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N-Nitrosamine Formation upon Chloramination and Chlorination of Cyanobacterial Strains

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Abstract

Blooms of blue-green algae (cyanobacteria) cause many water quality management issues. Water utilities oftentimes need to treat source waters laden with various cyanobacterial populations, which may contribute to the formation of harmful disinfection byproducts (DBPs), such as *N*-nitrosamines, in downstream drinking water treatment. This preliminary study investigated the formation of total and specific *N*-nitrosamines upon chloramination and chlorination of laboratory cultures of cyanobacteria. Two *Microcystis aeruginosa* strains (one confirmed microcystin producer and one non-toxin producing strain) were cultivated and harvested at different growth phases. Chloramination of cyanobacterial samples revealed that the toxin producing strain produced more *N*-nitrosamine precursors per dry cell weight than the non-toxin producing strain, and that the production of *N*-nitrosamine precursors from cyanobacteria was growth phase-dependent. Filtration of cyanobacterial samples through 0.7-µm glass fiber filters removed over 90% of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) precursors, which predominantly existed in the dissolved and/or colloidal phases. In contrast, filtration only removed around 50% of total *N*-nitrosamine (TONO) precursors, highlighting the importance of unknown precursors associated with cyanobacterial cells. Chlorination of cyanobacterial samples at varying chlorine doses led to enhanced TONO formation at and beyond the breakpoint, although the toxin producing strain released a significant amount of ammonium. The addition of ammonium chloride to non-toxin producing, low-ammonium cyanobacterial samples prior to chlorination did not result in statistically significant increases in TONO formation, suggesting that organic chloramines may play a role in the absence of *in situ* formation of inorganic chloramines during chlorination. Preliminary characterization of cyanobacterial samples using fluorescence spectroscopy and high resolution mass spectrometry revealed differences in optical and molecular properties of cyanobacterial organic matter derived at exponential and stationary growth phases.

Three Summary Points of Interest

- **The toxin producing *Microcystis aeruginosa* strain produced more *N*-nitrosamine precursors per dry cell weight than the non-toxin producing strain;**
- ***N*-nitrosamine precursors originated from both particulate and dissolved and/or colloidal fractions of cyanobacterial organic matter;**
- **In situ formation of organic chloramines likely played a role in *N*-nitrosamine formation upon chlorination of cyanobacterial cultures.**

Keywords
***N*-Nitrosamines; Precursors; Cyanobacteria; Chloramination; Chlorination**

Introduction

Freshwater harmful algal blooms (HABs) represent one of the greatest water quality threats to public health and aquatic ecosystems. Over the past few years, the frequency, magnitude, and duration of HAB events have increased in U.S. inland freshwater systems. For example, more than 200 HAB events were reported for various ponds, reservoirs, and lakes across New York in 2017, and over 100 beaches were closed for at least part of the summer. HABs occur when colonies of blue-green algae, or cyanobacteria, develop to excessive levels while releasing toxins that have been implicated in human and animal illness or death.

HABs pose significant threats to drinking water safety because cyanobacteria not only release cyanotoxins (e.g., microcystins)[5–7] of immediate risks but also react with chemical disinfectants applied during drinking water treatment to form carcinogenic disinfection byproducts (DBPs) of greater long-term public health concern. Previous research has shown that cyanobacteria-derived DBP precursor materials exhibit different reactivity towards disinfectants (e.g., free chlorine and chloramines) than those derived from other natural or anthropogenic sources[8–10] and that cyanobacteria-derived precursors contribute to the formation of both regulated (e.g., trihalomethanes and haloacetic acids) and non-regulated DBPs, including *N*-nitrosamines. Nevertheless, comparatively limited information exists regarding the identities of cyanobacteria-derived *N*-nitrosamine precursors and the specific formation pathways of *N*-nitrosamines.

N-Nitrosamines are a group of nitrogenous organic contaminants present in wastewater or formed during drinking water disinfection[12]. *N*-Nitrosodimethylamine (NDMA) is by far the most studied *N*-nitrosamine species in the field of environmental engineering because of its known carcinogenicity and frequent detection in chlorinated and chloraminated drinking water and wastewater[13,14]. Besides NDMA, a few other *N*-nitrosamines species have been routinely targeted in past studies, including *N*-nitrosomorpholine (NMOR), *N*-nitrosodipropylamine (NDPA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosodibutylamine (NDBA), and *N*-nitrosodiethylamine (NDEA). However, NDMA and the few other *N*-nitrosamines of current interest only constitute a fraction of the larger pool of total *N*-nitrosamines (TONO) in source waters and wastewater, while the rest of *N*-nitrosamines in the TONO pool remains largely unidentified[15].

In practice, water utilities oftentimes need to treat source waters containing numerous algal species with constantly changing environmental factors that also impact algal growth[24,25,27]. Yet, previous studies have largely ignored the potential effects of growth phase and toxin-producing ability of cyanobacteria on DBP formation. For instance, most studies extracted algal organic matter from single cyanobacterial strains at early exponential growth phase in light of the easiness of cultivation[21–23]. However, the metabolism of algae varies at different growth phases and among different strains, leading to the production of distinct profiles of primary and secondary metabolites that may in turn serve as DBP precursors during disinfection[6,10,24–26].

The overall goal of this 1-year study is to characterize the formation patterns of *N*-nitrosamines from two cyanobacterial strains under controlled chloramination and chlorination conditions. Our hypothesis is that the composition and reactivity of *N*-nitrosamine precursors vary with the growth phase and toxin-producing ability of cyanobacteria.

Results & Discussion

Laboratory cultivation of model cyanobacterial strains

Two cultures of *Microcystis aeruginosa*, LB 2385 and LB 2386, were purchased from the Culture Collection of Algae at the University of Texas at Austin. LB 2385 is a confirmed microcystin producer[28], while LB 2386 is a non-toxin-producing strain. Cyanobacterial strains were cultured in a temperature and light controlled environmental chamber. The chamber temperature was maintained at 30 °C. The LED light setting was programmed to provide 12 hours of light exposure followed by 12 hours of darkness. Cyanobacterial growth was monitored by periodically measuring the absorbance of aliquots of cell suspensions at 600 nm using a UV-visible spectrophotometer. The measured optical density (OD) was correlated with the cell dry weight (cdw), yielding a cdw-to-OD conversion factor of 0.278 for LB 2385 and 0.272 for LB 2386, respectively. The concentration of ammonium in cyanobacterial samples was measured by the HACH salicylate method at

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the end of cultivation. The concentration of ammonium was 5.6 mg/L for LB 2385 and 0.02 mg/L for LB 2386, respectively. The higher ammonium level measured in LB 2385 likely resulted from the decomposition of algal organic matter[29].

Figure 1 shows the growth curves of two different batches of cyanobacterial strains, which were cultivated for 60 days (Batch 1) and 21 days (Batch 2), respectively, to allow for comparison of the release of *N*-nitrosamine precursors at different growth phases. The growth rate during the exponential growth phase was 0.384 mg/mL-day for LB 2385 and 0.393 mg/mL-day for LB 2386, respectively, for the 60-day cultivation. Similarly, the growth rate was 0.416 mg/mL-day for LB 2385 and 0.466 mg/mL-day for LB 2386, respectively, for the 21-day cultivation.

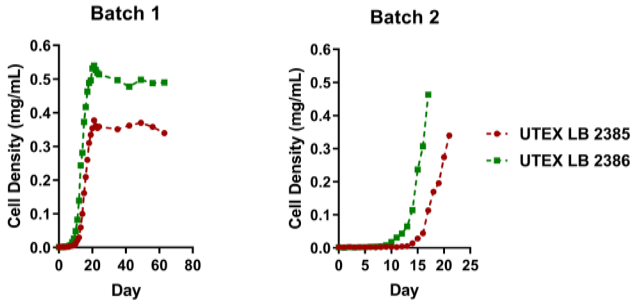

Figure 1. Growth curves of Microcystis aeruginosa strains.

N-Nitrosamine formation patterns

Cyanobacterial samples were subjected to standardized chloramination and chlorination tests to evaluate the formation patterns of *N*-nitrosamines. For chloramination, preformed monochloramine (NH_2Cl) at the concentration of 140 mg/L (as Cl_2) was applied following the Formation Potential test protocol described previously[30]. The high NH_2Cl concentration was chosen to maximize the conversion of all potential cyanobacteria-derived precursors into *N*-nitrosamines such that the *N*-nitrosamine formation potential could be compared across different experimental conditions. Preformed NH_2Cl solution was spiked into 100 mL of agitated cyanobacterial samples, which were then held in the dark at 20 °C for a reaction time of 7 days. For chlorination, free chlorine (Cl_2) solution was spiked into 100 mL of agitated cyanobacterial samples to achieve a predetermined initial chlorine concentration. The Cl_2 residual was measured after 24 hours using the DPD method. For both chloramination and chlorination experiments, samples were buffered at pH 7.0 with 10 mM phosphate buffer.

Figure 2 illustrates the level of *N*-nitrosamines measured in different chloraminated samples. Chloramination of LB 2385 and LB 2386 was performed with unfiltered ("UF", group A in Figure 2) and filtered ("F"; group B in Figure 2) samples taken during the exponential growth phase as well as unfiltered samples taken during the stationary growth phase ("aged"; group C in Figure 2). The measured *N*-nitrosamine concentration was converted to NDMA equivalents and further normalized by the cell density (i.e., expressed in the unit of nmol as NDMA/g cell) to facilitate comparisons among different samples. LB 2385 produced TONO (35 nmol as NDMA/g cell) even in the absence of NH_2Cl ("Raw"; group D in Figure 2), while LB 2386 did not form a measurable amount of TONO, indicating that certain primary or secondary metabolites of LB 2385 likely constituted unknown *N*-nitroso compounds. Comparing *N*-nitrosamine concentrations within each sub-group also revealed that LB 2385 generally produced a higher level of *N*-nitrosamine precursors. However, it remains unclear to what extent the production of these precursors is regulated by genetic and metabolic diversity of cyanobacteria and whether the *mcy* gene cluster (which produces microcystins) plays a role.

Filtration of cyanobacterial samples through 0.7-µm glass microfiber filters reduced the level of TONO precursors by 46% for LB 2385 and 58% for LB 2386, respectively. On the other hand, filtration reduced the level of NDMA precursors by up to 95% for LB 2385 and 92% for LB 2386, respectively. Likewise, filtration reduced the level of NPYR precursors by 90% for LB 2385 and 97% for LB 2386, respectively. Given that the 0.7-µm filters have a pore size smaller than a cyanobacterial cell (3.0-5.0 µm)[31], these results suggest that NDMA precursors were mainly associated with cyanobacterial cells, presumably intracellular organic materials, concurring with observations from a previous study[21], while TONO precursors were derived from both intracellular and extracellular materials.

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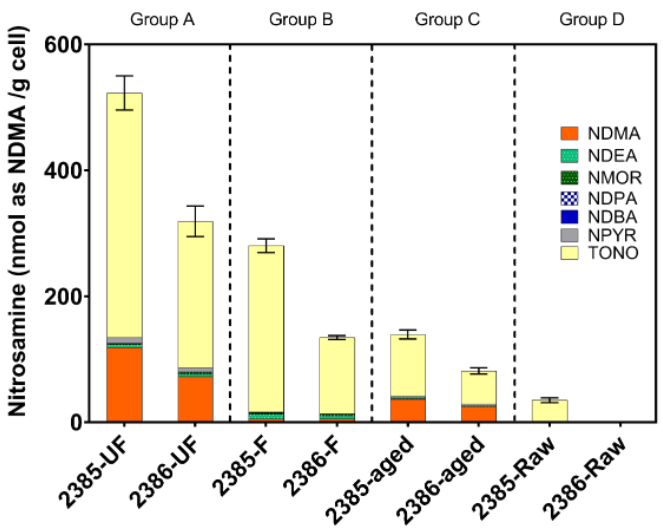

Figure 2. N-Nitrosamine concentrations in chloraminated samples.

The levels of TONO and NDMA precursors in "aged" cyanobacterial samples (i.e., cultivated for two months; group C in Figure 2) were ~75% and ~67% lower than those in fresher cyanobacterial samples (i.e., cultivated for less than three weeks; group A in Figure 2). Since cyanobacterial cells in group C exhibited a slightly lower growth rate than those in group A, these results point to the potential impacts of cyanobacterial cell age on the production of *N*-nitrosamine precursors.

Figure 3 shows the level of *N*-nitrosamines measured in a series of chlorinated samples. Chlorination of LB 2385 was performed at various Cl_2 doses to simulate different chlorine to ammonium-N ratios around the breakpoint ($[Cl_2]$:$[NH_4\text{-N}]$ ratio of ~7.6)[32,33]. No NDMA precursors were detected at all Cl_2 doses. Prior research suggests that NDMA precursors are most likely derived from intracellular organic materials. The fact that NDMA was not detectable suggests that the applied Cl_2 doses were not sufficiently high to lyse cells and release intracellular NDMA precursors. On the other hand, the concentration of TONO ranged from 20-200 nmol as NDMA /g cell, highlighting the extracellular origin of TONO precursors. The TONO concentration in chlorinated samples was ~4%-40% of that in chloraminated samples, suggesting that the Cl_2 dose is a key factor driving TONO formation from cyanobacteria.

Earlier mechanistic work has suggested that *N*-nitrosamine formation is enhanced in breakpoint chlorination[33]. To probe the formation mechanism of *N*-nitrosamine in cyanobacterial samples, LB 2386 was amended with NH_4-N ($[NH_4\text{-N}]$ = 5.60 mg/L) and chlorinated. Compared to the samples without NH_4-N addition ($[NH_4\text{-N}]$ = 0.02 mg/L), the concentration of *N*-nitrosamines formed in NH_4-N amended samples did not show a statistically significant increase. Previous studies have reported that organic nitrogen derived from cyanobacteria is an important precursor pool for organic chloramine formation during chlorination[34,23]. The reaction rate constants of Cl_2 with amino acids/organic amines are much higher than that with NH_4-N[35]. Furthermore, organic chloramines have proven to serve as intermediates of DBPs during chlorination[36]. These results suggest that organic chloramine rather than inorganic chloramines likely formed upon chlorination of cyanobacterial samples, and that organic chloramine may play an important role in *N*-nitrosamine formation.

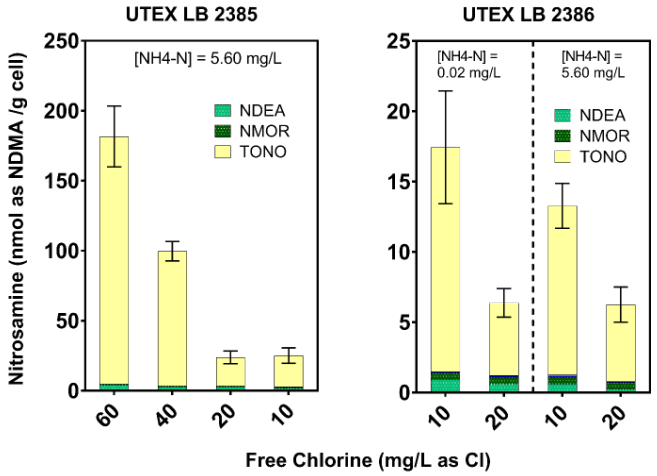

Figure 3. N-Nitrosamine concentrations in chlorinated samples.

Characterization of AOM

To further characterize the properties of cyanobacteria-derived *N*-nitrosamine precursors, non-target screening of cyanobacterial samples was performed on a liquid chromatograph-high resolution mass spectrometer to identify mass spectral features that might be responsible for differences in *N*-nitrosamine formation among samples. In addition, fluorescence excitation-emission matrix (EEM) spectra of raw, chlorinated, and chloraminated samples were recorded on an Aqualog spectrofluorometer to examine changes in fluorescent properties of cyanobacteria-derived organic matter.

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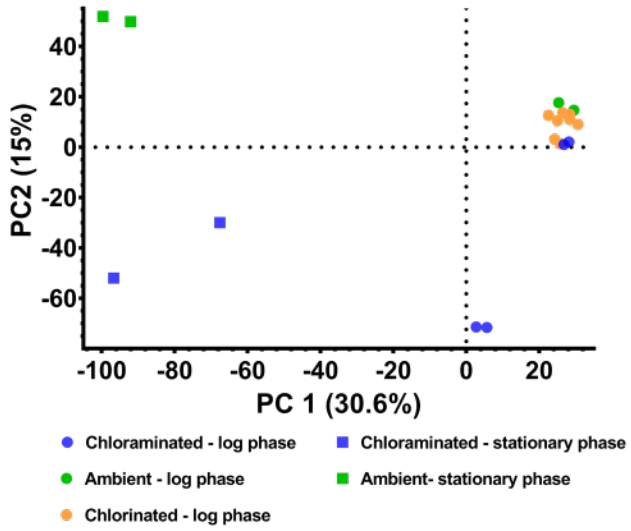

Figure 4. PCA of mass spectral features in cyanobacterial samples.

Figure 4 illustrates the principle component analysis (PCA) of mass spectral features identified in cyanobacterial samples. Component PC 1 (30.6%) is mainly driven by the "freshness" of cyanobacterial samples, while component PC 2 (15.0%) mainly differentiates the extent of sample treatment (ambient / chloraminated / chlorinated). This analysis highlighted the impact of growth stage on the mass spectral features in cyanobacterial samples.

Figure 5 shows a volcano plot of all mass spectral features detected in a LB 2385 sample during its stationary growth phase versus a LB 2385 sample during its exponential growth phase. Over 1000 mass spectral features (green area) showed a significant decrease in peak intensities during the exponential phase compared to the stationary phase. Considering the relatively low concentration of *N*-nitrosamine precursors during the stationary phase, these mass spectral features are likely unknown *N*-nitrosamine precursors, but further work is warranted to confirm their structural properties.

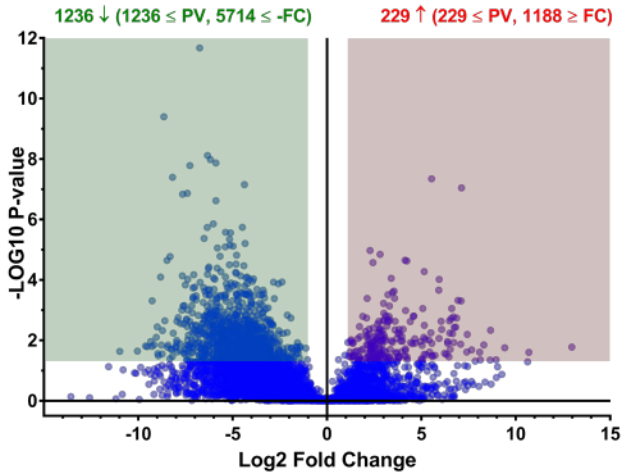

Figure 5. Volcano plot of mass spectral features detected in toxin- at stationary vs. log phase. PV : p-value (= 0.05); FC: fold change (=1).

Figure 6 shows the EEMs of LB 2385 samples upon chloramination (Figure 6-1), chlorination with 60 mg/L Cl_2 (Figure 6-2), and chlorination with 10 mg/L Cl_2 (Figure 6-3), as well the EEM of raw LB 2385 sample (Figure 6-4). The normalized region-specific EEM volume at excitation (250-400) / emission (280-380) increased successively from Figure 6-1 to Figure 6-4, reflecting the increasing abundance of the soluble microbial byproduct-like material based on previous literature[37]. The abundance of this portion of DOM in these samples is negatively correlated to the amount of oxidant (chloramine/chlorine) and levels of TONO formed, indicating a potential pool of TONO precursors in AOM.

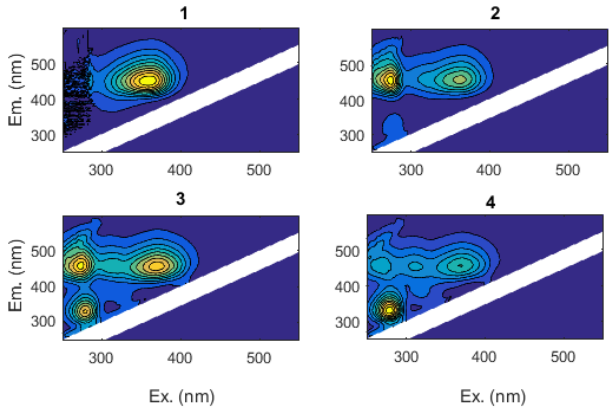

Figure 6. EEMs of LB 2385 samples under different treatment conditions.

Being qualitative and preliminary, these results may still provide new insights into the properties of *N*-nitrosamine precursors to guide further investigation.

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Policy Implications

To date, no federal standards are established to regulate *N*-nitrosamines in drinking water; however, the U.S. EPA has included five *N*-nitrosamines on the Contaminant Candidate List 4, and is considering nationwide regulatory limits on these contaminants. Fifteen States in the U.S., such as California, Massachusetts, and New Jersey, have set drinking water and groundwater guidelines for NDMA, but not New York. Given the increasing frequency, magnitude, and geographic extent of HAB events in New York State, water utilities relying on HAB-impacted source waters may find balancing algal toxin removal and *N*-nitrosamine control a challenging task. We advocate for comprehensive assessment of *N*-nitrosamine formation potential in New York freshwater lakes that serve as public water supplies.

Methods

A solid-phase extraction (SPE) protocol adapted from previous research[38] was used to extract *N*-nitrosamines from cyanobacterial samples to maximize the recoveries of *N*-nitrosamines with a range of polarities. The final SPE extracts were concentrated by rotary evaporator and reconstituted prior to analysis. The concentration of total *N*-nitrosamines in samples was determined by a chemiluminescence assay[39] and reported as NDMA equivalents. The concentrations of specific *N*-nitrosamine species (e.g., NDMA, NPYR, NMOR) were determined by an isotope-dilution method by liquid chromatography-high resolution mass spectrometry.

Outreach Comments

No direct outreach was conducted. However, the research team has established a strong collaborative relationship with the Citizens Statewide Lake Assessment Program (CSLAP) over the course of this project.

Student Training

This project directly supported part of the dissertation work of one Syracuse University Ph.D. student, who will continue working on an expanded study in Fall 2019. Although not supported, two Syracuse University undergraduate students will participate in the Fall 2019 research. The project also supported a local high school student for summer research during Summer 2018.

Notable Awards & Achievements

Not applicable.

Publications/Presentations

Pu, C.; Zeng, T., Formation of *N*-nitrosamines upon chloramination and chlorination of cyanobacterial strains. *Gordon Research Conference Drinking Water Disinfection By-Products*, South Hadley, MA, 2019.

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Appendices (if needed)

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