized and extracted with 5% aqueous acetic acid, after which the supernatant was applied to an Oasis HLB cartridge (0.5 g, Waters, Milford, Massachusetts, USA) that had been previously conditioned with MeOH (10 mL) and distilled water (10 mL). For extracellular MC-LR analysis, the filtered water was applied directly to an HLB cartridge, which was subsequently eluted with MeOH, and the eluate containing the toxin was then collected. The MC-containing fraction was evaporated to dryness and the residue was redissolved in MeOH (200 μL). The resulting solution was injected into a high-performance liquid chromatograph (HPLC) for analysis.

Analytical methods

The samples were analyzed for OD₄₀₅ nm, chl. a, intra- and extracellular MC-LR.

The cell concentration of Microcystis was substituted for OD₄₀₅ nm because there is a high correlation between OD₄₀₅ nm and the cell concentration of M. ichthyoblabe strain (TAC95). The OD₄₀₅ nm was measured using a spectrophotometer (V-540 UV/VIS, Jasco, Tokyo, Japan), and the chl. a concentration was determined from UNESCO equations (UNESCO 1966). Measurement of MC-LR concentrations was conducted according to the method described by Xie et al. (2007). For intracellular MC-LR analysis, the samples were filtered through Whatman GF/C filter paper (GF/C, Whatman, Kent, UK) and the chlorophyll was extracted using 90% acetone (7 mL). The absorbance of the extracts was measured at 630, 645, 663 and 750 nm using a spectrophotometer (V-540 UV/VIS, Jasco, Tokyo, Japan), and the chl. a concentration was determined from UNESCO equations (UNESCO 1966).

For chl. a analysis, the samples were filtered through glass filter paper (GF/C, Whatman, Kent, UK) and the chlorophyll was extracted using 90% acetone (7 mL). The absorbance of the extracts was measured at 630, 645, 663 and 750 nm using a spectrophotometer (V-540 UV/VIS, Jasco, Tokyo, Japan), and the chl. a concentration was determined from UNESCO equations (UNESCO 1966).

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump coupled to a SPD-10A set at 238 nm, an SPD-M10A photodiode array detector, a C-R 6 A integrator, and an ODS column (Cosmosil 5C18-MS-II; 4.6×150 mm, Nakalai, Japan). The sample was separated using a mobile phase consisting of methanol: 0.05 M phosphate buffer (pH 3.0, 58:42) that was applied at a flow rate of 1 mL/min. The MC concentration was quantified against MC-LR standards (Kanto Ltd, Japan).
RESULTS AND DISCUSSION

The effect of added alum on the release of microcystin (the flask experiment)

The results showed that the cell concentration of Microcystis differed significantly between the control and the treatments (Figure 1). The cell concentration in the maximum treatment group was significantly lower (nearly zero) than that of the control during the experiment (Figure 1(a) and (c)). These findings resulted from coprecipitation with aluminium hydroxide (Al(OH)₃). When aluminium sulfate is added to water, it is immediately hydrated. A progressive series of hydrolysis reactions then occur, leading to the formation of aluminium hydroxide, a colloidal, amorphous floc. These flocs are able to adsorb large amounts of particulate organic matter such as cells and detritus (Cooke et al. 2005).

Interestingly, in the half-maximum treatment, the cell concentration was low until day 4, after which it increased gradually to almost the same concentration as the control (Figure 1(b)). Based on these findings, it can be inferred that the alum dose was not sufficient to coprecipitate cells and suppress Microcystis growth for a long period of time in the half-maximum treatment. A similar result was observed in the follow-up experiment.

It seemed that the extracellular MC-LR concentration in the control and treatment groups were dissimilar (Figure 2). While the release of MC-LR was not observed in other treatments (Figure 2(a) and (b)), a large amount of MC-LR exceeding by two-fold that of the other treatment was released to the water in the maximum treatment group after 1 day when alum was added (Figure 2(c)). Moreover, precipitated Microcystis cells turned from dark green to light blue after 2 days in response to the maximum treatment. Cyanobacteria have phycocyanin as well as chl. a, which are both photosynthetic pigments. Cell lysis causes the release of phycocyanin and a characteristic color change from green to blue (Jones & Orr 1994; Harada et al. 2009). Many studies of cyanobacteria removal by alum treatment have reported that alum does not cause cell damage or toxin release (Peterson et al. 1995; Chow et al. 1999; Drikas et al. 2001). However, the results of the present study – MC-LR and phycocyanin release – indicate that alum treatment caused damage to the Microcystis cells.

A comparison of microcystin release in response to alum treatment in microcosms with and without sediment (the microcosm experiment)

Chl. a concentration was found to differ considerably among the treatments (Figure 3). Specifically, chl. a concentration decreased to less than 20% 1 day after the alum treatment.
in both microcosms with and without sediments. These findings indicate that Microcystis cells coprecipitated with the aluminium hydroxide. In addition, chl. a concentration decreased continuously in the microcosm that contained the sediment but did not receive the alum treatment. These findings were similar to the variations in intracellular MC-LR concentrations that were observed in the chl. a concentration. The intracellular MC-LR concentration, which was below the detection limit at 2 days after treatment decreased to less than 10% at 1 day after treatment with alum in both microcosms with and without sediments.

The extracellular MC-LR concentration increased continuously in the microcosm that received the alum treatment but did not contain sediment (Figure 4, grey circles). These findings are similar to the results of the flask experiment, and indicate that the Microcystis cells were damaged by the alum, and subsequently released a large amount of MC-LR which is over 80% of the initial concentration.
intracellular MC-LR concentration. Therefore, release of a large amount of toxin needs to be very carefully monitored with alum treatment for toxic cyanobacterial removal.

Interestingly, the extracellular MC-LR concentration increased for 2 days after treatment in the microcosms that contained the sediment (Figure 4, black and white circles). This increase might have been due to cell lysis of Microcystis by microbes present in the sediment. Various microbes such as bacteria and fungi are well known to have the ability to lyse cyanobacterial cells (Daft et al. 1975; Mitsutani et al. 1987; Yamamoto et al. 1995). Moreover, high levels of algal lysing microbes have been observed in the sediment throughout the year (Yamamoto & Suzuki 1990). In this study, since the sediment was collected from a small eutrophic pond that was subject to an annual Microcystis bloom, it could be expected that Microcystis lysing microbes exist in considerable quantities in the sediment.

The extracellular MC-LR concentration decreased in the microcosms that contained the sediment from day 2 (Figure 4, black and white circles). These findings indicate that there may be microcystin degrading microbes existing in the sediment. Microcystin is known to be degraded in natural environments, although it is not easily degraded by chemical treatment (Maruyama et al. 2004). Park et al. (2001) and Maruyama et al. (2006) reported that the microcystin degrading bacteria, Sphingosinicella microcystinisivorans, completely degraded MC-LR and -RR within 6 days. Additionally, Holst et al. (2003) demonstrated that indigenous microorganisms in the sediment were capable of degrading microcystin. Microcystin primarily exists inside cyanobacteria cells (intracellular MC) in lake water, while only a small quantity exists in filtered lake water (extracellular MC). It is possible that extracellularly released MC with Microcystis lysing microbes was decomposed rapidly (Park et al. 1998), if microcystin degrading microbes were present, as the observed concentration of MC in the microcosms that contained the sediment were much lower than in the microcosm that did not contain the sediment.

The initial MC-LR release rate (from start to day 2) was 9 μg L⁻¹ day⁻¹ in the microcosm that received alum treatment but did not contain sediment, this value was lower than the mean release rate of 17 μg L⁻¹ day⁻¹ (based on the values measured from start to day 7). It was assumed that the reaction between aluminium hydroxides and Microcystis cells took some time because rapid and slow mixing was not conducted. Moreover, the initial MC-LR release rate was 19 μg L⁻¹ day⁻¹ in the microcosm that contained sediment but did not receive the alum treatment, which was higher than that of the microcosm that received the alum treatment but did not contain sediment. The initial release rate was the highest (36 μg L⁻¹ day⁻¹) in the microcosm that received the alum treatment and contained the sediment, while it was about the same as the sum of the release rate in the microcosm that received the alum treatment but did not contain the sediment (17 μg L⁻¹ day⁻¹) and that contained the sediment but did not receive the alum treatment (19 μg L⁻¹ day⁻¹). These findings indicate that the MC-LR release was caused by the lysis of Microcystis cells by microbes as well as cell damage in response to alum treatment.

From the results of the plask experiment, small dose (half maximum) treatment is not effective to precipitate Microcystis cells and large dose (maximum) treatment causes cell damage and releases amounts of toxins. Moreover, from the results of the microcosm experiment, in the condition that the sediment is in existence, microcystin was degraded partly by microcystin degrading microbes. Therefore, alum treatment is not suitable particularly for situations in which sediment is not present because any released toxins are not degraded perfectly by microbes.

**CONCLUSION**

The effects of alum treatment on toxic Microcystis cells were evaluated over a period of 30 days. The removal of Microcystis cells differed in response to different doses of alum applied, indicating that effective removal of the Microcystis cells requires a sufficient dose of alum. More importantly, it was previously reported that alum treatment does not cause cyanobacteria cell damage or the release of cyanotoxin. However, the results of the present study showed that alum treatment caused cell damage and the release of large amounts of MC-LR to the water.
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REFERENCES


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