Cyanotoxin impact on microbial-mediated nitrogen transformations at the interface of sediment-water column in surface water bodies

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A B S T R A C T

Harmful cyanobacterial blooms produce lethal toxins in many aquatic ecosystems experiencing eutrophication. This manuscript presents results on the effects of cyanotoxins on the aerobic microbial communities residing at the interface of sediments and water columns with the ammonia-oxidizing bacteria (AOB) as the model microbial community. Microcystin-LR (MC-LR), a heavily researched cyanotoxin variant, was used as the model cyanotoxin. To measure cyanotoxin influence on the activity of nitrifying microbial communities, an enriched culture of AOBs collected from an ongoing partial nitrification-nitritation reactor was examined for its exposure to 1, 5 and 10 μg/L of MC-LR. The nitritation kinetics experiment demonstrated MC-LR's ability at 1, 5, and 10 μg/L concentrations to prevent ammonium oxidation with statistically significant differences in nitritation rates between the blanks and spiked samples (One-way ANOVA, p < 0.05). Significantly decreased dissolved oxygen (DO) consumption during oxygen update batch tests demonstrated toxin's influence on AOB's oxidizing capabilities when exposed to even lower concentrations of 0.75, 0.5, and 0.25 μg/L of MC-LR in a separate set of experiments. Based on competitive kinetics, the MC-LR inhibition coefficient—the concentration needed to produce half-maximum inhibition of the mixed community AOBs was determined to be 0.083 μg/L. The stress tests proved the recovery of nitritation to some extent at lower MC-LR concentrations (1 and 5 μg/L), but significant irreversible inhibition was recorded when the AOB population was exposed to 10 μg/L MC-LR. The comparisons of amoA gene expressions corresponded well with nitrifying kinetics. All concentrations of MC-LR spiking were determined to produce a discernible impact on the AOB nitritation rate by either destroying the bacterial cell or immediately inhibiting the amoA gene expression.

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1. Introduction

Over the last few decades, the growth of harmful algal blooms (HABs) in many aquatic systems has become a prevalent problem due to increased agricultural runoff, wastewater treatment plant effluents and other nonpoint source discharges with high concentrations of nutrients getting into lakes (Heisler et al., 2008). While algal and cyanobacterial biomass occur naturally in aquatic ecosystems, cyanobacterial blooms are amplified by anthropogenic activities, such as excess agricultural, point and nonpoint run offs (Paerl et al., 2011; Paerl and Otten, 2013). Cyanotoxins, which are cyanobacterial secondary metabolites, are one of the most potent contaminants found in freshwater ecosystems (Stewart et al., 2008). Human, ecological and microbial health is affected by the toxins produced by cyanobacteria. Microcystin-LR (MC-LR) is mainly a dangerous cyanotoxin, known for accumulating in the liver of humans, livestock, and other animals (Lone et al., 2015).

As recent studies demonstrate how climate change correlates to cyanobacterial blooms (Wells et al., 2015; Griffith and Gobler, 2020), cyanotoxin's ecological impact has become a predominant question in relation to lake water post-bloom conditions (Dalu and Wasserman, 2018). While lake water conditions during algal blooms are toxic to the surrounding plant, animal, and microbial species, long term effects of cyanobacterial blooms are more indefinite. Moreover, cyanotoxin strains have appeared throughout the water column, sediment, marine life, and even tertiary plants in...
Within the bacterial processes, the aerobic process that converts ammonium-nitrogen \( (NH_4^+ - N) \) to nitrates \( (NO_3^- - N) \) and nitrates \( (NO_3^- - N) \) through oxidation is referred to as nitrification, and the essential component of the overall N cycle \( (Dasgupta et al., 2020; Podder et al., 2020a) \). Partial nitrification (nitrification), \( NH_4^+ - N \) oxidation to \( NO_2^- - N \), is the first step in the two-step nitrification process mediated by autotrophic ammonia-oxidizing bacteria (AOB) \( (Peng et al., 2017) \). \( NH_4^+ - N \) oxidation by AOBs is mediated through the expression of ammonia monoxygenase \( (amoA) \) as an enzymatic catalyst under aerobic conditions \( (Podder et al., 2020b; Rotthauwe et al., 1997) \). This process actively occurs at the sediment-water interface; due to in-situ aerobic conditions and nutrient accumulation on the top of sediments \( (Hong et al., 2019; Wu et al., 2013) \). Since MC-LR settles down to sediments through the water column after cyanobacterial cell death, the toxins will establish contact with the sediment-water interface, especially with aerobically respiring communities such as the nitrifying community. Wind-induced agitation, water currents, and human activities further aid lake water mixing, thereby enhances cyanotoxin exposure and oxygen content at the water-sediment interface \( (Chen et al., 2008) \). Regardless, factors such as MC-LR that inhibit microbial-mediated nitrification among AOB could potentially disrupt nitrogen cycling within the lake environment \( (Beman et al., 2011; Peng et al., 2017) \). However, the interactions between pre-existing microbiota and cyanotoxins within the sediment-interface layer have yet to be fully interpreted. Therefore, a detailed understanding is needed about the effect MCs could pose on aerobic microbial communities participating in critical ecological processes and residing at the interface.

The overall goal of this research was to evaluate the effect of selected concentrations of MCs on aerobic microbiota, potentially residing at the interface of sediments and the water column. We selected nitrification, aerobic \( NH_4^+ - N \) oxidation to nitrate, as the model aerobic process and an essential component of the overall nitrogen cycle. Early study has studied the treatment of a high density of cyanobacteria on AOBs \( (Peng et al., 2017) \). However, the impact of cyanotoxins on the nitrifying community alone was not adequately studied. The objectives of this study were a) to determine AOB community response from immediate exposure to microcystin by measuring and analyzing nitrification rates through \( NH_4^+ - N \) oxidation; b) measure the cyanotoxins’ residual effect on AOB with a stress study in the effort to facilitate our comprehension of the cyanotoxins’ fate within microbial communities after their initial introduction. Insight is provided on how cyanotoxins are altering the AOB nitrification and oxygen uptake rates, whether the toxin is inhibiting the \( amoA \) gene expressions or irreversibly damage the bacterial cells.

## Materials and methods

### 2.1. Nitration kinetic experiment under cyanotoxin toxicity

A mixed community of AOBs from an ongoing enrichment was used to conduct cyanotoxin toxicity experiments on biological ammonium oxidation to nitrate rates and \( amoA \) gene expressions. The details of the AOB enrichment reactor can be found in Kotay et al. (2013) and this AOB enriched reactor has been in operation consistently over the last 8 years \( (Gupta and Goel, 2019) \). Quantification of the mixed AOB community in the nitrification reactor suggests \( Nitrosomonas europaea \) as the dominant species \( (62 \pm 7\% \) of total community). To conduct toxicity experiments, biomass samples were taken from the ongoing enriched reactor and washed three times with deionized water for residue nutrient removal before using it for experiments. Washed biomass was purged through a 20-gauge needle to break the biomass pellet and disperse it. The total suspended solids (TSS), volatile suspended solids (VSS) and initial \( NH_4^+ - N \) and \( NO_2^- - N \) concentrations were measured immediately after dispersion. The bulk biomass was then spiked with 2–3 mg/L of \( NH_4^+ - N \), well mixed and divided into eight equal aliquots of 100 mL each in 125 mL acid-washed Wheaton® serum bottles. Of these eight serum bottles, four were spiked with a known concentration of MC-LR, and the rest were used as the control for four different time steps. This marked the beginning of toxicity experiments and initial \( NH_4^+ - N \) and \( NO_2^- - N \) were measured at zero time. Additionally, for this study, three concentrations of MC-LR \( (1, 5, \text{and } 10 \mu g/L) \) were used to run three different sets of experiments to reflect typical concentrations of MC-LR in surface waters during algal blooms. Based on the guidelines by World Health Organization, the “low risk” category for MC has been set at 10 \( \mu g/L \) for recreational water, and this category was commonly found for algal-bloom water bodies \( (Lindon and Heiskary, 2009; Kohlihepp, 2015) \). The experiment sets were first conducted with 10 \( \mu g/L \) MC-LR and subsequently continued at lower concentrations of 5 and 1 \( \mu g/L \). Serum bottles were sealed airtight using a 20 mm butyl rubber Teflon faced septa \( (Fischer, USA) \), and 20 mm aluminum crimp caps. They were aerated and mixed continuously by purging air through a 20-gauge needle to the liquid and with another unconnected needle for the air to exit the headspace of bottles. Samples were taken from the control and toxin spiked set at 0.5, 1, 2 and 3 h and, the contents of each bottle were tested for \( NH_4^+ - N \), \( NO_2^- - N \) and \( NO_3^- - N \) concentrations. \( NH_4^+ - N \) was measured using HACH Nitrogen-Ammonia Reagent Set TNT Kit \( (Hach, USA) \) and, \( NO_2^- - N \) and \( NO_3^- - N \) were measured on an Ion Chromatograph \( (Metrohm 883 Basic IC plus) \) for anion detection \( (EPAP method 300) \) \( (Pfaff, 1993) \), respectively. Triplicate samples \( (each \text{ with } 10 \text{ mL volume}) \) of mixed liquor from each bottle were also obtained at each time step to measure TSS and VSS following the USEPA method 1684. The rest of the sample volume remaining in the bottle was used for microbial analysis at each time step.

### 2.2. Stress study

Stress tests were conducted to evaluate whether MC-LR toxicity to AOBs is reversible or irreversible. Toxicity tests were conducted in a similar fashion, detailed in section 2.1. After 3 h of incubation with toxins, the biomass samples that were spiked with different concentrations of MC-LR were washed twice with DI water to remove any residual MC-LR in the bulk solution or toxins potentially stuck to the biomass. The washed biomass samples were spiked with 2–3 mg/L of \( NH_4^+ - N \) and the experiment was conducted with 20-gauge needle airtight using a 20 mm butyl rubber Teflon faced septa, following the USEPA method 1684.
continued further to monitor the $\text{NH}_4^+ - N$ oxidation in the absence of MC-LR. The analysis for $\text{NH}_4^+ - N, \text{NO}_2^- - N, \text{NO}_3^- - N, \text{TSS}, \text{VSS}$, and microbial activities were determined as detailed in section 2.1 of this manuscript earlier.

2.3. Cyanotoxin inhibition kinetics evaluation

MC-LR inhibition coefficient was estimated experimentally by exposing the nitrifying biomass to four different concentrations (0.25 $\mu$g/L, 0.5 $\mu$g/L, 0.75 $\mu$g/L, and 1 $\mu$g/L) of toxins in separate sets of experiments. A control experiment where the biomass was not subjected to any toxin was also run simultaneously. As the MC-LR spiked AOB feed solution was aerated for approximately 30 min, inhibition was measured as a reduction in the specific Oxygen Uptake Rate ($\text{sOUR}$) and described using a non-competitive inhibition model as shown by equation (1) (Chandran and Love, 2008).

The dissolved oxygen was measured using a HACH HQ40D Portable Multi Meter (Hach, USA).

$$\text{sOUR}_{\text{inh}} = \frac{\text{sOUR}_0}{1 + \frac{S_i}{K_i}}$$

(1)

$K_i$ is the inhibition coefficient (expressed as mg/L MC-LR), $\text{sOUR}_{\text{inh}}$ is the inhibited $\text{sOUR}$ (mg O$_2$/min), $\text{sOUR}_0$ is the $\text{sOUR}$ for the control solution without any MC-LR cyanotoxin (mg O$_2$/min), $S_i$ is the concentration of MC-LR (mg/L). $K_i$ was estimated using a linear regression model as follows:

$$\frac{1}{\text{sOUR}_{\text{inh}}} = \frac{1}{\text{sOUR}_0} + \frac{S_i}{\text{sOUR}_0 \times K_i}$$

(2)

2.4. Microbial analysis

2.4.1. DNA and mRNA extraction

Bacterial DNA and RNA were extracted from AOB biomass from both the blank and MC-LR spiked mixed liquors under different conditions. Biomass was concentrated by filtering through a 0.45 $\mu$m filter paper (Millipore, USA). DNA was extracted using a PowerSoil® DNA Isolation Kit (Qiagen, Germany). RNA was obtained using a PureLink® RNA mini kit (Life Technology, USA). Following RNA extraction, residual genomic DNA was removed from total RNA using an on-column PureLink DNase set (Life Technologies, USA). The concentrations and quality of DNA and RNA were checked using Nanodrop 2000c (ThermoFisher, USA) and stored at $-80 \degree C$. To be specific, samples with a 260/280 ratio of around 1.8 (pure DNA) and 2.0 (pure RNA) were selected for downstream analysis. After the extraction of RNA, reverse transcription was immediately conducted to convert 0.4 $\mu$g RNA to cDNA in each sample using the SuperScript® VILOTM cDNA synthesis kit (Life Technology, USA). They were further used as templates for gene expression analysis.

2.4.2. Real-time Polymerase Chain Reaction

Real-time Polymerase Chain Reaction (RT-PCR or qPCR) was conducted to quantify the functional ammonia-oxidizing gene, ammonium monooxygenase ($\text{amoA}$) of the AOB (Rotthauwe et al., 1997). The purpose was to normalize the ammonia-oxidizing rates or oxygen uptake rates based on functional gene copies, in addition to biomass (measured by VSS). The normalization based on functional gene copies has been applied in previous studies (Ma et al., 2019). The primers applied to target the $\text{amoA}$ gene were the amoA_1 F/amoA_2R listed in Table 1 (Rotthauwe et al., 1997). All reactions were carried out in a total volume of 20 $\mu$L containing 10 $\mu$L AB Power SYBR® Green Master Mix, 1 $\mu$L of each primer (10 $\mu$m), 5 $\mu$L of DNA template and 3 $\mu$L of nuclease-free water. Plasmids were used as a positive control and series diluted 10 times using ultrapure water to the concentrations of 10$^8$ copies/$5\mu$L to 10$^3$ copies/$5\mu$L. The amplification efficiency was tested to be higher than 80% during the entire process. Beforehand, plasmids were cloned from reactor biomass using TOPO® TA cloning kit/TOP10 (ThermoFisher, USA) and followed by E. coli incubation until the plasmids can be extracted using Zappy® Plasmid Miniprep Kit (Zymo Research, USA). qPCR was conducted using a QuantStudio 3 Real-Time PCR system (ThermoFisher, USA) in the process of 2 min at 50 $\degree C$, 5 min at 94 $\degree C$; then 40 cycles consisting of 60 s at 94 $\degree C$ (denaturation), 90 s at 60 $\degree C$ (annealing), 90 s at 72 $\degree C$ (elongation), and a final cycle consisting 10 min at 72 $\degree C$. All samples, including plasmids (positive controls), were conducted in triplicates.

2.4.3. Gene expression

Under cyanotoxin stress, the expressions of $\text{amoA}$ were also relatively quantified. The gene expression experiment was tested in a QuantStudio 3 Real-Time PCR system (ThermoFisher, USA) using the $\Delta \Delta CT$ method, developed by Schmittgen and Livak (2008). The equation was detailed below, and relative quantification ($RQ$) was calculated as $2^{-\Delta \Delta CT}$. The $RQ$ of reference sample was normalized to 1 based on this method.

$$\Delta \Delta CT = [(\text{CT}_{\text{target gene}}) - \text{CT}_{\text{internal control gene}})] \text{Sample A} - [(\text{CT}_{\text{target gene}}) - \text{CT}_{\text{internal control gene}})] \text{Reference Sample}$$

(3)

- $\text{CT}$ represents the number of cycles taken for the fluorescent signal of the reporter dye to cross an arbitrarily placed threshold.
- $\Delta \Delta CT$ represents the comparative $\text{CT}$ method. It is calculated based on the above equation to compare the expression level difference between control and treatment samples, as well as normalizing target genes based on the internal control genes.

In this study, the sample extracted at $t = 0$ in each batch study was used as the reference sample for other time steps (0.5 h, 1 h, 2 h, 4 h).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used in qPCR and gene expression analysis.</th>
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<tr>
<td>Target</td>
<td>Primers</td>
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<tr>
<td>$\text{amoA}$ gene</td>
<td>amoA_1 F</td>
</tr>
<tr>
<td>AOB 16S gene</td>
<td>amoA_2R</td>
</tr>
<tr>
<td>amoA_1 F</td>
<td>CTO 189F A/B</td>
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<tr>
<td>amoA_2R</td>
<td>CTO 189F C</td>
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<td>RT1r</td>
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2 h, and 3 h). Within each sample, the C\textsubscript{T} for the functional gene (amo\textsubscript{A} gene) was obtained and normalized by the 16S rRNA gene copy numbers of AOB (the internal control gene). The amo\textsubscript{A} gene and 16S rRNA gene of AOB in samples were targeted by primers amo\textsubscript{A}_1 F/amo\textsubscript{A}_2 R (Rothhauwe et al., 1997) and CTO 189f (A/B/C)/RT1r (Hermannsson and Lindgren, 2001), respectively. Primers applied are listed in Table 1. Two master mix solutions were created for each primer set, in which 10 \mu L AB Power SYBR® Green Master Mix, 1 \mu L of each primer (10 \mu M), 1 \mu L of DNA template and nuclease-free water were added to make a total volume of 20 \mu L. The qPCR process was modified to target both the amo\textsubscript{A} gene and the AOB 16S rRNA gene using the VeriFlex temperature control mode during the annealing process. It was followed by 2 min at 50 °C, 5 min at 94 °C, then 40 cycles consisting of 60 s at 94 °C (denaturation), 90 s at 56 °C for amo\textsubscript{A} gene and 60 °C for AOB 16S rRNA gene (annealing using VeriFlex mode), 90 s at 72 °C (elongation) and a final cycle consisting 10 min at 72 °C. The experiment was conducted in triplicates. The average, max, and min RQ values were plotted for each batch of samples in the results section.

2.4.4. Live and dead analysis
To evaluate the lethal effects of MC-LR, live and dead analysis was conducted to have a qualitative estimation of the membrane integrity at population level after the exposure to 1, 5, and 10 \mu g/L of MC-LR during a 3-h period. In this experiment, bacterial cells were stained with light-sensitive BacLight® dyes containing Propidium Iodide and SYTO 9 nucleic acid stain (ThermoFisher, USA), and were examined under an Olympus® BX51 light microscope (Olympus, USA). 10 \mu g/L of Propidium Iodide and 10 \mu L SYTO 9 master stocks were combined to create a working stock in the dark. Afterward, 2 \mu L of the working stock was mixed with 198 \mu L of Ultra-Pure Water to create the master mix. Next, a 0.22 \mu m polycarbonate filter (GVS Filter Technology, UK) with filtered AOB biomass on it was placed onto a glass slide and dabbed with Kim wipes to remove excess water. The filter was left in place for 3–5 min and later removed with a pair of tweezers. A plastic well was placed on the microscope slide to encompass the removed filter area, and 200 \mu L of the master mix was added inside the well. The mix was left on the slide in a dark room for 20 min. Later, the plastic well was removed with tweezers, and the area was cleaned with Mili-Q water. One drop of Dabco®-33-LV solution (Sigma-Aldrich, USA) was added to the area and covered with a micro cover glass, sealed and placed under the microscope with 100X magnification.

2.5. Statistical analysis
One-way ANOVA was applied to detect the changes in nutrient concentrations between blank and treatment groups for each set of kinetic and stress studies using Rstudio software v3.5.1 (Team, 2013). The variations of MC-LR concentrations on amo\textsubscript{A} gene expressions at the kinetic and stress status were also compared using one-way ANOVA. Significance was determined at an alpha level of 0.05 (p<0.05). SigmaPlot v 10.0 from Systat Software, Inc., San Jose California USA, was applied for plotting the figures.

3. Results and discussion
3.1. Effect of MC-LR on nitrification
Enriched biomass comprised mostly of AOBs was subjected to different concentrations of MC-LR and, decreases in NH\textsubscript{4}+ – N and increases in NO\textsubscript{2}− – N concentrations were recorded as a function of time. Since the biomass was obtained from an enriched ammonia-oxidizing bioreactor, the presence of nitrite oxidizers was not expected, and it was confirmed through periodic nitrate measurements. NO\textsubscript{2}− – N concentrations in serum bottles were found to be below detection limits. The first set of experiments was conducted with 10 \mu g/L MC-LR to evaluate the effect of the toxin on nitrifying biomass. The results are plotted in Fig. 1 in different panels for NH\textsubscript{4}+ – N increase (panel A) and NO\textsubscript{2}− – N decrease (panel D) for 10 \mu g/L MC-LR concentration. As evident from panel A in Fig. 1, absolutely no oxidation of the NH\textsubscript{4}+ – N was recorded at the 10 \mu g/L MC-LR concentration. On the other hand, noticeable nitrification activities were recorded with a consistent decrease in NH\textsubscript{4}+ – N with a corresponding increase in NO\textsubscript{2}− – N concentrations in the control experiment (panel D in Fig. 1). Significant differences were found among nutrient concentration changes between controls and 10 \mu g/L MC-LR spiked batch tests (One-way ANOVA, p < 0.05). With the observation that 10 \mu g/L toxin concentration caused complete inhibition of nitrification, subsequent batch tests were conducted with lower MC-LR concentrations of 5 \mu g/L and 1 \mu g/L. The results of these experiments for NH\textsubscript{4}+ – N concentration are plotted in panels B and C in Fig. 1. Surprisingly, the mixed community of AOBs did not exhibit any NH\textsubscript{4}+ – N oxidation at 5 and 1 \mu g/L MC-LR concentrations, demonstrating severe inhibition of nitrification at even 1 \mu g/L. On the other hand, the NH\textsubscript{4}+ – N oxidation in corresponding control batch tests shown in panels E and F of Fig. 1 is evident and convincing with corresponding increases in NO\textsubscript{2}− – N concentrations. The live and dead analysis further depicted the deadly effects of MC-LR at 1, 5 and 10 \mu g/L concentrations. However, it is also evident from Fig. S1 that not all bacteria are killed even at 10 \mu g/L (Fig. S1). In most algal bloom conditions, MC concentrations are between 1 and 10 \mu g/L in surface waters (Song et al., 2007; Liu et al., 2011; Preece et al., 2017; Turner et al., 2018). While the World Health Organization recommends limiting microcystin concentration to 10 \mu g/L in water for recreation purposes (Bartram and Chorus, 1999), the effects of MC-LR on AOB are visible at significantly lower concentrations in this study.

It is observed that the direct exposure of the AOB population to MC-LR at 1 \mu g/L concentration has similar inhibition effects as 10 \mu g/L. Additionally, in all treatment spiked with MC-LR, there was, in fact, a slight increase in NH\textsubscript{4}+ – N concentrations. This was surprising as well as un-explanatory at this point. Microcystins could introduce variability to nitrogen transformations occurring within freshwater ecosystems, and N removal rates have been more unpredictable in bloom-dominated ecosystems (Peng et al., 2017). One possible reason could be the occurrence of dissimilatory reduction of NO\textsubscript{2}− – N to NH\textsubscript{4}+ – N (Bu et al., 2017). As the reduction of functional transcripts under the toxicity could be one of the main factors causing inhibitory nitration rates (Kapoor et al., 2016b; Kim et al., 2016), some other genes may be simultaneously activated during the nitrification inhibition as bacteria tried to acquire nutrients from the surrounding environment. The corresponding decreases in NO\textsubscript{2}− – N concentrations were also recorded in toxin spiked samples with respect to NO\textsubscript{2}− – N concentrations present at the beginning of experiments. On the other hand, a consistent decrease in NH\textsubscript{4}+ – N with a corresponding increase in NO\textsubscript{2}− – N were recorded in all un-spiked (control) samples. However, the DNRA theory remains speculative at this time, and perhaps similar experiments in the future would benefit from the simultaneous measurement of DNRA activity.

In the control bottles which were not spiked with MC-LR, the NH\textsubscript{4}+ – N oxidation rates were 0.68 ± 0.16, 0.17 ± 0.17, 0.54 ± 0.19 and 0.57 ± 0.11 mg/L in three different control bottles tested between time periods of 0–0.5, 0.5–1, 1–2 and 2–3 h, respectively.
The corresponding increases in $\text{NO}_2^- - N$ concentrations were $0.35 \pm 0.37$, $0.44 \pm 0.13$, $0.61 \pm 0.18$ and $0.33 \pm 0.37$ mg/L, respectively. With normalization, the 3-h nitritation rates were measured as $0.0035 \pm 0.0009$ gNgVSS$^{-1}$hour$^{-1}$ and $7.44 \pm 2.38E-07$ mgL$^{-1}$copy$^{-1}$hour$^{-1}$ for the control group. Since the nitrifying biomass was obtained from an ongoing reactor primarily oxidizing $\text{NH}_4^+ - N$ to $\text{NO}_2^- - N$ (e.g., nitritation), no significant change of $\text{NO}_3^- - N$ was measured in the blank or sample bottles. This established the fact that nitritation achieved exclusively from AOB activity. Above all, there were no significant nitritation activities detected for the toxin-spiked group, demonstrating that MC-LR was severely toxic/inhibitory to nitrifiers even at low concentrations.

While other researches have corresponded the low DO concentrations with inhibited nitritation rates and high ammonium flux in sediment (McCarthy et al., 2008; Abell et al., 2011), the $\text{NH}_4^+ - N$ oxidation in our study was connected with a relatively high DO concentration throughout the experiment. Therefore, in this research, limited oxygen levels may not likely be a factor impeding AOB activity, as the $\text{NH}_4^+ - N$ concentrations in control batch tests decreased by 45–60% in comparison to spiked sample batch tests (discussed later).

### 3.2. Nitritation recovery potential after MC-LR spiking

The direct spiking experiments clearly indicated that nitration was severely inhibited with MC-LR concentration as low as 1 mg/L. From batch experiments, it was not clear whether the toxin effects on nitrifying bacteria were lethal/irreversible or reversible. This question is important as it will inform whether the nitration activity would restore to its normal conditions after algal blooms have occurred and cyanotoxins have degraded. To evaluate whether the effect was inhibitory, e.g., reversible, batch tests were further conducted with nitrifying biomass that was first subjected to MC-LR exposure and then washed twice to get rid of any residual toxin.

The $\text{NH}_4^+ - N$ and $\text{NO}_2^- - N$ concentrations with different exposed MC-LR concentrations are plotted in Fig. 2. Similar to the nitritation kinetics experiment, all three samples in the stress study initially experienced an inhibited nitritation rate, which demonstrates the lasting effect of the MC-LR (Fig. 2). As expected, $\text{NH}_4^+ - N$ decreased and $\text{NO}_2^- - N$ increased in control batch tests. Surprisingly, a trend of $\text{NH}_4^+ - N$ decrease and $\text{NO}_2^- - N$ increase was also observed for groups in which the nitrifying biomass was previously exposed to 1, 5 and 10 mg/L MC-LR but reused after thoroughly washing with DI water (Fig. 2). It appears that the rate of $\text{NH}_4^+ - N$ oxidation in sample bottles, in which the biomass was originally exposed to the toxin, was slower than the rates in their corresponding control batch tests (One-way ANOVA, $p < 0.05$). These results demonstrate that cyanotoxin toxicity is reversible to some degree. Higher nitritation rates (changes in $\text{NH}_4^+ - N$ and $\text{NO}_2^- - N$ concentrations) were detected at 1 and 5 mg/L but not at 10 mg/L. Especially in bottles where biomass was spiked with MC-LR and washed, the $\text{NH}_4^+ - N$ decreases were 0.80, 1.00, and 0.53 mg/L at 1, 5, and 10 mg/L exposure between periods of 0–3 h, respectively. With normalization, the 3-h nitritation rates were estimated to be $0.0015$, $0.0019$ and $0.0010$ gNgVSS$^{-1}$hour$^{-1}$ and $1.71E-06$, $2.47E-06$.

![Fig. 1. (A–C) NH$_4^-$ N concentrations versus time at 1, 5, 10 µg/L MC-LR respectively and (D–F) NO$_2^-$ N concentrations versus time at 1, 5, 10 µg/L MC-LR in nitrification kinetics study. Each data point is based on triplicate measurements.](image-url)
and 3.82E-07 mgL⁻¹ copy⁻¹ hour⁻¹ for the group exposed to 1, 5 and 10 μg/L, respectively. These results demonstrate that the nitrification inhibition by the model cyanotoxin is reversible after contacting with MC-LR for 3 h at some optimum MC-LR concentrations. AOBs were speculated to recuperate from the initial MC-LR spiking at lower concentrations, which was also proved by the gene expression analysis for an increased nitrification potential (relatively higher gene expression) after exposure to 1 and 5 μg/L MC-LR (Fig. 4 D, E). The lower concentration (1 μg/L and 5 μg/L) may show better recovered nitrifying activities after exposure. This indicates that the presence of toxins during heavy harmful algal blooms (HABs) will affect ecosystem functioning by interfering with important processes such as nitrogen cycling. However, this negative effect is reversible to some extent meaning that the ecosystem functioning can be restored once the bloom is disappeared and toxins are degraded. Previous studies also demonstrate that the ecosystem function can usually be restored by the degradation of cyanotoxins after blooms have disappeared (Koreivienė et al., 2014; Rastogi et al., 2015). AOB recovery may be a direct response to ambient cyanotoxin levels, as well as the level of NH₄⁺ – N present in the solution; these conditions indirectly impact the microbial physiology, functions, and community composition (Giaramida et al., 2013).

Similar to our study, the resilience of AOBs was also indicated by previous studies, albeit in the soil environment. When evaluated through a 90-day trial, the nitrification potential of soils irrigated with 50 and 100 μg/L of MC-LR experienced similar N transformations trends compared to the control (no toxin applied) while soils containing relatively lower MC-LR (5–20 μg/L) concentrations experienced higher nitrification potentials (Corbel et al., 2015a). Just as amoA gene expression ultimately increased in the stress nitritation study in this study, a rise in the relative abundance of the amoA gene was also observed when the AOB containing soil was irrigated with low concentrations of MC-LR for a 14-day period in the previous study (Corbel et al., 2015b). Moreover, after a moderate density of cyanobacteria (10⁶ cells/L) dominated by Microcystis was introduced to an AOB community in a laboratory microcosm, the amoA gene expression values were determined to be greatest relative to the high-density cyanobacteria (10⁸ cells/L) and untreated microcosm (Peng et al., 2017). The resilience of AOB is similarly depicted through nitrification recovery after exposure to metal (Ruyters et al., 2013). Another study evaluating bacterial resilience exposed microbial communities to heat stress over an extended period of time also demonstrated the AOB’s increased nitrification potential rates (Epelde et al., 2014). All of the above analyses suggested that microcystin exposure may induce greater amoA gene expression in AOBs even if their nitrification potential is low.

In contrast, the addition of relatively high concentrations of MC-LR (10 μg/L) caused crushing effects on the AOB’s cellular membrane and reduced the amoA gene expression levels due to the direct killing effect (Fig. 4 F and S1). Previous studies also reported that the harmful effect is visible when exposed to relatively higher concentrations of MC-LR at a regular frequency. For example, the significant inhibitory effects were previously observed with the treatment of a high density of cyanobacteria on AOB (Peng et al., 2017). This also suggests cyanotoxin’s immediate effect on AOB cultures and long-term adverse effects at a high density of cyanobacteria. While AOB is recognized to recover at lower concentrations of MC-LR exposr and as observed in this study as well, other studies analyzing the effects of MC contaminated irrigation on overall plant morphology demonstrated a decreased quality and
yield of crops with regular expoer to 10 μg/L of MC-LR or higher (Lee et al., 2017; Zhu et al., 2018). The cyanotoxin may reduce the harvest by affecting the N cycle of the soil bacterial communities. It was also found that high concentrations of MC-LR (>10 μg/L) significantly decreased soil potential nitrification rate, together with the decline of amoA gene abundance and these past results are very much in agreement with our study where we also used 10 μg/L MC-LR to study inhibitory effects on AOBs.

3.3. Determination of toxin inhibition coefficient

MC-LR toxicity to nitrifying activity was also evaluated by conducting oxygen uptake tests with MC-LR spiked in batch tests. The results of the oxygen uptake rate experiments complement the inhibited NH₄⁺ – N oxidation in the nitritation batch experiments. While the final DO concentration in the control experiment where the AOB biomass was not spiked with MC-LR decreased to 55% of the initial dissolved oxygen concentration due to active nitrification, the DO concentration mostly remained unchanged in 1 μg/L MC-LR spiked tests throughout the 30-min experimental period indicating inhibited AOB activity. Furthermore, amoA was down-regulated at 5 and 10 μg/L MC-LR concentrations correlating with low or no ammonium oxidation at these spiked concentrations (Fig. 4 B, C).

For five sets of experiments (0, 0.25, 0.5, 0.75, and 1 μg/L MC-LR), VSS and amoA gene copy numbers were in the range of 60–373 mg VSS/L and 1.60E–06 to 3.08E+06 gene copies per ml of mixed liquor respectively. Oxygen uptake rates are normalized based on amoA gene copy numbers for accurate estimation. As the concentration of MC-LR increased, the oxygen uptake rates decreased simultaneously. Normalized specific oxygen uptake rates (sOUR) were −4.45E-08 mg O₂ min⁻¹ copy⁻¹ under non-spiked conditions (e.g. MC-LR = 0 μg/L) and decreased to −1.33E-11 mg O₂ min⁻¹ copy⁻¹ as the MC-LR dosing increased to 1 μg/L. There was no significant decrease in SOUR once the spiked MC-LR concentration exceeded 1 μg/L. The inhibition coefficient was calculated from the normalized DO concentrations and was determined to be 0.083 μg/L MC-LR based on the linear regression with a coefficient of regression to be 0.87 (Fig. 3). Although complete inhibition was observed at 1 μg/L, inhibition effect occurred at even lower MC-LR concentrations. It also indicated MC-LR’s severe inhibitory effects compared to heavy metals, such as Cd (inhibition coefficient = 0.76 mg/L), Ni (inhibition coefficient = 1.9 mg/L), Zn (inhibition coefficient = 17.29 mg/L) or cyanide (inhibition coefficient = 0.12 mg/L) towards ammonium inhibition (Kapoor et al., 2016a, 2016b).

3.4. Ammonium monoxygenase (amoA) gene expressions

In all batch studies, the relative quantification of the AOB’s amoA gene was obtained through the ΔΔ Ct method for spiked samples and controls at different sampling time periods. Basically, the gene expressions (control and treatment groups) at different sampling points is relatively quantified based on time 0 (RQ = 1 for time 0), so that control and treatment groups are comparable at each sampling point. The higher RQ demonstrates relatively higher gene expression activities. The relative quantification (RQ) of amoA gene for AOBs in direct contact nitrification study is shown in Fig. 4 A-C. In general, comparable to the control, MC-LR spiked samples experienced a lower average RQ of the amoA gene at all measured time for samples containing 5 and 10 μg/L of MC-LR (Fig. 4 B, C). These results corresponded with the suppression of NH₄⁺ – N oxidation measured (Fig. 1B and C). The RQ values for the amoA gene are more variable in the samples containing 1 μg/L than the other samples having different concentrations (Fig. 4 A). Especially, the solution containing 1 μg/L of MC-LR observed relatively higher average RQ values compared to control for the first 2 h, although the oxidation rates were suppressed (Fig. 1A) and do not correlate with the gene expression here. It is predicted that the AOBs may still try to recover its function under 1 μg/L MC-LR concentration by enhancing gene expressions. Except for a few outliers, the highest RQ was observed at time 0.5 or 1 h and decreased afterward in all batches.

During the stress study, the RQ values of the amoA gene are also highlighted for controls/samples, and differences in expression levels are depicted (Fig. 4D–F). The stressed nitritation study’s gene expression procedure indicated greater RQ values for the samples exposed to 1 and 5 μg/L of MC-LR compared to their respective controls, as bacteria work on recovering their functions (Fig. 4 D, E). However, the sample exposed to 10 μg/L yielded a relatively low gene quantification compared to the control and other spiked MC-LR concentrations in the stress study (One-way ANOVA, p < 0.05) (Fig. 4 F). It is surprising to see that the gene expression was even enhanced after MC-LR exposure and recovery at lower concentrations (1 and 5 μg/L). It may indicate that the stress response was activated under adverse conditions, and some gene expressions were upregulated afterward (Qin et al., 2018). At the higher concentration (10 μg/L), lethal or irreversible effects may have occurred and made the AOB activity irreversible. These results well corresponded with the relatively higher recovered nitrification kinetics detected after exposure to 1 and 5 μg/L of MC-LR when compared with 10 μg/L.

3.5. Live and dead analysis

Live and Dead Analysis was conducted to detect the lethal effects caused by different concentrations of MC-LR and have a qualitative study of toxin effects on the bacterial population. Fig. S1 provided examples of live and dead analysis conducted in the study; cells staining with green and red were considered to be live and dead, respectively. The vast majority of the bacterial cells exposed to only 1 μg/L of MC-LR seem viable. However, relatively larger percentages of the AOB cultures exposed to 5 and 10 μg/L of MC-LR experienced cell death or destroyed cellular membranes, as indicated by the red or orange illumination. It is noticeable that lethal or membrane destroy may occur under all applied concentrations. However, even the set with 10 μg/L contact for 3 h does not seem to kill all bacteria, instead, to inhibit their functions. The
3.6. Implications of MCs effect of harmful algal blooms

MC-LR’s immediate impact on AOB activity may also relate to factors that promote *Microcystis* dominance and toxin production in freshwater environments (Reeders et al., 1998; Gilbert (2017); Hampel et al., 2018). MC-LR’s influence on AOB in lake sediments may vary depending on situ environmental conditions. To specify, DO, pH, ammonium, and nitrate concentrations are highly correlated with *Microcystis* abundance and toxin-producing capacity, further altering the given ecosystem’s microbial diversity (Raven et al., 1992; Wilhelmi et al., 2011; Giamarida et al., 2013; Su et al., 2017; Dalu and Wasserman, 2018; Li et al., 2019). Predominant microcystin-producing cyanobacteria, such as *Microcystis* sp., are incapable of fixing N2 and prefer to feed on other forms of nitrogen (Monchamp et al., 2014; Shan et al., 2019). Still, most cyanobacteria (including *Microcystis*) rely on the regeneration of ammonium to help preserve their own bacterial-bloom conditions (Lee and Cho, 2006; Hampel et al., 2018), suggesting toxin’s inhibition of nitrification can serve as a mechanism to compete with other ammonia-oxidizers. While cyanobacteria have the capacity to uptake varying forms of nutrients at lower concentrations, they can also survive in more extreme environmental conditions compared to their microbial counterparts (Gilbert et al., 2016). In contrast to cyanobacteria, other bacterial species are usually more affected by the availability and changing of N forms. Although it is established that nitrogen transformations occur rapidly near the sediment-water interface, $NH_4^+$ – N and $NO_2^-$ – N concentrations throughout the entire ecosystem are highly influenced by cyanobacterial blooms (Gardner and McCarthy, 2009). Nevertheless, AOB growth is highly dependent on $NH_4^+$ – N concentrations, signifying nutrient concentrations, microbial composition, and toxin levels’ considerable influence on them (Verhamme et al., 2011). Regardless, factors such as MC-LR that inhibit microbial-mediated nitrification among AOB could potentially disrupt nitrogen cycling within the environment (Beman et al., 2011).

The stress study may be well applied to predict the effects on nitrifying activities after the ambient cyanotoxin being degraded. Cyanobacteria bloom may have significant effects on the ecosystem’s nitrogen cycle through the effects on cell physiology and gene expression activities (Gardner and McCarthy, 2009; Giamarida et al., 2013). Results from the nitritation kinetics, stress study, and gene expression analysis further confirmed that the amoA gene could exhibit visible differences in expression rates between controls and samples within a laboratory setting. Although experimental results mostly correlate the abundance of the amoA gene with the level of nitrification and demonstrate that the quantity of amoA gene expressed may possess a measurable role in nutrient transmission within natural environments, the functional gene expression abundance does not always directly reflect processes in lake ecosystems (Hampel et al., 2018). Exceptions could be when bacteria upregulated the functional gene expression, while the function was still inhibited by toxicity. It could be similar to what we found when the mixed liquor directly contacted with 1 μg/L MC-LR. Confounding factors within the laboratory setting may have also deviated from typical nitrifying community conditions found in lake sediments due to different AOB community composition (Wu et al., 2013).
It further established that the degradation or remediation of MCs would benefit and boost the bacterial community restoration based on our study. While there is limited research on the fate of cyanotoxins, microcystin biodegradation in the water column, and the sediment-interface can be considered a significant removal pathway for the toxin (Chen et al., 2008). Particular species of bacteria (Sphingomonas sp., Arthrobacter sp., Brevibacterium sp. and Rhodococcus sp.) occupying the same sediment as Microcystis can biodegrade these toxins (Chen et al., 2008; Ho et al., 2010; Garamida et al., 2013). Yet, cyanotoxin exposure to other bacterial communities is mostly overlooked. As MC-producing cyanobacteria proliferate, their influence on nutrient transmissions may produce more predominant changes to freshwater ecology, comparable to what other species are doing to mitigate the toxin problem (Song et al., 2007; Hämple et al., 2018).

While cyanotoxin concentrations were not measured over the course of the experimental procedures, MC- LR is determined to persist in sediment several weeks after the decomposition of the cyanobacteria (Lahti et al., 1997). It is acknowledged that biodegradation helps minimize MC accumulation; however, MC concentrations are still known to exceed risk thresholds as they bioaccumulate in sediment, soils, and crops (Corbel et al., 2015a; Lvizou et al., 2017). A study measuring the accumulation of microcystin in the terrestrial soils determined that soils exposed to 1, 10, 100, and 1000 μg/L of MCs from lake water accumulated equivalent MC concentrations in dry soil samples (Cao et al., 2017). The dominance of MC-producing cyanobacteria is not only affecting nutrient cycling but a number of other lake ecosystem characteristics such as water temperature, light availability, and food web structure (Reeders et al., 1998). These factors allow for more significant quantities of cyanobacteria to continually reintroduce toxics to the sediment-interface. Therefore, measures are usually needed to combat the dense blooms of cyanobacteria and achieve the restoration of lakes (Stroom and Kardinaal, 2016).

4. Conclusions

The experiment detected the influence of MC-LR on partial nitrifying communities at 1, 5, and 10 μg/L. Overall, all concentrations of MC-LR spiking were determined to produce a discernible impact on the AOB nitratation rate and amoA gene expression. The harmful effect of MC-LR was even seen as low as 0.25 μg/L as it started to hinder oxygen uptake by AOB. It further provides an insight into how cyanobacterial harmful blooms interfere with the nitrogen cycle in aquatic ecosystems because aerobic nitrification is an important component of the overall nitrogen cycle. The cyanotoxin’s direct and residual influence on the nitritation activity and amoA gene is suggestive of how microcystin interacts with nitrifiers in freshwater ecosystems with and after cyanobacterid degradation. The rate recovery experiments suggest that nitratation may take quite a while to recover to its full strength after toxic blooms have occurred.

Author statement

The first author HL contributed to lab scale experiments and data analysis. Undergraduate student MH helped with batch tests and gene expressions analysis. AP contributed in writing and reviewing the manuscript. VC contributed in oxygen uptake experiments. MB contributed in writing and reviewing the first draft of this manuscript. The corresponding author RG supervised the whole study along with data analysis and writing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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