The effect of coagulation with aluminum sulfate as in-lake treatment on toxic *Microcystis* cells

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The effect of coagulation with aluminum sulfate as in-lake treatment on toxic *Microcystis* cells

Abstract

The presence of cyanobacteria in reservoirs, lakes, and rivers is a worldwide environmental health issue because some cyanobacterial strains produce toxins, as well as taste and odor compounds, as secondary metabolites. Aluminum salt has long been used in several water treatment or lake restoration. Most studies have only investigated cyanobacteria removal by alum treatment during the coagulation process in water treatment processes.

Although humans do not directly ingest cyanobacteria, they might be regularly exposed to sub-lethal dosages of extracellular microcystin in drinking water or several water recreational activities derived from contaminated lakes and reservoirs. Therefore, the release of toxins by the cyanobacteria removal treatment including adding alum should be considered not only in the conventional water treatment but also in lake treatment.

Therefore, in this study, the effect of alum treatment on toxic *Microcystis* cells was evaluated in several experiments: (1) the long term effect of alum coagulation in the flask experiment, (2) the microcosm experiment designed to simulate the conditions found in common lakes and reservoirs, (3) The effect of aluminum hydroxide on *Microcystis* cells through the analysis of precipitates.

The cell concentrations in supernatant were significantly decreased at 1 day after
adding alum resulted from co-precipitation with aluminum hydroxide, and the extracellular MC-LR concentration increased continuously after adding alum with maximum dose both in the flask and in the microcosm experiments. Moreover, precipitated cells were surrounded or coated with aluminum hydroxide floc, and cell membrane was torn, which was observed under a SEM. Therefore, it could be concluded that *Microcystis* cells have seriously damaged by alum treatment at the maximum dose. Moreover, alum treatment caused damage to bacteria existed in the sediment as well as *Microcystis* cells.

Many studies of the removal of cyanobacteria by alum treatment have reported that alum does not cause any cell damage and resultant toxin release. However, it should be considered that the treatment time which means the reaction between alum and cyanobacterial cells. Common water treatments did not exceed a maximum of 24 hours, meanwhile it might take more than a few months in-lake treatment. Therefore, it could be concluded that alum treatment is not suitable for removing toxic cyanobacterial bloom in lakes, reservoirs, and ponds, because floc would remain for a long time in the sediment.


Chapter 1

General introduction

1.1 Cyanobacteria bloom and cyanotoxins

Cyanobacteria frequently dominate the freshwater phytoplankton community in eutrophic waters, and have the ability to produce toxins as well as taste and odor compounds. Cyanotoxins have been reported to cause adverse effects and even death in wild and domestic animals (Dawson 1998, Carmichael 2001, Huisman et al. 2005), and these compounds have also been found to pose a significant hazard to human health (Yu 1995, Ueno et al. 1996, Pouria et al. 1998).

Cyanotoxins can be classified according to toxicity (Carmichael 1992, Huisman et al. 2005) as hepatotoxin (microcystins, nodularin and cylindrospermopsin) or neurotoxin (anatoxin-a, anatoxin-a(s) and saxitoxins) as well as their chemical structures (Kaebernick and Neilan 2001, Pietsch et al. 2002) as cyclic peptides (microcystin and nodularin), alkaloids (anatoxin-a, anatoxin-a(s), saxitoxin, cylindrospermopsin, aplysiatoxins and lyngbyatoxin-a) and lipopolysaccharides. The toxicity, activity and genera to produce of these toxins are listed Table 1-1. Microcystins and nodularin which are representative hepatotoxins inhibit protein phosphatases type 1 and type 2A resulting in excessive phosphorylation (Kaebernick and Neilan 2001). Neurotoxins, Anatoxin-a and anatoxin-a(s) inhibit transmissions at the neuromuscular junction (Carmichael 1992, Kaebernick and Neilan 2001).
Several species of commonly occurring cyanobacteria such as *Microcystis*, *Anabaena* and *Oscillatoria* produce microcystins, which are a type of cyanotoxin (Carmichael 2001, Kaebernick and Neilan 2001, Holst et al. 2003). Microcystin-LR (MC-LR) is cyclic heptapeptides contained the amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda), with arginine and leucine (Fig. 1-1), which is regarded as one of the most toxic microcystins. Indeed, it has been reported that the LD$_{50}$ of MC-LR is approximately 50 μg per kg body weight in mouse (Sivonen and Jones 1999), and time-to-death from a lethal oral dose in rodents is variable, around 6-20 hours (Kaebernick and Neilan 2001). Moreover, the World Health Organization (WHO) published a guideline value for microcystins in drinking water, based on MC-LR of 1 μg L$^{-1}$ (WHO 1998).

1.2 Aluminum coagulant

When aluminum salts are added to water, aluminum ions are readily hydrolyzed depending on the solution pH (Fig. 1-2). The hydrolysis reactions proceed to the formation of aluminum hydroxide (Cooke et al. 2005), the following occurs:

$$\text{Al}^{3+} + \text{H}_2\text{O} \rightleftharpoons \text{Al(OH)}^{2+} + \text{H}^+ \quad (\text{Eq. 1})$$

$$\text{Al(OH)}^{2+} + \text{H}_2\text{O} \rightleftharpoons \text{Al(OH)}_2^+ + \text{H}^+ \quad (\text{Eq. 2})$$

$$\text{Al(OH)}_2^+ + \text{H}_2\text{O} \rightleftharpoons \text{Al(OH)}_3(\text{am}) + \text{H}^+ \quad (\text{Eq. 3})$$

Where (am) = amorphous.

Furthermore, at increasing pH levels above 8.0, aluminum hydroxide lead to the formation of the aluminate ion:
\[
\text{Al(OH)}_3 + \text{H}_2\text{O} \rightleftharpoons \text{Al(OH)}_4^- + \text{H}^+ \quad \text{(Eq. 4)}
\]

Aluminum hydroxide is of very low solubility and a colloidal and amorphous precipitate at intermediate pH values (pH 6 to 8), and it has high coagulation and phosphorus adsorption properties (Holz and Hoagland 1999, Duan and Gregory 2003, Cooke et al. 2005, Anderson and Berkowitz 2010).

Iron and calcium also has long been used to remove phosphorus in the wastewater treatment, but they have been much less common rather than aluminum as in-lake treatment. Depending on oxidation reduction potential and pH, inorganic iron in the lake or pore water takes oxidized ferric (Fe\(^{3+}\)) or reduced ferrous (Fe\(^{2+}\)) ion. Ferric ions have the high coagulation and phosphorus adsorption properties such as aluminum. When dissolved oxygen concentration decreased, however, ferric ions are reduced to ferrous ions, phosphorus is released again because of soluble ferrous ions (Wetzel 2001, Cooke et al. 2005, Jančula and Maršálek 2011). In my previous coagulant experiment, the flocs produced by calcium were very small and not heavy enough to precipitate (not shown). In addition, the abundance of zooplankton might be decreased by lime (calcium hydroxide) treatment which increase the pH in lakes above 10, and likely affect other organisms such as benthic invertebrates (Mohamed 2001).

Based on these chemical background, aluminum salt has long been used in drinking water production, industrial and domestic wastewater treatment, sludge treatment or lakes and reservoirs (Duan and Gregory 2003, Cooke et al. 2005).
1.3 Aluminum application as in-lake treatment

Because internal or external phosphorus increase in lakes caused unpleasant algal blooms, the control of phosphorus loading is very important to improve the water quality. Therefore, the primary purpose of aluminum application as in-lake treatment which is to lower phosphorus concentration in lake by precipitation of phosphorus from water column, and or by inactivation of phosphorus in the lake sediments (Hansen et al. 2003, Cooke et al. 2005).

Since 1970’s, it began to be reported that aluminum salts were applied to improve water quality according to removal of phosphorus in water column or retarding release of phosphorus in the lake sediments. Welch and Cooke (1999) evaluated effectiveness and longevity of aluminum treatments at controlling sediment P release and improving lake water quality in 21 lakes or lake basins across the U. S. that treated prior to 1987. Internal loading rate as well as Chl.a were reduced in most lakes regardless of the lake type (polymictic or dimictic).

And these researches have been continuously conducted until recently (Lewandowski et al. 2003, Anderson and Berkowitz 2010, Huser 2012, Egemose et al. 2013). Furthermore, Moore et al. (2009) applied alum in lake with a unique microfloc alum injection system, and post-turnover Secchi depths have all improved and volume-weighted total phosphorus concentrations also have generally declined following alum injection.

As described above, aluminum application in lakes has been used to remove phosphorus from water column (precipitation) and or to retard release of phosphorus in the lake sediments. Therefore, in these studies, the effect of aluminum on each phytoplankton was little considered. Surely, some studies have investigated the variation in biomass of each
species (Lelkova et al. 2008, Moore et al. 2009) or the transition of dominant species (Paul et al. 2008, Jaworska et al. 2009) in phytoplankton, but it is considered as a result of reduction of phosphorus loading by aluminum treatment.

1.4 Aluminum application as water treatment

The primary purpose of aluminum application as water treatment is dissimilar to in-lake treatment. In the industrial and domestic wastewater treatment, aluminum, iron or calcium have been used to reduce total phosphorus concentrations in the final effluent to very low levels, however, in the drinking water treatment, aluminum has been used to remove particulate matters such as phytoplankton from treating water.

Moreover, unlike in-lake treatment, it has been investigated that the effect of used chemicals or treatment processes in the drinking water treatment on each phytoplankton (Lin et al. 1971, Henderson et al. 2008). Particularly, as described above, several strains of cyanobacteria have the ability to produce cyanotoxins as secondary metabolites which have been implicated in human illness or death, in some serious cases (Ueno et al. 1996, Jochimsen et al. 1998). Therefore, the intact removal of cyanobacteria is very important in the drinking water treatment, and these researches paid attention to the effect of chemicals or treatment processes on cyanobacteria.

In the most of studies relating the effect of the coagulation with aluminum on cyanobacteria, it was concluded that the aluminum treatment did not cause the cell damage or lysis, and toxin release (Table 1-2). Particularly, Peterson et al. (1995) suggested that several water treatment chemicals fall into three categories of potential hazard to effective water
treatment. Chemicals including hydrogen oxide, copper sulfate, chlorine etc. caused physiological toxicity, and release of cellular components. On the other hand, the category represented a low hazard group including ferric chloride and aluminum sulfate did not cause physiological toxicity, caused little or no release of cellular components at realistic treatment concentrations. There were the studies that cyanotoxin was released by coagulation with aluminum salts, however, released cyanotoxins were negligible in comparison with another treatment (Lam et al. 1995, Schmidt et al. 2002).

1.5 A purpose of this study

Aluminum as in-lake treatment also could be used to remove of phytoplankton or detritus to some extent from water column by coagulation and entrapment in the aluminum hydroxide, a secondary objective (Cooke et al. 2005, Jančula and Maršálek 2011).

Although humans do not directly ingest cyanobacteria, they might be frequently exposed to sub-lethal dosages of extracellular microcystin in drinking water or several water recreational activities derived from contaminated lakes and reservoirs (Lam and Prepas 1997).

Therefore, the release of dissolved organic matters including toxins by the cyanobacteria removal treatment including adding alum should be considered not only in the conventional water treatment but also in lake treatment. Furthermore, 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 during a long period due
to settling, which could have a continuous effect on the water column being subject to treatment.

In this study, three types of experiments were conducted: (1) the flask experiment to confirm long term effect on the variation in cell concentration and the release of microcystin, (2) the microcosm experiment which designed to simulate the conditions found in common lakes and reservoirs to confirm the cell damage and the microcystin release, and to evaluate the effect on other bacteria derived from the sediment as well as Microcystis cells, (3) the precipitate analysis to determine whether Microcystis cells were certainly damaged or not, to investigate the effect of aluminum treatment as in-lake treatment on cyanobacteria, toxic Microcystis cells.
**Figure 1-1.** The chemical structure of microcystin-LR.
Figure 1-2. The concentrations of aluminum species as a function of pH in equilibrium with the amorphous aluminum hydroxides (redrawn from Duan and Gregory, 2003).
Table 1-1. The toxicity and activity of several cyanobacterial toxins (from Kaebernick and Neilan 2001\(^{(a)}\), Huisman et al. 2005\(^{(b)}\) and Falconer 2008\(^{(c)}\))

| Toxins          | Toxicity \(^{(a, c)}\)  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toxicity (^{(a, c)}) (LD(_{50}) *, μg kg(^{-1}))</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Microcystins</td>
<td>50~300</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodularins</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Cylindrospermopsins</td>
<td>2100 (24h)</td>
</tr>
<tr>
<td></td>
<td>200 (5~6d)</td>
</tr>
<tr>
<td>Anatoxin-a</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td>≤ 100 (i.v.)**</td>
</tr>
<tr>
<td>Anatoxin-a(s)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Saxitoxins</td>
<td>8~10</td>
</tr>
<tr>
<td></td>
<td>3.4 (i.v.)**</td>
</tr>
</tbody>
</table>

* Toxicity determined intraperitoneal dosing in mouse.

** Intravenous dosing (i.v.)
Table 1-2. The effect of aluminum salt as water treatment on cyanobacteria cells.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cyanobacteria</th>
<th>Treatment</th>
<th>Toxin release or cell lysis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory</td>
<td>Aphanizomenon flos-aquae</td>
<td>AS</td>
<td>Did not</td>
<td>Peterson et al. (1995)</td>
</tr>
<tr>
<td>Laboratory</td>
<td>Anabaena circinals</td>
<td>AS</td>
<td>Did not</td>
<td>Velzeboer et al. (1995)</td>
</tr>
<tr>
<td>Laboratory</td>
<td>Microcystis aeruginosa</td>
<td>AS</td>
<td>A slight release</td>
<td>Lam et al. (1995)</td>
</tr>
<tr>
<td>Laboratory</td>
<td>Microcystis aeruginosa</td>
<td>AS, conventional processes (AS)</td>
<td>Did not</td>
<td>Chow et al. (1999)</td>
</tr>
<tr>
<td>Laboratory</td>
<td>Various natural cyanobacteria</td>
<td>AS, AS+CH</td>
<td>Did not</td>
<td>Mohamed (2001)</td>
</tr>
<tr>
<td>Laboratory</td>
<td>Microcystis aeruginosa,</td>
<td>C/F/DAF (PAC)</td>
<td>Did not</td>
<td>Teixeira et al. (2010)</td>
</tr>
<tr>
<td>Pilot plant</td>
<td>Various natural cyanobacteria</td>
<td>Conventional processes</td>
<td>Did not</td>
<td>Lahti et al. (2001)</td>
</tr>
<tr>
<td>Pilot plant</td>
<td>Microcystis aeruginosa</td>
<td>Conventional processes (AS)</td>
<td>Did not</td>
<td>Drikas et al. (2001)</td>
</tr>
<tr>
<td>Pilot plant</td>
<td>Various natural cyanobacteria</td>
<td>Conventional processes (AS, PAC)</td>
<td>Did not</td>
<td>Jurczak et al. (2005)</td>
</tr>
<tr>
<td>Pilot plant</td>
<td>Various natural cyanobacteria</td>
<td>F (AS) and S-FL</td>
<td>Did not</td>
<td>Hoeger et al. (2005)</td>
</tr>
</tbody>
</table>

AS: aluminum sulfate (Al₂SO₄·nH₂O), CH: calcium hydroxide (Ca(OH)₂), PAC: poly aluminum chloride (AlₙCl(₃n-m)(OH)ₘ),

F: flocculation, S-FL: sand filtration, C: coagulation, DAF: dissolved air flotation
References


Hansen, J., Reitzel, K., Jensen, H.S. and Andersen, F.Ø. (2003) Effects of aluminum, iron,
oxygen and nitrate additions on phosphorus release from the sediment of a Danish softwater lake. Hydrobiologia 492(1-3), 139-149.


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Chapter 2

The long effect of alum coagulation on toxic *Microcystis* cells in the flask experiment

*(Water Science and Technology: Water Supply, 2012)*

2.1 A purpose

As described in chapter 1, aluminum salt has been used not only in lakes and reservoirs, but also in drinking water production, industrial and domestic wastewater treatments.

However, the application of alum for removal of toxic cyanobacterial blooms in natural lakes, reservoirs and recreational ponds using alum is necessary to evaluate different from the application in the typical water treatment processes. 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. One the other hand, in natural aquatic ecosystems, the precipitate is present in the sediment during a long period due to settling, which could have a continuous effect on the water column being subject to treatment.

Therefore, this study was conducted for 30 days in the flask experiment, to evaluate
the long term effect of alum treatment.

2.2 Materials and methods

2.2.1 Microcystis suspension preparation

A unialgal culture of *Microcystis ichthyoblabe* strain TAC95 (Tsukuba Algal Collection, National Museum of Nature and Science, Tokyo, Japan) was grown in 10 L of MA medium (*Microcystis aeruginosa* medium, Table 2-1) at 23 ± 1 °C under illumination at ca. 16 μmol m$^{-2}$ s$^{-1}$ and a 12:12 h light:dark cycle. *Microcystis* cultures were harvested during the late exponential growth phase or the early stationary growth phase. *M. ichthyoblabe* strain TAC95 produces only microcystin-LR (Yokoyama and Park 2003).

To remove the buffer and phosphate that are included in MA medium (Ichimura 1979), a cultivated *Microcystis* suspension was centrifuged at 2100 × g for 15 min. The supernatant was then discarded and the *Microcystis* cells were washed with tap water that had been dechlorinated by aeration overnight. All processes were conducted in triplicate.

2.2.2 Preparation of chemicals

Aluminum sulfate (anhydrous), which is a Cica Reagent that produced by KANTO Chemical Co., Inc. in Tokyo, Japan (>92% purity, Al$_2$(SO$_4$)$_3$, MW 342.15 g), was used as a coagulant. Distilled water was used for the preparation of alum stock solution.
2.2.3 Jar test

The form of aluminum in water is dictated by pH. Based on previously published results using alum treatment, maintaining pH ≥ 6.0 should prevent toxicity from dissolved monomeric Al (Pilgrim and Brezonik 2005). It was concluded that the alum concentration to be pH 6.0 is the maximum which does not cause toxicity by monomeric Al species, therefore, maximum dose was added in this study.

The jar-test was conducted for calculating the concentration of alum to be added in the experiment. 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. Maximum dose to be pH 6.0 in this flask experiment was 14 mg L\(^{-1}\) as Al.

2.2.4 The flask experiment

First of all, the experiment of 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 aluminum-floc could exist and effect continuously in case of alum treatment in natural aquatic systems. The flask experiment was conducted using nine flasks with a volume of 1000 mL. Rinsed Microcystis suspension of 800 mL was carefully added to each flasks. The maximum dose of alum (14 mg L\(^{-1}\) as Al) was added to three flasks, while half of the maximum dose (7 mg L\(^{-1}\) as Al) was added to another three flasks and the remaining three flasks were left 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 m² s⁻¹ in a 16:8 h light:dark cycle. This experiments were conducted in triplicate.

The optical density at 405 nm (OD₄₀₅nm) was measured every day, and samples (50 mL) were taken for analysis of the 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.

2.2.5 Analytical methods

Optical density at 405nm (OD₄₀₅nm)

Optical density at 405nm was used as Microcystis cell concentration, because it has been confirmed experimentally that there is a good correlation between OD₄₀₅nm and the cell concentration of Microcystis ichthyoblabe strain TAC95.

The absorbance of samples were taken from supernatant were measured at 405 nm using a spectrophotometer (V-540 UV/VIS, Jasco, Tokyo, Japan).

The extracellular MC-LR concentrations

Measurement of MC-LR concentrations was conducted according to the method previously described by Xie et al. (2007). The filtered water of 50 mL was applied directly to an Oasis HLB cartridge (0.5 g, Waters, Milford, MA, USA) that had been previously conditioned with methanol (10 mL) and distilled water (10 mL). It was subsequently eluted with methanol, and the elute containing the toxin was then collected. The MC-containing fraction was evaporated to dryness and the residue was reconstituted in methanol (200 μL). The resulting solution was analyzed by high-performance liquid chromatography (HPLC).

The HPLC system consists 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-R6A integrator, and an ODS column (Cosmosil 5C18-MS-II; 4.6 mm × 150 mm, Nacalai Tesque, 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⁻¹. The MC concentration was quantified against MC-LR standards (Kanto Ltd., Japan).

2.3 Results

2.3.1 The cell concentrations (based on OD₄₀₅nm)

The results of OD₄₀₅nm during 30 days showed a different variations among the three treatment groups. First, the typical growth curve from exponential to early death phase was observed in the control (Fig. 2-1). On the other hand, regardless of adding dose, the cell concentrations in the alum treating groups were significantly decreased from 1 day after adding alum.

Interestingly, in the half-maximum treatment, the cell concentration was low until day 4, after which it increased gradually to almost the same level as the control, meanwhile, in the maximum treatment, the cell concentrations of nearly zero was maintained during the experiment (Fig. 2-1).

2.3.2 The extracellular MC-LR concentrations

The high concentration of the extracellular MC-LR was determined in the maximum treatment from 1 day after adding alum (Fig. 2.2, (c)), meanwhile, it was not detected in the
control and half-maximum treatment until day 2 (Figs. 2-2 and 2-3).

In addition to, the extracellular MC-LR concentrations in the control and half-maximum treatment also increased although the concentrations were consistently lower than the maximum treatment over time (Figs. 2-2, (a) and (b)).

2.4 Discussion

The initial cell concentrations in the alum treating groups were significantly decreased from 1 day after adding alum. It resulted from coprecipitation with aluminum hydroxide ($\text{Al(OH)}_3$). When aluminum sulfate is added to water, it is immediately hydrated. A progressive series of hydrolysis reactions then occur, leading to the formation of aluminum 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).

The cell concentration in the half-maximum treatment was increased gradually from 5 day after the treatment (Fig. 2-1, gray circles), meanwhile, the cell concentration in the maximum treatment was awfully lower (nearly zero) throughout the 30 days (Fig. 2-1, black circles). The alum treatment with low dose, the half-maximum dose in this study seemed to be difficult to expect the precipitation effect of $\text{Microcystis}$ cells for a long time. Moreover, in previous studies, the repeated small dose treatment of alum (0.25 mg L$^{-1}$ as Al day$^{-1}$, 30 days) in artificial small pond have no effect on the improvement of water quality such as the decease of phosphate or chl.a concentrations (unpublished). It could be concluded that alum dose above a certain level is required for the effective removal or growth inhibition of $\text{Microcystis}$ cell caused by aluminum species.
A large amount of MC-LR was released to the water in the maximum treatment from 1 day after adding alum, meanwhile, the release of MC-LR was not observed in the control and half-maximum treatment until day 2 (Fig. 2-3). The extracellular MC-LR concentration after 1 day when alum with maximum dose was added was approximately 61% based on the concentration determined at day 30. The release of the toxin more than 50% could only be caused by the cell damage. In addition, since the extracellular MC-LR concentration at day 5 (5 day after adding alum with maximum dose) amounted to approximately 93%, it might be considered that most of intracellular toxin was released to the extracellular water with serious cell damage less than one week after the treatment.

The extracellular MC-LR concentrations in the control and half-maximum treatment were also determined (Figs. 2-2, (a) and (b)). Based on the extracellular MC-LR concentration of the maximum treatment determined at day 30, the concentrations in the control and half-maximum treatment were 56 and 78%, respectively, meanwhile, based on the cell concentration increased three times more than the initial cell concentration, they were just 10~15%. First of all, due to the long experimental period of 30 days, the growth phase of Microcystis cells has reached death phase in the control, microcystin might be released from naturally lysed cells. As a result of lysis that occur in old cultures and aged natural water blooms, extracellular microcystin concentrations increased to about 10% of the total (Jüttner and Lüthi 2008). Also, it might be caused by the natural release. It has been reported that extracellular portion in several toxins such as cylindrospermopsin and saxitoxin varied from 10~15% to 50~60% depending on the growth phase (Chiswell et al. 1999, Hawkins et al. 2001, Ho et al. 2012). Therefore, it could not be considered that the extracellular MC-LR concentrations in the control and half-maximum treatment were not a similar level with the maximum treatment.
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 and Orr 1994, Harada et al. 2009).

Therefore, the results of the considerable increase of the extracellular MC-LR concentration and the color change to light blue of precipitated cells indicate that alum treatment caused damage to the *Microcystis* cells.
Table 2-1. The composition of MA medium for culture of *Microcystis ichthyoblabe* strain TAC 95.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (mg L⁻¹)</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>50</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>40</td>
</tr>
<tr>
<td>MgCl·6H₂O</td>
<td>50</td>
</tr>
<tr>
<td>β-Na₂ glycerophosphate</td>
<td>100</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>5</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>5</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.5</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>5</td>
</tr>
<tr>
<td>NaMoO₄·2H₂O</td>
<td>0.8</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>20</td>
</tr>
<tr>
<td>Bicine</td>
<td>500</td>
</tr>
<tr>
<td>pH</td>
<td>8.6</td>
</tr>
</tbody>
</table>
Figure 2-1. The variations in cell concentration based on $OD_{405nm}$ according to alum treatment during 30 days in the flask experiment. White circles: the control (no adding alum); gray circles: adding alum of half-maximum dose; black circles: adding alum of maximum dose. Error bar indicates + S.D. Day 0 means before the treatment.
(Continued)
Figure 2-2. The variations in cell and extracellular MC-LR concentrations according to alum treatment during 30 days in the flask experiment. (a): the control; (b): the half-maximum treatment; (c): the maximum treatment; the circles: the cell concentration; the shade bars: the extracellular MC-LR concentration. Error bar indicates ± S.D. N.D. means not detected. Day 0 means before the treatment. The extracellular MC-LR concentrations of (a) and (b) were not detected until day 2, and the extracellular MC-LR concentration of (c) was not detected only at day 0.
Figure 2-3. The variations in the initial extracellular MC-LR concentration in the flask experiment. (a): the control; (b): the half-maximum treatment; (c): the maximum treatment. Day 0 means before the treatment. N.D. means not detected. No analyzed at Day 4.
References


Jones, G. and Orr, P. (1994) Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by


Chapter 3

The effect of alum coagulation on toxic *Microcystis*

and other cyanobacteria-related bacteria

in microcosm experiment

(Ecotoxicology and Environmental Safety, 2013)

3.1 A purpose

Although humans do not directly ingest cyanobacteria, they might be regularly exposed to sub-lethal dosages of extracellular microcystin in drinking water or several water recreational activities derived from contaminated lakes and reservoirs (Lam and Prepas 1997). Therefore, the release of dissolved organic matters including toxins by the cyanobacteria removal treatment including adding alum should be considered not only in the conventional water treatment but also in lake treatment. Moreover, if alum coagulation is used as in-lake treatment, it should be considered that the effect of adding alum on the organisms in the lake sediment or water column such as several bacteria.

Therefore, this study was conducted to examine *Microcystis* cell damage and the microcystin release, and to evaluate the effect on other bacteria derived from the sediment as well as *Microcystis* cells using the alum treatment in the microcosm experiment designed to
simulate the conditions found in common lakes and reservoirs.

3.2 Materials and methods

3.2.1 Microcystis suspension preparation

A unialgal culture of *Microcystis ichthyoblabe* strain TAC95 was harvested during the late exponential growth phase or the early stationary growth phase.

To remove the buffer and phosphate that are included in MA medium (Ichimura 1979), a cultivated *Microcystis* suspension was centrifuged at $2100 \times g$ for 15 min. The supernatant was then discarded and the *Microcystis* cells were washed with tap water that had been dechlorinated by aeration overnight. All processes were conducted in triplicate.

3.2.2 Preparation of chemicals

Aluminum sulfate (anhydrous) was used as a coagulant. Distilled water was used for the preparation of alum stock solution.

3.2.3 Jar test

The jar-test was conducted for calculating the concentration of alum to be added in the microcosm and bottle experiments. Alum solution (100 mg Al L$^{-1}$) was added to a beaker containing 100 mL of *Microcystis* suspension with stirring. The pH value was recorded after adding 100 μL of alum, which was expected to result in pH 6 (Cooke et al. 2005).
The conditions of washed *Microcystis* suspension might not be the same in every experiment, due to the removing extent of buffer and phosphate in the washing process (as described 3.2.1). Particularly, maximum dose used in this experiment could be affected by buffering action of water, therefore, jar-test was conducted before every experiment, and added alum concentrations differed in each experiment (Table 3-1).

3.2.4 The microcosm experiment

The microcosm experiment was conducted to examine *Microcystis* cell damage from alum treatment, in common lake environments. A microcosm was constructed using three acryl chambers with a size of 320 × 180 × 150 mm (length × width × height). To mimic the environment common in lakes or reservoirs, sediment taken from a small eutrophic pond (Chikato-ike, Matsumoto, Japan) was added to two of the microcosm chambers to give a sediment bed volume of 320 × 180 × 40 mm (length × width × height, ca. 2 L), while the third chamber did not receive sediment.

Five liters of rinsed *Microcystis* suspension were added to each chamber. The microcosms were then allowed to stand overnight. Alum solution was subsequently added to two chambers (one with sediment and the other without sediment) at the maximum dose. All chambers were continuously mixed with a propeller placed in the upper middle portion of the microcosm to improve coagulation of the *Microcystis* cells and aluminum hydroxide (Al(OH)₃). The unique propeller was used to do not disturb the sediment. Experiments were conducted in a temperature-controlled room at 24 ± 1 °C, and were illuminated with fluorescent lights at 5 μmol m⁻² s⁻¹ under a 24 h light cycle for 7 days.

Similar experiments were conducted three times, because the microcosm experiment
could not be triplicated for some of experimental conditions such as a considerable amount of *Microcystis* suspension more than 50 L. The initial chl.a and added alum concentrations of each experiment are shown Table 3-1. The experimental results were very similar all three times.

3.2.5 **Identification and the effect of alum treatment on Microcystis-lysing bacteria**

To confirm the effect on *Microcystis*-lysing bacteria in the water by adding alum, water samples of 20 mL were collected from each microcosm on days 0 (before adding alum), 4 and 7. The number of *Microcystis*-lysing bacteria was determined using a modified double-layered agar technique previously described by Mitsutani et al. (1987). *M. ichthyoblabe* TAC95 was used as the host alga. A mixture of 4 mL host alga (MA medium) and 2 mL water sample was added to 0.05 g agar and overlaid on a MA 1% agar plate. Agar plates were incubated at 23 ± 1°C under illumination at 32 μmol m⁻² s⁻¹ in a 12:12 h light:dark cycle for two weeks. After incubation, the number of *Microcystis*-lysing bacteria was counted. The number of plaques per mL was expressed as plaque forming units (PFU). This experiment was conducted in triplicate.

3.2.6 **The effect of alum treatment on the activity of microcystin-degrading bacteria**

A water sample of 100 μL was taken from each microcosm and added to a microtube containing 300 μL of a minimal medium (Table 3-2) and pure MC-LR (3.0 mg L⁻¹) mixture. Because there was no carbon source in the minimum medium, microcystin-degrading bacteria could use MC-LR as a carbon source. The microtubes were incubated at 24 ± 1°C under illumination at 5 μmol m⁻² s⁻¹ in a 24 h light cycle for 3 days. Following incubation, the
microtubes were stored at -30°C until MC-LR analysis.

3.2.7 Analytical methods

Chlorophyll a

For chl.a analysis, the sample of 20 mL was filtered through glass filter paper (GF/C, Whatman, Kent, UK) and it was extracted using 90% acetone (7 mL). The absorbance of the extracts were 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 using UNESCO equations (UNESCO 1966).

The intra and extracellular Microcystin LR

For intracellular MC-LR analysis, the sample of 30 mL was filtered through Whatman GF/C filter paper and immediately frozen at -30 °C before being freeze-dried. The freeze-dried filter was then homogenized and extracted with 5% aqueous acetic acid, after which the supernatant was applied to a HLB cartridge.

For extracellular MC-LR analysis, the filtered water of 50 mL was applied directly to a HLB cartridge, which was subsequently eluted with methanol, and the elute containing the toxin was then collected. The MC-containing fraction was evaporated to dryness and the residue was reconstituted in methanol (200 μL). The resulting solution was analyzed by high-performance liquid chromatography (HPLC).
3.3 Results

3.3.1 The effect of the alum treatment on *Microcystis* cells in the microcosm experiment

The chl.a and intracellular MC-LR concentrations in the microcosms that received the alum treatment were significantly decreased at 1 day after adding alum (Fig. 3-1, gray and black circles).

Moreover, the extracellular MC-LR concentration increased in the microcosms that received the alum treatment (Fig. 3-2). Particularly, in the +alum microcosm (received alum treatment but did not contain sediment) after 7 days from adding alum, intracellular MC-LR was released an average of 80% into the extracellular environment.

Interestingly, in the +alum +sediment microcosm (contained sediment and received alum treatment), the extracellular MC-LR concentration increased for 3 days but decreased continuously thereafter. Moreover, in the +sediment microcosm (contained sediment but did not receive alum treatment), the extracellular MC-LR concentration was also decreased by approximately 99%.

3.3.2 The effect of the alum treatment on *Microcystis*-lysing bacteria

The plaque forming units (PFU) of *Microcystis*-lysing bacteria were found to differ considerably among treatments (Fig. 3-3). No plaques were observed during the experimental period in the +alum microcosm. In the +sediment microcosm, the plaques observed during the experiment period and the PFU counts were dramatically increased (Fig. 3-3, white circles). Interestingly, in the +alum +sediment microcosm, plaques were observed at day 0, whereas no plaques were observed at day 4 (Fig. 3-3, gray circles). Plaques were again
observed at day 7, however the PFU count was considerably lower than that of the +sediment microcosm.

3.3.3 The effect of the alum treatment on the activity of microcystin-degrading bacteria

The remaining MC-LR concentration after 3 days incubation was expressed as a percentage of the initial adding MC-LR concentration (3.0 mg L\(^{-1}\)). The residual MC-LR concentration in the +alum microcosm was not significantly changed during the experimental period. This likely indicates that there were no microcystin-degrading bacteria in the +alum microcosm (Fig. 3-4, (b)). Conversely, in the microcosms that contained sediment, the residual MC-LR concentration was decreased (Figs. 3-4, (a) and (c)). In particular, MC-LR was decreased more than 85% in the +sediment microcosm. This indicated that MC-LR was degraded by microcystin-degrading bacteria that were presented in the sediment. The authors of this study also noted that the activity of degrading bacteria was lower in the microcosm that received the alum treatment compared to the microcosm that did not receive alum treatment.

3.4 Discussion

Although the initial chl.a and added alum concentrations of each microcosm experiment differed (Table 3-1), the experimental results were very similar all three times.

The cell concentration based on the intracellular MC-LR and chl.a concentrations
was significantly decreased at 1 day after adding alum (Fig. 3-1). This resulted from co-precipitation with aluminum hydroxide \((\text{Al(OH)}_3)\), which produces a colloidal amorphous floc in the neutral pH range.

The extracellular MC-LR concentration increased continuously after adding alum with maximum dose in the microcosm experiment (Fig. 3-2, black circles) as well as the flask experiment as described in chapter 2. In particular, the extracellular MC-LR concentration on 7 days after adding alum was equivalent to approximately 80% of the initial intracellular MC-LR concentration. Therefore, it could be concluded that Microcystis cells have seriously damaged by alum treatment at the maximum dose.

Many studies of the removal of cyanobacteria by alum treatment have reported that alum does not cause any cell damage and resultant toxin release. Peterson et al. (1995) suggested that alum did not cause physiological toxicity, and caused little or no release of cellular components. Chow et al. (1999) reported that the addition of alum did not cause cell damage leading to the release of MC-LR. Drikas et al. (2001) also reported that the addition of alum had no significant effect on cell viability, and flocculation did not cause additional release of MC-LR into the water. Finally, Jurczak et al. (2005) reported that alum treatment can be effective in removing intracellular microcystins through the removal of intact cyanobacterial cells.

However, most studies described above were investigated the effect of alum coagulation in water treatment process. It would be considered that one of the biggest differences between above-described studies and this study was ‘treatment time’ which means the reaction between alum and cyanobacterial cells. Treatment time of above-described studies did not exceed a maximum of 24 hours. Although treatment time differs depending on the treatment facility, coagulation/flocculation and sedimentation processes in general take
approximately 6 hours. On the other hand, when alum was added in lakes and reservoirs, floc (coagulates of aluminum hydroxide and cyanobacterial cells) was precipitated in lake sediment, it would be remain for a long time in lake system. The impact of a long remaining time of floc could be described as the continuous increase of extracellular MC-LR concentration after adding alum (Fig. 3-2, black circles). To use alum as the in-lake treatment, therefore, the long ‘remaining time’ of floc should be considered.

More seriously, alum treatment does not remove aqueous cyanotoxin. While cyanobacterial cells were removed, extracellular MC-LR was not effectively removed by alum treatment (Pietsch et al. 2002, Hoeger et al. 2005, Jurczak et al. 2005). To confirm the coagulation/precipitation of MC-LR with alum treatment, pure MC-LR after adding alum were analyzed simply, there was no decrease (not shown). Therefore, dangerously large amounts of released MC-LR might remain in the water column following alum treatment.

The effect of alum treatment on sediment bacteria, such as cyanobacteria-lysing bacteria and microcystin-degrading bacteria, was observed in the microcosms that contained sediment, while any activity of the bacteria has not been observed in the microcosms that only added alum (Figs. 3-3 and 3-4). The chl.a and intracellular MC-LR concentrations decreased continuously in the +sediment microcosm (Fig. 3-1, white circles). The decrease of *Microcystis* cell concentrations in the microcosm that contained sediment can be attributed to cell lysis by cyanobacteria-lysing bacteria. Various microorganisms are known to have the ability to lyse cyanobacterial cells (Daft et al. 1975, Mitsutani et al. 1987, Yamamoto et al. 1993). High levels of algae-lysing bacteria have been observed in sediment throughout the year (Yamamoto and Suzuki 1990).

In this experiment, the extracellular MC-LR concentration also decreased in the microcosms that contained sediment. As previously described, alum do not have the ability to
remove aqueous MC-LR, and microcystin is not easily degraded by chemical treatment (Maruyama et al. 2004). Therefore, it is likely that released MC-LR was degraded by microcystin-degrading bacteria. A large group of microcystin-degrading bacteria may be prevalent in natural waters and sediments (Holst et al. 2003). The microcystin-degrading bacterium *Sphingosinicella microcystinivorans* completely degraded MC-LR and -RR within 6 days (Park et al. 2001, Maruyama et al. 2006).

The PFU of *Microcystis*-lysing bacteria in the +alum +sediment microcosm on day 7 was greatly lower than that of the +sediment microcosm, with no plaques observed on day 4 (Fig. 3-3, gray circles). Moreover, microcystin-degrading activity of bacteria was lower during the experimental period in the microcosms that received alum treatment than in those that did not (Fig. 3-4, (a) and (c)). Rothrock Jr et al. (2008) suggested that the addition of alum to poultry litter potentially shifts the microbial populations from bacterially-dominated to being dominated by fungi. Therefore it could be concluded that alum treatment had an effect on cyanobacteria-lysing bacteria and microcystin-degrading bacteria as well as *Microcystis* cells.
**Table 3-1.** The initial chl.a and intracellular MC-LR concentration and added maximum alum dose in the microcosm experiments which were conducted three times.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Chl.a conc. (μg L(^{-1}))</th>
<th>Intracellular MC-LR conc. (μg L(^{-1}))</th>
<th>Maximum alum dose (mg L(^{-1}) as Al)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st.</td>
<td>27 ± 5</td>
<td>5 ± 2</td>
<td>2.7</td>
</tr>
<tr>
<td>2nd.</td>
<td>196 ± 2</td>
<td>97 ± 29</td>
<td>15.0</td>
</tr>
<tr>
<td>3rd.</td>
<td>264 ± 44</td>
<td>316 ± 46</td>
<td>48.0</td>
</tr>
</tbody>
</table>
Table 3-2. The composition of minimal medium for measuring the activity of microcystin-degrading bacteria.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (g L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH(_4)NO(_3)</td>
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</tr>
<tr>
<td>K(_2)HPO(_4)</td>
<td>1.50</td>
</tr>
<tr>
<td>Na(_2)HPO(_4)·12H(_2)O</td>
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</tr>
<tr>
<td>MgSO(_4)·7H(_2)O</td>
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<td>FeSO(_4)·7H(_2)O</td>
<td>0.01</td>
</tr>
<tr>
<td>CaCl(_2)·2H(_2)O</td>
<td>0.01</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>
Figure 3-1. The variations in chl.a and intracellular MC-LR concentrations following alum treatment in the microcosm experiments. (a): chl.a concentration, (b): intracellular MC-LR concentration. Black circles: received alum treatment but did not contain sediment; white circles: contained sediment but did not receive alum treatment; gray circles: contained sediment and received alum treatment. Error bar indicates ± S.D. Day 0 means before treatment.
Figure 3-2. The variations in extracellular MC-LR concentrations following alum treatment in the microcosm experiments. Black circles: received alum treatment but did not contain sediment; white circles: contained sediment but did not receive alum treatment; gray circles: contained sediment and received alum treatment. Error bar indicates ± S.D. Day 0 means before treatment.
Figure 3-3. Variations in the plaques of *Microcystis*-lysing bacteria following alum treatment in the microcosm experiment. Black circles: received alum treatment but did not contain sediment; white circles: contained sediment but did not receive alum treatment; gray circles: contained sediment and received alum treatment. Error bar indicates ± S.D. Day 0 means before treatment.
Figure 3-4. Variations in the residual MC-LR concentrations as a result of the activity of microcystin-degrading bacteria in the microcosm experiment. (a): +sediment (contained sediment but did not receive alum treatment), (b): +alum (received alum treatment but did not contain sediment), (c): +alum +sediment (contained sediment and received alum treatment). Day 0 means before treatment.
References


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Chapter 4

The effect of alum precipitates on *Microcystis* cells

through the analysis of precipitates

4.1 A purpose

From the results of the flask and microcosm experiments (chapters 2 and 3), it was found that *Microcystis* cells were damaged and subsequently a large amount of microcystin was released by the alum treatment with the maximum dose. It has been known that microcystin is released when *Microcystis* cells are damaged; therefore the massive release of microcystin is very important marker for cell damage.

However, in order to conclude for a certainty that cyanobacterial cells are damaged by alum treatment, it should be supposed that more definite and/or lots of evidences are required. Therefore, in this study, precipitated *Microcystis* cells were analyzed to determine whether *Microcystis* cells were certainly damaged or not.
4.2 Materials and methods

4.2.1 Microcystis suspension preparation

A unialgal culture of *Microcystis ichthyoblabe* strain TAC95 was harvested during the late exponential growth phase.

A cultivated *Microcystis* suspension was centrifuged at $2100 \times g$ for 15 min. The supernatant was then discarded and the *Microcystis* cells were washed with tap water that had been dechlorinated by aeration overnight. All processes were conducted in triplicate.

4.2.2 Preparation of chemicals

Aluminum sulfate stock solution (2000 mg Al L$^{-1}$) was prepared by dissolving anhydrous aluminum sulfate (Kanto Chemical Company, Tokyo, Japan) in distilled water.

4.2.3 Jar test

Alum solution (100 mg Al L$^{-1}$) was added to a beaker containing 100 mL of *Microcystis* suspension with stirring. The pH value was recorded after adding 100 μL of alum, which was expected to result in pH 6 (Chow et al. 1999). Jar-test was conducted before the experiment, added maximum alum dose was 35 mg L$^{-1}$ as Al.

4.2.4 The bottle experiments to investigate the effect of alum on precipitated *Microcystis* cells

A bottle experiment was conducted using 16 polypropylene bottles with a volume of
2 L. One and a half liters of rinsed *Microcystis* suspension was carefully added to each bottle. The maximum dose (35 mg L\(^{-1}\) as Al) of alum was added to eight bottles, while half of the maximum dose (17 mg L\(^{-1}\) as Al) was added to the remaining eight bottles. Rapid mixing (200 rpm) for 1 min was followed by 3 min of slow mixing (50 rpm) with a magnetic stirrer.

The experiment was conducted in a temperature-controlled room at 24 ± 1 °C, and was illuminated with fluorescent lights at 5 μmol m\(^{-2}\) s\(^{-1}\) under a 24 h light cycle for 7 days. One bottle from each treatment was destructively-sampled each day, and the precipitate was separated from the supernatant using a siphon. The chl.a and intracellular MC-LR concentrations were both analyzed from the supernatant and precipitates, while extracellular MC-LR analyzed only from the supernatant.

### 4.2.5 The effect of alum on precipitated *Microcystis* cell morphology

Two bottles with a volume of 2 L were prepared, one and a half liters of rinsed *Microcystis* suspension was carefully added to each bottle. The maximum dose (42 mg L\(^{-1}\) as Al) of alum was added to one bottle, while the other bottle was left untreated as a control. Rapid mixing (200 rpm) for 1 min was followed by 3 min of slow mixing (50 rpm) with a magnetic stirrer.

The experiment was conducted in a temperature-controlled room at 24 ± 1 °C, and was illuminated with fluorescent lights at 5 μmol m\(^{-2}\) s\(^{-1}\) under a 24 h light cycle. After 3 days from adding alum, the precipitate was separated from the supernatant using a siphon. 10 mL of precipitates was used to analyze the cell morphology.

Extracellular MC-LR concentration in the supernatant was also analyzed, which was very similar with previous experimental results (not shown).
4.2.6 Analytical methods

Chlorophyll a

For chl.a analysis, the sample of 20 mL was filtered through glass filter paper (GF/C, Whatman, Kent, UK) and it was extracted using 90% acetone (7 mL). The absorbance of the extracts were 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 using UNESCO equations (UNESCO 1966).

The intra and extracellular Microcystin LR

For intracellular MC-LR analysis, the sample of 30 mL was filtered through Whatman GF/C filter paper and immediately frozen at -30 °C before being freeze-dried. The freeze-dried filter was then homogenized and extracted with 5% aqueous acetic acid, after which the supernatant was applied to a HLB cartridge.

For extracellular MC-LR analysis, the filtered water of 50 mL was applied directly to a HLB cartridge, which was subsequently eluted with methanol, and the elute containing the toxin was then collected. The MC-containing fraction was evaporated to dryness and the residue was reconstituted in methanol (200 μL). The resulting solution was analyzed by high-performance liquid chromatography (HPLC).

The scanning electron microscopy (SEM)

10 mL of precipitates was used to analyze the cell morphology. The samples were
dehydrated through a graded series of ethanol from 50 to 100%, and then they were transferred to t-butyl alcohol. The containers containing precipitates in t-butyl alcohol were placed in a refrigerator, the butyl alcohol was then frozen within a few minutes. The containers were transferred into a vacuum evaporator (JFD-310, JEOL, Tokyo, Japan), and were freeze dried for 1 hour. The dried samples were coated with osmium using a sputter coater (Osmium coater Neo-AN, Meiwafosis Co., Ltd., Tokyo, Japan). The cell morphology was observed under a scanning electron microscope (JSM-7600F, JEOL, Tokyo, Japan).

4.3 Results

4.3.1 The effect of alum on precipitated Microcystis cells

The variations in the chl.a and intracellular MC-LR concentrations of the supernatant in the bottle experiments were similar to the results observed in the microcosm experiments. At 1 day after adding alum, the chl.a and intracellular MC-LR concentrations were decreased in excess of 95% (Fig. 4-1). This decrease resulted from co-precipitation, as described previously. Interestingly, in the half-maximum treatment, although present in very low concentrations, the chl.a and intracellular MC-LR concentrations began to increase at day 5 (Fig. 4-1, gray circles).

The extracellular MC-LR concentration in the supernatant also increased significantly in the maximum treatment sample such as the result of the +alum treatment in the microcosm experiment (Fig. 4-2, black circles). Interestingly, in the half-maximum treatment, the extracellular MC-LR concentration was lower than that in the maximum treatment; corresponding to approximately 10% of the extracellular MC-LR concentration in
the maximum treatment (Fig. 4-2).

The variations of chl.a and intracellular MC-LR concentrations of the precipitates were greatly different between the maximum and half-maximum treatments (Fig. 4-3). There was very little decrease in the chl.a concentration in the half-maximum treatment (Fig. 4-3, (A) gray bars), whereas the concentration decreased approximately 50% in the maximum treatment (Fig. 4-3, (A) black bars). The intracellular MC-LR concentrations were decreased in both the maximum and half-maximum treatments; however, the decreased MC-LR concentrations were 99% and 49%, respectively (Fig. 4-3 (B)).

4.3.2 The effect of alum on precipitated *Microcystis* cell morphology

*Microcystis* cells in the control were kept intact and no lysis. There was also observed a lot of dividing cells (Fig. 4-4, (a) and (b)).

In the alum treatment, on the other hand, the number of observed cells was remarkably less than the control. Precipitated *Microcystis* cells were surrounded or coated with aluminum hydroxide floc, the most of which were damaged (Fig. 4-4, (c) ~ (e)). There was observed the cell in which cell membrane was torn (Fig. 4-4, (c) and (d)). In addition, even worse, there was observed the cell in which the protoplasm in the cell was released, and which a trace of cell coated by aluminum hydroxide was barely remained (Fig. 4-4, (e)).

4.4 Discussion

The results of the supernatant in the bottle experiment were very similar to the results
in the previous flask and microcosm experiments that the decrease of chl.a and intracellular MC-LR concentration and the continuous increase of the extracellular MC-LR concentration (Figs. 4-1 and 4-2). It is known that cyanotoxin is released when the cell was damaged, therefore, the release of cyanotoxin is one of the most important evidence for the cell damage. It was certain through several experiments that the alum treatment with maximum dose caused the cell damage and subsequent release of cyanotoxin.

Furthermore, the decrease of the chl.a and intracellular MC-LR concentrations in the precipitates in the bottle experiment have supported the hypothesis of this study that *Microcystis* cells are damaged by alum treatment of maximum dose. Although chl.a is a photosynthetic pigment that is not easily released from inside the cell (Hall and Rao 1999), the chl.a concentration decreased approximately 50% in the maximum treatment (Fig. 4-3, black bars). Moreover, precipitated *Microcystis* cells changed from dark green to light blue after adding alum at the maximum dose in the bottle experiment as well as the flask and microcosm experiment. As previously described, cell lysis causes the release of phycocyanin and a characteristic color change from green to blue (Jones and Orr 1994, Harada et al. 2009). The decreasing of chl.a and intracellular MC-LR concentrations of precipitates (Fig. 4-3) and the releasing of phycocyanin identified a characteristic color change, therefore, are reliable evidence of *Microcystis* cell damage, along with MC-LR release.

Interestingly, serious cell damage was not observed in the half-maximum treatment. Although present at very low levels, in the half-maximum treatment, the chl.a and intracellular MC-LR concentrations in the supernatant began to increase at day 5 (Figs. 4-1, gray circles). A similar result was observed in the flask experiment (Fig. 2-1), the cell concentration in half-maximum treatment was very low until day 4, and then increased gradually to almost the same level as the control. Moreover, the chl.a concentration in the
precipitates decreased very little in the half-maximum treatment (Fig. 4-3, gray bars), and the variations in extracellular MC-LR concentration in the supernatant and intracellular MC-LR concentration in the precipitates were much smaller rather those than in the maximum treatment. These results indicated that low-dose alum treatment did not cause the serious cell damage; therefore, it could concluded that low alum dose might be not sufficient to remove thoroughly cells from water column by co-precipitation and suppress *Microcystis* cell growth for a long period.

It is also important to understand the mechanisms of toxicity of aluminum on *Microcystis* cells. Few have investigated the toxic effects of aluminum on phytoplankton or microbial cells. Sun et al. (2013) confirmed with SEM analysis that *Microcystis aeruginosa* cells began to lyse after 2 d and were almost completely damaged after 4 d in the system with both coagulant and stirring. Malecki-Brown et al. (2010) reported that the aluminum concentration in plants was elevated 50 times, while the biomass were induced in the submerged aquatic vegetation in alum-treated mesocosms. Submerged aquatic vegetation absorbs the majority of its nutrients into the shoots directly from the water column, in a manner similar to that of phytoplankton or aquatic microorganisms. Pettersson et al. (1986) concluded that aluminum toxicity in cyanobacteria is due to intracellular accumulation of Al, which occurs via passive diffusion. Moreover, aluminum binds to cell membranes, causing changes in membrane configuration and membrane bound enzyme activities, such as ATPase (Pettersson et al. 1986, Greger et al. 1992, Malecki-Brown et al. 2010). Adding aluminum also caused precipitation of aluminum and phosphate in cell walls, covering the cell surface and blocking ion uptake (Greger et al. 1992). Pettersson et al. (1985) investigated the intracellular localization of aluminum by X-ray microanalysis. They showed that aluminum was bound in cell walls and phosphate granules, which might influence the generation of phosphate and energy and disturb the translocation of other ions.
It is likely that some of the toxic mechanisms described above occurred in this study. First of all, it could be seen that cell membrane became hard or were torn, because *Microcystis* cells were coated with aluminum hydroxide floc. This was confirmed through the results of SEM analysis (Fig. 4-3, (c) and (d)). After all, it could be considered that these damaged cells by alum are lysed, the traces of cells released protoplasm are barely remained (Fig. 4-3, (e)). In addition, based on the release of MC-LR and photosynthesis pigments by alum treatment with maximum dose, it might be possible that changes occurred in membrane configuration, and cell surfaces could have been covered with the precipitation of aluminum and phosphate.

The various analyzed results of precipitated *Microcystis* cells such as SEM analysis in this study provided the more definite evidence that the alum treatment cause the cell damage.
Figure 4-1. Variations in the chl.a and intracellular MC-LR concentrations of the supernatant following alum treatment in the bottle experiment. (a): chl.a concentration, (b): intracellular MC-LR concentration. Gray circles: half-maximum treatment, black circles: maximum treatment. Day 0 means before treatment.
Figure 4-2. Variations in the extracellular MC-LR concentration of the supernatant following alum treatment in the bottle experiment. Gray circles: half-maximum treatment, black circles: maximum treatment. Day 0 means before treatment.
Figure 4-3. Variations in the chl.a and intracellular MC-LR concentrations of the precipitates following alum treatment in the bottle experiment. (A): chl.a concentration, (B): intracellular MC-LR concentration. Gray bars: half-maximum treatment, black bars: maximum treatment.
Figure 4-4. The SEM micrographs of *Microcystis* cells without and with alum treatment. (a) and (b): control (without alum treatment), (c)~(e): alum treatment. Black arrow: aluminum hydroxide, red arrow: damaged *Microcystis* cell.
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Chapter 5

General discussion and conclusions

5.1 The long term effect of alum coagulation on cyanobacteria cells

Many studies of the removal of cyanobacteria by alum treatment have reported that alum does not cause any cell damage and resultant toxin release (Table 1-2). Peterson et al. (1995) suggested that alum did not cause physiological toxicity, and caused little or no release of cellular components. Chow et al. (1999) reported that the addition of alum did not cause cell damage leading to the release of MC-LR. Drikas et al. (2001) also reported that the addition of alum had no significant effect on cell viability, and flocculation did not cause additional release of MC-LR into the water. Finally, Jurczak et al. (2005) reported that alum treatment can be effective in removing intracellular microcystins through the removal of intact cyanobacterial cells.

However, *Microcystis* cells were damaged, and a large quantity of microcystin-LR were subsequently released to water column by alum treatment with maximum dose (Figs 2-2 and 3-2) both in the flask experiment and in the microcosm experiment. In addition, intracellular microcystin-LR was released extracellular water column more than 80% within 7 days.

It would be considered that one of the biggest differences between previous studies
and this study was ‘treatment time’ which means the reaction between alum (aluminum hydroxide) and cyanobacterial cells. Treatment time of previous studies did not exceed a maximum of 24 hours. Although treatment time differs depending on the treatment facility, coagulation/flocculation and sedimentation processes in general take approximately 6 hours. On the other hand, when alum was added in lakes and reservoirs, floc (coagulates of aluminum hydroxide and cyanobacterial cells) was precipitated in the lake sediment, it might remain for a long time of several months or more in lake system.

Furthermore, cyanobacteria cell damage and toxin release by alum with long treatment time might be a serious problem in the water treatment sludge management processes as well as in-lake treatment. Because, sludge supernatant is recycled to the head of the water treatment processes, particularly since conventional water treatment is largely ineffective for the removal of extracellular metabolites (Ho et al. 2012).

5.2 The effect of alum coagulation on other cyanobacteria-related bacteria

The primary purpose of aluminum application as in-lake treatment is to lower phosphorus concentration in lake by precipitation of phosphorus from water column, and or by inactivation of phosphorus in the lake sediments (Hansen et al. 2003, Cooke et al. 2005). However, if alum is used as in-lake treatment for not only reducing phosphorus but also coagulating organic particulate matter, it should be considered that the effect of adding alum on the organisms such as several bacteria in the lake sediment or water column. Therefore, it was evaluated that the effect of alum treatment with maximum dose for long treatment time on cyanobacteria-related bacteria derived from the sediment as indirect indicator.
No plaques were observed at 4 day after adding alum, and plaques were again observed at day 7, however, the PFU count was considerably ten times lower than that of the microcosm that did not received alum treatment, meanwhile, plaques were a similar level in the microcosms that contained sediment before the treatment (Fig. 3-3). Moreover, microcystin-degrading activity of bacteria was lower during the experimental period in the microcosms that received alum treatment than in those that did not (Fig. 3-4).

Although it is not possible to assert that alum treatment caused doubtless the cell lysis of cyanobacteria-lysing bacteria and microcystin-degrading bacteria like *Microcystis* cells, however, it could be concluded that alum treatment caused a certain damage on these bacteria.

### 5.3 The confirmation of cell damage by alum treatment through the analysis of precipitates

From the results of the flask and microcosm experiments (chapters 2 and 3), it was found that *Microcystis* cells were damaged and subsequently a large amount of microcystin was released by the alum treatment with the maximum dose. However, in order to obtain more certain and or lots of evidences that cyanobacterial cells are damaged by alum treatment, precipitates as well as supernatant were analyzed.

Although chl.a is a photosynthetic pigment that is not easily released from inside the cell (Hall and Rao 1999), intracellular chl.a was released approximately 50% (Fig. 4-3) as well as the intracellular MC-LR was nearly released to extracellular water in the precipitates by alum treatment with maximum dose.
It could be confirmed through the scanning electron microscopy analysis that Microcystis cells were damaged or further lysed by alum treatment with maximum dose. The number of observed cells was remarkably less than the control. Precipitated Microcystis cells were surrounded or coated with aluminum hydroxide floc. There was observed the cell in which cell membrane was torn, and a trace of cell coated by aluminum hydroxide was barely remained (Fig. 4-4). It was considered that cyanobacterial cell membrane coated with aluminum hydroxide was hardened, after all, which was torn, and most of intracellular protoplasm with cyanotoxin and photosynthetic pigments were released through this portion. Furthermore, it might be thought that aluminum binds to cell membranes, causing changes in membrane configuration and membrane bound enzyme activities (Pettersson et al. 1986, Greger et al. 1992, Malecki-Brown et al. 2010).

5.4 The effect of alum dose regarding treatment efficiency and cell damage

To evaluate the effect of alum dose, alum of half-maximum dose was added in the flask and bottle experiments. The cell concentration in supernatant in the flask experiment was low until day 4, after which it increased gradually to almost the same level as the control (Fig. 2-1). In the bottle experiment, also, although present at very low levels the chl.a and intracellular MC-LR concentrations in the supernatant began to increase at day 5 (Fig. 4-1), moreover, the chl.a and intracellular MC-LR concentrations in the precipitates were hardly changed during the experiment (Fig. 4-3). The alum treatment with low dose, the half-maximum dose in this study seemed to be difficult to expect the precipitation effect of Microcystis cells for a long time. It could be concluded that alum dose above a certain level is
required for the effective inhibition of *Microcystis* cell growth caused by aluminum species.

**5.5 The suggestions about the alum treatment as in-lake treatment**

Aluminum sulfate has several advantages that can be easily available, quick and inexpensive rather than other various treatments for removal of cyanobacterial bloom. However, based on the results of this study, the alum treatment with the maximum dose cased serious damage to toxic *Microcystis* cells and subsequently a large amount of microcystin was released. Therefore, it could be concluded that alum treatment with maximum dose is not suitable to remove toxic cyanobacterial bloom in lakes, reservoirs and ponds. Particularly, it might be required more attention that using aluminum coagulant in an artificial ponds that do not exist the sediment, because released cyanotoxin by alum treatment remain in water column for a long term without any degradation (Figs. 3-2 and 4-2).

Nevertheless, it could be suggested that several ways about the removal of cyanobacterial bloom using alum. The first is to investigate the dominant species of cyanobacterial bloom occurred in lakes or reservoirs before the treatment. When non-toxic cyanobacteria species are dominant, alum treatment can be used without any concern about the toxins release.

On the other hand, though the dominant species are toxic, because the toxin release was lower than maximum dose, meanwhile the effect of growth inhibition was short-lived in the half-maximum dose, alum might be able to use based on careful consideration of adding dose. Moreover, if the methods to remove toxins are added after adding alum, it is possible to use alum treatment. There could be suggested as the additional methods that adding toxin-
degrading bacteria in lake water column after alum treatment or adding air flotation device during alum treatment to remove immediately and easily suspended flocs.

5.6 Conclusions

Results of this study suggested that alum treatment with maximum is not suitable to remove toxic cyanobacterial bloom in artificial reservoirs and ponds that do not contain sediment, because a large amount of microcystin was released into the water. However, low alum dose might be not sufficient to remove thoroughly cells from water column by co-precipitation and suppress Microcystis cell growth for a long period. Moreover, it might be considered that alum treatment caused a certain damage to the organisms such as several bacteria in the lake sediment or water column. Accordingly, natural cyanobacterial cell lysis or microcystin degradation by these bacteria became underactive, the residual microcystin concentration in the lake water would not be considered safe for wild and domestic animals and humans.
References


Appendix

The effect on the growth of *Microcystis* cells of small-scale oscillating mixing
Introduction

Cyanobacterial blooms are often occurred during vertically stratified conditions in mainly summer. Several factors that caused Microcystis bloom have been suggested that high nutrients loading, higher light and water temperature, stability of the water column and a low N:P ratio (Moisander et al. 2002, Huisman et al. 2005, Reynolds 2006).

Because buoyancy is crucial for Microcystis, artificial mixing have been used to reduce (Visser et al. 1996, Huisman et al. 2005). Changes in turbulent mixing or artificial destratification caused a dramatic shift in phytoplankton species composition from Microcystis to sinking diatoms and green algae (Huisman and Sommeijer 2002, Huisman et al. 2004, Antenucci et al. 2005). Moreover, Microcystis colonies was broken up under turbulent mixing (O’Brien et al. 2004).

However, the significance of small scale turbulent has been a relatively neglected factor in phytoplankton ecology (Regel et al. 2004), the effects of small scale oscillation or turbulent on phytoplankton including cyanobacteria were rarely reported. Therefore, this study investigated the effect of small scale oscillating mixing on the growth of toxic Microcystis.

Materials and methods

Microcystis suspension preparation

A unialgal culture of Microcystis ichthyoblabe strain TAC95 (Tsukuba Algal Collection, National Museum of Nature and Science, Tokyo, Japan) was grown in 10 L of
MA medium at 23±1 °C under illumination at ca. 16 μmol m⁻² s⁻¹ and a 12:12 h light:dark cycle. *Microcystis* cultures were harvested during the late exponential growth phase or the early stationary growth phase.

**The oscillating mixing device**

It was used the oscillating mixing device which has two axes of horizontal and diagonal with a length of approximately 4 cm made from silicon (Fig. X-1). The device was rotated by a motor connected above. Interestingly, the water flow was turned depending on the rotating direction (Fig. X-5).

**Growth inhibition of *Microcystis* cells with several rotating apparatuses**

First of all, to simply examine the inhibition effect of this device, three treatments were prepared: (1) control (no mixing), (2) the oscillating mixing device and (3) magnetic stirrer used commonly. 6 L of *Microcystis* suspension were added each acryl chambers with a size of 320 × 180 × 150 mm (length × width × height). Rotational speeds of the oscillating mixing device and magnetic stirrer were 168 and 170 rpm, respectively. Experiments were conducted in a temperature-controlled room at 22 ± 1 °C, and were illuminated with fluorescent lights at 5 μmol m⁻² s⁻¹ under a 24 h light cycle for 12 days. The optical density at 405 nm (OD₄₀₅nm) was measured every day.

In the second experiment, for the same rotational position, the motors were connected in all treatments: (1) a propeller and (2) a bar magnet of a similar size to the device, and (3) the oscillating mixing device. 6 L of *Microcystis* suspension were added each acryl chamber. Rotational speeds that a propeller, a magnet and the oscillating mixing device were
166, 161 and 171 rpm, respectively, and they were rotated counterclockwise. Experiments were conducted in a temperature-controlled room at 22 ± 1 °C, and were illuminated with fluorescent lights at 5 μmol m⁻² s⁻¹ under a 24 h light cycle for 6 days. OD₄₀₅nm was measured every day.

**The optimum rotational speed for growth inhibition of Microcystis cells**

It was measured that growth inhibition of Microcystis cells by the different rotational speed of the oscillating mixing device. The experiment was conducted two times: (1) 50, 150 and 250 rpm, (2) 100, 150, 200 rpm. As described above, the water flow was turned depending on the rotating direction, therefore, devices in all treatments were rotated in a counter-clockwise direction. Experiments were conducted in a temperature-controlled room at 22 ± 1 °C, and were illuminated with fluorescent lights at 5 μmol m⁻² s⁻¹ under a 24 h light cycle for 6 days. OD₄₀₅nm was measured every day.

**The effect of oscillating mixing on the cell damage and microcystin release**

It was examined that oscillating mixing caused whether the cell damage or growth inhibition. Moreover, it was investigated that the effect of rotating direction in the oscillating mixing device, because which caused the inverse water flow (Fig. X-5). They were prepared four treatments: (1) a propeller, (2) a bar magnet, (3) the oscillating mixing device with a clockwise rotation, and (4) the oscillating mixing device with a counter-clockwise rotation. Rotating direction was counterclockwise in all treatment. Experiments were conducted in a temperature-controlled room at 22 ± 1 °C, and were illuminated with fluorescent lights at 5 μmol m⁻² s⁻¹ under a 24 h light cycle for 6 days. OD₄₀₅nm was measured every day, and
samples (30 mL) were taken for analysis of the extracellular MC-LR before the rotation (day 0), and at days 1, 3, 5 and 7.

**Analytical methods**

**Optical density at 405nm (OD$_{405nm}$)**

Optical density at 405nm was used as *Microcystis* cell concentration, because it has been confirmed experimentally that there is a good correlation between OD$_{405nm}$ and the cell concentration of *Microcystis ichthyoblabe* strain TAC95.

The absorbance of samples taken from supernatant were measured at 405 nm using a spectrophotometer (V-540 UV/VIS, Jasco, Tokyo, Japan).

**The extracellular MC-LR concentrations**

Measurement of MC-LR concentrations was conducted according to the method previously described by Xie et al. (2007). The filtered water of 50 mL was applied directly to an Oasis HLB cartridge (0.5 g, Waters, Milford, MA, USA) that had been previously conditioned with methanol (10 mL) and distilled water (10 mL). It was subsequently eluted with methanol, and the elute containing the toxin was then collected. The MC-containing fraction was evaporated to dryness and the residue was reconstituted in methanol (200 μL). The resulting solution was analyzed by high-performance liquid chromatography (HPLC).

The HPLC system consists 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-R6A integrator, and an ODS column (Cosmosil 5C18-MS-II; 4.6 mm ×150 mm, Nacalai Tesque, 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\(^{-1}\). The MC concentration was quantified against MC-LR standards (Kanto Ltd., Japan).

**Results**

**Growth inhibition of* Microcystis* cells with the oscillating mixing device**

Both in two times experiments, the cell concentration was decreased in the oscillating mixing, meanwhile it was increased in the other treatments (Figs X-1 and X-2, black circles). The cell concentrations in a magnet treatment was increased regardless rotational position (Figs X-1 and X-2, gray circles).

**The optimum rotational speed on growth inhibition of* Microcystis* cells**

The cell concentrations decreased in the treatments with 150 and 200 rpm, meanwhile it increased in the treatment with 50 rpm (Figs X-4, upper). And, there was almost no difference in the cell concentrations between 100, 150 and 200 rpm (Figs X-4, lower). Although there was very little difference, it could be considered that the optimal rotational speed on growth inhibition of* Microcystis* cells is 150 rpm.

**The effect of oscillating mixing on the cell damage and microcystin release**

Similar to the previous results, the cell concentrations in the oscillating mixing
devices decreased, meanwhile, the cell concentrations in a propeller and a magnet increased (Fig. X-6). Moreover, the difference in cell concentration was not observed between counter-clockwise and clockwise in the oscillating mixing devices (Fig. X-6).

The rapid increase of extracellular MC-LR concentrations was not observed in all treatments (Fig. X-7). Nevertheless, extracellular MC-LR concentration at day 7 when oscillating mixing device was rotated with counter-clockwise direction was two times higher rather than other rotating apparatuses including oscillating mixing device with clockwise rotation (Fig. X-7).

**Discussion**

Interestingly, in rotating with a propeller and a bar magnet, in contrast, the cell concentrations were increased. Moreover, when the oscillating mixing was rotated at 50 rpm, although it a growth was slower rather than other apparatuses, the cell concentration increased. Surely, a certain speed of shaking or stirring is required for a successful laboratory incubation of phytoplankton including cyanobacteria. It might be considered that all type of mixing does not cause the growth inhibition of cyanobacteria.

It was confirmed that the growth of *Microcystis* cells was inhibited by oscillating mixing over 100 rpm, the optimal rotational speed on growth inhibition of *Microcystis* cells is 150 rpm (Fig X-4). The oscillation frequency was 2.5 Hz when the oscillating mixing device was rotated at 150 rpm. Regel et al. (2004) evaluated that the effect of small-scale turbulence on the physiology of *Microcystis aeruginosa* using a vertically oscillating grid, as a result, oscillation frequency of 3 and 4 Hz deleteriously affected *M. aeruginosa* by decreasing cell
esterase activity and causing cell death. Affected frequency in this study was lower than Regel et al. (2004), it might be due to the cell size, because M. ichthyoblabe is known to be smaller than M. aeruginosa. Based on these results, it could be concluded that a certain oscillating mixing caused the deleterious effect on Microcystis cells.

Furthermore, the cell concentration in the oscillating mixing device began to decrease after approximately 48 h. It suggested that Microcystis cells have tolerance of mixing for short period in the order of days (Regel et al. 2004).

The oscillating mixing device caused the growth inhibition, however, did not cause the release of microcystin (Fig X-7). Although extracellular MC-LR concentration at day 7 when oscillating mixing device was rotated with counter-clockwise direction was higher than other rotating apparatuses, it could be seen that a release of this level was much lower than the chemical treatment such as aluminum, chloride, copper sulfate (Peterson et al. 1995, Chow et al. 1999, Han et al. 2013).

Conclusions

This study investigated the effect of the growth inhibition on toxic Microcystis cells using the oscillating mixing device. The growth of Microcystis cells was inhibited by oscillating mixing over 100 rpm, optimal rotational speed was 150 rpm, 2.5 Hz. Moreover, the release of microcystin was not observed by oscillating mixing. Therefore, the oscillating mixing device would be useful to remove toxic Microcystis cells without the toxin release.
**Figure X-1.** The photographs on the oscillating mixing device.
**Figure X-2.** The variation in OD$_{405\text{nm}}$ as the cell concentration in the first experiment. White circles: the control (no mixing); gray circles: stirring with a bar magnet (at the bottom); black circles: oscillating mixing device. Day 0 means before treatment.
Figure X-3. The variation in OD\textsubscript{405nm} as the cell concentration in the second experiment. All treatments had same rotational position. White circles: rotating with a propeller; gray circles: rotating with a bar magnet; black circles: rotating with oscillating mixing device. Day 0 means before treatment.
**Figure X-4.** The variation in OD$_{405\text{nm}}$ by the rotational speed. The experiment was conducted two times (upper and lower). White triangles: 50 rpm; black circles: 150 rpm; gray squares: 250 rpm, white circles: 100 rpm, gray circles: 200 rpm. Day 0 means before treatment.
Figure X-5. Schematic diagram of the water flow by rotating direction in the oscillating mixing device. The water moves downward by oscillating mixing into rotating counter-clockwise direction (left), meanwhile it moves upward by oscillating mixing into rotating counter-clockwise direction (right).
Figure X-6. The variation in OD$_{405\text{nm}}$ as the cell concentration by several rotating apparatuses. White circles: rotating with a propeller; gray circles: rotating with a bar magnet; left semi-filled circles: rotating with oscillating mixing device with counter-clockwise rotation; right semi-filled circles: rotating with oscillating mixing device with clockwise rotation. Day 0 means before treatment.
**Figure X-7.** The variation in OD$_{405nm}$ and extracellular MC-LR concentration by several rotating apparatuses. (a): a bar magnet; (b): a propeller; (c): oscillating mixing device with counter-clockwise; (d): oscillating mixing device with clockwise. Black circles: OD$_{405nm}$; gray bar: extracellular MC-LR concentration. Day 0 means before treatment.
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