EPA Public Access

Author manuscript

Environ Sci Technol. Author manuscript; available in PMC 2020 July 26.

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Published in final edited form as:

Environ Sci Technol. 2018 August 07; 52(15): 8252–8262. doi:10.1021/acs.est.8b00034.

Susceptibility of the Algal ion of Environmental Exposure to Microcystin and Aflatoxin as a Toxin Microcystin-LR to UV/Chlorine Process: Comparison with Chlorination

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Abstract

Microcystin-LR (MC-LR), an algal toxin (cyanotoxin) common in sources of drinking water, poses a major human health hazard due to its high toxicity. In this study, UV/chlorine was evaluated as a potentially practical and effective process for the degradation of MC-LR. Via mass spectrometry analysis, fewer chlorinated-MC-LR products were detected with UV/chlorine treatment than with chlorination, and a transformation pathway for MC-LR by UV/chlorine was proposed. Different degrees of rapid degradation of MC-LR were observed with varying pH (6–

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The authors declare no competing financial interest.

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10.4), oxidant dosage ($0.5\text{--}3\text{ mg L}^{-1}$), natural organic matter ($0\text{--}7\text{ mg L}^{-1}$), and natural water sources. In contrast to the formation of primarily chloroform and dichloroacetic acid in deionized water where MC-LR serves as the only carbon source, additional chlorinated disinfection byproducts were produced when sand filtered natural water was used as a background matrix. The UV/chlorine treated samples also showed quantitatively less cytotoxicity in vitro in HepaRG human liver cell line tests than chlorination treated samples. Following 16 min (96 mJ cm^{-2}) of UV irradiation combined with 1.5 mg L^{-1} chlorine treatment, the cell viability of the samples increased from 80% after exposure to 1 mg L^{-1} MC-LR to 90%, while chlorination treatment evidenced no reduction in cytotoxicity with the same reaction time.

Graphical Abstract

Introduction

The widespread occurrence of cyanobacteria harmful algal blooms (HABs) in sources of drinking water has resulted in significant challenges for safeguarding water bodies globally. In 2016, severe HABs occurred in more than 20 states in the U.S., from Florida to California, in both inland lakes and coastlines.(1) Certain cyanobacteria can produce a diverse group of toxic metabolites named algal toxins (cyanotoxins). Microcystins (MCs) are a large group (>150 variants) of cyanotoxins common to varied species of cyanobacteria and are geographically diverse.(2) MCs are cyclic heptapeptides differing primarily in amino acid sequence (aa_2 and aa_4), methylation, hydroxylation, and epimerization. MC-LR (named for the substitution of leucine, L, and arginine, R, Supporting Information (SI) Figure S1), is the most common and studied variant. The primary mode of toxicity of MCs is inhibition of protein phosphatases.(3) Increased risk of renal-function impairment due to environmental exposure to MC-LR has been recently reported based on the investigation of >5000 people in rural southwest China.(4) In 2015, the USEPA issued health advisory levels of $0.3\text{ }\mu\text{g L}^{-1}$ MCs for children under 6, and $1.6\text{ }\mu\text{g L}^{-1}$ for other ages.(5) In August, 2014, a “do not drink” order was issued to the residents of the city of Toledo, Ohio, for nearly three days after MCs were detected in finished water.(6) The presence of MCs in finished drinking water is a public concern all over the world,(7–9) indicating cases of failure of conventional treatment processes for removal of such toxins from water, and the need for practical and efficient alternatives or more barriers to be implemented into the water treatment regimen.

Chlorine is the most widely used oxidant for pre- and post-disinfection and has also been widely used for the removal of organic contaminants during water treatment.(10,11) Multiple cyanotoxins have been shown to be degraded successfully by chlorine.(12) However, the elimination rate depends on treatment conditions, including the pH which directly affects the speciation and reactivity of chlorine in water.(13) A high disinfection CT (disinfectant concentration $\times$ contact time) value was thus proposed to ensure the elimination of MCs.(13) However, toxic disinfection byproducts (DBPs) such as trihalomethanes (THMs) and haloacetic acids (HAAs), may be generated through chlorination with synthetic and naturally occurring organic chemicals in water.(14) On the other hand, germicidal UV has been gradually adopted as an alternative by drinking water treatment plants (DWTPs) to inactivate chlorine-resistant microorganisms. Although UV is also capable of eliminating certain organic contaminants, a long irradiation time is required, resulting in high energy consumption and associated cost.(15,16) UV and chlorine may instead be integrated as an advanced oxidation process (AOP) to enhance disinfection and organic chemical degradation. In the UV/chlorine, aqueous chlorine undergoes photolysis to generate reactive oxygen species (ROS) such as hydroxyl radical ($\text{HO}^\bullet$), oxide radical anion ($\text{O}^{\bullet-}$), excited singlet oxygen ($\text{O}(^1\text{D})$), and ozone (O_3),(14,17) as well as reactive chlorine species (RCS) including chlorine radical ($\text{Cl}^\bullet$), chlorine oxide radical ($\text{ClO}^\bullet$), chlorohydroxyl radical ($\text{ClOH}^{\bullet-}$), and dichloride radical anion ($\text{Cl}_2^{\bullet-}$).(18) Among those species, $\text{HO}^\bullet$ is known to be highly reactive toward many organic chemicals,(19) whereas RCS are more selective for electron rich compounds, such as trimethoprim, phenol, quinoline, and N,N-dimethylaniline at near diffusion-controlled rates.(20–22) $\text{Cl}_2^{\bullet-}$ is typically less reactive toward organic compounds than $\text{Cl}^\bullet$.^{23, 24} The role of $\text{ClO}^\bullet$ in UV/chlorine system is an area of active research in recent years due to its high concentrations under treatment conditions and relatively high reaction rates toward several structures, such as phenoxide ions and dimethoxybenzenes.(18,25,26) Since MCs typically contain both diene and phenyl ring moieties which would react with various ROS/RCS generated through UV/chlorine treatment, MCs are expected to be susceptible to this process in DWTPs.

In this study, the UV/chlorine process was employed for the removal of MC-LR. The major degradation contributing factors, that is, UV direct photolysis, chlorine and/or radical species, were identified using various radical probes. Transformation pathways were further revealed based on the detected products from tandem mass spectrometry analysis. The effects of light sources (i.e., regular mercury low pressure UV lamps vs UV-based light emitting diodes (UV-LEDs)), pH, natural organic matter (NOM), and water matrices were evaluated. Formation of regulated chlorinated DBPs was monitored in both presence and absence of natural water matrix. Lastly, the toxicity of treated samples was also assessed in vitro using HepaRG human liver cell line. The outcome of this study provided a useful assessment on the UV/chlorine process which could be potentially applied especially during an algal bloom event to prevent the cyanotoxins from entering the finished water.

Materials and Methods

Chemicals

Sources of chemicals are provided in SI Text S1.

Photolysis Experiments

Most of the UV_{254 nm} irradiation experiments were performed in a laboratory scale collimated beam system with two 15 W UV_{254 nm} lamps (Cole-Parmer, IL) mounted on the top. The average fluence rate through the reaction solution was determined to be 0.10 mW cm⁻² by ferrioxalate actinometry and was monitored regularly with a calibrated radiometer (ILT1700, with XRD (XRL) 140T254 probe, International Light, Co., MA). Light scavenging due to other water constituents was considered, with UV fluence corrected accordingly.(27) Collimated UV-LEDs (PEARLBEAM, AquiSense Technologies, KY), emitting 255 nm (intensity at 0.03 mW cm⁻²), 285 nm (intensity at 0.12 mW cm⁻²), and 365 nm (intensity at 3.05 mW cm⁻²) were also tested in this study both individually and as combined. In a typical experiment, the solution was prepared at 1 mg L⁻¹ (1 μM) MC-LR, 5 mM phosphate buffer (pH 6.0 and 7.4) or borate buffer (pH 9.0 and 10.4), and treated with 1.5 mg L⁻¹ chlorine, unless stated otherwise, and was divided shortly into two aliquots. One was transferred into a Pyrex Petri dish (10 × 60 mm) with a quartz cover for UV irradiation, and the other remained in an amber vial as the dark control. The total solution volume was 10 mL. At the desired reaction time or UV fluence, a 0.1 mL aliquot was sampled and quenched with 100 mg L⁻¹ ascorbic acid (AA), unless stated otherwise, before analysis. Free and total chlorine concentrations were monitored by HACH DR2800 spectrophotometer (Loveland, CO) using DPD method. Residual H₂O₂ was determined by iodometric spectrophotometry.(28)

Analytical Methods

The analytical methods to measure MC-LR, transformation products, and DBPs are detailed in SI Text S2.

In Vitro Cytotoxicity

In mammals including humans, the liver is the main target organ of MCs. HepaRG (human hepatoma, a trademark of BioPredic International, Saint Grégoire, France) cell line has been touted as the best alternative to primary human hepatocytes and established liver cell lines. (29) Differentiated HepaRG retains most liver functions such as expression of high level of P450 activity and all nuclear receptors compared to other popularly used HepG2 and Fa2N-4 cell lines.(30,31)

The toxicity of treated samples was assessed using HepaRG hepatocytes in vitro. Cell cytotoxicities were assessed microscopically and biochemically. Microscopic examination of cell cytotoxicity requires experience and can be subjective. The biochemical test, the XTT cell proliferation assay, provided quantitative results and was statistically analyzed in this study. Water samples were concentrated in a SpeedVac System (Thermo Fisher Scientific, Inc., KY), resuspended in Toxicity Medium (TM, Williams' Medium E supplemented with glutamine and HepaRG toxicity medium supplement; Life Technologies, CA) in the same volume as the water sample, and filter-sterilized (sterile 0.45 μm PTFE syringe filter, Thomas Scientific, NJ). Terminally differentiated cryopreserved HepaRG cells were obtained from Life Technologies (Carlsbad, CA). HepaRG cells were thawed, seeded, maintained and tested for toxicity according to the manufacturer's procedure (Life Technologies HepaRG Cell User Guide). Cells were seeded onto a sterile flat-bottom

collagen coated 96-well plate (4×10^4 cells per well) and cultivated in a humidified 5% CO₂ incubator at 37 °C. On day 7, confluent cells were exposed to treated water samples and returned to a humidified 5% CO₂ incubator at 37 °C. Controls included TM only, TM with 1 mg L⁻¹ MC-LR, and TM with 1.5 mg L⁻¹ Cl₂+AA (ascorbic acid). Only 5 mg L⁻¹ AA was used as the reaction quencher to minimize its effect on the subsequent sample analysis. After 6 h, cells were evaluated for cytopathic effects using a microscope; afterward, the cells were further incubated for the biochemical analysis. After 48 h exposure, all controls and treatments were washed with prewarmed William's Medium E six times, added fresh media and then assayed with XTT cell proliferation kit (ATCC, Manassas, VA). According to the manufacturer's procedure (ATCC XTT Cell Proliferation Assay Kit Instructional Manual), the specific absorbance of the sample = $A_{475 \text{ nm(} \text{Test)}} - A_{475 \text{ nm(} \text{Blank)}} - A_{660 \text{ nm(} \text{Test)}}$. The cell viability was calculated by normalizing the specific absorbance of TM. The student's *t* test was used to determine a *p*-value with an alpha of 0.05 as the cutoff for significance. If the *p*-value is less than 0.05, the null hypothesis is rejected (e.g., no difference between the untreated control and treated samples), indicating that a significant difference does exist. The *t* test was performed only with the biochemical quantitative analysis.

Results and Discussion

Degradation of MC-LR by UV/Chlorine

The decomposition of MC-LR by UV_{254 nm} photolysis, chlorine, and the combined UV/chlorine process was tested. As shown in Figure 1, at pH 7.4, 65% of 1 mg L⁻¹ MC-LR was removed in 15 min by 1.5 mg L⁻¹ chlorine (pseudo first-order rate constant $k_{\text{Cl}_2} = 0.0614 \text{ min}^{-1}$, $R^2 = 0.9979$), whereas UV irradiation alone eliminated roughly 20% MC-LR in 16 min (96 mJ cm⁻², $k_{\text{UV}} = 0.0204 \text{ min}^{-1} = 0.0035 \text{ cm}^2 \text{ mJ}^{-1}$). In contrast, UV/chlorine achieved a complete removal of 1 mg L⁻¹ MC-LR in 16 min ($k_{\text{UV/Cl}_2} = 0.2426 \text{ min}^{-1} = 0.0414 \text{ cm}^2 \text{ mJ}^{-1}$, $R^2 = 0.9999$), and 5 μg L⁻¹ MC-LR in 5 min (30 mJ cm⁻², $k_{\text{UV/Cl}_2} = 0.3385 \text{ min}^{-1} = 0.0578 \text{ cm}^2 \text{ mJ}^{-1}$, $R^2 = 0.9995$). The free chlorine decay after 16 min was 18% and 10% in UV/chlorine and dark chlorination, respectively, as shown in SI Figure S2. In the case of 1 mg L⁻¹ MC-LR, the molar ratio of chlorine to MC-LR was 21:1; while in the case of 5 μg L⁻¹ MC-LR, the chlorine was more than 4000 times in excess. The significant difference in the orders of magnitude caused varied pseudo first-order rate constants, which agreed with the findings of He et al. (2012)(15) and Mash and Wittkorn (2016).(32)

The synergistic degradation by UV/chlorine was probably caused by the efficient generation of ROS and RCS, especially HO•, Cl•, Cl₂•⁻, and ClO•. Considering the decay of chlorine, the removal rates of MC-LR by UV, Cl₂, HO•, and RCS at different time intervals were calculated (Text S3)(25) and shown in SI Figure S3. At 960 mJ cm⁻² of UV/chlorine exposure, the contribution of UV, Cl₂, HO•, and RCS for MC-LR removal was 8%, 27%, 39%, and 26%, respectively.

Radical scavengers, that is, tert-butanol (TBA), nitrobenzene (NB), and bicarbonate (HCO₃⁻), with known rate constants with the aforementioned radicals (SI Table S2), were added individually into the reaction solutions to examine more specifically the contribution of the reactive species to the degradation of MC-LR (SI Text S4). Results of their inhibition

effects at different concentrations are shown in SI Figure S4. $\text{Cl}^\bullet$ and $\text{ClO}^\bullet$ were found to be the two main RCS aiding the degradation of MC-LR. The contributions of different reacting components were further estimated as 8.5%, 25.4%, 42.5%, 11.1%, and 13.3% for UV, Cl_2 , $\text{HO}^\bullet$, $\text{Cl}^\bullet$, and $\text{ClO}^\bullet$, respectively. $\text{HO}^\bullet$ is the most important oxidizing species in this process at neutral pH.

UV-LED, a recently developed semiconductor light source, can be installed flexibly for disinfection and contaminant elimination in various locations. A bench-scale collimated LED system emitting monochromatic UVC light (255 nm) was tested to evaluate the degradation of MC-LR by UV/chlorine (SI Figure S5). A comparison with the traditional UV lamp system found identical destruction rates with the same light intensity ($\approx 0.03 \text{ mW cm}^{-2}$). Compared with the conventional UV lamp, both low voltage source requirements and the mercury-free construction make UV-LEDs more energy-saving and environmental-friendly. Only three min of irradiation was needed to remove 1 mg L^{-1} MC-LR at pH 7.4 when all LEDs with different wavelengths (255, 285, and 365 nm) were switched on. No synergistic effect from combined wavelengths of UV to activate chlorine for the degradation of MC-LR was observed.(33) The quantum yield of $\text{HO}^\bullet$ from HOCl is 1.4 at 254 nm,(34) and 1.0 at 308 nm.(35) The quantum yields from OCl^- at 254, 313, and 365 nm are 0.278, 0.127, and 0.08, respectively.(17) Therefore, although the peak of molar absorptivity of aqueous chlorine at pH 7.4 (containing a mixture of HOCl and OCl^-) is around 300 nm (SI Figure S6), the quantum yields of $\text{HO}^\bullet$ from both HOCl and OCl^- are higher at 254 nm than at around 310 nm.(17,34) As a result, though chlorine activated by UVB (285 nm) or UVA (365 nm) was more effective to degrade MC-LR than UVC in terms of time because of their higher light intensities, UVC was more effective in terms of UV fluence which is more applied in field (Table 1). Therefore, the rest of the study was conducted using conventional collimated UVC lamps for optimum chlorine activation.

With increasing oxidant dosage, UV/chlorine treatment can generate more radicals. By varying the initial chlorine input from 0 to 3.0 mg L^{-1} , the reaction time based elimination rates of MC-LR increased linearly (SI Figure S7). According to eqs 1–4, the concentration of $\text{ClO}^\bullet$ should increase with more chlorine input. Wu et al. (2017)(36) reported that the level of $\text{HO}^\bullet$ remained the same while that of the RCS ($\text{Cl}^\bullet$, $\text{Cl}_2^{\bullet-}$, and $\text{ClO}^\bullet$) increased with increasing chlorine as indicated by the kinetic modeling study. Thus, it can be expected that the contributions of chlorine and RCS was more promoted to degrade MC-LR at a higher chlorine dosage. The slope of the dose–response curve by UV/chlorine is approximately 2.5 times higher than that by chlorination alone. In DWTPs where a UV fluence rate of higher than 0.1 mW cm^{-2} is typically used, a higher slope by UV/chlorine can be obtained and a larger difference between UV/chlorine and chlorination alone can be expected. Overall, to reach the same degradation rate of MC-LR in clean water, UV/chlorine required only 1/3 of chlorine dosage than chlorination alone (SI Figure S8).(17,34,37,38,39)

Degradation Products

The products of MC-LR degradation by both UV/chlorine and chlorination treatment were investigated by tandem mass spectrometry and are summarized in SI Table S3. Due to the complexity of the MC-LR structure, isomeric transformation products can be formed following reactions at different sites of the molecule which are indistinguishable by mass alone. In some cases, these can be distinguished by differences in chromatographic retention times (RT, SI Figure S9). In other cases, an analysis of both the full-scan mass spectrum to identify parent masses, and a selected reaction monitoring (SRM) analysis to identify those products which can generate a fragment of m/z 135.0 (corresponding to the Adda moiety, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid) can provide additional information related to the location of transformations of the microcystin molecule. In particular, products identified at a specific retention time which did not generate an SRM product at m/z 135.0 were considered likely to contain substitutions or transformations on the Adda moiety. Note that there could be compounds that were unstable under the analytical conditions. Due to the unavailability of standards for these transformation products, in addition to an inability to directly compare peak areas to concentration, their relative stability under ionization or fragmentation conditions cannot be determined. This means in particular that it could be unlikely to use the absence of a product from LC/MS/MS analysis to eliminate a potential reaction pathway or product. Therefore, the pathway is preliminarily illustrated in this manuscript based on the detected products.

Of the reactive species identified in Degradation of MC-LR by UV/Chlorine of this study, $\text{HO}^\bullet$ can react via hydrogen abstraction, hydroxyl addition, and electron transfer pathways. Reactions with $\text{Cl}^\bullet$ are known to proceed primarily via chlorine addition and hydrogen abstraction processes, with the possibility of electron transfer.(23,24) In contrast, $\text{ClO}^\bullet$ oxidizes organic compounds mainly through electron transfer.(26) However, because each of these reactive species was demonstrated to play a role in the degradation of MC-LR by UV/chlorine, it is difficult to distinguish the participation of individual radicals in generation of specific products. LC/MS/MS analysis identified products from photoisomerization, hydroxylation, chlorination, hydration, and bond cleavage processes, leading to the proposed transformation pathways shown in Figure 2. Based on analysis of the product mixtures, as the duration of UV/chlorine treatment increased, the concentration of most of the identified products increased up to 16 min of exposure (96 mJ cm^{-2}). The followed depletion of MC-LR and degradation/further transformation to unidentified products resulted in a decrease in their residuals (Figure 3b and SI Figure S10). In contrast, in the dark chlorination process, these products were generally stable for the duration of the experiment. While the individual response ratios of these transformation products are unknown and thus cannot be directly related to concentration, an examination of the total peak area of products with m/z above 500 is presented in Figure 3a as a reference to show the general trend of the products.

More than five distinct products with m/z 1011.5 (corresponding to addition of a single oxygen to MC-LR) were identified from the mass spectrometry analysis. By comparing the full scan and the SRM chromatograms (SI Figure S9a) for the 1011.0–1012.0 mass range and the 1011.5 $\rightarrow$ 135.0 m/z transition, it was observed that multiple peaks (RT 3.3, 3.4, and 3.6 min) were present in the full scan analysis, but were absent in the SRM analysis, suggesting the transformation of the phenyl ring or diene of the Adda moiety (which are responsible for the 135 m/z product quantified in SRM mode). Other products were observed in both full scan and SRM analysis, some of which could correspond to isomers formed from hydroxylation of the double bond in the Mdha (methyl dehydroalanine). Mechanistically, the observation of multiple products transformed by addition of a single oxygen atom can be attributed to a $\text{HO}^\bullet$ addition onto the double bond to form an alkyl radical, followed by the reaction with O_2 to produce peroxy radical. Subsequent transformation into the enol-MC-LR (product **a**), followed by tautomerization, could yield aldehyde-MC-LR (product **b**, proposed mechanism in SI Scheme S1). A similar mechanism can be proposed for the hydroxylation of either side of the diene bond in Adda, where one hydroxyl group could be added onto C_5 or C_7 (products **c** and **c'**) to produce a ketone-MC-LR with a conjugated π bond (products **d** and **d'**). Alternatively, the alkyl radical in Mdha and allylic radical in Adda could react with another $\text{HO}^\bullet$ to form several diol-MC-LR products (m/z 1029.5, **e**, **f**, and **g**, SI Scheme S1). Dehydration of the two adjacent hydroxyl groups at C_6 – C_7 of the products could result in bond cleavage to produce the ketone (m/z 835.4, product **h**).⁽⁴⁰⁾ Additionally, hypochlorous acid (HOCl) can be added across any of the double bonds of MC-LR to generate product **i** (m/z 1047.5) with or without UV irradiation. The alkene hydration product **j** (m/z 1013.5) was also observed in both processes.

Another site susceptible to oxidation is the aromatic ring. An aromatic radical cation can be generated via electron transfer from $\text{HO}^\bullet$ or $\text{ClO}^\bullet$ and transformed to a hydroxycyclohexadienyl radical intermediate, which could also be produced by direct $\text{HO}^\bullet$ addition to the aromatic ring (SI Scheme S2). The product **k** with m/z 1011.5 could be formed following hydroxylation primarily on the ortho and para positions of the phenyl ring, and would correspond to one of the Adda-transformed products from the SRM analysis.⁽⁴¹⁾ Monohydroxyl-MC-LR (product **k**) could also undergo hydration at one of the diene bonds to generate product **l** (m/z 1029.5). Product **l** was not stable during UV/chlorine among all of the m/z 1029.5 products (SI Figure S9b), where the SRM and full scan chromatograms were similar indicating these products were not formed from species attacking the phenyl ring. The product **l** was readily attacked by extra $\text{HO}^\bullet$ to yield product **m** (m/z 1045.5), because hydroxylation increases the electron density and thus the reactivity of the aromatic system (SI Scheme S3).⁽⁴²⁾ The diol-MC-LR product **g** could form trihydroxyl-MC-LR product **n** (m/z 1045.5), which could also lose the Adda moiety to produce product **h** (m/z 835.4). The products that have also been detected in other AOPs are listed in SI Table S3.

Similar to hydroxylation on the aromatic ring, the aromatic radical cation formed by electron transfer could react with Cl^- to produce a chlorocyclohexadienyl radical⁽²³⁾ (SI Scheme S4). Alternatively, $\text{Cl}^\bullet$ could also be added directly to the aromatic ring resulting in the formation of an identical chlorocyclohexadienyl radical intermediate. Subsequent reaction

with dissolved O₂ could yield peroxy radical and eventually monochloro-MC-LR product **o** (m/z 1029.5). Dechlorination of the aromatic ring could be achieved by direct UV photolysis of the C–Cl bond; thus the chlorination of MC-LR was reversible in UV/chlorine system(43) which is consistent with the observation in SI Figure S9b with product **o** to be unstable during UV/chlorine. Furthermore, monochloro-MC-LR could be attacked on the aromatic ring by HO• to form chlorohydroxycyclohexadienyl radical intermediates, which could subsequently produce hydroxyl-MC-LR with m/z 1011.5 (product **k**) (SI Scheme S5). Alternatively, hydration of the diene bond on product **o** led to the detection of another product with m/z 1047.5 (product **p**), susceptible to dechlorination and hydroxylation to form product **i** (m/z 1029.5).

Most of the above products were also found when MC-LR was subject to chlorine in dark conditions (SI Table S3). However, the formation of what were identified as geometric isomers of MC-LR was only observed following UV irradiation; these two products, at m/z 995.5, were distinguishable from MC-LR solely by the retention time, and were not observed in any of the samples that were not irradiated. MC-LR is known to undergo UV-induced photoisomerization of one or both of the unsaturated positions of the Adda moiety to produce (4)-*E*,(6)-*Z* or (4)-*Z*,(6)-*E* MC-LR isomers, generating isomers with identical mass and fragmentation patterns to MC-LR but slight shifts in retention time.(44) On this basis we attribute these observed products to geometric isomers of MC-LR. It has been reported that the arginine moiety is also susceptible to hydroxyl radicals to lose the guanidine group,(45,46) or react with chlorine to form N–Cl bonds.(47,48) However, no transformation products representative of guanidine loss were observed, whereas N-chlorinated products were not distinguishable from chlorine adducts elsewhere in the molecule with our methodologies.

The products generated during chlorination are in agreement with published studies;(47–51) however, the mechanism of the chlorination process has not been fully elucidated. Special attention has been paid to the species and amounts of chlorinated MC-LR products observed in both UV/chlorine and chlorination alone processes, owing to their potential toxicity.(51) These two processes appeared to share similar products, as shown in SI Table S3, which may partially be due to the strong contribution of chlorination in the UV/chlorine process. The monochloro-dihydroxyl-MC-LR (m/z 1063.5) was only found in reaction in the absence of UV irradiation, and more peaks of monochloro-monohydroxyl-MC-LR (m/z 1047.5, products **i** and **p**) were identified under dark chlorination conditions. Among all the chlorinated MC-LR products which have distinct peaks, higher peak areas were observed following exposure to 1.5 mg L⁻¹ chlorine solution than under UV/chlorine (Figure 3b). After 2 h of exposure to chlorine, the levels of chlorinated products remained unchanged in dark; while under UV irradiation chlorinated species were observed to decrease to below detection limit. One explanation is that UV irradiation resulted in faster degradation which precluded exhaustive substitution processes. Alternatively, the irradiation may have enhanced dehalogenation pathways through photocleavage of C–Cl bonds. The amounts of other major nonchlorinated products (m/z 1011.5 and 1029.5) followed similar trends, and small product **h** (m/z 835.4) reached the same concentration after 1 h oxidation by UV/chlorine and dark chlorination (SI Figure S10). The difference between the trends of

transformation products in UV/chlorine and dark chlorination suggests that UV/chlorine AOP was more effective at removing intermediate oxidation/substitution products.

The products generated through the oxidation of MC-LR contained a high level of amino acids, benzene rings, phenols, and methyl ketones, which are important THM and HAA precursors.(49,52) The formation of THMs (including chloroform (CF), bromodichloromethane (BDCM), dibromochloromethane (DBCM), and bromoform (BF)) and HAAs (including monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), monobromoacetic acid (MBAA), and dibromoacetic acid (DBAA)) were monitored. After a series of oxidation steps, the degraded MC-LR produced some chlorinated DBPs, such as CF and DCAA (Figure 3a). Other DBPs were not detected in this study. Both the concentrations of CF and DCAA from MC-LR in DI water were higher following UV/chlorine treatment, probably because of the faster rates of degradation/oxidative transformations under those conditions. When the treatment time increased from 30 min (180 mJ cm^{-2}) to 2 h (720 mJ cm^{-2}), the yields of CF and DCAA (i.e., molar concentration of DBPs normalized to molar concentration of decayed MC-LR) in UV/chlorine increased from 1.84% to 3.68%, and 1.55% to 5.32%, respectively; whereas for chlorination without UV exposure, the yields of CF and DCAA increased from 1.68% to 2.51%, and 0.78% to 2.67%, respectively. To evaluate the effects of DBPs formation following UV/chlorine treatment, samples were held in the dark for 24 h following treatment. During this period both CF and DCAA concentrations increased significantly (SI Figure S11). However, with longer UV/chlorine exposure time, there could be a lower level of DBP precursors and a lower chlorination residual for further dark reactions. Thus, the final DBPs formation after 24 h was reduced following well the trend of residual free chlorine after UV irradiation, suggesting both the UV/chlorine exposure and the dosage of chlorine played an important role in DBPs formation in these cases.

Cytotoxicity Evaluation

Because the UV/chlorine treatment results in formation of chlorinated products, it was important to investigate whether there was a potential for the generation of species with increased cytotoxicity, particularly since the toxicity of chlorinated MCs are not well studied. In this study, an in vitro cell cytotoxicity assay was developed using differentiated HepaRG human liver cells exposed to treated water samples. Cell cytotoxicities were assessed microscopically and biochemically using XTT cell proliferation assay kit.

Morphological changes, including cell rounding, swelling, clumping, blebbing, and cells detaching, were readily observed under a microscope after 6 h of exposure to 1 mg L^{-1} MC-LR (SI Figure S12). Highly refractile amorphous clumps were also observed at the early stages of exposure. Reduced cytotoxicity was observed for exposure to 1 mg L^{-1} MC-LR treated with $1.5 \text{ mg L}^{-1} \text{ Cl}_2$ in dark for 16 min (SI Figure S12c), which is similar to that by $0.5 \text{ mg L}^{-1} \text{ Cl}_2$ with 96 mJ cm^{-2} UV irradiation (SI Figure S12d), suggesting that the incorporation of UV treatment with chlorine reduced the amount of oxidant required to achieve the same reduction in cytotoxicity. The degradation of MC-LR by $1.5 \text{ mg L}^{-1} \text{ Cl}_2$ with UV for 96 mJ cm^{-2} UV irradiation revealed no cell cytotoxicity (SI Figure S12e), in

agreement with the MC-LR degradation kinetics and the level of chlorinated MC-LR products formed in different systems.

The biochemical assay required 48 h exposure compared with about 6 h exposure for the microscopic analysis. Figure 4 shows the quantitative cytotoxic effects of the treated water. The cell viability of 1 mg L⁻¹ MC-LR was 20% lower than the control sample. Chlorine (1.5 mg L⁻¹) treatments of MC-LR showed no reduction in cytotoxicity in 16 min ($p = 0.77$) and slight increase in viability after 30 min of reaction ($p = 0.06$). With 16 min (96 mJ cm⁻²) UV and 1.5 mg L⁻¹ chlorine treatment, when MC-LR was reduced to below detection limit, the observation of 90% viability ($p = 0.01$) indicated that there was certain cytotoxicity associated with the transformation products. After 30 min (180 mJ cm⁻²) of UV/chlorine exposure, the cell viability reached 98% ($p = 2.7 \times 10^{-4}$), consistent with the trend of MC-LR and the total transformation products (Figure 3a). Although UV + 0.5 mg L⁻¹ Cl₂ removed MC-LR at a similar rate to 1.5 mg L⁻¹ Cl₂ (SI Figure S8), the former treatment is likely to cause more extensive product degradation via radical processes, resulting in the observed faster decrease in cytotoxicity. Furthermore, the cell viabilities of UV/chlorine with different chlorine concentrations at 180 mJ cm⁻² UV were comparable ($p = 0.89$), although the detected MC-LR level concentration was higher with less chlorine (SI Figure S8), potentially resulting from the formation of more DBPs at higher chlorine dose.

Effects of pH and NOM in MC-LR Degradation

The pH in most US inland lakes experiencing HABs is in the range of 8–10, and increases with the severity of the blooms. In contrast, chlorination is only generally effective removing MC-LR at pH < 8 due to the lower reactivity of ClO⁻ which predominates at elevated pH values.(13) To examine if the addition of UV irradiation can overcome this limitation, the effect of pH on MC-LR degradation was tested (Figure 5), noting that the dominant species of MC-LR does not change in the range of pH 6–10.4 because the p*K*_a's of MC-LR are 2.09, 2.19, and 12.48.(53) As expected, the degradation rate decreased with increasing pH (7.4), consistent with the lower radical quantum yield of OCl⁻ photolysis as compared with HOCl (0.97 vs 1.45, respectively).(54) In addition, the consumption of HO[•] and Cl[•] by OCl⁻ was faster than by HOCl (eqs 1–4), leading to fewer reactive radicals remaining in the system for the oxidation of MC-LR. Nevertheless, UV/chlorine degraded 1 mg L⁻¹ MC-LR to below detection limit with 120 mJ cm⁻² at pH 9, suggesting that this process was effective under wider pH ranges than direct chlorination. The degradation of a large variety of organic contaminants by UV/chlorine has been reported to be faster at acidic pH.(34,54,55) However, slower kinetics at pH 6 was observed for MC-LR elimination, which has been proposed to result from protonation of amine moieties of the arginine moiety.(48)

The presence of NOM in natural water is highly problematic for chlorination due to additional oxidant demand and the potential formation of harmful DBPs. In UV/chlorine process, NOM could inhibit MC-LR removal by competing for UV photons and oxidative radical species. It has been reported that NOM reacts with HO[•], Cl[•], and ClO[•] at the rates of 2.5×10^4 , 1.3×10^4 , and 4.5×10^4 (mg L⁻¹)⁻¹ s⁻¹, respectively, thus contaminants with degradation dominated by HO[•] such as MC-LR may be somewhat less affected by NOM than those with degradation dominated by ClO[•].(22) This was demonstrated when only 15%

MC-LR was removed following chlorination in the dark after 32 min, in the presence of 7 mg L⁻¹ NOM (Figure 6 and SI Figure S13), in contrast to approximately 60% removal by UV/chlorine treatment (equivalent to 169 mJ cm⁻² after considering UV absorption of NOM). Consumption of chlorine was also larger in the presence of NOM, with less than 0.02 mg L⁻¹ of free chlorine and 0.15 mg L⁻¹ of total chlorine detected; therefore, the degradation does not follow pseudo first-order kinetics.

MC-LR Degradation in Field Water Matrices

Various components in natural waters such as pH, NOM, alkalinity, and inorganic ions can influence the overall degradation efficiency of contaminants by AOPs. Thus, source waters experiencing HABs were tested to validate the efficacy of UV/chlorine system. In this study, water samples were collected from the lake buoy (BUOY) and DWTP intake (WTPI) of Lake Harsha, Ohio during the bloom season in 2016. After prefiltration through a 0.45 µm pore size membrane, the water samples were spiked with 5 µg L⁻¹ MC-LR. The water quality information for these samples is listed in SI Table S4. Compared to the control data in Figure 1, MC-LR was barely removed with treatment using 1.5 mg L⁻¹ chlorine, whereas only half of the 5 µg L⁻¹ toxin was removed by UV/chlorine (SI Figure S14), probably due to the high DOC levels in the water matrices. A chlorine dosage of 4 mg L⁻¹ resulted in 60% removal within 8 min in the presence of UV light in the BUOY sample. The reaction was halted because of a lack of chlorine residual. In contrast to the 1.5 mg L⁻¹ chlorine samples, for the 4 mg L⁻¹ chlorine dosage samples, the impact of DOC levels appeared to be more significant, with reduced DOC in the WTPI resulting in much faster degradation rates than in the higher DOC BUOY sample.

Water samples from different treatment stages in Greater Cincinnati Water Works (GCWW, SI Table S4), were tested to evaluate if the location within the treatment train would influence the outcome (SI Figure S15). In raw water, neither chlorination nor UV/chlorine achieved any satisfactory toxin degradation. Sand filtration, which reduced DOC concentrations to 2.09 mg L⁻¹, improved the treatment efficiencies by both processes. With the strong augmentation of UV transmittance and reduction of DOC by GAC (UVT = 98.8% and DOC = 0.43 mg L⁻¹), MC-LR decomposition rate in GAC effluent was found to be comparable with that in DI water. In conclusion, GAC is an effective pretreatment process to improve the degradation of MC-LR by AOPs including UV/chlorine by reducing the amount of DOC in competition for radicals.

Formation of DBPs was further evaluated using sand filtrated samples from GCWW as a background matrix in this study. Samples were spiked with 5 µg L⁻¹ MC-LR, treated by UV + 1.5 mg L⁻¹ chlorine for no more than 400 mJ cm⁻², and left subsequently in dark for 24 h. Different from the case in DI water (SI Figure S11), CF and BDCM were dominant THMs, and major HAAs were found to be DCAA and TCAA (Figure 7). The speciation of DBPs in natural water was more dependent upon the NOM in the water matrices, while in DI water it was influenced by MC-LR. The chlorine residual decreased with a longer UV/chlorine exposure, resulting in the slight decrease in the formation of DBPs after 24 h dark reaction. Reducing the chlorine level to 0.5 mg L⁻¹ resulted in fewer DBPs formation, indicating that the amounts of DBPs are highly dependent on chlorine dose. When the effluent of GAC

spiked with $5 \mu\text{g L}^{-1}$ MC-LR was treated by UV+ 1.5 mg L^{-1} chlorine with 24 h subsequent chlorination, levels of DBPs were much lower than in the effluent of sand filtration, and did not change much with UV/chlorine exposure (SI Figure S16). In all cases, the total DBPs measured were below the USEPA regulatory levels, which are $80 \mu\text{g L}^{-1}$ for total THMs and $60 \mu\text{g L}^{-1}$ for total HAAs.(56)

Engineering Implications

UV/H₂O₂ is one of the most widely accepted AOPs for water treatment and reuse; however, H₂O₂ is not sufficiently effective in producing HO[•] and leaves a high amount of residual H₂O₂. Considering that chlorine is typically dosed as a final process for disinfection and needs to be maintained in the distribution system, this residual H₂O₂ could increase chlorine demand by $2.09 \text{ mg L}^{-1} \text{ Cl}_2$ per $\text{mg L}^{-1} \text{ H}_2\text{O}_2$.(57) leading to a rise in cost and risk of DBPs formation in distribution systems. The cheaper oxidant, chlorine, is more readily activated by UV. The formation of halogenated DBPs is the most serious concern in using UV/chlorine for drinking water treatment. Compared to chlorination alone, UV/chlorine may either increase or decrease the formation of THMs and HAAs depending on the matrix and treatment conditions.(14) Most of the studies only compared the DBPs formed with an identical concentration of chlorine in UV and dark conditions;(58–60) however, to reach the same degradation rate of the target contaminants, the addition of UV could allow for a reduction in chlorine dosage, thus the comparison could be better performed with less oxidant input in UV/chlorine than in chlorination. Lower DBPs formation might be possible under these conditions but needs to be carefully evaluated. In this study 2/3 of chlorine could be saved by coupling UV with chlorination to remove the same amount of MC-LR in DI water, and more than 2/3 of the DBPs were reduced in filtered water samples. Additional chlorine may be dosed before discharging the treated water into distribution systems for disinfection, and the final DBP formation needs further evaluation.

In addition, the results using UV-LEDs (SI Figure S5) show that a variety of wavelengths of UV may activate chlorine to remove, at a different extent, MCs. Since UV-LED technology has been in very quick development in recent years, and its intensity and stability are expected to improve, large-scale UV-LED application combined with chlorine is highly promising for water treatment. For the utilities which have already installed UV facilities and which dose chlorine following UV disinfection process to maintain a chlorine residual through distribution systems, it might be beneficial to add chlorine before the UV unit, particularly during an algal bloom event, to prevent the MC-LR from entering the finished water. The UV and chlorine doses required in UV/chlorine treatment to degrade MCs from environmental level to below USEPA health advisory value are easily achievable by utilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The project was supported by a Harmful Algal Bloom Research Initiative grant from the Ohio Department of Higher Education. X. Duan is thankful to the Grants-in-Aid of Research from Sigma Xi Society University of Cincinnati Chapter, and Summer Research Fellowship from University of Cincinnati Research Council. We also

appreciate Greater Cincinnati Water Works for providing water samples in the treatment train. D.D.D. also acknowledges support from the University of Cincinnati through a UNESCO co-Chair Professor position on “Water Access and Sustainability” and the Herman Schneider Professorship in the College of Engineering and Applied Sciences. The U.S. Environmental Protection Agency, through its Office of Research and Development collaborated in the research described herein. It has been subjected to the Agency’s peer and administrative review and has been approved for external publication. Any opinions expressed in this paper are those of the author(s) and do not necessarily reflect the views of the Agency, therefore, no official endorsement should be inferred. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Figure 1. Degradation of MC-LR by UV, chlorination, and UV/chlorine. $\text{UV}_{254 \text{ nm}}$ fluence rate = 0.1 mW cm^{-2} , $[\text{Cl}_2]_0 = 1.5 \text{ mg L}^{-1}$, pH 7.4 maintained by 5 mM phosphate buffer.

Figure 2.
Proposed transformation pathway of MC-LR during UV/chlorine.

Figure 3.

Time-dependent peak areas for (a) MC-LR, sum of products with $m/z > 500$, and DBPs, and (b) major chlorinated products identified in mass spectrometry. UV_{254 nm} fluence rate = 0.1 mW cm⁻², [MC-LR]₀ = 1 mg L⁻¹, [Cl₂]₀ = 1.5 mg L⁻¹, in DI water.

Figure 4.

HepaRG human liver cell toxicity. UV_{254 nm} fluence rate = 0.1 mW cm⁻², [MC-LR]₀ = 1 mg L⁻¹, [AA] = 5 mg L⁻¹, in autoclaved DI water.

Figure 5.

Effect of pH on the degradation of MC-LR by UV/chlorine and chlorination. UV_{254 nm} fluence rate = 0.1 mW cm⁻², [MC-LR]₀ = 1 mg L⁻¹, [Cl₂]₀ = 1.5 mg L⁻¹, 5 mM phosphate buffer (pH 6.0 and 7.4) or borate buffer (pH 9.0 and 10.4).

Figure 6.

Effect of NOM in degradation of MC-LR by chlorine and UV/chlorine. $[\text{MC-LR}]_0 = 1 \text{ mg L}^{-1}$; $[\text{Cl}_2]_0 = 1.5 \text{ mg L}^{-1}$, pH 7.4.

Figure 7.

Formation of DBPs in sand filtered water samples from GCWW spiked with 5 μg L⁻¹ MC-LR, treated by UV/chlorine with respective exposure, and subsequently held in the dark for 24 h. [Cl₂]₀ = 1.5 mg L⁻¹ in solid columns and 0.5 mg L⁻¹ in patterned columns.

Table 1.

Degradation Rate of MC-LR by Different UV-LED Wavelengths

	255 nm (UVC)	285 nm (UVB)	365 nm (UVA)
Peak wavelength (nm)	256	285.7	366.1
Average intensity (mW cm ⁻²)	0.03	0.12	3.05
Pseudo-first order rate constant (min ⁻¹)	0.1310	0.3065	0.3968
Pseudo-first order rate constant (cm ² mJ ⁻¹)	0.0772	0.0561	0.0017