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Oxidation of the Cyanobacterial Hepatotoxin Microcystin-LR by Chlorine Dioxide: Influence of Natural Organic Matter

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Cyanobacteria (blue-green algae) are known producers of cytotoxic, hepatotoxic, and neurotoxic compounds with severe acute and chronic effects on vertebrates. Successful removal of these toxins in drinking water treatment is therefore of importance for public health. In the present work the oxidation of the cyanobacterial hepatotoxin microcystin-LR (MC-LR) by chlorine dioxide (ClO_2) was studied at natural microcystin concentrations (10 μg L^{-1}) and normal ClO_2 dosages (1 mg L^{-1}) in the absence and presence of natural organic matter (NOM). ClO_2 was found to be rapidly consumed by fulvic and humic acids, leaving less residual ClO_2 to oxidize MC-LR. Predicted decrease rates in MC-LR concentration correlated highly with experimental data both in pure water and in the presence of NOM. Rate constants determined at high ClO_2 and MC-LR concentrations in pure water could be used to predict the oxidation of MC-LR at natural concentrations. Toxicity tests with a protein phosphatase inhibition assay on reaction solutions and high-performance liquid chromatography fractions revealed that PP1 enzyme inhibition emerged only from intact MC-LR, while the oxidation products, dihydroxy isomers of MC-LR, were nontoxic even at unnaturally high concentrations.

Introduction

The strong oxidizing capacity of chlorine dioxide (ClO_2) makes this chemical useful in several technical applications, e.g., in treatment of wastewaters and in bleaching of textiles and cellulose. Since ClO_2 effectively inactivates viral, bacterial, and protozoan pathogens, it has also for a long time been used as a disinfectant/oxidant in drinking water treatment, where its use has several advantages in comparison with chlorination (*1*). Undoubtedly, the most important advantage is that ClO_2 does not produce harmful trihalomethanes (THMs) in the reaction with natural organic matter (NOM), and the formation of other halogenated organic byproducts is strongly reduced (*2*−*4*). ClO_2 disinfection/oxidation has thus become an alternative to chlorination for waterworks using surface water as raw water, although chlorine (Cl_2, $HOCl/OCl^-$) is still by far the most frequently used disinfectant in both pre- and posttreatment of drinking water (*5*).

The main concern with the use of ClO_2 in drinking water treatment has been the unknown health effects of organic oxidation products originating from NOM, and the formation of chlorite (ClO_2^-) and chlorate (ClO_3^-) from ClO_2. While ClO_2^- and ClO_3^- are suspected of causing hemolytic anemia and other health effects (*6*), there is so far no evidence that the organic oxidation products are harmful at concentrations found in finished drinking water (*7*). The formation of ClO_2^- and ClO_3^-, however, restricts the dosage of ClO_2 to a maximum of ca. 1−2 mg L^{-1}, since many countries have regulations on the maximum concentration of ClO_2^- in finished drinking water, e.g., 1.0 mg L^{-1} in the United States. In drinking water treatment, typically 50−70% of ClO_2 is reduced to ClO_2^-, while 30% is converted to ClO_3^- and chloride (Cl^-) (*1*, *8*).

When utilized in drinking water treatment, ClO_2 is mainly added as a predisinfectant/oxidant, followed by, e.g., chlorination or chloramination in the posttreatment step. Since the NOM concentration prior to the preoxidation step may be as high as several milligrams per liter, it is obvious that the added ClO_2 may rapidly be consumed, leaving only a small or no residual ClO_2 concentration. The impact of ClO_2 oxidation on harmful micropollutants such as microcystins in drinking water treatment might therefore be negligible. For an example of micropollutant removal by ClO_2 in natural waters, see ref *9*.

Microcystins are cyclic heptapeptide hepatotoxins, produced by strains of several cyanobacterial genera frequently found in eutrophied freshwaters. More than 70 analogues of microcystins have been identified (*10*), microcystin-LR (MC-LR) being the most abundant and also the most toxic (LD_{50} = 50 μg kg^{-1}, mouse ip) (*11*). The molecular basis of microcystin toxicity is through the inhibition of eukaryotic serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) (*12*, *13*). In mammals, acute exposure to high levels of microcystins will cause lethal liver hemorrhage or liver failure, while chronic exposure to low levels exhibits tumor-promoting activity and is possibly carcinogenic (*14*−*17*). Due to the health hazards caused by microcystins, the World Health Organization (WHO) has set a provisional guideline value for MC-LR in drinking water to 1.0 μg L^{-1} (*18*). This guideline value is based on the acute toxicity of MC-LR, and it does not take into account possible long-term exposures to microcystins at low concentrations.

Typical dissolved microcystin concentrations in surface waters rich in toxic cyanobacteria are 0.1−10 μg L^{-1}, but the concentration can be much higher if a major bloom is breaking down (*11*). Since dissolved microcystins readily pass conventional treatment methods, i.e., coagulation/flocculation, clarification, and sand filtration (*19*, *20*), other measures must be taken to degrade or remove these toxins, e.g., chemical oxidation or granular activated carbon (GAC) filtration.

While there is much scientific information available on microcystin oxidation by ozone (O_3), potassium permanganate ($KMnO_4$), monochloramine (NH_2Cl), and chlorine, see, e.g., refs *21*−*24*, little is known about microcystin oxidation by ClO_2. We recently showed that MC-LR is oxidized to dihydroxy isomers of MC-LR in the reaction with ClO_2,

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TABLE 1. Some Important Characteristics of the Waters Used

	[DOC] (mg L^{-1})	abs (254 nm)[a]	SUVA[b] (L mg^{-1} m^{-1})
Milli-Q	0.06	0	0
Lake Littois water (0.2 μm filtered)	7.7	0.135	1.75
fulvic acid (No.FA, 100 mg L^{-1}, 0.2 μm filtered)	50.1 (*28*)	0.448[c]	4.47
humic acid (No.HA, 100 mg L^{-1}, 0.2 μm filtered)	51.5 (*28*)	0.476[c]	4.62

[a] With a 1 cm quartz cuvette. [b] Specific UV absorbance. [c] Diluted 5 times.

and that the toxicity with respect to PP1 inhibition is lost in the reaction (*25*). The overall rate constant for the reaction between MC-LR and ClO_2 was, however, modest, $k = 1.24$ M^{-1} s^{-1} at 293 K and pH 5.65 in pure water, suggesting that ClO_2 is not a suitable oxidant for the degradation of microcystins in drinking water treatment (*25*).

The aim of this work was to study the reaction between MC-LR and ClO_2 at natural microcystin concentrations and normal ClO_2 dosages in the absence and presence of NOM, to evaluate the validity of previously determined overall rate constants by comparing experimental results with theoretical calculations, to test the toxicity of individual oxidation products, and to study the competitive reaction between ClO_2 and fulvic and humic acids to give a more complete picture of microcystin oxidation by ClO_2.

Experimental Section

Chemicals. MC-LR was from the same batch as in ref *25*. ClO_2 was prepared according to ref *25*. $NaClO_2$ was of technical grade, while other chemicals were of analytical grade or higher.

ClO_2 Quantification. ClO_2 concentration in stock solutions was determined by direct spectrometry at 358 nm prior to use, using a molar absorptivity of 1200 M^{-1} cm^{-1} (*26*). Residual ClO_2 concentrations in the experiments were determined by the lissamine green B (LGB) method at 616 nm (*27*).

Natural Organic Matter. Diluted lake water from the eutrophic Lake Littois, SW Finland, and the IHSS reference samples of Nordic aquatic fulvic acid (No.FA; code IR105F) and Nordic aquatic humic acid (No.HA; code IR105H) were used as representatives of NOM in the experiments. Fulvic and humic acid stock solutions (100 mg L^{-1}) were prepared by dissolving 10 mg of substance in 100 mL of Milli-Q water, followed by magnetic stirring for 18 h in darkness and filtration through 0.2 μm GHP hydrophilic polypropylene membrane filters (Pall Life Sciences). Stock solutions (150 mg L^{-1}) of No.FA and No.HA have reported DOC concentrations of 75.1 and 77.3 mg L^{-1}, while the carbon content obtained by elemental analysis is 53.1% and 54.6%, respectively (*28*, *29*). ^{13}C NMR data and IR spectra of No.FA and No.HA are available on the IHSS Web site (*30*). Water from Lake Littois was collected in mid-August 2004, prefiltered through Whatman GF/A filters, and stored at 278 K in darkness. The lake water was filtered through 0.2 μm GHP hydrophilic polypropylene membrane filters (Pall Life Sciences) prior to use. The DOC concentration of the lake water was determined with a Shimadzu TOC 5000A analyzer after the 0.2 μm filtration. Some important characteristics of the waters used are given in Table 1.

Reactions at Natural Concentrations and Normal Dosages. Reactions between MC-LR and ClO_2 in Milli-Q water, diluted lake water, and fulvic acid solutions were carried out in 250 mL borosilicate glass bottles filled to the neck with 300 mL of solution. The initial concentrations of MC-LR and ClO_2 were 10 μg L^{-1} and 1 mg L^{-1}, respectively. An 80 μL portion of MC-LR stock solution was added to the bottles prior to the addition of 180−200 μL of ClO_2 stock solution. Mixing was achieved by shaking the bottles after addition of MC-LR and by gently inverting the bottles 10 times after addition of ClO_2. The bottles were incubated in a thermostated water bath at 293 K in darkness. The reaction between MC-LR and ClO_2 was stopped after 0, 5, 10, 20, 30, and 40 h (or after 0, 1, 3, 5, and 10 h in some experiments) through the addition of 80 μL of ascorbic acid stock solution (0.1 mg μL^{-1}). Solid-phase extractions were carried out immediately when the reactions were stopped. The reactions in diluted lake water and in fulvic acid solutions were carried out at pH 7.0, buffered with 10^{-2} M KH_2PO_4/10^{-2} M K_2HPO_4. In each test series, a control sample was prepared and treated in the same way except for the addition of ClO_2 and ascorbic acid. Protein phosphatase inhibition assay (PPIA) on 500 μL subsamples from the reactions carried out in Milli-Q water was done according to ref *25*.

Solid-Phase Extraction. A 3 mL portion of methanol was added to each test bottle after the reaction had been stopped with ascorbic acid. The bottles were shaken, and the SPE cartridges (Isolute SPE columns, 1 g of C18(EC)/6 mL) were conditioned with 10 mL of methanol followed by 10 mL of Milli-Q water. The samples were applied via Teflon tubes at a flow rate not exceeding 10 mL min^{-1}. The cartridges were washed with 4 mL of 20% methanol and dried by flushing with air for 2 min. MC-LR was eluted with 4 mL of 100% acetonitrile (ACN) + 0.05% trifluoroacetic acid (TFA) into borosilicate test tubes. The solvent was evaporated at 323 K under a gentle flow of Ar or N_2. The residue was resuspended in 500 μL of 75% methanol, transferred to 0.7 mL Eppendorf tubes, and centrifuged for 10 min at 10000*g*. Finally, 200 μL of the solution was pipetted into polypropylene HPLC vials, and the remaining MC-LR concentration was determined by HPLC according to ref *25*.

Recovery Test. To assess for losses of MC-LR in the solid-phase extraction, a recovery test was performed. MC-LR was spiked to 300 mL of Milli-Q water at concentrations of 1, 3, 5, 7, 9, and 10 μg L^{-1} in triplicate. SPE was performed as described above, and the recovery (average ± standard deviation) was obtained after HPLC analysis according to ref *25*. Another test was performed to verify that the presence of NOM did not alter the recovery of MC-LR in the SPE. MC-LR was spiked to 300 mL of Milli-Q water, diluted lake water ([DOC] = 5 mg L^{-1}), and fulvic acid solution (10 mg L^{-1}) at a concentration of 10 μg L^{-1} in triplicate. SPE was carried out after 0, 20, and 40 h of contact time, and the recovery was obtained after HPLC analysis according to ref *25*. In addition, it was possible to verify the recovery from the control samples in the experiments.

Stopped Flow Measurements. An Applied Photophysics SX.18MV stopped flow reaction analyzer with a dead time of 2 ms was used to study the initial reaction between ClO_2 and fulvic and humic acids. ClO_2 solution at a concentration of 20 mg L^{-1} and fulvic or humic acid solution at a concentration of 20 mg L^{-1} buffered to pH 7.0 (2×10^{-2} M KH_2PO_4/2×10^{-2} M K_2HPO_4), pH 9.0 (2×10^{-2} M $NaHCO_3$/2×10^{-3} M Na_2CO_3), or pH 10.0 (2×10^{-2} M $NaHCO_3$/2×10^{-2} M Na_2CO_3) were introduced into the two burets prior to rapid injection into the reaction cuvette at equal volumes. The data acquisition system (SpectraKinetic Workstation v4.46) recorded the decrease in absorbance at 358 nm during the initial 100 s of the reaction between ClO_2 and fulvic or humic

acid with a sampling rate of 100 ms. The instrument was thermostated to 293 K, and all measurements were done in triplicate.

PPIA on HPLC fractions. For the PPIA on HPLC fractions, the reaction between MC-LR and ClO_2 was carried out in a 1.8 mL HPLC borosilicate glass vial at 293 K and pH 5.65. The initial concentrations of ClO_2 and MC-LR were 50.0 mg L^{-1} (0.741 mmol L^{-1}) and 36.9 mg L^{-1} (0.037 mmol L^{-1}), respectively. MC-LR (932 μL of stock solution) was added to the vial prior to the addition of ClO_2 (47 μL of stock solution). Mixing was achieved by inverting the vial twice after each addition. The final volume in the vial was 1500 μL. At $t = 30$ min, the reaction was stopped by pipetting 15 μL of ascorbic acid stock solution (0.1 mg μL^{-1}) into the vial. The remaining MC-LR concentration in the sample was determined by HPLC according to ref *25*. Separation of the reaction products was performed on a Merck Hitachi LaChrom HPLC system (degasser, low-pressure gradient pump, auto sampler, column oven, UV/DAD detector) using a Merck Purospher Star RP-18e column (5 μm particles, 250 × 4 mm + 4 × 4 mm guard column). The column oven temperature was 313 K, the injection volume was 80 μL, and the flow rate was 0.75 mL min^{-1}. The eluent program (eluent A, Milli-Q water + 0.5% formic acid; eluent B, 100% ACN) was 0 min 25% B, 35 min 45% B, 45 min 90% B, 47 min 90% B, 47.1 min 25% B, 70 min stop. The 0.5 min fractions were collected into 1.5 mL Eppendorf tubes using a Pharmacia FRAC-100 fraction collector. PPIA was performed on the 2.0−30.0 min fractions in triplicate according to ref *25*.

Results and Discussion

In a previous work we determined the overall (second-order) rate constant k for the reaction between MC-LR and ClO_2 under pseudo-first-order conditions ($[ClO_2] \gg$ [MC-LR]) to be 1.24 M^{-1} s^{-1} at 293 K and pH 5.65 in pure water (*25*). On the basis of previous findings (*25*, *26*), it can be assumed that the reaction between MC-LR and ClO_2 follows second-order kinetics, being first-order in both MC-LR and ClO_2 concentrations. The rate law for the decrease in MC-LR concentration can thus be expressed by

$$-\mathrm{d[MC\text{-}LR]}/\mathrm{d}t = k[\mathrm{MC\text{-}LR}][ClO_2] \quad (1)$$

Integration of eq 1 gives, on the assumption that the concentration of ClO_2 is constant

$$\ln([\mathrm{MC\text{-}LR}]/[\mathrm{MC\text{-}LR}]_0) = -k[ClO_2]_0 t \quad (2)$$

Rewritten, eq 2 can be expressed by

$$[\mathrm{MC\text{-}LR}] = [\mathrm{MC\text{-}LR}]_0 e^{-k[ClO_2]_0 t} \quad (3)$$

Further, the half-life of MC-LR at any (constant) ClO_2 concentration can be calculated from the overall rate constant k according to

$$t_{1/2} = (\ln 2)/k[ClO_2]_0 \quad (4)$$

Hence, at a constant ClO_2 concentration of 1 mg L^{-1}, which is a typical dosage of ClO_2 in preoxidation of surface waters, the theoretical half-life of MC-LR is 10.5 h for $k = 1.24$ M^{-1} s^{-1}. This relatively long half-life suggests that ClO_2 is not a suitable oxidant for the degradation of microcystins in drinking water treatment. We have evaluated this half-life value, and thereby also the validity of the previously determined overall rate constant, by studying the reaction between MC-LR and ClO_2 at a natural concentration of MC-LR, i.e., 10 μg L^{-1}, and a normal dosage of ClO_2, i.e., 1 mg L^{-1}, in pure water at 293 K and pH 5.65. At these concentrations ClO_2 is in great excess (ClO_2:MC-LR molar ratio 1475:1), which

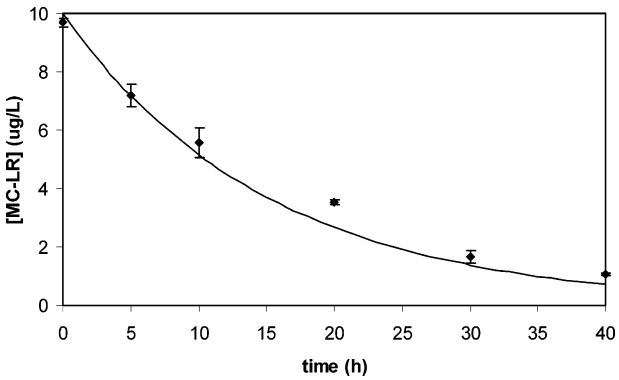

FIGURE 1. Comparison between experimental (◆) and predicted (solid line) decrease rates in MC-LR concentration over 40 h at 293 K and pH 5.65 in pure water. Experimental values have been corrected for 86% recovery. Initial concentrations of MC-LR and ClO_2 were 10 μg L^{-1} and 1 mg L^{-1}, respectively (average ± standard deviation, $n = 3$).

means that the concentration of ClO_2 is approximately constant during the experiment. Equations 2−4 are thus applicable. As evident from Figure 1, there is a fairly good agreement between experimentally obtained and (according to eq 3) predicted decrease rates in MC-LR concentration over 40 h. We consider the slight difference observed mainly to be a consequence of the uncertainty in the 86% recovery correction of the experimental data. In the recovery test performed in Milli-Q water at an MC-LR concentration range of 1−10 μg L^{-1}, the recovery of MC-LR was 85.9 ± 6.6% (average ± standard deviation, $n = 18$). Other factors that possibly could affect the decrease rate in MC-LR concentration were the escape of small amounts of ClO_2 into the air-filled space in the bottles, the degradation of ClO_2 in the reaction with MC-LR, and the disproportionation of ClO_2 into ClO_2^- and ClO_3^- in the reaction with OH^-. At prevailing conditions, however, these processes should only marginally retard the decrease rate in MC-LR concentration, and they were therefore not corrected for. In a linear regression analysis, the correlation between experimental and predicted data in Figure 1 was high ($r = 0.997$), although the slope of the regression line deviated by 8% from 1.

Although eq 3 can be used to predict the decrease rate in MC-LR concentration in pure water when the ClO_2 concentration is approximately constant, the situation will be totally different in the presence of ClO_2-consuming NOM. Consequently, a correction for the decrease in ClO_2 concentration must be made to eq 3:

$$[\mathrm{MC\text{-}LR}] = [\mathrm{MC\text{-}LR}]_0 e^{-k(\int [ClO_2]\, \mathrm{d}t)} \quad (5)$$

where $\int [ClO_2]\, \mathrm{d}t$ is the ClO_2 exposure, i.e., ClO_2 concentration integrated over time. As will be shown later, eq 5 can be used to predict the decrease rate of [MC-LR] in the presence of NOM when k (determined in pure water) and the ClO_2 exposure are known. Let us first briefly consider the competitive reaction between ClO_2 and NOM.

NOM is a complex mixture of organic substances, such as simple hydrocarbons, amino acids, proteins, polysaccharides, lipids, nucleic acids, and humic substances. Many of these substances are not (or are only slightly) reactive with ClO_2, while others contain functional groups, such as deprotonated phenolic moieties, that are highly reactive (*26*). In most surface waters, the most abundant dissolved NOM is fulvic and humic acids. These complex polyphenolic macromolecules are known to rapidly consume ClO_2 in the initial stage of the reaction, with the main oxidation products

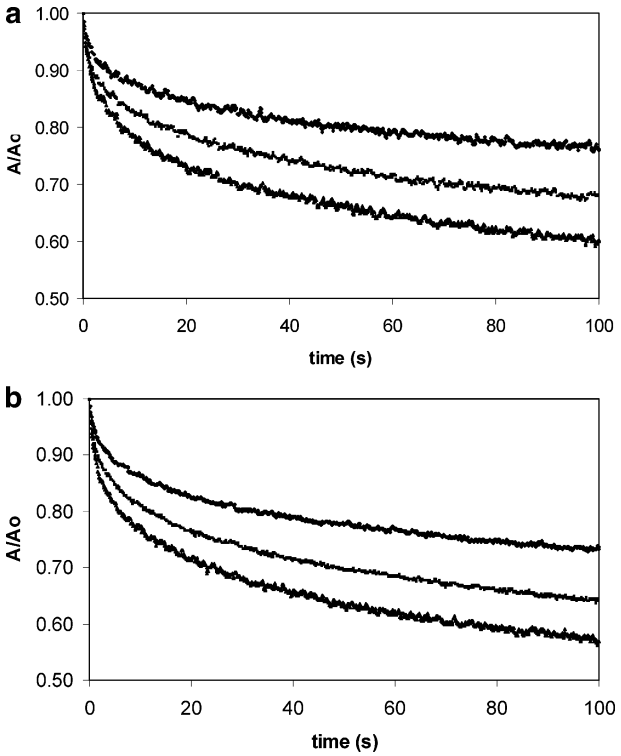

FIGURE 2. Decrease in relative absorbance at 358 nm vs time when ClO_2 at a concentration of 10 mg L^{-1} reacted with fulvic acid (a) and humic acid (b) at a concentration of 10 mg L^{-1} at 293 K and pH 7.0, 9.0, and 10.0 (from top to bottom).

formed being quinones, hydroquinones, aldehydes, and carboxylic acids (*7*).

We have studied the initial phase of the reaction between ClO_2 and fulvic and humic acids with respect to ClO_2 consumption by a stopped flow technique over 100 s. As expected from the polyphenolic character of fulvic and humic acids, ClO_2 was rapidly consumed, and the reaction was strongly pH dependent. The consumption of ClO_2 was about 10 times higher at pH 10 compared to pH 7 over 100 s, and the ClO_2 depletion rate was found to be slightly higher in humic acid solutions than in fulvic acid solutions at all pH values (Figure 2). The higher depletion rate in humic acid solutions is in agreement with the higher degree of aromaticity of No.HA compared to No.FA (*31*).

Since fulvic and humic acids rapidly consumed ClO_2, it was of interest to study the oxidation of MC-LR by ClO_2 in the presence of fulvic acid, and predict the decrease rate in MC-LR concentration by eq 5. As exemplified in Figure 3, where MC-LR (initial concentration 10 μg L^{-1}) was oxidized by ClO_2 (initial concentration 1 mg L^{-1}) in fulvic acid solution (1 mg L^{-1}) over 10 h at 293 K and pH 7.0, there was a good agreement between experimental and predicted decrease rates in MC-LR concentration. In the prediction, the ClO_2 exposure was calculated according to the dashed line in Figure 3, while k was 1.08 M^{-1} s^{-1} (*25*). In a linear regression analysis, the correlation between experimental and predicted data in Figure 3 was good ($r = 0.956$). The slope of the regression line deviated by 7% from 1. It is obvious that rate constants determined in pure water at high concentrations of reactants can be used to predict the oxidation of micropollutants in the presence of NOM if the oxidant exposure is known. These findings are in agreement with those by Huber et al., who predicted the oxidation of pharmaceutical traces by ClO_2 in natural waters (*9*).

We also studied the reaction between MC-LR (initial concentration 10 μg L^{-1}) and ClO_2 (initial concentration 1 mg L^{-1}) over 10 h at 293 K and pH 7.0 in 0.5 and 2.0 mg L^{-1} fulvic acid solutions. The remaining MC-LR concentrations (corrected for 86% recovery) after 10 h are given in Table 2. Interestingly, the remaining MC-LR concentration (μg L^{-1}) could be expressed as a linear function of the fulvic acid concentration (mg L^{-1}) with high correlation ($r = 0.999$):

$$[\text{MC-LR}] = 1.70[\text{fulvic acid}] + 5.63 \qquad (6)$$

According to eq 6, there would not have been any reduction in MC-LR concentration after 10 h if the fulvic acid concentration had been 2.57 mg L^{-1}.

The results of MC-LR oxidation by ClO_2 in diluted Lake Littois water are shown in Figure 4. In lake water diluted to 1 mg L^{-1} DOC and buffered to pH 7.0, the reduction in MC-LR concentration after 40 h was 76%, while the corresponding reductions at DOC levels of 3 and 5 mg L^{-1} were only 29%

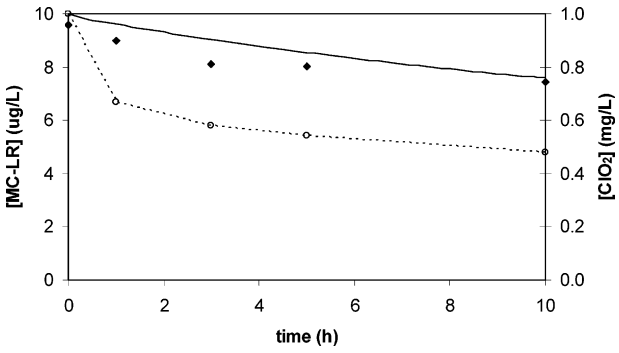

FIGURE 3. Comparison between experimental (◆) and predicted (solid line) decrease rates in MC-LR concentration over 10 h at 293 K and pH 7.0 in 1 mg L^{-1} fulvic acid solution. Experimental MC-LR concentrations have been corrected for 86% recovery. Initial concentrations of MC-LR and ClO_2 were 10 μg L^{-1} and 1 mg L^{-1}, respectively. Open symbols (○) represent the residual ClO_2 concentration, determined by the LGB method.

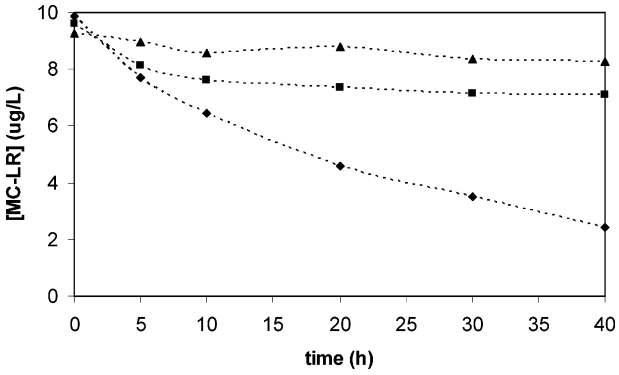

FIGURE 4. Decrease rate in MC-LR concentration over 40 h at 293 K and pH 7.0 in Lake Littois water diluted to 1, 3, and 5 mg L^{-1} DOC (from bottom to top). Initial concentrations of MC-LR and ClO_2 were 10 μg L^{-1} and 1 mg L^{-1}, respectively. MC-LR concentrations have been corrected for 86% recovery.

TABLE 2. Remaining MC-LR Concentrations after 10 h of Reaction with ClO_2 in Fulvic Acid Solutions

fulvic acid concn (mg L^{-1})	remaining MC-LR concn (μg L^{-1})	fulvic acid concn (mg L^{-1})	remaining MC-LR concn (μg L^{-1})
0	5.57[a]	1.0	7.44
0.5	6.50	2.0	8.98

[a] In pure water at pH 5.65.

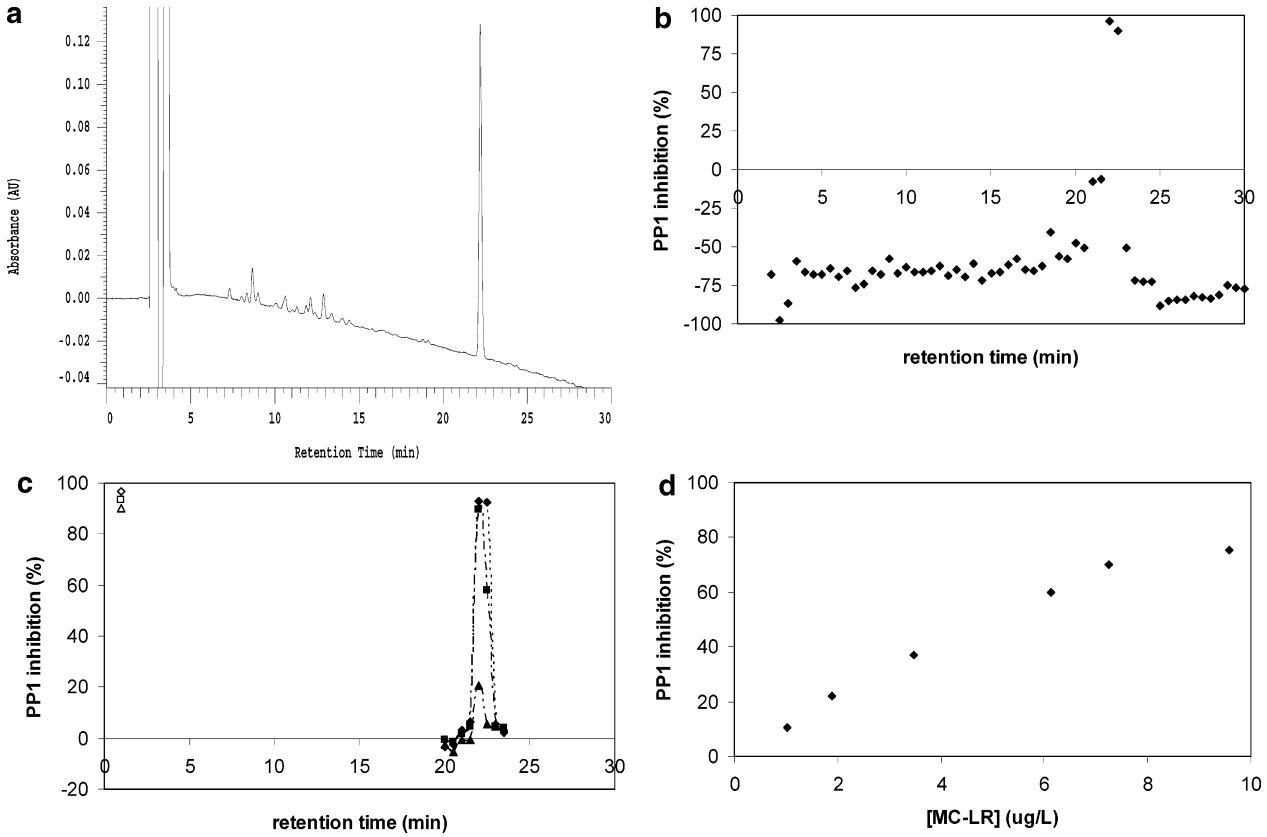

FIGURE 5. (a) HPLC chromatogram of MC-LR (RT = 22 min) and its oxidation products (RT = 7−14 min) detected at 238 nm after 30 min of reaction with ClO_2, PP1 inhibition (b) of collected HPLC fractions and (c) of the reaction solution diluted 10−1000 times (open symbols, upper-left corner) together with eight selected HPLC fractions diluted 10−1000 times (filled symbols), and (d) PP1 inhibition vs remaining concentration of intact MC-LR as determined by HPLC after solid-phase extraction. The data points represent (from left to right) 40, 30, 20, 10, 5, and 0 h of reaction time. MC-LR concentrations have been corrected for 86% recovery.

and 17%, respectively. As a comparison, there was only a 19% reduction in MC-LR concentration in a 2 mg L^{-1} fulvic acid solution (DOC 1 mg L^{-1}) after 40 h. The more efficient reduction in MC-LR concentration in diluted lake water compared to that in fulvic acid solution of the same DOC concentration was expected, since as the specific UV absorbance indicates (Table 1) the aromaticity of the lake water was significantly lower than that of the fulvic acid solution. Besides humic substances, the lake water thus contained nonaromatic constituents such as polysaccharides, peptides, and lipids that are not very reactive with ClO_2.

Our results show that the use of ClO_2 as a predisinfectant/oxidant in drinking water treatment will have only a small or negligible impact on dissolved microcystins if these toxins are present in the raw water. This should especially be the case in raw water with high specific UV absorbance, i.e., raw water rich in fulvic and humic acids. If predisinfection/oxidation with ClO_2 is followed by chloramination (*32*) in the posttreatment step, dissolved microcystins might to a large extent pass the treatment process unless other measures that readily degrade or remove microcystins are taken (e.g., ozonation (*33*) or GAC filtration (*19*, *20*)). An issue that should be of even greater concern for drinking water producers is the release of cell-bound microcystins after predisinfection/oxidation with ClO_2 (*34*), since most of the microcystin content in a toxic bloom is intracellular. To avoid the release of cell-bound toxins, it might be a better alternative to apply ClO_2 predisinfection/oxidation after removal of the cyanobacterial cells through, e.g., sand filtration.

In this work we have also further studied the toxicity of the main oxidation products formed in the reaction between MC-LR and ClO_2. In our previous work we found a positive correlation between PP1 inhibition and increasing concentration of intact MC-LR in the MC-LR/ClO_2 reaction solutions, and a close to zero inhibition of PP1 when all MC-LR had been oxidized (*25*). It was, however, not possible to test for PP1 inhibition of individual oxidation products with the applied method. Since it cannot be ruled out that individual oxidation products may, at least to some extent, possess PP1 inhibition when present at high concentrations, we wanted to clarify this. As demonstrated by, e.g., Liu et al. (*35*), it is possible to reveal the toxicity of individual oxidation products by performing the reaction at high concentrations, separate the oxidation products from each other and from intact MC-LR on a HPLC system, and then finally test the collected fractions using a PPIA.

In the oxidation experiment where PPIA was carried out on HPLC fractions, the initial concentrations of MC-LR and ClO_2 were 36.9 mg L^{-1} (0.037 mmol L^{-1}) and 50.0 mg L^{-1} (0.741 mmol L^{-1}), respectively. When the reaction was stopped at $t = 30$ min, 78% of MC-LR had been oxidized by ClO_2. Since 80 μL or 2.9 μg of microcystin (total mass of intact and oxidized MC-LR as MC-LR equivalents) was injected into the HPLC system, the microcystin concentration would be 7.8 mg L^{-1} if collected in a single fraction. Although distributed over several fractions, the microcystin concentration is still on the (sub)microgram per liter level. Hence, if individual oxidation products of MC-LR were to possess any inhibiting effect on PP1, it would be observed in the PPIA. As evident from Figure 5b, PP1 inhibition was observed only in the four HPLC fractions containing intact MC-LR (RT = 21−23 min). No PP1 inhibition was observed in the region where the oxidation products eluted (RT = 7−14 min) (Figure 5a). These peaks, not present before the oxidation, were

further analyzed by LC−MS. They all had an m/z value of 1029; i.e., they were dihydroxy isomers of MC-LR (*25*).

Due to the presence of ACN and formic acid in the HPLC fractions, but not in the positive and negative zero controls in the PPIA, the baseline appeared far on the negative y-axis. This artifact disappeared when the fractions were diluted 10−1000 times in Milli-Q water (Figure 5c). As evident from Figure 5c, the fraction containing most of the intact MC-LR (RT = 22.0 min) had to be diluted 1000 times before the inhibition dropped within the linear response range of the applied PPIA. As a comparison, PP1 inhibition caused by the 10−1000 times diluted reaction solution remained on approximately the same level (presented to the left in Figure 5c). It can thus be concluded that individual oxidation products of MC-LR, formed in the reaction with ClO_2, do not even at unnaturally high concentrations possess any PP1 inhibition.

We also performed PPIA on undiluted aliquots from the 40 h experiment (see above), where the initial concentrations of MC-LR and ClO_2 were 10 $\mu g\,L^{-1}$ and 1 $mg\,L^{-1}$, respectively. A positive correlation between PP1 inhibition and increasing concentration of intact MC-LR was obtained within the linear response range of the applied PPIA, and as previously demonstrated (*25*), the data points were approaching the origin of the coordinates (Figure 5d).

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