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Application of Edible Montmorillonite Clays for the Adsorption and Detoxification of Microcystin

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Abstract

Exposure to microcystins (MCs) in humans and animals commonly occurs through the consumption of drinking water and food contaminated with cyanobacteria. Although studies have focused on developing water filtration treatments for MCs using activated carbon, dietary sorbents to reduce the bioavailability of MCs from the stomach and intestines have not been reported. To address this need, edible calcium and sodium montmorillonite clays were characterized for their ability to bind MC containing leucine and arginine (MC-LR) under conditions simulating the gastrointestinal tract and compared with a medical-grade activated carbon. Results of in vitro adsorption isotherms and thermodynamics showed that binding plots for MC-LR on montmorillonites fit the Langmuir model with high binding capacity, affinity, Gibbs free energy, and enthalpy. The in silico results from molecular modeling predicted that the major binding mechanisms involved electrostatics and hydrogen bonds, and that interlayers were important binding sites. The safety and detoxification efficacy of the sorbents against MC-LR were validated in a battery of living organisms, including *Hydra vulgaris*, *Lemna minor*, and *Caenorhabditis*

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elegans. The inclusion of 0.05% and 0.1% montmorillonite clays in hydra media significantly reduced MC-LR toxicity and protected hydra by 60–80%, whereas only slight protection was shown with the heat-collapsed clay. In the *Lemna minor* assay, montmorillonites significantly enhanced the growth of lemna, as supported by the increase in frond number, surface area, chlorophyll content, and growth rate, as well as the decrease in inhibition rate. Similar results were shown in the *C. elegans* assay, where montmorillonite clays reduced MC-LR effects on body length and brood size. All 3 bioassays confirmed dose-dependent protection from MC-LR, validated the in vitro and in silico findings, and suggested that edible montmorillonites are safe and efficacious binders for MC-LR. Moreover, their inclusion in diets during algal blooming seasons could protect vulnerable populations of humans and animals.

Graphical Abstract

Keywords

adsorption isotherm; *Hydra vulgaris*; *Lemna minor*; *Caenorhabditis elegans*; molecular model; dietary sorbent

1. INTRODUCTION

Cyanobacteria, also known as blue-green algae, are photosynthetic microbes that occur in freshwater, brackish water, and oceans, and can cause major problems with water quality and the health of aquatic ecosystems.¹ The increasing global water temperature and rising nutrient levels due to intensive farming practices, sewage generation, and phosphate detergent usage have resulted in an extended blooming season and the production of highly active cyanotoxins in concentrations exceeding safe limits for human consumption and recreational use.^{2,3} Oral ingestion of contaminated drinking water and food, especially fish and shellfish, accounts for 80% of human exposures to cyanotoxins.^{4,5} Among cyanotoxins, microcystin (MC) containing the amino acids leucine (L) and arginine (R) (MC-LR) is the most deadly and common toxicant that represents 56–70% of the MCs in U.S. and Canadian water samples. A guideline value of 1 mg/L for MC-LR in drinking water has been issued by the World Health Organization.^{5,6}

Considerable efforts have been made to develop remediation techniques since conventional water treatments are ineffective in removing extracellular cyanotoxins that are dissolved in the water.^{7–10} The best-studied and most frequently used remediation strategies for water deploy activated carbon filtration, nanofiltration, ozonation, and chlorination.^{8,10,11} Especially, powdered activated carbon (PAC) has been used as an effective sorbent and one of the major treatment methods for the removal of extracellular cyanotoxin in most Australian water treatment plants.^{10,12–16} However, the adsorption of MC-LR onto carbon surfaces proceeds slowly, requiring more than 12 h of water treatment for the complete removal.¹⁷ Additionally, these PAC treatments work for water, but are not used to remove microcystins from contaminated food. Since microcystins are also commonly found as contaminants of food, there is a need for effective dietary treatments that will reduce human and animal exposures from the diet. On the basis of the literature, microcystins are adsorbed strongly to sediment and are difficult to recover^{18,19} and the adsorption is more effective by montmorillonite clays than other clay minerals.^{6,20} Montmorillonite clay is the mineral in the smectite group of phyllosilicates with a stable porous structure, high expandability, specific surface area, and cation exchange capacity due to negative charge on the siloxane surface, enabling its application as a toxin binder.^{21–26} Using dietary montmorillonite clay as a detoxification strategy for MC-LR is a logical spinoff from our previous human clinical trials in the U.S. and Africa, where we reported that quality-controlled montmorillonite inclusion in the diet and drinking water was effective in binding a foodborne mycotoxin in the gastrointestinal tract and was safe for consumption in adults and children.^{27,28} Therefore, in this study, we characterized the binding interactions of MC-LR on the surfaces of montmorillonites by in vitro adsorption equilibrium analyses and thermodynamics, and in silico computational modeling.

Since cyanobacterial bloom is a transient and intermittent phenomenon, there is a critical need for rapid screening tools to determine the toxicity of polluted water and the efficacy of detoxification treatments. Therefore, in this study, we also included a battery of 3 living organisms to validate the safety and detoxification efficacy of sorbent inclusion in the culture medium. Previously, the morphological response of *Hydra vulgaris* has been widely used to indicate the toxicity of environmental aqueous samples.^{29–32} This method has been

used alongside an in vitro gastrointestinal model,³³ mammalian cell culture model,³⁴ and in silico molecular dynamics simulations^{35–37} for screening purposes. *Lemna minor* is an aquatic floating plant and a recommended species to monitor water pollutants in ecotoxicity studies.³⁸ The toxicological testing protocols for lemna have been well-established^{7,39} and widely applied in toxicity evaluations in the pesticide registration process.^{40,41} Previous studies with *Caenorhabditis elegans* have repeatedly and consistently shown a high degree of correlation with mammals, suggesting *C. elegans* can be included as a biomonitor in early safety testing and as a component in an integrated toxicity testing strategy.^{42,43} The combination of these bioassays was used to enhance our ability to validate the in vitro and in silico findings and their application in living organisms.

In this study, we have characterized and optimized MC-LR/sorbent binding parameters and interaction mechanisms using (1) in vitro adsorption isotherms and thermodynamics under conditions simulating the gastrointestinal tract model, (2) in silico modeling to delineate mechanisms of the sorption, and (3) bioassays in hydra, lemna, and *C. elegans* as ecotoxicological models to validate the safety and detoxification efficacy of sorbent treatments.

2. MATERIALS AND METHODS

2.1. Reagents and Materials.

High-performance liquid chromatography (HPLC) grade acetonitrile and formic acid were purchased from Fisher Scientific (Waltham, MA). MC-LR standard (purity 95%) was purchased from Cayman Chemical (Ann Arbor, MI) and stored at -20°C . Calcium montmorillonite (CM) clay was obtained from BASF (Ludwigshafen, Germany) with a total surface area of approximately $850\text{ m}^2/\text{g}$, an external surface area of approximately $70\text{ m}^2/\text{g}$, a cation exchange capacity equal to 89.2 cmol/kg , and a pH_{PZC} equal to 8.8.^{34,44} Its chemical characterization by X-ray powder diffraction (XRD), thermogravimetric analysis (TGA), and scanning electron microscope (SEM) was previously published.^{34,45,46} CM clay was heated at 200°C for 30 min and 800°C for 1 h.⁴⁷ After the heating process, the total surface area of the collapsed CM clay decreased to $77\text{ m}^2/\text{g}$, which was similar to the external surface area of the parent CM.⁴⁴ This was indirect evidence that the heating process resulted in effective dehydroxylation of the siloxane surface and significantly collapsed the interlayer spacing. The physicochemical properties of sodium montmorillonite (SM) obtained from Halliburton (Houston, TX) were previously published.⁴⁸ The generic formula for montmorillonite clays is $(\text{Ca},\text{Na})_{0.3}(\text{Al},\text{Mg})_2\text{Si}_4\text{O}_{10}(\text{OH})_2 \cdot n\text{H}_2\text{O}$. Quality control of montmorillonite clays used in our studies has been routinely performed. Composition and particle size were consistent from lot to lot and representative samples were tested for environmental contaminants including polychlorinated dibenzo-p-dioxins/furans (PCDDs/PCDFs) and heavy metals (e.g., As, Cd, Hg, and Pb) following standard USEPA protocols (e.g., Method 6010B and 7471A) to ensure compliance with federal and international regulations.^{49,50} Medical grade PAC, purity >99%, was obtained from General Carbon Corporation (Paterson, NJ). It is labeled as a virgin PAC derived from a selected grade of coconut shell with $1100\text{ m}^2/\text{g}$ surface area, 5% moisture, pH_{PZC} equal to 9.57, and zeta potential of -31 mV measured at pH 7 and 25°C .^{51,52} Clays and carbons were sieved at 100 mesh to achieve a uniform particle size equal to, or less than $149\text{ }\mu\text{m}$.

2.2. Microcystin Analysis.

The concentration of MC-LR was measured by a HPLC system composed of a Waters 1525 binary mixing pump, a 717plus autosampler, and 2487 UV–Vis detector (Waters Corporation, Milford, MA).⁵³ The spectrophotometric system was fully enclosed to ensure high spectral resolution and wavelength accuracy at 238 nm. The separation was achieved by a Symmetry C18 ($4.6 \times 150 \text{ mm}^2$, $5 \mu\text{m}$) column. The injection volume was $10 \mu\text{L}$ with a flow rate of 0.6 mL/min . The mobile phase was 60% acetonitrile with 0.1% formic acid in water. The retention time for MC-LR was 10.2 min. A standard curve containing 7 points was prepared for concentrations ranging from 0.02 to 6 ppm (mg/L) ($r^2 = 0.995$). The limit of detection was 50 ppb ($\mu\text{g/L}$). Blank controls and calibration solutions were prepared daily and run before every set of experiments to ensure minimal system error.

2.3. Adsorption Isotherms.

Pure powdered MC-LR was dissolved in methanol at 1 mg/mL as a stock solution. Appropriate aliquots of the stock solution were separately transferred by a glass syringe to yield 5 ppm standard toxicant solution in pH 2 and pH 7 water. The concentration was set based on previous literature^{12,54} and the optimal ratio of sorbent/toxin to achieve equilibrium (saturation) on isotherm plots. Sorbent at 10 mg/L was then added to a concentration gradient from 5% to 100% of a 5 mL toxicant solution without changing the solution pH. Controls included 5 mL of blank water solution, toxicant solution, and sorbent suspension in water. To simulate mixing, samples containing sorbent and toxicant in pH 2 and pH 7 were vibrated at 37°C and 1000 rpm using an IKA electric shaker (VIBRAX VXR basic, Werke, Germany) for 2 and 48 h, respectively. The reaction time was set to mimic the average digestion duration in the human stomach and intestines and to reach an equilibrium of adsorption based on the shape of isotherm plots. In the thermodynamic study, all samples including controls and sorbent/toxicant mixtures were agitated at three temperatures (4°C , 24°C , and 37°C) to determine the interaction energy expressed as Gibbs free energy and enthalpy. The sorbent/toxicant complex was then separated from the solution by centrifugation at $2000g$ for 20 min, and the supernatant was analyzed by HPLC for the residual concentration in the solution.

2.4. Data Calculations and Curve Fitting.

The quantity of bound MC-LR was calculated by the concentration difference between control and test groups and expressed as mol/kg of sorbents on the isotherm plots. Table-Curve 2D (Systat Software, Inc., Palo Alto, CA) and R code were used to plot adsorption data and derive values for the variable parameters. The R code was used to calculate adsorption values and fitness to standard models based on maximum likelihood estimation, and standard errors and confidence bands calculated using the information matrix method.^{55,56} The adsorption isotherms were plotted by the Langmuir model using mean values of observed data points and 95% confidence bands from triplicate analyses. The Langmuir isotherm describes monolayer adsorption onto a surface with a finite number of identical sites and uniform energies of adsorption. The ideal situation for the Langmuir isotherm assumes that the adsorption sites have the same energy.⁵⁷ This means that the surface is mostly homogeneous and there is no interaction between the adsorbed species.

$$\text{Langmuir model } q = Q_{\max} \frac{K_d C_w}{1 + K_d C_w} \quad (1)$$

where q = the amount of MC-LR adsorbed (mol/kg), $Q_{\max}$ = maximum binding capacity (mol/kg), K_d = Langmuir distribution constant, and C_w = equilibrium concentration of MC-LR (mol/L).⁴⁴ To calculate the dimensionless K_e° from K_d , the following equation was used:

$$K_e^\circ = \frac{K_d \times [\text{adsorbate}]^\circ}{\gamma} \quad (2)$$

where γ is the coefficient of activity, $[\text{adsorbate}]^\circ$ is the standard concentration of the adsorbate = 1 mol/L, and K_e° is the thermodynamic equilibrium constant.⁵⁸

Adsorption parameters coupled with the Gibbs free energy equation and van't Hoff equation were used to calculate free energy (G°) and enthalpy (H):

$$\Delta G = \Delta G^\circ + RT \ln K_e^\circ \quad (3)$$

$$\Delta H = \frac{-R \ln \left(\frac{K_{d2}}{K_{d1}} \right)}{\left(\frac{1}{T_2} \right) - \left(\frac{1}{T_1} \right)} \quad (4)$$

where R (gas constant) = 8.314 J/mol/K and T (absolute temperature) = 273 + t (°C).

G will be zero for an adsorption system in equilibrium. A negative G° indicates the adsorption is thermodynamically favorable and will go in the forward adsorption direction ($K_e^\circ > 1$). If G° has a positive value, then the adsorption process will not be favored and the adsorption will not be significant ($K_e^\circ < 1$).⁵⁸

2.5. Molecular Modeling.

The molecular models for montmorillonite clay and MC-LR were drawn in ISIS Draw 2.0 (MDL Information Systems, Inc., Hayward, CA) and then imported into HyperChem 8.0 (HyperCub, Inc., Gainesville, FL). All chemical structures were energy-minimized using the semiempirical quantum mechanical AM1 method in HyperChem 8.0. The unit cell coordinates of muscovite were used to construct the models.⁵⁹ These coordinates were then converted to orthogonal coordinates and the unit cells defining the clay structure were replicated in three-dimensional space using the symmetry operations for a $C2/c$ space group.^{56,60} The d_{001} spacing of the clay model was set to 21 Å.³⁷ Microcystin was shown to be randomly oriented within the interlaminar region.

2.6. Hydra Assay.

Hydra vulgaris were obtained from Environment Canada (Montreal, QC) and maintained at 18 °C. Using a hydra classification method,⁶¹ the morphology of the hydra was rated over time as an indicator of solution toxicity. The morphological scoring of hydra was classified using a dissecting microscope based on a 10–0 point scale, where a score of 10

represented normal, healthy hydra and a score of 0 represented disintegrated (dead) hydra.⁶¹ Previous studies have reported MC-LR altered expression of liver cytochrome P450 (e.g., CYP 2E1) in mice, suggesting a possible involvement of the enzyme with its metabolism.⁶² Therefore, a metabolism activation package (MAP) was included in the hydra assay. MAP was standardized and consisted of 2.4 $\mu\text{g/mL}$ mice hepatic microsomal cytochrome P450, 225 μM NADPH, and 25 μM MgCl_2 .⁶³ Varied concentrations of MC-LR from 2.5 to 20 ppm were exposed to hydra to determine dose-dependent toxicity, and MC-LR at the minimum effective dose that resulted in 100% mortality of hydra in 92 h was included in a detoxification study and treated with sorbents. Sorbent treatments included SM, CM, and collapsed CM at 0.1% and 0.05% inclusion rates. The toxicant/sorbent complex in hydra media containing MAP was mixed at 1000 rpm for 2 h and centrifuged at 2000g for 20 min before exposure to hydra in Pyrex dishes. Three hydra colonies were included in each group and exposed to 4 mL of test media at 18 °C. The average score for each group was used to determine the toxicity rating at each time point (0, 4, 20, 28, 44, 68, and 92 h).

2.7. Lemna Assay.

Lemna minor (duckweed) was purchased from AquaHabit (Chatham, England). The plant was cultured with cool white fluorescent lights (400 ft-c intensity) at a light-to-dark cycle of 16 h/8 h and a mean temperature of 25 °C. A mineral growth medium for *Lemna minor* was prepared based on previous literature.⁶⁴ Three colonies of 3-frond lemna plants were randomly selected and incubated in Pyrex dishes closed with loose-fitting lids for 7 days. Lemna was exposed to varying doses of MC-LR from 10 to 30 ppm to determine toxicity. For the detoxification study, MC-LR solution at 15 ppm was treated with 0.1% and 0.15% CM and SM for 7 days. Lemna was inspected daily for frond number and surface area of surviving plants and analyzed by ImageJ (NIH, Bethesda, MD). On day 7, the plants were removed from individual dishes and homogenized in 1.5 mL 80% acetonitrile. The chlorophyll content was extracted after 48 h (4 °C, dark) and measured by UV–Vis scanning spectrophotometry (Shimadzu UV-1800, Kyoto, Japan) at 663 nm. Growth rate and inhibition % were calculated based on standard OECD guidelines:^{39,64}

$$\text{growth rate} = \frac{\text{Log } 10(\text{final frond no.}) - \text{Log } 10(\text{initial frond no.})}{\text{days}} \quad (5)$$

$$\text{inhibition of growth \%} = 100 \times \left(1 - \frac{\text{frond no. in the treatment}}{\text{frond no. in the control}} \right) \quad (6)$$

2.8. C. elegans Assay.

C. elegans wildtype N2 (Bristol) and *E. coli* NA22 and OP50–1 strains were purchased from the Caenorhabditis Genetics Center (CGC, University of Minnesota). *C. elegans* were grown on 8P media (25 g/L bactoagar, 20 g/L bactopectone, 500 μM KPO_4 , 13 μM cholesterol in 95% ethanol, 1 mM CaCl_2 , and 1 mM MgSO_4). *C. elegans* was seeded with 8×10^8 cells/mL *E. coli* NA22 (maintained in 16 g/L tryptone 10 g/L yeast extract, and 85.5 mM NaCl grown to $\text{OD}_{600} = 1$) and maintained at 18 °C as previously described.⁶⁵ Age synchronized populations of nematodes were obtained by washing with bleaching solution

(0.55% NaOCl and 0.5 M NaOH) to isolate pure egg cultures; once eggs were obtained, they were washed with M9 solution (68 mM NaCl, 20 mM KH₂PO₄, and 40 mM Na₂HPO₄) and incubated for 18 h on a rocking platform.⁶⁵

After the incubation period, a population of approximately 2000 nematodes at larva stage 1 (L1) was used per group throughout this study. This amount was achieved by counting the number of nematodes from 3 small samples (2 μ L aliquots) of the worm suspension, and then the size of the entire synchronization yield and the volume required to hold 2000 nematodes were calculated. For toxin exposures, L1 nematodes were transferred to 1.5 mL microcentrifuge tubes and incubated with 50 μ L *E. coli* OP50–1 (maintained in 10 g/L tryptone, 5 g/L yeast extract, 171.1 mM NaCl, and 343.9 μ M streptomycin grown to OD₆₀₀ = 1) and varying concentrations of MC-LR (40 to 320 ppb) for 24 and 48 h in K-medium complete solution, prepared as previously described.⁶⁶ For the detoxification study, a 160 ppb MC-LR solution was treated with 0.1% and 0.2% CM and SM at 1000 rpm for 2 h and centrifuged at 2000g for 20 min. The supernatants were exposed to *C. elegans* in microcentrifuge tubes for 24 and 48 h. After exposure, *C. elegans* in both toxin control and clay treatment groups were washed three times with K medium complete solution and transferred to Nematode growth media (NGM) (17 g/L bactoagar, 2.5 g/L bactopectone, 51.3 mM NaCl, 1 mM CaCl₂, 1 mM MgSO₄, 500 μ M KPO₄, 12.9 mM cholesterol in 95% ethanol, 2.275 μ M nystatin, and 343.9 μ M streptomycin), seeded with *E. coli* OP50–1 and incubated for 48 h before body length and brood size quantification. Body length was measured using an OMAX stereomicroscope and OMAX ToupView camera software after nematodes were paralyzed with 25 mM sodium azide.⁶⁷ Relative body length was calculated as the percentage of the K-medium control, which was adjusted as 100%. Brood size was quantified by isolating a single L4 hermaphrodite on a seeded NGM plate and transferring the nematode to a new plate every day for 3 days; offspring were counted at the L4 stage (approximately 84 h after original plating of the hermaphrodite). Brood size was calculated from total offspring from the three plates; hermaphrodites that crawled off the plate during the assay were not included.⁶⁸

2.9. Statistical Analysis.

All experiments included blanks and negative controls and were independently triplicated. A one-way ANOVA followed by a posthoc Tukey test was used to determine statistical significance. $Q_{\max}$ and K_d from isothermal and thermodynamic analyses, toxicity scores from the hydra assay, frond number and surface area of lemna, and body length and brood size of *C. elegans* were calculated for standard deviation and p -value. Bonferroni correction was used for multiple test corrections.⁶⁹ Results were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

Clays have been used as ancient medicine for diarrhea, cholera, bacterial infections, and mitigation of poisonings.^{70,71} For centuries, eating clay has been a global practice that exists among humans as well as numerous animal species, including nonhuman primates, birds, butterflies, elephants, bats, dogs, cats, various isopods, etc. These clays have been widely used as mycotoxin binders and anticaking agents and have been tested for safety

and efficacy in long-term feeding studies.^{28,45,72,73} Preliminary screening studies were performed to determine the best sorbents for MC-LR. Sorbents tested included naturally occurring clays, acid processed clays, and clays amended with ferrihydrite, thiamine, carnitine, or choline (data not shown). Among all sorbents tested, parent montmorillonite clays similar to those used in our previous human clinical trials consistently delivered the highest binding percentage for all concentrations of MC-LR. Moreover, this is in alignment with the literature where MC-LR is adsorbed more effectively by montmorillonite clays than other clay minerals in deionized water systems.^{6,20} Therefore, CM and SM clays were further tested for their binding efficacy and safety as potential therapies for microcystin.

3.1. Adsorption Analyses Simulating the Stomach.

Adsorption isotherms were conducted under conditions simulating the stomach, using MC-LR in pH 2 aqueous solution reacted for 2 h at 37 °C. Isothermal adsorption onto medical grade powdered activated carbon and montmorillonite clays were plotted using a Langmuir model based on their good correlation coefficients (r^2) and mean squared error (MSE), curved shape, and a plateau, suggested that the active binding sites were saturable and homogeneous (Figure 1A). These findings supported earlier studies showing that the Langmuir model most accurately described the MC-LR adsorption on montmorillonite clay.^{53,54}

The binding capacity ($Q_{\max}$) for SM and CM clays were equal to 0.29 mol/kg and 0.22 mol/kg, which were higher than that of PAC ($Q_{\max} = 0.04$ mol/kg) (Table 1). The high binding percentage in the preliminary screening study, high binding capacity, affinity, and free energy of adsorption indicated that CM and SM were the most effective binders for MC-LR. Specifically, the higher hydration energy of sodium ions in SM induced its swelling and expansion in water, facilitating the attraction of large molecules such as microcystins into the interlayer. Furthermore, montmorillonite clays were heat-treated to dehydroxylate and collapse their interlayer spacing. The adsorption onto collapsed montmorillonite was significantly decreased, with a binding capacity of only 0.02 mol/kg (Figure 1A). This indirectly suggested that interlayers are the major binding sites for MC-LR with minor binding on the edges and basal surfaces. Since the clay interlayers are negatively charged due to cation substitution in the clay sheet structure, the MC-LR binding to clay interlayers possibly involved electrostatic interaction and cation exchange^{18,54} with the positive guanidinium moiety on MC-LR.

To investigate the tightness of the binding interaction, thermodynamic studies were conducted at 3 temperatures (4 °C, 24 °C, and 37 °C) to calculate the Gibbs free energy (G°) and enthalpy (H°). The free energy indicates the spontaneity of the adsorption process and enthalpy indicates either a physisorption or chemisorption mechanism. The Gibbs free energy values for MC-LR binding onto CM and SM at 3 temperatures were negative with an absolute value ranging from 32.1 to 42.3 kJ/mol, indicating that the adsorption was thermodynamically favorable and spontaneous in the forward direction for the formation of the bound complex. The adsorption of MC-LR on the surfaces of CM and SM at 4 and 24 °C is shown in Figure 1B,C. Enthalpy values derived from the van't Hoff equation are equal to -35.0 ± 1.9 kJ/mol for CM and -41.6 ± 15.9 kJ/mol SM. These

high absolute values of enthalpy ($|H| > 20$ kJ/mol) indicated that MC-LR was chemisorbed tightly to the clay surfaces, which is consistent with the Langmuir model derived from the isothermal analysis and a possible electrostatic interaction and cation exchange at the negative clay interlayers. Compared to previous work in the literature showing MC-LR binding with montmorillonite,^{53,54} our CM and SM clays showed higher free energy, enthalpy, and capacity values, possibly due to their higher total surface area and large d_{001} interlayer spacing.

All of the correlation coefficient (r^2) values for the Langmuir model were greater than 0.87, MSE values were low, standard deviations were all lower than 4%, and all points had small deviations from the plots supporting the validity of the Langmuir models for describing the MC-LR adsorption process. Controls, including blanks and MC-LR without sorbents, were performed and confirmed that MC-LR precipitation did not occur and was not associated with MC-LR sorption.

A molecular model simulating the interaction of MC-LR and montmorillonite clays is shown in Figure 2. After energy minimalization, the primary reaction groups on MC-LR are carboxylate groups associated with the glutamic acid and methylaspartic acid groups ($pK_a = 2.09$ and 2.19) and the amine associated with the arginine group ($pK_a = 12.5$). The model demonstrates the major binding forces associated with hydrogen bonds and electrostatic interactions, which were also predicted from in vitro isothermal and thermodynamic studies. Cation exchange and water-bridging interactions may also make minor contributions to MC-LR adsorption onto montmorillonites.^{6,18}

3.2. Adsorption Analyses Simulating the Intestines.

Adsorption isotherms were conducted in pH 7 water at 37 °C for 48 h to simulate conditions in the intestines. As shown in Figure 3 and Table 1, CM and SM in the intestinal model showed lower Q_{max} values (0.14 mol/kg and 0.18 mol/kg, respectively), K_d , and free energy compared to pH 2. This aligns with previous findings that acidic solutions are optimal for MC-LR adsorption.⁵³ The lower binding of MC-LR at pH 7 (in the intestines) may be explained by the two deprotonated carboxyl groups and the positive guanidinium group, resulting in a net negative charge in MC-LR. The negative MC-LR is repulsed by the negatively charged clay interlayer surfaces, but can also form surface bonds via cation bridging and ligand exchange reactions.⁶ This suggests that the stomach is the major site of MC-LR binding, and the remaining unbound MC-LR may continue the adsorption process in the intestines.

3.3. Hydra Assay.

Hydra vulgaris is very sensitive to environmental toxins and has been widely used to indicate the toxicity of water pollutants. As shown in Figure 4A, the morphology response of hydra to MC-LR at concentration gradients between 2.5 ppm –20 ppm was dose-dependent, where 2.5 ppm MC-LR showed minor toxicity on the last day, while 20 ppm MC-LR showed rapid toxicity and complete mortality. Therefore, 15 ppm MC-LR was included in the sorbent treatment study to validate the efficacy and safety of sorbents. In Figure 4B, the inclusion of only 0.05% CM and SM showed significant protection of hydra at

60% and $67.7 \pm 5.77\%$ against MC-LR toxicity, respectively ($p < 0.01$). This reduced toxicity in hydra correlated with the reduced MC-LR concentrations at 10.3 and 8.03 ppm in CM and SM treatment groups as detected by HPLC. A collapsed CM clay at the same inclusion level only showed 10% protection with 14.7 ppm MC-LR residual in the hydra media. This is consistent with the in vitro isothermal results showing that CM and SM are effective binders for MC-LR and that the interlayer is the major binding site for MC-LR. Additionally, CM inclusion at a higher dose of 0.1% resulted in higher protection ($80 \pm 10\%$) and lower residual MC-LR concentration (7.9 ppm) in hydra media. These results supported our previous dosimetry study where sorbent treatment showed a dose-dependent reduction in toxicity.

3.4. Lemna Assay.

Lemna minor is an aquatic plant with well-established toxicological testing protocols that have been widely used in ecotoxicology studies. In our studies, lemna media promoted a positive increase in frond number by 4 leaflets after a 7-day growth period. Plants exposed to MC-LR produced fewer fronds than the lemna media controls in a dose-responsive manner. Specifically, only 2 fronds were produced at 10 ppm MC-LR, and 30 ppm MC-LR completely stopped frond production and disintegrated 1 frond (Figure 5A). Similar toxicity results are shown regarding the surface area of surviving plants where exposures to MC-LR slowed down and even reversed the increase in surface area (Figure 5B). The chlorophyll-a content was extracted and detected using a UV–Vis scanning spectrophotometer on day 7. As shown in Figure 5C, the decreased chlorophyll-a content correlated with MC-LR concentrations, which is aligned with visual observations that exposed fronds were chlorotic with brown edges and altered morphologies, and produced fewer daughter fronds. The frond number on each day was used to calculate an average growth rate and inhibition percentage. Similar to the above phenotypes, higher concentrations of MC-LR (e.g., 30 ppm) showed a lower growth rate (1.32 ± 0.716) and a higher inhibition (38.5%) (Figure 5D). All of the parameters in lemna response are highly correlative, suggesting that lemna is a sensitive model to indicate MC-LR toxicity.

In the sorbent treatment study, exposure to 15 ppm MC-LR stopped the growth of lemna in terms of frond production and the surface area of surviving plants. The inclusion of 0.15% SM showed the most significant increase in lemna frond number, followed by 0.15% CM, 0.1% CM and 0.1% SM (Figure 6A). For surface areas of lemna, both CM and SM treatments at 0.1% and 0.15% protected lemna from MC-LR toxicity and showed similar increases in surface area of 0.03 cm^2 on day 7 (Figure 6B). For chlorophyll-a content, CM and SM at 0.15% completely protected lemna and showed chlorophyll concentration similar to the lemna media control group (Figure 6C). Compared to the growth rate (1.32 ± 0.621) and inhibition percentage (33.3%) with exposure to 15 ppm MC-LR, treatments of CM and SM reduced MC-LR toxicity and delivered higher growth rates and lower inhibition (Figure 6D). The results of all growth parameters in lemna consistently showed that CM and SM at very low doses (0.1% and 0.15%) adsorbed MC-LR tightly and significantly protected lemna from the severe toxicity of MC-LR. Importantly, the inclusion of CM and SM at levels up to 0.15% showed no effects on the growth parameters and phenotypes, supporting the safety of sorbent treatments.

3.5. *C. elegans* Assay.

Caenorhabditis elegans is a reliable toxicological model as it is sensitive to a wide range of contaminants at environmentally relevant concentrations and its toxicity testing methods have been well-established.^{42,66} Our preliminary *C. elegans* studies on montmorillonite and other related clays have demonstrated that the inclusion of these clay materials at 0.2% had no adverse impact on the nematodes, which aligned with the literature.⁷⁴ As shown in Figure 7A, changes in body length are dose-dependent during exposure to MC-LR, with all doses reducing the length of the nematode. Body length reduction was enhanced by 48 h of exposure to all MC-LR concentrations versus the 24 h treatments. No incidences of nematode mortality during treatment were observed. Decreases in brood size after exposure to MC-LR were also observed in a dose-dependent manner after 24 and 48 h of exposure (Figure 7C). The relatively large standard deviation in the mean values of brood size is likely due to the primarily neuro- and hepatotoxicity of MC-LR.⁷⁵ Because of these observed changes, 160 ppb MC-LR was utilized in the sorbent treatment study to validate their efficacy.

Exposures to MC-LR treated with 0.1% or 0.2% of CM or SM in the nematode media for 24 h showed complete protection against the growth reduction induced by exposures to MC-LR for 24 h, and promoted growth in those nematodes comparable to media controls (~100%) (Figure 7B). The inclusion of 0.2% SM with 48 h of exposure showed the most significant increase in body length, followed equally by the 0.2% CM, 0.1% CM, and 0.1% SM groups, which still significantly protected the *C. elegans* from growth inhibition by 160 ppb MC-LR ($p < 0.01$) (Figure 7B). For brood size effects, treatment with only 0.1% SM significantly protects nematodes from reduced fertility induced by 24 h exposure to MC-LR. This protection is further enhanced by treatment with 0.2% CM and 0.2% SM that completely neutralized MC-LR toxicity in brood size and promoted an increase in brood size compared to control ($p < 0.01$) (Figure 7D). Enhanced brood size was also observed with 0.1% SM, 0.2% CM, and 0.2% SM, compared to exposure to MC-LR for 48 h. The increase in brood size in the nematodes after clay treatment may be contributed to the role of cationic ions such as calcium signaling in fertilization, sperm activation, and meiotic maturation of oocytes.^{76–78} These results indicate that the treatment with 0.1% or 0.2% of CM or SM for 2 h reduced the toxicity from exposure to MC-LR for 24 and 48 h in a dose-dependent manner. The *C. elegans* results supported our in vitro, in silico, hydra, and lemna results, indicating that CM and SM are effective binders of MC-LR and can reduce the toxicity of MC-LR in a dose-dependent manner. MC-LR is one of the most hydrophilic microcystins and, with its efficacy for clay surfaces, it is possible that other microcystins will show a similar behavior.¹⁹

4. CONCLUSIONS

Mitigation strategies for microcystins have focused mainly on the purification of contaminated water, with limited studies on the development of dietary strategies to mitigate microcystin exposures from contaminated drinking water and food. Microcystin can irreversibly and directly damage the liver within 15–60 min after exposure to a lethal dose. In this case, prophylaxis is important since treatment may have little, or no, health

benefits. Currently, rifampin, an antibiotic used to treat several types of bacterial infections, including tuberculosis, is the only therapeutic agent that has shown some potential for the treatment of microcystin intoxication.⁷⁹ On the basis of our previous results in rodents, no adverse effects on body weights, serum biochemistry, and histopathology, including the gastrointestinal tract, were observed following long-term ingestion of levels of clays as high as 2% of the diet.^{30,45} These results suggest no significant accumulation of montmorillonite. It may be possible that montmorillonite clays could be deployed in various foods, flavored water, snacks, condiments, or by capsules, tablets, nutritional supplements, etc. to protect humans and animals from MC-LR intoxication.

Cyanobacteria blooms (and the production of hazardous chemicals like MC-LR) can result from a variety of environmental factors including: nutrients, temperature, sunlight, ecosystem disturbances (turbidity), hydrology, and water chemistry. The combinations of factors that can trigger a sustained algal bloom are not well understood, but natural disasters such as hurricanes and floods that can mobilize and redistribute sediments and nutrients, as well as droughts that can cause decreased flows and ponding of water, may play a role to encourage the growth of algae. Further work is needed to clarify the mechanisms behind these algal blooms and to develop practical methods to mitigate their effects on health.

In this study, we characterized the ability of sodium and calcium montmorillonites to sorb MC-LR. Using adsorption isotherms and thermodynamics under conditions simulating the stomach and intestines, we validated the binding efficacy and tightness of MC-LR onto surfaces of montmorillonites based on: (1) high correlation to the Langmuir model, (2) high binding capacity and affinity, (3) high enthalpy and Gibbs free energy, and (4) negatively charged interlayer surfaces as the primary binding site. Computational modeling indicated that the major mechanisms of adsorption involved electrostatic interactions and hydrogen bonding. The safety and efficacy of detoxification for montmorillonite clays were confirmed in 3 living organisms (hydra, lemna, and *C. elegans*). Our results have shown that these montmorillonite clays have a notable potential for binding and detoxifying MC-LR and suggest that consumption of edible clay-based sorbents may significantly decrease human and animal exposures to MC-LR toxicity from contaminated water and food. Further work is warranted to confirm the ability of montmorillonites to decrease exposures to MC-LR and mixtures of microcystins from contaminated food and water.

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Figure 1.

Adsorption isotherms of MC-LR onto various sorbents at 37 °C and pH 2 (A), plotted using the Langmuir model. Adsorption isotherms of MC-LR onto CM (B) and SM (C) at 24 and 4 °C, pH 2, plotted by the Langmuir model. Data represent the mean adsorption (mol/kg) at each concentration, run in triplicate. Bands indicate 95% confidence intervals on the mean response.

Figure 2. Energy minimized molecular model snapshots of MC-LR binding onto montmorillonite clay with close-up views of electrostatic interactions and hydrogen bonding. Atom color code: red (O), yellow (Si), purple (Al), black (C), and blue (N).

Figure 3.

Adsorption isotherms of MC-LR onto CM and SM at 37 °C and pH 7, plotted by the Langmuir model. Data represent the mean adsorption (mol/kg) at each concentration, run in triplicate. Bands indicate 95% confidence intervals on the mean response.

Figure 4.

Hydra toxicity from MC-LR (A) and protection by sorbents at 0.05% and 0.1% (B). Hydra media with MAP are included as a control for comparison and showed constant scores of 10. Data represent the mean morphological score at each time point, run in triplicate (** p 0.01).

Figure 5. Dose-dependent toxicity of MC-LR on lemna frond number (A), surface area of surviving plants (B), chlorophyll content on day 7 (C), and growth rate (bar graph) and inhibition percentage (line) (D).

Figure 6. Protection against MC-LR toxicity by sorbents at 0.1% and 0.15% on lemna frond number (A), surface area of surviving plants (B), chlorophyll content on day 7 (C), and growth rate (bar graph) and inhibition percentage (line) (D).

Figure 7. Effect of varying concentrations of MC-LR on body length (A) and brood size (C) of *C. elegans*. Protection against MC-LR toxicity by sorbents at 0.1% and 0.2% on body length (B) and brood size (D) (* $p < 0.05$; ** $p < 0.01$).

Table 1.Parameters and Correlation Coefficients of Adsorption Isotherms in the Langmuir Model^a

	CM					SM				
	$Q_{\max}$	K_d	G	r^2	MSE	$Q_{\max}$	K_d	G	r^2	MSE
pH2, 37 °C	0.22 ± 0.01	5.58 × 10 ⁶	-37.1	0.93	3.45 × 10 ⁻⁴	0.29 ± 0.01	1.33 × 10 ⁷	-42.3	0.96	3.64 × 10 ⁻⁴
pH2, 24 °C	0.23 ± 0.02	2.97 × 10 ⁶	-36.8	0.95	2.58 × 10 ⁻⁴	0.26 ± 0.01	3.27 × 10 ⁶	-37.7	0.98	9.60 × 10 ⁻⁵
pH2, 4 °C	0.23 ± 0.02	1.12 × 10 ⁶	-32.1	0.97	1.07 × 10 ⁻⁴	0.29 ± 0.01	2.34 × 10 ⁶	-33.8	0.98	1.12 × 10 ⁻⁴
pH7, 37 °C	0.14 ± 0.01	4.20 × 10 ⁶	-39.9	0.94	1.02 × 10 ⁻⁴	0.18 ± 0.01	2.21 × 10 ⁶	-37.7	0.98	4.49 × 10 ⁻⁵

^a $Q_{\max}$ (binding capacity, mol/kg) ± SD; K_d (binding affinity); r^2 (squared correlation coefficients); G (Gibbs free energy, kJ/mol); and MSE (mean squared error).