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Solar photo-Fenton treatment of microcystin-LR in aqueous environment: Transformation products and toxicity in different water matrices

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Abstract

Transformation products and toxicity patterns of microcystin-LR (MC-LR), a common cyanotoxin in freshwaters, during degradation by solar photo-Fenton process were studied in the absence and presence of two major water components, namely fulvic acid and alkalinity. The transformation products m/z 795, 835, 515/1030 and 532 can be formed through attack of $\text{OH}\cdot$ on the conjugated carbon double bonds of Adda. Transformation products with m/z 1010, 966 and 513 can be generated through the attack of $\text{OH}\cdot$ on the methoxy group of Adda. The transformation products m/z 783, 508 and 1012 can be originated from the attack of $\text{OH}\cdot$ on the cyclic structure of MC-LR. Transformation products (m/z 522, 1028, 1012, 1046 and 514) formed after hydroxylation of the aromatic ring with $\text{OH}\cdot$ were also identified in this study. The toxicity study revealed that fulvic acid and alkalinity strongly influence the toxicity profiles of solar photo-Fenton treated MC-LR. Fulvic acid enhanced the detoxification whereas low level total alkalinity ($1.8 \text{ mg L}^{-1} \text{ CaCO}_3$) inhibited the detoxification of MC-LR by solar photo-Fenton process as assessed by protein phosphatase-1 (PP-1) inhibition assay. This work provides insights on the utility of solar photo-Fenton destruction of MC-LR in water based on transformation products and toxicity data.

Graphical Abstract

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Appendix A. Supplementary data

Keywords

Cyanotoxins; Microcystin-LR; Solar photo-Fenton; Toxicity; Transformation product

1. Introduction

The frequent occurrence of harmful blooms of cyanobacteria is a serious public health concern. These harmful blooms can produce toxins (cyanotoxins) which are hazardous to human and ecological health. The most commonly encountered toxins produced by cyanobacteria in contaminated freshwaters are microcystins. Microcystins are cyclic heptapeptides which are potent hepatotoxins and tumor promoters. The main target organ is the liver, but the kidneys and gastrointestinal tract can be affected [[1], [2]]. Epidemiological studies in China, Serbia and the United States showed positive association of liver cancer and exposure to cyanotoxins [[3], [4], [5]]. Several incidents of acute wild and domestic animal poisoning and death as well as human intoxication have also been documented [[6], [7], [8], [9]]. Human fatalities have been reported in dialysis patients exposed to microcystins in Brazil in 1996 [10].

Microcystins have been detected worldwide in terrestrial and aquatic environments with concentrations as high as 1300 µg L⁻¹ [11,12]. Microcystin-LR (MC-LR) is the most well studied variant due to its ubiquity, abundance and toxicity. Several genera of cyanobacteria such as *Microcystis*, *Anabaena*, *Planktothrix*, and *Nostoc* have been shown to produce microcystins [13]. Environmental factors when harmful blooms will occur and cyanotoxins are produced have not been fully elucidated. Increased eutrophication and global climate change will most likely exacerbate harmful blooms of cyanobacteria increasing the likelihood of exposure to their toxins [14]. Humans are primarily exposed to microcystins orally either by accidentally drinking contaminated water recreationally or drinking improperly treated water. Microcystins are stable in natural waters with a half-life of 10 weeks [15]. The cyclic structure and novel amino acid composition render microcystins resistant to heat, chemical hydrolysis and biological oxidation [16].

The potential hazard of microcystins to human health calls for the continuous development of control programs for cyanobacteria and their toxins especially in drinking water treatment plants. Conventional treatment methods (*i.e.*, filtration, coagulation and sedimentation) have

shown limited efficacy for the remediation of cyanotoxin-contaminated water especially during harmful bloom events [17]. A group of alternative chemical technologies, advanced oxidation processes (AOPs), have been shown to be effective in degrading pollutants and toxins. AOPs utilize oxidants, catalysts and/or radiation, or their combination, to generate highly reactive, non-selective oxidizing radical species responsible for detoxifying organic compounds in water.

AOPs are highly efficient chemical treatment methods which are based on the reactions of radical species including hydroxyl radicals ($\text{HO}\cdot$), the second most powerful oxidant after fluorine (2.8 V *vs* standard hydrogen electrodes) [18]. AOPs have been shown to be capable of successfully degrading microcystins found in water [19,20]. The photo-Fenton process ($\text{Fe}^{2+}/\text{H}_2\text{O}_2/h\nu$) is among the most studied AOPs, as evidenced by the number of publications on this process [21]. The application of photo-Fenton process under solar irradiation has been also shown for a range of pollutants at both laboratory and pilot plantscales, eliminating the operational cost of artificial UV light sources [[22], [23], [24]]. Despite this potential of photo-Fenton process in water treatment, its application for the degradation of aqueous microcystins has been limited to a few research papers [[25], [26], [27]].

While AOPs are very efficient in degrading many environmental pollutants in water, total mineralization is expensive at high amounts of chemical reagents and prolonged irradiation times [28]. AOPs can be used for partial degradation and mineralization of pollutants to save operational cost, but this has the main drawback of generating transformation products that are more toxic than their parent compounds [29]. At this point, assessment of transformation products and toxicity gains importance in extending the understanding of the overall efficiency of AOPs for the degradation of environmental pollutants. A number of studies have been reported on the transformation products during degradation of microcystins by AOPs [[30], [31], [32], [33]].

However, toxicity tests are lacking on the biological activity of microcystin-containing waters subjected to AOPs [34,35]. Furthermore, to the best of our knowledge, the influence of major chemical components of freshwaters on transformation products and toxicity profiles has not been previously studied during degradation of microcystins by AOPs. Interpretation of transformation product and toxicity data in relation to chemical water characteristics can help increase understanding the suitability of a proposed treatment for the degradation of microcystins in freshwater environments.

This study focused on the analysis of transformation products and assessment of toxicity during the degradation of MC-LR by the photo-Fenton process using a solar simulator in the absence and presence of HCO_3^- and fulvic acid, two major chemical components of freshwaters. In the first part of the study, baseline experiments were conducted to assess the impact of certain water quality and process parameters, such as pH, bicarbonatealkalinity, and the presence of isolated natural organic matter (NOM), on the performance of solar photo-Fenton process for the degradation of MC-LR. In the second part, transformation products were characterized and monitored over reaction time using liquid chromatography with quadrupole time-of-flight mass spectrometry (LC/Q-TOF MS) under the experimental

conditions established in the first part of the study. In the final part of the work, the biological activity of the treated samples was followed during solar photo-Fenton degradation of MC-LR by means of cell viability, namely protein content (PC), tetrazolium salt reduction (MTS) and neutral red (NR) cellular uptake, and PP-1 inhibition assays.

2. Materials and methods

2.1. Materials

Pure MC-LR (>95%, molecular weight = 995.17 g mol⁻¹) was purchased from CalBiochem. A 500 µM (~500 mg L⁻¹) stock solution of MC-LR was prepared by dissolving 0.5 mg of solid MC-LR with 1 mL of autoclaved Milli-Q water. This stock solution was diluted to 100 µM (~100 mg L⁻¹), and 1.0 µM toxin concentration was achieved by spiking 100 µL of 100 µM MC-LR solution in Milli-Q water. Milli-Q water with a conductivity of 0.05 µS cm⁻¹ produced by a Milli-Q Synthesis A10 system (Millipore) was used throughout the experiments. FeSO₄·7H₂O and H₂O₂ (50%, w/w) were obtained from Sigma and Fisher Scientific, respectively. Acetonitrile, trifluoroacetic acid and methanol were obtained from Fisher Scientific, and used to prepare mobile phases for the high performance liquid chromatography (HPLC) measurements. MC-LR and all reagents employed in the toxicity study were used without further purification. All chemicals for (bio)analytical and experimental procedures were at least of analytical grade.

2.2. Solar photo-Fenton photocatalytic experiments

In a typical experiment, MC-LR stock solution and H₂O₂ solution were added to a borosilicate glass Petri dish (Pyrex, 60 mm diameter×15 mm height) to a final volume of 10 mL, and enclosed with a quartz cover. The addition of FeSO₄·7H₂O solution was recognized as the starting point of the solar photo-Fenton process. In experiments where initial pH was to be set to predetermined values, H₂SO₄ and NaOH solutions with varying concentrations were used to adjust the pH. The solution was irradiated from the top with a 500-W Hg(Xe) lamp model 66142 (Newport-Oriel Instruments) as the simulated solar light source with an atmospheric attenuation (AM 1.5) filter (Newport-Oriel Instruments). The simulated solar light source delivered stable, low to medium power UV to NIR radiation in the wavelength range of 200–2500 nm with a maximum at 469.3 nm. The spectral output of the simulated solar light source in 200–800 nm wavelength range is given in Fig. S1. The light intensity was 70 mW cm⁻² and was measured with a radiant power meter (Newport Corporation). The solution was magnetically mixed at ambient temperature throughout the reaction. Samples (100 µL) were taken at specific time intervals, and immediately quenched with 100 µL of methanol for the chemical analyses and with 100 µL of 220 units mL⁻¹ catalase solution (Sigma, 51,100 units mg⁻¹) for the toxicity study.

2.3. Chemical analyses

2.3.1. Sample analysis on HPLC—MC-LR was monitored by a 1100 Series HPLC (Agilent Technologies) system equipped with a diode array detector and a Discovery HS C18 column (150 mm × 2.1 mm, 5 µm particle size, Supelco). The mobile phase consisted of 0.05% trifluoroacetic acid in acetonitrile: 0.05% trifluoroacetic acid in water (40:60, v/v)

with a flow rate of 0.2 mL min^{-1} . The detection wavelength and injection volume were set at 238 nm and 20 μL , respectively. The column temperature was maintained at 40 °C.

2.3.2. Sample analysis on LC/Q-TOF MS—The separation of reaction byproducts from solar photo-Fenton experiments was performed in Agilent 1290 infinity HPLC system (Agilent Technologies) equipped with a Zorbax Eclipse XDB C_{18} narrow-bore rapid resolution column ($2.1 \times 50 \text{ mm}$, $3.5 \mu\text{m}$, Agilent Technologies). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a gradient elution starting from 2% B for 1 min, followed by a linear increase to 95% B in the next 4 min, and back to 2% B in the last 0.1 min. The sample injection volume was set as 30 μL .

The transformation products of MC-LR were identified using an Agilent 6540 ultra-high definition accurate-mass Q-TOF MS with an electrospray ionization source. Mass spectra were obtained in positive ion mode in mass-to-charge ratios (m/z) between 500 and 1300. The obtained data were analyzed by Agilent MassHunter B.04.00 software providing a list of possible chemical formulas based on m/z (relative mass differences $\leq 5 \text{ ppm}$) and isotopedistribution. The structures of the byproducts were proposed according to their mass spectra, possible formulas and retention time.

2.4. Toxicity studies

2.4.1. PC and cell viability—Human hepatocellular carcinoma cells, Hep G2 (ATCC No. HB-8065), were maintained in Eagle's Minimum Essential Medium (EMEM, ATCC No. 30-2003) supplemented with 10% fetal bovine serum (ATCC No. 30-2020) and incubated in a humidified CO_2 chamber at 37 °C. Hep G2 cells were diluted to 2×10^5 cells mL^{-1} and plated in 200 μL volumes 24 h before exposure to MC-LR. The culture medium of the cells was then replaced by the medium with toxin, and incubated at 37 °C for 48 h. The culture without MC-LR was used as the control. All fluorescence and absorbance measurements were quantified using a FlexStation 3 Plate Reader (Molecular Devices, CA) spectrophotometer.

Total PC was used as a biomarker to assess the toxic effects of MC-LR and its transformation products [36]. The cell culture medium was removed, and 200 μL of 1 M NaOH was added to the cells to dissolve proteins. After incubation of the plate at 37 °C for 2 h, 180 μL of the NaOH solution was removed from the original plate and replaced with 180 μL of Coomassie Brilliant Blue G solution (0.01% Coomassie Brilliant Blue G, 4.7% ethanol (99.5%, Sigma) and 8.5% phosphoric acid (85%, Fisher)) in the original plate. After a 30 min incubation at room temperature, the absorbance was quantified at 620 nm.

The NR cellular uptake test was used to check cell viability. The old culture medium was removed, and 100 μL serum-free medium with $50 \mu\text{g mL}^{-1}$ NR was added to each well with the cells. After incubation of the plate at 37 °C for 3 h, cells were fixed with 200 μL of 1% formaldehyde with 1% CaCl_2 solution for 1 min at room temperature. The solution was removed from the original plate and replaced with 200 μL of acetic acid:ethanol:water (1:49:50, v/v/v) solution in the original plate in order to achieve the extraction of NR within the cells. The absorbance was measured at 540 nm.

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reduction test was used to assess cell metabolic activity. Diluted MTS solution (CellTiter 96 AQueous One Solution Cell Proliferation Assay reagent (Promega):fresh medium, 1:5, v/v) was added (120 μL) onto each well. After 3 h incubation at CO_2 incubator, the plate was mixed well then absorbance was measured at 490 nm using a spectrophotometer.

All experiments of the above-described assays were performed four times. The data from each assay were shown as the mean percentage of the results from the control experiments. Statistically significant differences ($p < 0.050$) were evaluated applying analysis of variance (ANOVA).

2.4.2. PP-1 activity—A RediPlate™ 96 EnzChek® serine/threonine phosphatase assay kit (ThermoFisher Scientific) was used to analyze the inhibition of PP-1 activity. PP-1 (New England Biolabs® Inc.) was diluted to 12.5 units mL^{-1} in a buffer of 50 mM tris hydrochloride at pH 7.0 containing 0.1 mM CaCl_2 , 125 $\mu\text{g mL}^{-1}$ bovine serum albumin, 0.05% Tween® 20, 2 mM dithiothreitol and 200 μM MnCl_2 . The buffer components were dissolved and diluted with sterile water, and stored at 4 °C until needed.

PP-1 solution (40 μL) was added to 10 μL of test samples diluted in 50 μL of the reaction buffer in a 96-well microplate. The plate was incubated at 37 °C for 30 min, and the fluorescence of 6,8-difluoro-7-hydroxy-4-methylcoumarin was measured (355 nm excitation and 460 nm emission) using a spectrophotometer. The dose-dependent activity of PP-1 against substrate (6,8-difluoro-4-methyl-umbelliferyl phosphate) was established to determine the optimal PP-1 concentration for MC-LR. The inhibition of enzyme activity was also measured at known MC-LR concentrations to establish the PP-1 inhibition curve.

3. Results and discussion

3.1. MC-LR treatability studies

In the first part of the study, MC-LR treatability studies were conducted to assess the efficiency of treatment with solar photo-Fenton process under different operational and water quality conditions. An initial MC-LR concentration of 1.0 μM ($\sim 1.0 \text{ mg L}^{-1}$) was selected to represent high microcystin concentrations in nutrient-rich fresh waters. In fact, microcystins have been shown to be primarily a concern in eutrophic and hypereutrophic systems with concentrations reaching up to 2.2 mg L^{-1} [37]. In addition, during large bloom events microcystins can reach concentrations in mg L^{-1} range. In fact, previous work by the Florida Harmful Algal Bloom Task Force [38] showed that microcystins can be found in drinking water sources at concentrations up to 8 mg L^{-1} . Fig. 1 presents the effect of initial H_2O_2 and Fe^{2+} concentrations on the rate constants for MC-LR degradation ($k_{\text{MC-LR}}$; in min^{-1}) whose kinetics was adequately fitted to pseudo-first-order equation. From Fig. 1a it can be seen that $k_{\text{MC-LR}}$ steadily increased from 0.0046 to 0.032 min^{-1} within the H_2O_2 concentration range 10-300 μM , after which it did not significantly change. Therefore, 300 μM was the initial H_2O_2 concentration of choice for the forthcoming experiments. The insignificant increase in $k_{\text{MC-LR}}$ between 300 and 400 μM could be accounted for by

increasing competition of H_2O_2 for $\text{HO}\cdot$ and thus its decomposition without oxidizing the target pollutant in the presence of excess H_2O_2 [21,26].

Fig. 1b shows the effect of initial Fe^{2+} concentration on $k_{\text{MC-LR}}$ for solar photo-Fenton process. From Fig. 1b it can be seen that Fe^{2+} addition greatly enhanced the $k_{\text{MC-LR}}$ even at its lowest concentration as compared to the case where no Fe^{2+} was added (solar light-driven photolysis of H_2O_2 , $k_{\text{MC-LR}} = 0.010 \text{ min}^{-1}$). This profound increase in $k_{\text{MC-LR}}$ is caused by the following main route of $\text{HO}\cdot$ generation in photo-Fenton process together with $\text{HO}\cdot$ originated from the photolysis of H_2O_2 ($\text{H}_2\text{O}_2 + h\nu \rightarrow 2 \text{HO}\cdot$):

From the experimental results it became evident that $k_{\text{MC-LR}}$ gradually increased from 0.032 to 0.075 min^{-1} as the initial Fe^{2+} concentration was raised from 0.10 to 0.40 mg L^{-1} (1.8 to $7.2 \text{ }\mu\text{M}$).

A series of solar photo-Fenton experiments was performed to assess the effect of pH on the degradation of aqueous MC-LR. The most suitable pH range for the removal of organic pollutants with Fenton and photo-Fenton processes has already been established as 2–5 [39,40]. However, the experiments were performed in the pH range ~6–9 since this is the pH of most lakes and rivers [41]. This also eliminates inevitable consumption of acids which creates a major obstacle for the real-scale application of photo-Fenton process. In fact, the application of photo-Fenton process at near neutral pH has also been reported in the scientific literature for efficient degradation of different types of micropollutants [[42], [43], [44]] and deserves investigation for the case of MC-LR. In the present study, however, increasing pH had a negative effect on $k_{\text{MC-LR}}$. The photo-Fenton reaction proceeded significantly faster ($k_{\text{MC-LR}} = 0.054 \text{ min}^{-1}$) at pH unadjusted water (pH 5.7) than at pH 7.4–9.0 ($k_{\text{MC-LR}} = 0.012 \text{ min}^{-1}$). The degradation of MC-LR was even incomplete above neutral pH values, reaching a minimum value of 12% at pH 9.0. Although solar photo-Fenton process was promising for the degradation of MC-LR in moderately acidic waters without modifying the natural pH, the effect of pH was found to be critical in the alkaline pH range.

The above study was performed at pH 7.0 with 5 and 10 mM phosphate buffer. The degradation rate constant for MC-LR obtained in 5 mM buffer solution with a pH of 7.0 ($k_{\text{MC-LR}} = 0.0056 \text{ min}^{-1}$) was about half of that obtained in Milli-Q water at same pH adjusted with NaOH ($k_{\text{MC-LR}} = 0.012 \text{ min}^{-1}$). As previously reported, the HPO_4^{2-} and H_2PO_4^- react with $\text{HO}\cdot$ with the second order rate constants of 1.5×10^5 and $2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, respectively [45]. Although the second-order rate constant for the reaction between $\text{HO}\cdot$ and MC-LR at pH 7 has been reported to be $1.1 \pm 0.01 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ [46], the molar concentration of phosphate used in the present study was 5000 times higher than that of MC-

LR (5 mM vs 1 μ M), that probably led to a competition between MC-LR and phosphate ions for $\text{HO}\cdot$. This argument was supported by another experiment which showed that when 10 mM phosphate buffer was used, an even lower $k_{\text{MC-LR}}$ of 0.0026 min^{-1} was obtained. To conclude, phosphate buffer acted as a $\text{HO}\cdot$ scavenger in the photo-Fenton system under the experimental conditions used.

The effect of alkalinity was also studied by subjecting the MC-LR samples to solar photo-Fenton's oxidation under different bicarbonate alkalinity levels. The results are presented in Fig. 2. In Fig. 2, the $k_{\text{MC-LR}}$ was negatively affected even by the lowest bicarbonate alkalinity of $1.8 \text{ mg CaCO}_3 \text{ L}^{-1}$. When the alkalinity of the solution was adjusted to $1.8 \text{ mg CaCO}_3 \text{ L}^{-1}$, the removal percentage (after 30 min) and $k_{\text{MC-LR}}$ were only 51% and 0.017 min^{-1} , respectively. According to the reaction (2) below, HCO_3^- reacts with $\text{HO}\cdot$ with the second-order rate constant of $8.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [47]:

which suggests that scavenging effect of HCO_3^- leads to some decline in degradation kinetics as a result of the lower reactivity of $\text{CO}_3^{\cdot-}$ with MC-LR compared to $\text{HO}\cdot$.

The effect of isolated NOM on the MC-LR degradation percentages (after 30 min) and $k_{\text{MC-LR}}$ was assessed by performing a series of solar photo-Fenton experiments using Milli-Q water fortified with 5 mg L^{-1} fulvic acid (FA) or humic acid (HA) and $1.0 \mu\text{M}$ MC-LR as shown in Fig. 3. In the absence of isolated NOM, MC-LR degradation percentage and $k_{\text{MC-LR}}$ were higher (100% and 0.075 min^{-1} , respectively) compared to those obtained in the presence of 5 mg L^{-1} HA (71% and 0.032 min^{-1} , respectively). NOM could act as $\text{HO}\cdot$ scavenger thereby lowering the number of free $\text{HO}\cdot$ to react with MC-LR. Lindsey and Tarr [48] demonstrated that the Fenton degradation of hydrophobic compounds including *o*-cresol, pyrene, phenol and phenanthrene, was hindered even in the presence of a very low concentration of dissolved NOM due to decreased concentration of $\text{HO}\cdot$ at the microenvironmental sites of the studied compounds. This could also explain the decreased percentage and $k_{\text{MC-LR}}$ of MC-LR degradation observed in the presence of HA (Fig. 3). On the other hand, the experiment with 5 mg L^{-1} FA indicated that the rate constant for MC-LR degradation was considerably higher than that in Milli-Q water without NOM. Previous studies using Fenton's reagent provided evidence that iron chelation by FA results in stabilization of Fe^{2+} at higher pH. Voelker and Sulzberger [49] observed that the effective rate constant of Fenton's reaction increased with increasing FA concentration and pH between 3–5 due to the formation of Fe^{2+} -fulvate complexes that react more rapidly with H_2O_2 than Fe^{2+} -aquo complexes. The authors also demonstrated that FA could contribute to the increase observed in the reaction rate constants by regenerating H_2O_2 and Fe^{2+} through a number of competing processes. Lindsey and Tarr [50] reported that addition of FA resulted in a slight decrease in $\text{HO}\cdot$ formation by Fenton's reaction at pH 3.1 where the highest yield of $\text{HO}\cdot$ was observed, although increased radical production was obtained with FA above this pH. Considering that our system was operated at the pH of Milli-Q water open to the atmosphere (pH 6.0), such an increase in MC-LR degradation rate during solar photo-Fenton process in the presence of FA could be explained by the above-given hypotheses. Another

important factor which can contribute to the observed higher MC-LR degradation rate in the solar photo-Fenton system in the presence of FA is their photosensitizing effect under direct sunlight leading to the formation of reactive oxygen species including HO $\cdot$, $^1\text{O}_2$ and H $_2\text{O}_2$ [[51], [52], [53]]. In order to assess the effect of FA on solar photolysis of MC-LR, an additional experiment was conducted using 1.0 μM MC-LR and 5.0 mg L^{-1} FA in Milli-Q water at pH 6.0. Addition of 5 mg L^{-1} FA to the reaction solution resulted in an increase in the MC-LR degradation from 2% to 24% after 60 min direct solar photolysis, suggesting the contribution from the reactive oxygen species to the MC-LR degradation to some extent. When the solar photo-Fenton experiment was carried out in the presence of 5 mg L^{-1} HA, 5 mg L^{-1} FA and 96 mg L^{-1} NaHCO $_3$ (Total alkalinity = 57 $\text{mg CaCO}_3 \text{ L}^{-1}$), the MC-LR degradation percentage and rate constant were the lowest achieved (Fig. 3). The opposite trend observed between the experiments in the presence of HA and FA may be explained by the fact that HA molecules are typically larger and generally contain more aromatic character than FA molecules which in turn may compete for HO $\cdot$ and play the role of HO $\cdot$ scavenger and reduce the degradation kinetics of MC-LR [48].

3.2. Effect of water constituents on MC-LR transformation products

Transformation products generated during solar photo-Fenton treatment of MC-LR were elucidated, and the effect of two important water constituents, namely FA and NaHCO $_3$, was assessed on their identities and formation/degradation patterns. It should be noted here that identification of all transformation products in the treated samples was not possible because of the low concentration of MC-LR (1.0 μM) selected for the studies. Transformation products formed during solar photo-Fenton degradation of MC-LR were identified and followed against treatment time using LC/Q-TOF MS. The most suitable reaction conditions established in Section 3.1. (300 μM H $_2\text{O}_2$; 0.40 mg L^{-1} (7.2 μM) Fe $^{2+}$) were used.

Table 1 presents the results on Milli-Q water (A), Milli-Q water + FA (B), and Milli-Q water + NaHCO $_3$ (C). Possible chemical structures of these transformation products are presented in Fig. 4. Twenty compounds were detected, 18 of which were identified as transformation products formed during solar photo-Fenton treatment of MC-LR (m/z 996). Hydroxylation of the benzene ring of Adda amino acid and dihydroxylation of the diene bonds of the Adda followed by oxidative cleavage are commonly regarded as the initial stages of MC-LR degradation by HO $\cdot$ [32,54]. The first aromatic hydroxylation is followed by a second and third one which yields the intermediates C9, C8 and C6 with m/z of 506(+2H $^+$)/1012, 1028 and 522 (+2H $^+$), respectively. The transformation product C6 which was formed only in Milli-Q water, is believed to be the product of triple hydroxylation of the aromatic ring as has also been suggested by Antoniou et al. [33]. It should be noted here, however, that the –OH groups have been tentatively placed in the aromatic ring in Fig. 4. More specific fragmentation studies need to be done in order to find their exact locations. On the other hand, since both the Adda chain and –OH groups are *o*-, *p*-directors [55], the chemical structure given in Fig. 4 could be suggested to be more preferentially generated. The absence of C6 in samples containing FA and NaHCO $_3$ could be explained by the higher reactivity of C6 with oxidizing species than its precursors and its low concentrations generated in these samples [56].

Three peaks with the same nominal mass ($M = 1028.5542$) were detected in the total ion chromatogram, which were deduced to be positional isomers. The products C7, C15, and C18 with $m/z = 515 (+2H^+)/1030 (+H^+)$ could be related to 1,2- and 1,4-addition to the conjugated carbon double bonds of Adda. The positions of $-OH$ groups could be at bonds C_4-C_5 , C_6-C_7 , or C_4-C_7 of Adda depending on the stability of the allylic radical ($RCH = CH - R_2C\cdot$) which is formed following the incorporation of the first $HO\cdot$ into the double bond. The product ($M = 1028.5542$) having $-OH$ groups at C_6-C_7 positions rapidly isomerizes to the more stable tautomer of ketone-MC-LR and follows a series of oxidative induced bond cleavage mechanistic steps at bonds C_6-C_7 of Adda, transforming to the ketone-derivative intermediate product C3 with m/z 835 (*cyclo*[$C_8H_{11}NO_2$ -Glu-Mdha-Ala-Leu-MeAsp-Arg-H]) in all the studied water samples. The intermediate product C2 with m/z 783, which corresponds to alterations of the MC-LR structure in both the conjugated carbon double bonds of Adda and the Mdha, was also common for all the studied water matrices, and most likely originated from degradation of the cyclic structure of C3. The intermediate product C1 with m/z 795 (*cyclo*[$C_5H_7NO_2$ -Glu-Mdha-Ala-Leu-MeAsp-Arg-H]), an aldehyde-derivative which was formed in all the studied water samples, is also generated through oxidative cleavage at C_4-C_5 bond in residual Adda of C3 as has also been suggested by other authors [30,31,54]. The product C1 could also be directly formed from the oxidative cleavage occurred at bond C_4-C_5 of the product ($M = 1028.5542$) having $-OH$ groups at C_4-C_5 positions. The product C1 has been reported to be free from toxicity produced by the intact Adda amino acid [32]. Therefore, it was speculated that the biologically active portion of MC-LR could be removed to some extent regardless of the water samples studied. Oxidation of the Mdha to aldehyde and cleavage of the $R_2HC-COR$ bond led to the generation of the intermediate product C5 which was not isolated in water containing FA.

Overall, the transformation products m/z 795 (C1), 835 (C3), $515(+2H^+)/1030$ (C7, C15 and C18) and 532 (C14) can be formed through the attack of $OH\cdot$ on the conjugated carbon double bonds of Adda. Transformation products with m/z 1010 (C12), 966 (C13) and $513(+2H^+)$ (C20) can be generated through the attack of $OH\cdot$ on the methoxy group of Adda. The transformation products m/z 783 (C2), $508(+2H^+)$ (C5) and 1012 (C11) can be originated from the attack of $OH\cdot$ on the cyclic structure of MC-LR. Transformation products (m/z $522(+2H^+)$ (C6), 1028 (C8), $506(+2H^+)/1012$ (C9), 1046 (C10) and $514(+2H^+)$ (C19)) can be formed after hydroxylation of the aromatic ring with $OH\cdot$.

Figs. S2–S4 present the temporal variation of peak volumes as followed by LC/Q-TOF MS during course of degradation of MC-LR by solar photo-Fenton process in Milli-Q water with and without FA and $NaHCO_3$. In Milli-Q water without and with FA (Figs. S2 and S3), the majority of the identified transformation products were first observed after 2.5 min, and peaked in volume after 5–7.5 min of solar photo-Fenton treatment where 85–92% of MC-LR were eliminated from the reaction solution. In both water samples no transformation products were detected after 60 min treatment. Although not statistically significant, peak volumes obtained in Milli-Q water were typically lower than those detected in Milli-Q water with FA which could be correlated with the lower k_{MC-LR} value in the former one. Although the majority of the identified transformation products started to be generated after 7.5 min of solar photo-Fenton treatment when applied in Milli-Q water with $NaHCO_3$, they were still detectable after 60 min reaction, and most of these products (m/z 835, 515, 795 and 1028)

continued to increase in volume even when the treatment was ended (Fig. S4). This was most likely caused by the slow degradation kinetics of MC-LR observed in Milli-Q water with NaHCO_3 where about 32% of MC-LR was still available after 60 min solar photo-Fenton process.

3.3. Effect of water constituents on toxicity patterns during MC-LR degradation

3.3.1. Cell-based toxicity evaluation—Human hepatocellular carcinoma cells Hep G2 (ATCC No. HB-8065) were initially used to determine cell viability (PC, MTS and NR cellular uptake) exposed to different MC-LR concentrations. Upon exposing Hep G2 cells to concentrations of untreated MC-LR at 0.50 and 1.0 μM , all the three cell viability parameters did not significantly change (data not shown), suggesting that MC-LR was not cytotoxic for the selected concentrations. Therefore, an initial MC-LR concentration of 1.0 μM was selected for the treatment experiments to examine possible cytotoxic effects caused by transformation products.

Changes in the selected cell viability parameters during solar photo-Fenton treatment of MC-LR were followed as affected by the presence of 5.0 mg L^{-1} FA and 3.0 mg L^{-1} NaHCO_3 (Total alkalinity = 1.8 mg L^{-1} CaCO_3). The results are given in Fig. 5. PC and MTS reduction capability did not significantly change during the course of solar photo-Fenton reaction when applied to MC-LR sample regardless of the water sample treated (Fig. 5a and b). On the other hand, the NR assay was most sensitive to MC-LR exposure (Fig. 5c), a result also seen in a recent cytotoxicity study with cylindrospermopsin [57]. The cell viability in Milli-Q water with FA decreased to 64 and 67% of control according to the NR assay after 2.5 and 10 min solar photo-Fenton treatment, respectively, where the majority of the detected transformation products (C1, C7, C9, C11, C12 and C16; Fig. S3) peaked in volume as assessed by LC/Q-TOF MS. Therefore, the breakdown products with nominal masses of 794, 945, 1009, 1011 and 1029 could be at least in part responsible for the decrease in cell viability observed during first 10 min of solar photo-Fenton treatment in Milli-Q water with FA. However, it should be noted that the entire toxicity pattern could not be related to the detected breakdown products since the cell viability remaining after 60 min solar photo-Fenton treatment in Milli-Q water with FA was not significantly different than after 10 min ($p > 0.050$) although no byproducts were evidenced in chromatogram after 60 min treatment. This latter finding suggests that there could still be unidentified breakdown products persisting in treated Milli-Q water with FA, which exert cytotoxic effect toward Hep G2 cells. Similar to the case in Milli-Q water with FA, cell viability as determined by the NR assay also decreased to 68% of control within 7.5 min of solar photo-Fenton treatment of MC-LR in Milli-Q water with NaHCO_3 after which it only slightly changed (Fig. 5c). After 60 min treatment where 68% of MC-LR was eliminated from the reaction solution, cell viability was still significantly lower than that of control (73% of control; $p < 0.050$) in correlation with the presence of intermediate products (C1, C2, C3, C5, C7, C8, C9, C12 and C20; Table 1 and Fig. S4) which were refractory to further degradation in Milli-Q water with NaHCO_3 and accumulated with treatment time. On the other hand, the NR assay showed that cell viability was typically higher than that of control when solar photo-Fenton process was applied to Milli-Q water (Fig. 5c), suggesting no cytotoxic effect was evident in treated MC-LR after 60 min associated with its transformation products.

Overall, FA and alkalinity can greatly influence NR uptake patterns in Hep G2 cell line exposed to MC-LR treated with solar photo-Fenton.

3.3.2. PP-1 inhibition—While MC-LR was found to be non-cytotoxic based on PC, MTS and NR assays within the concentration range studied, the major concern is type 1 and type 2A protein phosphatase (PP-1 and PP-2A) inhibition [1,16]. Although solar photo-Fenton treatment resulted in complete MC-LR degradation in the absence and presence of NOM, the formation of byproducts could be still of critical importance in terms of their potential to inhibit PP-1 and PP-2A. However, since identification of all individual reaction byproducts and assessment of their individual biological activities is a challenging task particularly for high molecular weight compounds like MC-LR in presence of complex organic molecules such as FA, analysis of the inhibition activity in the overall solution could help to evaluate the toxic potential of the treated solutions. Keeping all these in mind, the PP-1 inhibition was determined to evaluate the biological activity of the treated solutions. A standard curve of PP-1 inhibition was first prepared within the range of the residual MC-LR concentration in the solar photo-Fenton treated solutions (Fig. S5). Over 85% inhibition of the PP-1 enzyme occurred at MC-LR concentrations $> 0.25 \mu\text{M}$. The linear region of the inhibition curve appeared between 0.016 and 0.062 μM MC-LR with IC_{50} of 0.053 μM .

The PP-1 inhibition was followed over the course of reaction in MC-LR samples treated in Milli-Q water, Milli-Q water with FA and Milli-Q water with NaHCO_3 as shown in Fig. 6. According to the results from treatment experiments, the inhibition decreased to 65 and 28% after 60 min reaction when the solar photo-Fenton experiment was conducted in Milli-Q water without and with FA, respectively. Considering that MC-LR was completely degraded in the solar photo-Fenton systems performed in Milli-Q water without and with FA (Fig. 3), the respective PP-1 inhibitions of 65 and 28% must be associated with breakdown products which could not be detected after 60 min using LC/Q-TOF MS. The solar photo-Fenton process most rapidly detoxified MC-LR in the presence of 5 mg L^{-1} FA in parallel to the highest rate constant for MC-LR degradation achieved in Milli-Q water with FA (0.30 min^{-1} ; Fig. 3), and the resulting PP-1 inhibition after 60 min did not indicate the formation of considerably toxic transformation products. In fact, the residual PP-1 inhibitions measured in the studied water samples at the end of treatment inversely correlated to the rate constants for MC-LR degradation achieved in these samples. This was most evident in Milli-Q water with NaHCO_3 where about 50% MC-LR degradation was obtained after 60 min solar photo-Fenton treatment with a $k_{\text{MC-LR}}$ of 0.017 min^{-1} and PP-1 inhibition of 92%. Overall, it could be concluded that FA and alkalinity content of water samples could have a major role in determining levels of inhibitory effect during solar photo-Fenton treatment of MC-LR.

4. Conclusions

The elucidation of transformation products and toxicity during solar photo-Fenton degradation of MC-LR in the absence and presence of two major water components, namely fulvic acid and alkalinity are important in assessing the utility of the technology. LC/Q-TOF MS results showed that four sites on MC-LR were responsible for the interaction with $\text{OH}^\bullet$, including the aromatic ring (a), conjugated double bonds of Adda (b), cyclic structure (c), and methoxy group of Adda (d). Total alkalinity adjusted with NaHCO_3 was the most

critical component of water which decreased the percentage and rate constant of MC-LR degradation and hindered detoxification. Transformation products slowly but steadily increased in peak volume when the solar photo-Fenton process was applied in the presence of low level bicarbonate alkalinity (Total alkalinity = 1.8 mg L⁻¹ CaCO₃). The kinetic and toxicity studies showed that fulvic acid increased the rate constant of MC-LR degradation and the level of detoxification (as assessed by PP-1 assay) by solar photo-Fenton process.

This study verified the importance of assessment of AOPs in environmentally relevant aqueous matrices containing NOM and alkalinity. This is the first study where transformation product and toxicity profiles during degradation of MC-LR by solar photo-Fenton process were discussed in relation with water constituents. Hence, it provides a fundamental support for the efficacy assessment of MC-LR degradation in environmentally relevant aqueous matrices by solar photo-Fenton process based on transformation product and toxicity data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AOPs	advanced oxidation processes
FA	fulvic acid
HA	humic acid
HO	hydroxyl radicals
HPLC	high performance liquid chromatography
$k_{\text{MC-LR}}$	rate constant for MC-LR degradation (min ⁻¹)
LC/Q-TOF MS	liquid chromatography with quadrupole time-of-flight mass spectrometry
m/z	mass-to-charge ratios
MC-LR	microcystin-LR
MTS	tetrazolium salt reduction
NOM	natural organic matter
NR	neutral red

PC	protein content
PP-1	protein phosphatase-1

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Fig. 1.

The *pseudo*-first-order rate constants of MC-LR removal as a function of initial H_2O_2 (a) and Fe^{2+} (b) concentrations for solar photo-Fenton process. Initial experimental conditions: MC-LR = $1.0 \mu\text{M}$; Fe^{2+} = 0.10 mg L^{-1} ($1.8 \mu\text{M}$) (in Fig. 1a); H_2O_2 = $300 \mu\text{M}$ (in Fig. 1b); pH = 5.7.

Fig. 2.

Influence of alkalinity on the *pseudo*-first-order rate constants of MC-LR removal. Initial experimental conditions: MC-LR = 1.0 μ M; H₂O₂ = 300 μ M; Fe²⁺ = 0.40 mg L⁻¹ (7.2 μ M); without pH adjustment.

Fig. 3.

Percentages and *pseudo*-first-order rate constants of MC-LR degradation in Milli-Q water with and without isolated NOM. Initial experimental conditions: MC-LR = 1.0 μM ; FA = 5.0 mg L^{-1} ; HA = 5.0 mg L^{-1} ; NaHCO₃ = 96 mg L^{-1} (Total alkalinity = 57 $\text{mg CaCO}_3 \text{ L}^{-1}$); H₂O₂ = 300 μM ; Fe²⁺ = 0.40 mg L^{-1} (7.2 μM); without pH adjustment.

Fig. 4.
Proposed chemical structures of the MC-LR transformation products identified by LC/Q-TOF MS.

Fig. 5.

The Protein content (a), tetrazolium salt reduction (b), and neutral red uptake (c) of MC-LR sample in different aqueous matrices with different treatment time. CW: Clean water, CW + FA: Clean water with 5 mg L⁻¹ FA, CW + SB: Clean water with 3 mg L⁻¹ NaHCO₃ (Total alkalinity = 1.8 mg L⁻¹ CaCO₃). Initial experimental conditions: MC-LR = 1.0 μM; H₂O₂ = 300 μM; Fe²⁺ = 0.40 mg L⁻¹ (7.2 μM).

Fig. 6.

Temporal evolution of PP-1 inhibition during solar photo-Fenton degradation of MC-LR in CW, CW with 5.0 mg L⁻¹ FA, and CW with 3.0 mg L⁻¹ NaHCO₃ (Total alkalinity = 1.8 mg L⁻¹ CaCO₃). Initial experimental conditions: MC-LR = 1.0 μM; H₂O₂ = 300 μM; Fe²⁺ = 0.40 mg L⁻¹ (7.2 μM).

Table 1.

Transformation products identified via LC/Q-TOF MS analyses during solar photo-Fenton treatment of MC-LR in Milli-Q water (A), 5.0 mg L⁻¹ FA in Milli-Q water (B), and 3.0 mg L⁻¹ NaHCO₃ (total alkalinity = 1.8 mg CaCO₃ L⁻¹) in Milli-Q water (C).

Compound	Retention time (min)	Formula	Experimental mass (m/z)	Sample
MC-LR	3.140	C ₄₉ H ₇₄ N ₁₀ O ₁₂	995.5599 (+H ⁺)	A, B, C
C1	2.254	C ₃₄ H ₅₄ N ₁₀ O ₁₂	795.4001 (+H ⁺)	A, B, C
C2	2.311	C ₃₃ H ₅₄ N ₁₀ O ₁₂	783.4002 (+H ⁺)	A, B, C
C3	2.364	C ₃₇ H ₅₈ N ₁₀ O ₁₂	835.4278 (+H ⁺)	A, B, C
C4	2.591	C ₄₇ H ₇₆ N ₈ O ₁₆	505.2762 (+2H ⁺) 1027.5453 (+H ₃ O ⁺)	A
C5	2.755	C ₄₈ H ₇₄ N ₁₀ O ₁₄	508.2794 (+2H ⁺)	A, C
C6	2.786	C ₄₉ H ₇₄ N ₁₀ O ₁₅	522.2741 (+2H ⁺)	A
C7	2.769	C ₄₉ H ₇₆ N ₁₀ O ₁₄	515.2843 (+2H ⁺)	A, B, C
C8	2.869	C ₄₉ H ₇₄ N ₁₀ O ₁₄	1027.5417 (+H ⁺)	A, C
C9	2.871	C ₄₉ H ₇₄ N ₁₀ O ₁₃	506.2778 (+2H ⁺) 1011.5421 (+H ⁺)	A, B, C
C10	2.897	C ₄₉ H ₇₆ N ₁₀ O ₁₅	1045.547 (+H ⁺)	A
C11	3.000	C ₄₉ H ₇₄ N ₁₀ O ₁₃	1011.5505 (+H ⁺)	A, B
C12	3.064	C ₄₉ H ₇₂ N ₁₀ O ₁₃	1009.5341 (+H ⁺)	A, B, C
C13	3.336	C ₄₆ H ₇₄ N ₁₀ O ₁₁	965.543 (+Na ⁺)	A, B
C14	2.733	C ₄₉ H ₇₈ N ₁₀ O ₁₆	532.2896 (+2H ⁺)	A, B, C
C15	2.878	C ₄₉ H ₇₆ N ₁₀ O ₁₄	1029.5560 (+H ⁺)	A, B, C
C16	3.136	C ₄₆ H ₇₆ N ₁₀ O ₁₁	967.5615 (+Na ⁺)	A, B
C17	3.120	C ₄₉ H ₇₄ N ₁₀ O ₁₃	1011.5481 (+H ⁺)	C
C18	2.925	C ₄₉ H ₇₆ N ₁₀ O ₁₄	515.2835 (+2H ⁺)	C
C19	2.967	C ₄₉ H ₇₄ N ₁₀ O ₁₄	514.2752 (+2H ⁺)	C
C20	2.882	C ₄₉ H ₇₂ N ₁₀ O ₁₄	513.2694 (+2H ⁺)	C