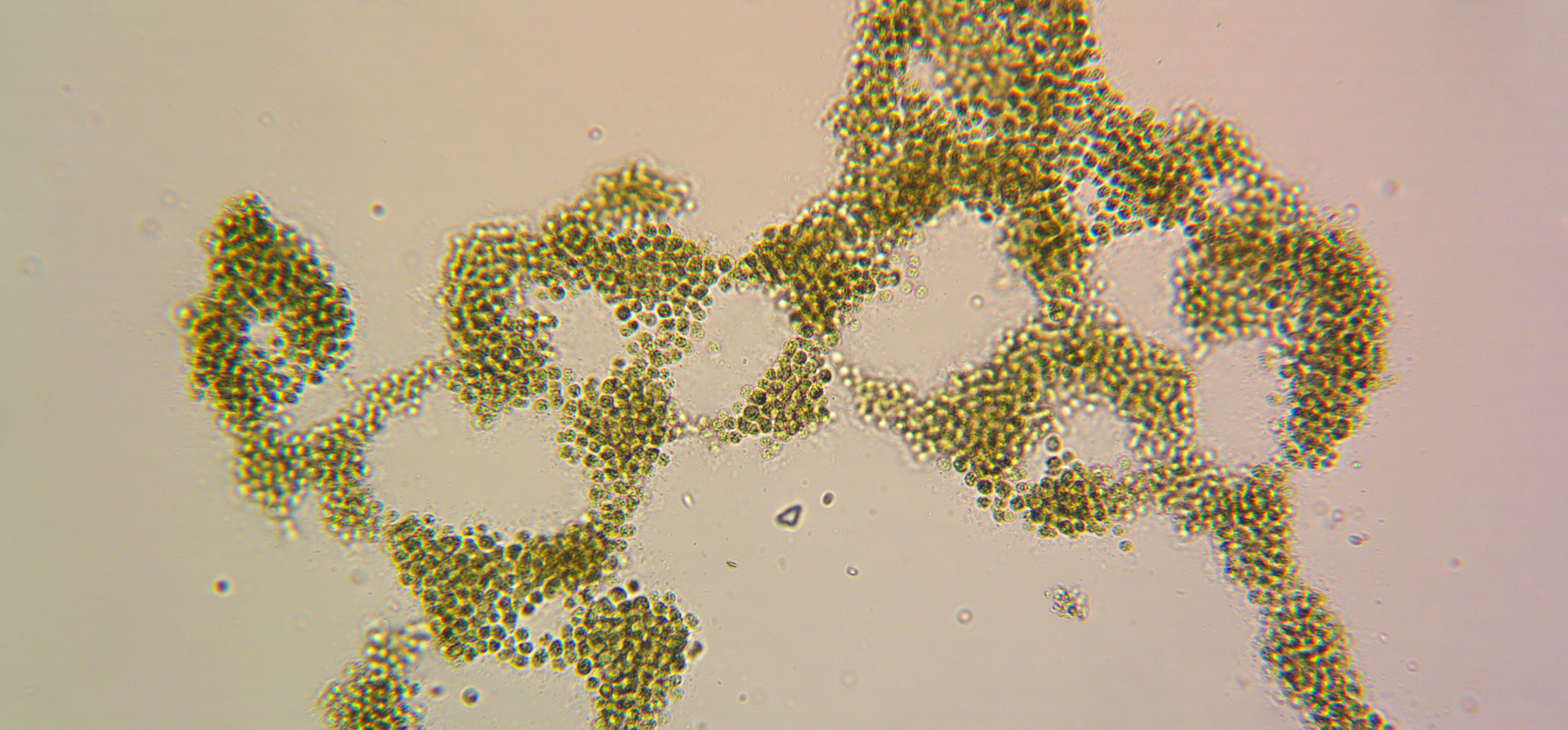

Image caption: *Microcystis aeruginosa* under a microscope, photographed by Brad Austin at the Arkansas Water Resources Center.

Mechanisms, Kinetics and Toxicity of Microcystin-LR Biodegradation by Free and Immobilized Enzymes

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Abstract: Microcystin-LR (MC-LR) is among the most problematic algal toxin in America, with several outbreaks and appearances in fresh water supplies in recent years. We investigated a new strategy for remediation of MC-LR that combines linearization of the toxin using microcystinase A, MlrA, enzyme with rejection of linearized byproducts using membrane filtration. An active MlrA enzyme was produced from a heterologous host, and the enzyme was able to degrade MC-LR and convert it to linearized MC-LR. The linearized MC-LR along with some undegraded cyclic ones were filtered through composite hydrophobic and highly negative membranes made of 95% polysulfone with 5% sulfonated PEEK. We found that a SPEEK membrane could reject or adsorb the vast majority of cyclic MC-LR and virtually all linearized MC-LR. The partial success of modified membranes and reactive processes suggests that a physical barrier membrane with additional functionality (e.g., charge, reactivity) may result in enhanced MC-LR remediation. The PSf/SPEEK membrane was effective at rejecting or adsorbing 97.4% cyclic MC-LR and virtually all linear MC-LR. MC-LR was found to reversibly adsorb to the PSf/SPEEK membranes, with desorption of MC-LR occurring when the membranes were soaked in a methanol/water solution. This is highly advantageous because it provides a method of removing MC-LR from feed waters via reversible adsorption to membranes that can then be cleaned and reused.

Key Points:

- The MlrA enzyme was overexpressed in *Escherichia coli* and purified via a His-tag with 95% purity.
- MlrA was successful at linearizing approximately 86% of the MC-LR over 24 hours.
- Membrane filtration tests showed rejection of 97.4% of cyclic MC-LR and virtually all linearized MC-LR, with adsorption to the membranes being the main rejection mechanism.
- This study demonstrates a novel strategy of remediation of microcystin-tainted water, combining degradation of the toxin with removal by membrane filtration.

Introduction

Enzymatic remediation allows an engineered catalysis system (e.g., enzyme-immobilized catalytic membranes), in comparison to whole-organism bioremediation of cyanotoxins. Our approach is to use enzyme-mediated catalysis to target microcystin degradation and removal from water specifically. The research focuses on proof-of-concept experiments to demonstrate enzyme-driven microcystin-LR (MC-LR) degradation from contaminated synthetic water solutions. The overall goal of this research is to work toward membrane-mediated biocatalysis, an innovative approach where membranes provide structural support and physical separation of contaminants, while enzymes enable catalytic degradation of contaminants.

Methods

MlrA Expression and Experiments

Strain and plasmid construction

Escherichia coli (E. coli) chemically competent BL21 (DE3) was purchased from New England Biolabs Inc. and used as a host to produce targeted recombinant protein. The pET-21a (+) plasmid was purchased from Novagen Life Technologies. A DNA fragment encoding to MlrA was designed and purchased from Integrated DNA Technologies (Coralville, IA). After plasmid construction, recombinant pET-MlrA plasmid was transformed into E. coli Bl-21 cells by heat shock method.

Media, growth, and expression of recombinant MlrA enzyme

Ampicillin contained in Luria-Bertani (LB) broth was used for all plates and seed cultivations. A single colony of E. coli BL21 containing cloned pETmlrA plasmid pick up from agar plate was used to inoculate 10 ml of LB media supplied with 75 µg/mL ampicillin as an initial growth seed culture. The culture was incubated at 37°C with shaking at 200 rpm overnight. This experiment was performed by using 2.5 mL of overnight growth to inoculated 1 L supplemented with 75 µg/mL ampicillin. The growth was incubated at 37°C with 200 rpm shaking speed. Isopropyl β-D-1-thiogalactopyranoside (IPTG) with final concentration of 0.5 mM was used to induce recombinant plasmid at the mid-exponential phase of growth when the optical density 1 reached ~0.6 units (at 600 nm). After 4 hours from induction, cells were harvested to proceed in further experiments.

Cell lysate preparation and Hisx6-tag MlrA purification

After expression was completed, cells were harvested by centrifugation at 4,500 × g for 45 minutes. Cell pellets were resuspended in 10 mM sodium phosphate buffer, pH 7.4 and subjected to sonication on ice with a Qsonica sonicator on a 20 second burst cycle (power 10). The cell homogenate was centrifuged to clarify the cell lysate. Supernatants were collected to proceed in further experiments such as tested for enzyme activity and purification. The Fast Protein Liquid Chromatography system from ÄKTA Amersham Pharmacia Biotech was used to purify the cell lysate. Hisx6-tag is designed to be in the C-terminal of MlrA enzyme to facilitate purification through Immobilized Metal Affinity Chromatography (IMAC). Cobalt loaded HiTrap IMAC FF column (Co-NTA) was used to purify expressed protein. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was used to ensure that the targeted protein expressed and produced properly by the host and to confirm the purity.

Microcystin-LR Degradation Activity of MlrA

The MlrA cyanotoxin degradation activity was tested by incubating MlrA with microcystin-LR. 2 mL solution of 100 µg/L MC-LR and 216 mg/L MlrA in phosphate buffer (5 mM) was incubated in a glass scintillation vial for 24 hours at room temperature. 500 µL samples were taken at 0, 4, and 24 hours after initial mixing and transferred to glass HPLC vials. The reaction in each HPLC vial was stopped by 50 µL of 5% acetic acid. Samples were stored at 4°C until analysis. The linearization of MC-LR was monitored by LC-MS.

LC-MS Methods for Detecting MC-LR

Cyclic and linearized MC-LR were detected using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). A LCMS-8040 Triple Quadrupole Liquid Chromatograph Mass Spectrometer (Shimadzu, Columbia, MD, USA) was used to analyze the samples. A Shimadzu C18 column (2.1 x 50 mm, 1.9 µm particle size) was used for all samples except for those from membrane filtration experiments, for which a Waters Acquity UPLC peptide HSS T3 column (1x100 mm, 1.8 µm particle size) was used. An injection volume of 10 µL was used and the column was held at 40°C. The mobile phases were water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The series of gradients were as follows: the column was initially balanced with 20% acetonitrile for 1 minute, then increased from 20% to 80% acetonitrile over 7 minutes, then held at 80% acetonitrile for 1 minute, then returned to 20% acetonitrile and held for 1 minute.

For mass spectrometry, electrospray ionization was used. Full scans were performed for m/z 400-1200 in positive ion mode. Additionally, for the experiments involving filtration of MC-LR and its degradation products through a membrane, single-ion monitoring was used for m/z 498.5 and 995.7 (2+ and 1+ charge states for the cyclic MC-LR) and for m/z 507.4 and 1013.5 (2+ and 1+ charge states for linear MC-LR).

Membrane Fabrication and Experiments

Sulfonation of SPEEK

The sulfonation procedure was adapted from literature (Eke et al., 2018) and modified as discussed here. In the method, 25 g of PEEK granules were heated in a vacuum oven at 100°C for 24 hours. They were then crushed into fine powder, which was dissolved in concentrated sulfuric acid in a 90:10% (H_2SO_4:PEEK) at room temperature for 48 hours, then precipitated out of the solution using cold deionized water. The precipitant was neutralized and dried overnight in a vacuum oven at 100°C.

Membrane Formation

The membranes were formed via non-solvent induced phase separation. In this method, once the membrane is cast, it is immersed into a non-solvent where a phase transition takes place (Dong et al., 2021). The membrane formed contains a polymer rich surface (active rea) and a polymer poor pore structure suitable for ultrafiltration (Jung et al., 2016; Dong et al., 2021). The non-solvent utilized was water. The dope solution was prepared by dissolving polysulfone (PSf) blended with SPEEK (95:5%) into N-Methyl-2-pyrrolidone solvent to make 79:21% NMP:SPEEK-PSf. The blended dope solution was then spread onto a glass plate using a doctor blade (0.4 mm) and exposed to air for 15 seconds before being immersed in de-ionized water. The formed membrane was then stored in de-ionized water (Eke et al., 2018; Dziga et al., 2019).

Adsorption and Desorption of Microcystin-LR on Membrane

The adsorption and desorption of MC-LR was tested by incubating MC-LR with a PSf/SPEEK membrane to adsorb, then adding methanol to desorb. A ¼ section of a PSf/SPEEK membrane (~4.33 cm^2) was incubated with 100 µg/L MC-LR in phosphate buffer (5 mM) in a glass scintillation vial for 24 hours at room temperature. 500 µL samples were taken at 0, 4, and 24 hours after initial mixing and transferred to glass HPLC vials. Before taking the sample at 24 hours, 667 µL methanol was added to the scintillation vial (bringing the concentration of methanol to 40% by volume and swirled to mix for 5 minutes. Samples were combined with 50 µL of 5% acetic acid to maintain consistency with other tests. Samples were then stored at 4°C and analyzed by LC/MS.

Membrane Filtration

The by-products of enzymatic degradation of MC-LR were filtered through the PSf/SPEEK membranes using a dead-end filtration cell from ®Millipore, MA USA with max operating pressure 5 bar. To provide the required pressure drop for flow, nitrogen gas was employed under a constant pressure of 4.1 bars (60 psi). Pre-compaction of the membrane with D-Ionized water was carried out followed by filtration of the byproducts at the end of the 24-hour degradation study. The filtrate after MC-LR degradation, which was the membrane feed solution, included degradation by-products, potentially MC-LR, methanol, phosphate buffer (containing disodium hydrogen phosphate and potassium phosphate dibasic), acetic acid, and MlrA enzyme solution. The reaction volume was concentrated by four times for all components while maintaining the same reactant concentration ratios. Samples were analyzed by LC/MS.

Results and Discussion

Protein Expression and Inclusion body Formation

E. coli containing cloned pETMlrA plasmid was expressed in a normal condition after optical density 1 reached ~0.6 units (at 600 nm) by using 0.5 mM IPTG. Results shows highly expressed band at ~42 kD (Figure 1, lane 2 and 3), matching the MlrA molecular weight which was designed in this study.

The purity of MlrA eluted in 125 mM imidazole was estimated to be about 95% while less purity of MlrA eluted in 62.5 mM imidazole due to metal weak binding proteins. This purity assessment confirmed by SDS-PAGE as a general detection of total protein. As shown in Figure 1, a single band represents MlrA enzyme in 125 mM was detected. These results prove the full length of MlrA expression and purity.

Microcystin-LR Degradation Activity of MlrA

MC-LR was treated with MlrA in a phosphate buffer (5 mM) solution. LC/MS was used to analyze samples taken

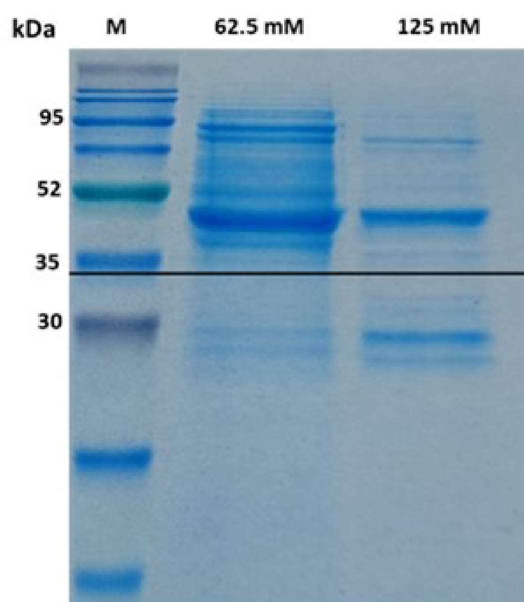

Figure 1: SDS-PAGE stained with Coomassie Brilliant Blue. 1 – molecular weight marker; 2- MlrA eluted with 62.5 mM imidazole; 3- MlrA eluted in 125 mM imidazole

at 0, 4, and 24 hours after initial mixing (Figure 2). At the start of the experiment, all MC-LR is present in the cyclic form (5.03 min). MS detected at peak for the cyclic MC-LR at m/z = 995.6. At 4 hours, the cyclic peak is reduced in height and an additional peak for linear MC-LR is present at 5.17 min. MS detected peaks for the linear MC-LR at m/z = 1013.8 (+1 charge state) (Bourne et al., 1996; Dziga et al., 2012) and m/z = 507.4 (+2 charge state). At 24 hours, the linear MC-LR peak is roughly 6x taller than the cyclic MC-LR peak, indicating that approximately 86% of the MC-LR has been linearized.

Adsorption and Desorption of Microcystin-LR on Membranes

Experiments were performed to determine the adsorption and desorption behavior of MC-LR to a PSf/SPEEK membrane. LC/MS was used to analyze samples taken at 0, 4, and 24 hours after initial mixing (Figure 3). Note that methanol was added just before the sample for 24 hours was taken. At t = 0 h, a cyclic MC-LR peak is visible at 5.06 min. At t = 4 h, the cyclic MC-LR peak is still present, but the height (vs. baseline) is reduced to 20.8% of the starting intensity, indicating that most of the MC-LR has adsorbed to the membrane. At t = 24 h, after methanol addition, the cyclic MC-LR peak height reverts to 73.6% of the starting intensity, indicating that methanol caused most of the adsorbed MC-LR to desorb.

These results are consistent with other studies that found that microcystin adsorbs to various types of plastic, but methanol can reduce adsorption. For example, Altaner et al. found that microcystin congeners dissolved in water adsorbed to polypropylene pipette tips, but methanol (≥ 40%) rectified the adsorption losses (Altaner et al., 2017).

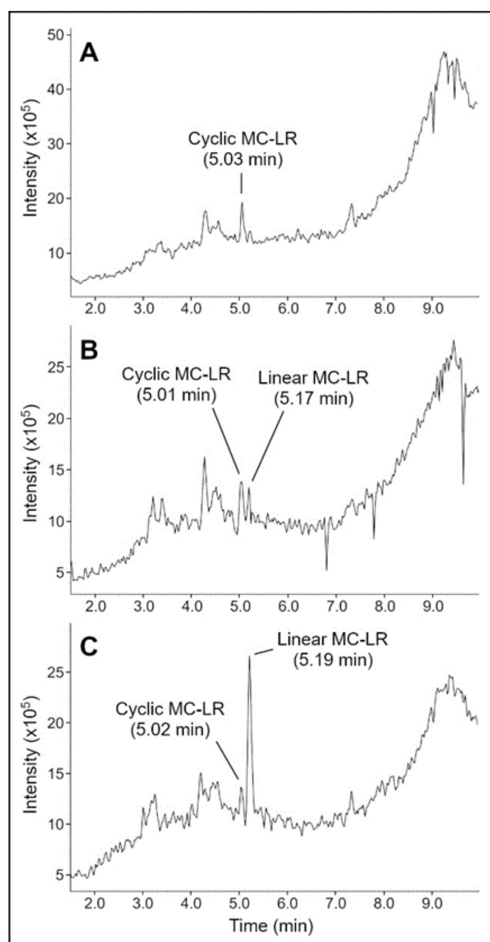

Figure 2: LC/MS analysis of the MC-LR as it is degraded by MlrA in an environment of phosphate buffer (5 mM), for samples taken 0 hours (A), 4 hours (B), and 24 hours (C) after initial mixing.

Filtration of MC-LR and Degradation Products Through Membrane

For filtration experiments, MC-LR was incubated with MlrA for 24 hours and the resulting solution was passed through a membrane to determine if MC-LR or any byproducts would be adsorbed or rejected by the membrane (Figure 3). For this experiment, a different UPLC column (peptide column) was used than in previously described experiments, so the MC-LR peaks occur at different times from those shown in Figures 2 and 3, where a C18 column

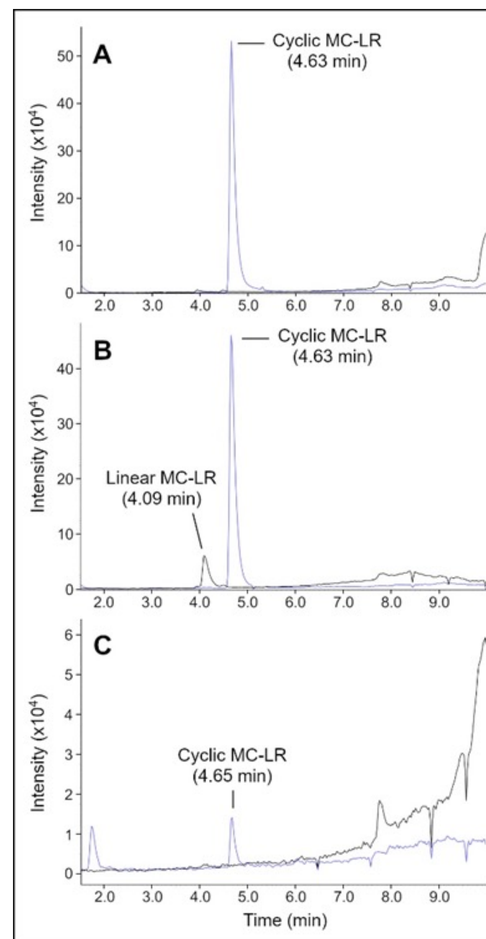

Figure 3: LC/MS analysis of the MC-LR solution incubated with MlrA, for samples taken at 0 hours (A), and 24 hours (B) after initial mixing, and for the solution after 24 hours filtered through a SPEEK membrane (C). Graphs for the cyclic MC-LR region (m/z 498.5) are in blue, while graphs for the linear MC-LR region (m/z 507.4) are in black.

was used. Additionally, single-ion monitoring was used to increase the clarity of the MC-LR peaks.

At t = 0 h (Figure 3A), a cyclic MC-LR peak is visible at 4.63 min. MS detected peaks for the cyclic MC-LR at m/z = 995.6 and 498.5 (+2 ion). At t = 24 h (Figure 3B), a cyclic MC-LR peak is still visible at 4.63 min but there is also a linear MC-LR peak at 4.09 min. MS detected peaks for cyclic MC-LR at m/z = 995.6 and 498.5 (+2 ion) and peaks for linear MC-LR at m/z = 1013.8 and 507.5 (+2 ion). At t = 24 h, the cyclic MC-LR peak is significantly taller than the linear MC-LR peak, indicating that much of the MC-LR was not degraded. This could be because of degradation of the MlrA enzyme over the course of long-term storage. The MlrA was stored at -20°C for approximately two years before being used for this experiment. Loss of MlrA activity can occur over the course of storage. For example, Wu et al. found that MlrA lost approximately 50% of activity at degrading nodularin, a related toxin, after 4 days of storage at 0°C (Wu et al., 2020). MlrA would be expected to lose activity at a slower rate at -20°C, but the loss could still be significant over the course of long-term storage.

For the (partially) degraded MC-LR solution filtered through the SPEEK membrane (Figure 3C), the peak for cyclic MC-LR at 4.65 min is much smaller than it was before filtration (2.6% of original height). Also, the peak for linear MC-LR is no longer visible. The small peak at 1.73 min is likely a contaminant or impurity and the peak was not visible in any other samples. The tall curve that escalades from ~7 min onwards is from the phosphate buffer. This curve is more clearly visible for this sample vs. other samples because the much shorter y-axis scale. In the mass spectrums for this sample, the MC-LR peaks were not distinguishable from background noise due to the significantly reduced concentration of MC-LR.

The LC-MS results of the filtrate indicate that the vast majority (97.4%) of cyclic MC-LR is adsorbed or rejected by the membrane, and virtually all linear MC-LR is adsorbed or rejected. The membrane could be more selective for linear MC-LR than cyclic MC-LR because the larger surface area of linear MC-LR enables stronger adsorption to the membrane. The results from the previous section indicate that the membrane could be soaked in methanol solution to desorb attached MC-LR and regenerate the membrane.

Conclusions

Predicted increases in surface water temperatures, combined with current agricultural practices, is expected to result in the continued expansion of algal blooms containing cyanobacteria, and enhanced public health risk due to algal toxins migrating into potable water. The most common cyanotoxins released during algal blooms are microcystins. Due to MCs cyclic peptide structure, removal via conventional treatment processes remains challenging. Enzymatic remediation allows an engineered catalysis system to potentially destroy these toxins. To investigate enzymatic degradation of MC-LR, MlrA enzyme was used here. In this study, an active MlrA enzyme was produced from a heterologous host, and the enzyme was able to degrade MC-LR and convert it to linearized MC-LR. The linearized MC-LR along with some undegraded cyclic ones were then filtered through composite hydrophobic and highly negative membranes made of 95% polysulfone with 5% sulfonated PEEK. During a dead-end filtration test, we found that a SPEEK membrane could reject or adsorb the vast majority of cyclic MC-LR and virtually all linearized MC-LR. The partial success of modified membranes and reactive processes suggests that a physical barrier membrane with additional functionality (e.g., charge, reactivity) may result in enhanced MC-LR remediation. The PSf/SPEEK membrane was effective at rejecting or adsorbing 97.4% cyclic MC-LR and virtually all linear MC-LR. MC-LR was found to reversibly adsorb to the PSf/SPEEK membranes, with desorption of MC-LR occurring when the membranes were soaked in a methanol/water solution. This is highly advantageous because it provides a method of removing MC-LR from feed waters via reversible adsorption to membranes that can then be cleaned and reused. Algal blooms are becoming more prevalent in the state of Arkansas, specifically Fayetteville. The results are steps toward water treatment in the region and nationally.

Acknowledgements

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