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Chlorination of *Microcystis aeruginosa* suspension: Cell lysis, toxin release and degradation

Min Ma[a,b], Ruiping Liu[a], Huijuan Liu[a], Jiuhui Qu[a,*]

[a] *Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China*
[b] *Graduate School of Chinese Academy of Sciences, Beijing 100039, China*

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ABSTRACT

Although the release of intracellular toxins after chlorination has been reported, the relation between cell lysis and the release and degradation of toxins during chlorination has not been well illustrated. This study used *Microcystis aeruginosa* to investigate the release and degradation behaviors of toxins after its exposure to chlorine at different doses for prolonged contact time. Scanning electron microscopy (SEM) analysis indicates no considerable change in the morphology for most algae cells. However, the release of intracellular K^+ and intracellular organic matter (IOM) did occur, and the significant K^+ release of 63% was observed even at chlorine exposure as low as 0.8 mg/L min. The damage of cell membrane was faster than the release of intracellular MCLR at all chlorine doses. Extracellular MCLR accumulated as a result of the fact that intracellular MCLR released from damaged cells faster than it was degraded by chlorine, especially at low dose of chlorine (0.8 mg/L). A maximum level of extracellular toxin at a contact time of 60, 30 and 1 min was observed at chlorine doses of 1.2, 1.6 and 2.0 mg/L, respectively. In addition, Cl_2 consumption (%), toxin release and degradation (%), and cells lysis (%) were well related.

1. Introduction

The proliferation of cyanobacteria is problematic and deteriorates source water quality. Algae cells excrete the release of intracellular metabolites, e.g., toxins, taste and odor substances, and proteins, into water during the growth. The release of intracellular organics also occurs in case of algae cells lysis. Release of these intracellular organics also occurs in case of algae cells lysis. This is of concern because cyanotoxins could be hepatoxic and cytotoxic [1,2].

On the other hand, the plentiful algae cells, together with the algogenic organic matter (AOM), show side effects on drinking water treatment processes. Coagulation is strongly affected in cases of algae bloom; and unfortunately it shows limited efficiency to remove algae cells due to the negatively charged algae cells [3] and the increased levels of AOM [3,4]. The elevated doses of coagulants may alleviate the negative effect of AOM on coagulation to some extent. However, this strategy brings forward other problems such as the increased cost and sludge yield.

Pre-oxidation is another strategy to enhance the removal of algae cells [5–7], among which pre-chlorination is the most widely used. However, the application of chlorine is limited by the concern of disinfection by-products formation [8–10]. In addition, pre-chlorination also damages cell membrane and leads to the significant release of intracellular metabolites including toxins [11]. Although chlorine may effectively degrade microcytins [12], the removal efficiency of toxins depends on the chlorine dose, contact time, pH, and the presence of chlorine-consumption substances [1,13,14].

Effect of chlorine on cyanobacteria cell during chlorination has been well studied. These studies were conducted with natural waters [11,15,16] or buffered reagent-grade water [16,17], usually at pH 6.8–10.7. However, for experiments conducted with natural waters, the presence of natural organic matters (NOM) would also consume chlorine and thus meddle in the chlorination process of algae. Additionally, high level of phosphate, applied in test with phosphate-buffered reagent-grade water [16,17], made the matrix like brackish or salt water rather than freshwaters. Yet many algae blooms occurred in freshwater lakes, thus it is meaningful to study the effect of chlorine on algae under a low salinity condition.

Additionally, although these studies aforementioned have addressed the release and degradation kinetics of toxins including microcystins from cyanobacteria cell during chlorination, no model has been established to demonstrate the relativity between chlorine decay, cell lysis and toxins release and degradation. Knowledge of the relationships between these factors is important for the optimization of chlorination strategy to control the risk of microcystins thereafter. The contribution of authors to these themes is presented in this paper.

* Corresponding author. Tel.: +86 10 62849160; fax: +86 10 62923558.
E-mail address: jhqu@rcees.ac.cn (J. Qu).

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The objectives of this study are: (1) to investigate the effect of chlorination on algae cells and the release of intracellular substances without any possible interference which derive from NOM and phosphate; (2) to illustrate the kinetics of toxin release and degradation; (3) and to indicate the relationship among chlorine decay, cell lysis, and toxin release and degradation.

2. Materials and methods

2.1. Materials and reagents

NaClO was used for chlorination and its stock solution was prepared by diluting the bleach to Milli-Q water and stored at 4 °C in darkness. Its content of free chlorine was determined by N,N-diethyl-*p*-phenylenediamine photometry method [18].

As one of the dominant species of algae blooms in China [19], *Microcystis aeruginosa* was used as a model cyanobacteria. An axenic strain of *M. aeruginosa* (No. FACHB-905) previously described [20] was used in this study, and was cultured in BG-11 [21] medium. This *M. aeruginosa* strain produces only microcystin-LR (data not shown). Full details of growth conditions are presented in Supporting information.

2.2. Chlorination experiment

Stock algae suspensions were centrifuged for 10 min at 6000 rpm (Allegra X-22R, Beckman, USA) and the supernate was discarded. The cell pellet was washed three times and resuspended in deionized water to a cell density of 2.2×10^6 cells/mL. The initial pH of the algae suspension was 5.3 due to the dissolution of carbon dioxide into the solution. The cell lysis caused by the change of osmosis is negligible (data not shown). Chlorine was added from the stock solution into 400 mL of algae suspension. Because experiments were conducted with unbuffered deionized water, at chlorine dose of 0.8, 1.2, 1.6 and 2.0 mg/L, the final pH of solutions varied within 5.8–6.3. It can be seen that within this pH range the dominate halogen species was hypochlorous acid (HOCl) [22].

Samples were sufficiently stirred during the chlorination process. Before each pre-determined time, 100 mL of sample was withdrawn from the bulk sample and divided into two subsamples: 5 mL for cell number measurement by OD_{680} analysis; the remaining sample for free chlorine analysis by titration with a chlorine amperometric titrator (AutoCAT 9000, HACH). At specified contact times, residual chlorine was quenched with Na_2SO_3. Samples for scanning electron microscopy (SEM) analysis (20 mL) were withdrawn from the bulk sample and centrifuged for 10 min at 6000 rpm. The precipitates were retained for SEM analysis. Full details of SEM procedure and its pre-treatment are shown in Supporting information.

Remaining sample was filtered through a 0.45 μm GFC filter (Whatman, UK). The supernate was for subsequent analysis, which included concentration of K^+, ultraviolet absorbance at 254 nm (UV_{254}) and extracellular toxin. Concentration of K^+ and UV_{254} was measured by an inductively coupled plasma optical emission spectrometer (OPTIMA 2000, PerkinElmer, UK) and a U-3010 UV/vis spectrophotometer (Hitachi Co., Japan), respectively. Cell pellet was subjected to three freeze/thaw cycles before the subsequent pre-treatment for intracellular toxin analysis. See Supporting information for the methods of microcystin extraction and analysis. All analyses were conducted in duplicate. Standard error and ANOVA analysis were calculated by software SPSS 13.0.

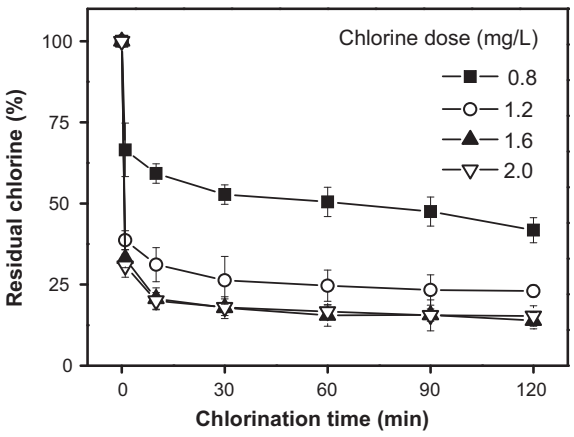

Fig. 1. Effect of chlorination of *M. aeruginosa* on residual chlorine. Cell density: 2.2×10^6 cells/mL.

3. Results and discussion

3.1. Residual chlorine

There was a rapid decay of chlorine in the first 10 min followed by a more gradual decrease over the remaining chlorination (Fig. 1). This trend was similar to the results in other studies [15,17], and the significant decrease of chlorine is attributed to its reaction with different materials such as cell membrane and the intracellular substances after chlorine penetrating into algae cells.

Moreover, the consumption of chlorine (%) increased with the initial dose of chlorine. At chlorine dose of 0.8 mg/L, chlorine residue of 42% could be anticipated. In contrast, the lowest chlorine residues of 14–15% (0.2–0.3 mg/L) were achieved at initial doses of chlorine of 1.6–2.0 mg/L. This can be explained by that the higher chlorine doses contributed to the more significant release of chromophoric compounds (Fig. 4b), which was highly reactive toward free chlorine [23].

3.2. Chlorination of algae suspensions

3.2.1. Effect of chlorination on algae cell damage

SEM images present the limited extent of cell lysis or distortion of cell membrane after chlorination. For the majority of *M. aeruginosa* cells, chlorine caused no severe deformation to cell membrane. Before chlorination, algae cells showed spherical shapes and smooth surfaces with some filaments on the outside of cells (Fig. 2a), which may be attributed to the naturally released metabolites. Interestingly, for most algae cells, no distinct alteration of morphology as previously reported [24,25] was observed even after the exposure to chlorine dose of 2 mg/L for 120 min (Figs. 2b and c). This further confirms the suggestion by Lin et al. [15] that chlorine ruptured the cells but not to the extent of complete disintegration. It was noted that the extensive damage of cell membrane for some cells was observed (Fig. 2d), and this was in accordance with previous studies [24,25]. Generally, chlorination did not severely alter the algae morphology in this study. Previous studies mainly presented some specific cells whereas this study showed the plentiful algae cells with the magnification of 5000, and this might be the attribution for the difference between the results in literature and those in this study [24,25].

Although SEM images showed the seemingly intact algae cells, chlorination did lead to a ruptured membrane [11]. K^+ is mainly stored as enzyme activator after being absorbed into the vacuole of cell [26], and the release of K^+ can indicate the integrity of cell

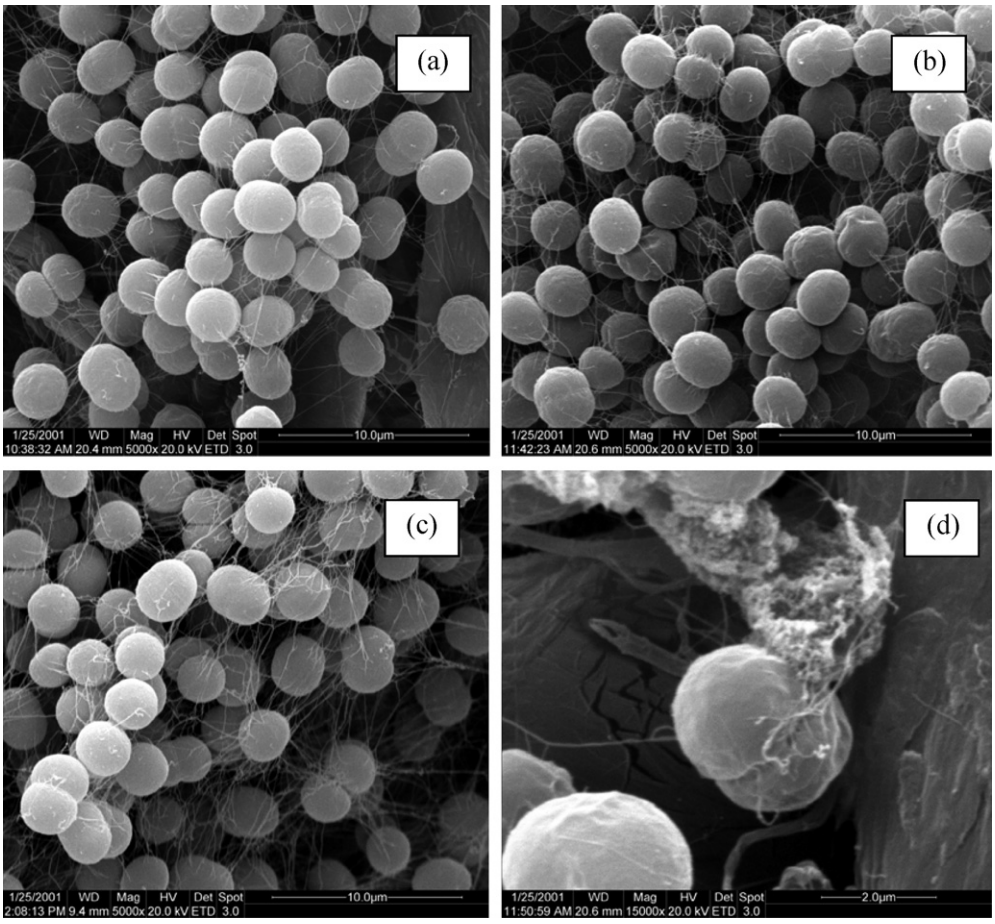

Fig. 2. SEM micrographs of *M. aeruginosa* before and after chlorination. (a) 0 mg/L Cl_2, ×5 K, (b) 1.2 mg/L Cl_2, 120 min, ×5 K, (c) 2.0 mg/L Cl_2, 120 min, ×5 K, and (d) 0.8 mg/L Cl_2, 120 min, ×15 K. Algae cell density: 2.2×10^6 cells/mL.

membrane [27]. The release of K^+ after being exposed to chlorine is illustrated in Fig. 3, and it is noted that the slight ratio of K^+ release in the control sample has been subtracted. The release of K^+ was much more rapid in the initial minutes than that in the remaining period. Furthermore, no substantial increase of K^+ release was observed at chlorine dose of above 1.2 mg/L.

To describe the kinetics of cell rupture, a first-order reaction model (Eq. (1)) can be applied in terms of chlorination, assuming a similarity between the damage kinetics of algae cell and bacteria cells [11].

$$\frac{N_{CT}}{N_0} = e^{-k(CT)} \quad (1)$$

where CT is the chlorine exposure; N_{CT} and N_0 equal the numbers of integral cells at a chlorine exposure of CT and 0; and k means the first order rate constant. Due the fast inactivation of algae cell by chlorine, only estimated rates of 2.7×10^3, 5.5×10^3, 3.8×10^4 and $6.1 \times 10^4\ M^{-1}\ s^{-1}$ were derived at chlorine doses of 0.8, 1.2, 1.6 and 2.0 mg/L, respectively. These algae cell rupture rates were much higher than the observed rates reported by Daly et al. [11] ($670\ M^{-1}\ s^{-1}$), Lin et al. [15] (790 and $1100\ M^{-1}\ s^{-1}$) and Zamyadi et al. (1400 and $1600\ M^{-1}\ s^{-1}$). These distinct results should be attributed to the different water matrix and pH. In this study, chlorination of *M. aeruginosa* was conducted without any interference such as NOM. Additionally, pH in this study was lower than those in literatures aforementioned (usually 6.8–8), which was associated with a higher oxidation ability of chlorine [22].

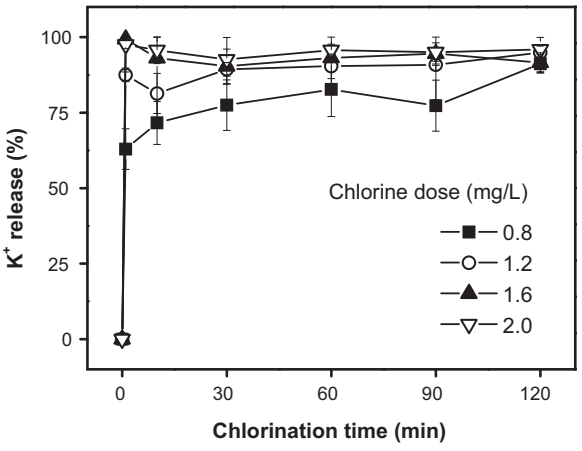

Fig. 3. Effect of chlorination of *M. aeruginosa* on the release of K^+. Cell density: 2.2×10^6 cells/mL. K^+ release in the control sample has been subtracted.

The ANOVA analysis was performed on the ratios of K^+ release in aforementioned experiments at chlorine doses of 0.8–2.0 mg/L and the effect of chlorine consumption on the ratios of K^+ release during chlorination was observed to be significant ($R^2 = 0.738$, $F = 61.908$, $P = 0.000 < 0.05$). The high F value indicates that the percentage of damage cells, i.e., the ratio of K^+ release, varied significantly with chlorine doses and contact time.

The underlying mechanism involves in the inactivation of microbial by chlorine remains unclear in terms of molecular-level reaction. Several mechanisms such as membrane-crossing have

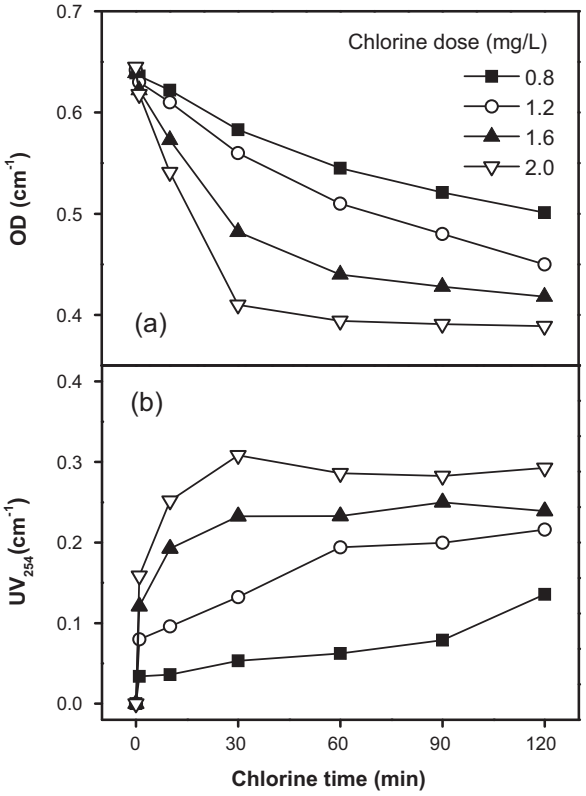

Fig. 4. Effect of chlorination of *M. aeruginosa* on (a) OD_{680} and (b) UV_{254}. Cell density: 2.2×10^6 cells/mL. Error bars were not shown in (b) for the consideration of readability.

been proposed to explain the inactivative effect of chlorine on algae cells. Venkobachar et al. [28] proposed that the inactivation of algae cells was achieved by the penetration of chlorine into cell membranes and the damage of the intracellular organelles thereafter. The discrepancy between unchanged cell morphology and metabolites release in this study supported this assumption.

3.2.2. Effect of chlorination on the release of AOM

The variation of chlorophyll-*a* and UV_{254} can indicate the release of intracellular organics. Chlorophyll-*a*, biliprotein pigments (phycobilins), and phycocyanin contribute to the blue green color of the cyanobacteria [29]. The optical density at 680 nm (OD_{680}) for *M. aeauginosa* suspensions showed positive correlation with the cell number [30], and in this study this value was used to quantify the biomass and to indirectly measure the cell integrity. Fig. 4a indicates that OD_{680} of *M. aeruginosa* decreased with either elevated chlorine doses or prolonged contact time. At chlorine doses of 0.8 and 1.2 mg/L, the OD_{680} values decreased steadily with longer chlorination time. At higher chlorine doses of 1.6 and 2.0 mg/L, however, the rapid decrease of OD_{680} values in the initial 15 min followed with a more gradual decrease was observed. This suggests that the lysis of algae cells was relative to the decreased cell density after chlorination.

The ANOVA analysis was performed on the percentage of OD_{680} decrease from tests processed under chlorination at 0.8–2.0 mg/L. At these chlorine doses, the chlorine consumption was also positively correlated with the percentage of OD_{680} decrease to some extent ($R^2 = 0.515$, $F = 23.320$, $P = 0.000 < 0.05$), but the significance value was less than that of K^+ release. The rate of OD_{680} decrease was lower than that of intracellular K^+ release, and this can be attributed to that the damaged cells also contain pigments, which also showed absorption at wavelength of 680 nm. Thus, the actual efficiency of chlorination on the inactivation of *M. aeruginosa* could be masked [7].

On the other hand, despite of the decrease in OD_{680} (Fig. 4a), chlorination could not remove algae cells. This is consistent with the results of Lin et al. [15]. This indicated a decrease in the correlation between the level of pigments in algae cells and the quantity of algae cells, which was mainly contributed by the degradation of pigments by chlorine. Results of UV–vis scan spectra on the AOM indicated the species transformation of AOM after chlorination (Fig. 5). As being indicated, the OD_{680} values decreased with the progressing of chlorination; however, the absorbance at wavelength of below 422 nm increased accordingly. This may be an indicator on the degradation of AOM during chlorination.

3.3. Effect of chlorination on toxin release and degradation

To assess the release of intracellular toxin from algae cells and the overall degradation of toxin by chlorine, the concentrations of intracellular and extracellular toxins were quantified after exposure to four chlorine doses at each pre-determined contact time. Prior to chlorination, the bulk solution at desired concentration of *M. aeruginosa* cells contained the total intracellular MCLR concentration of 105.9 μg/L, and the levels of extracellular MCLR and MCRR were below the detection limit of HPLC method. The release of intracellular toxin was not observed in the control sample.

At chlorine dose of 0.8 mg/L, the continuously steady decrease of intracellular MCLR was observed with prolonged contact time (Fig. 6a). The extracellular MCLR could be detected after 30 min,the concentrations of which increased with prolonged time. The concentrations of extracellular MCLR achieved to be 12.8 μg/L at 120 min, and this corresponded to a 48% decrease in the amount of intracellular MCLR. Although the release of intracellular MCLR contributed to the increased level of extracellular MCLR, chlorine is effective to degrade the released MCLR thereafter. The degradative behaviors of microcystin toxins by chlorine have been well investigated before [1,14]. In this study, this net increase of extracellular toxin levels was observed, and this indicates the higher rate of MCLR release than that of MCLR degradation in this case. This agrees with the results of Daly et al. [11]. However, different results were reported by Ding et al. that no buildup of MCLR derived from the chlorination, indicating slower release than chemical oxidation [17]. This inconsistency might be attributed to the difference of pH, which affects the equilibrium between HOCl and hypochlorite ion (OCl^-). The experiments by Ding et al. [17] were conducted at pH 7.6, higher than those of Daly et al. [11] and in this study, with relatively slower kinetics of cell inactivation and toxin release due to the much greater disinfection potential of HOCl compared to OCl^- [31].

At chlorine dose of 1.2 mg/L, there was a rapid decrease in the level of intracellular MCLR during the initial minutes and the amount of intracellular component was reduced by 98% at 60 min (Fig. 6b). The extracellular MCLR was below detection limit at 30 min and then achieved to be 7.7 μg/L at 60 min. However, the level of extracellular MCLR slightly reduced to 6.6 μg/L at 120 min. This was attributed to no further release of intracellular toxin after 60 min contact.

Similar trend was observed at chlorine dose of 1.6 mg/L except that the more significant decrease of intracellular MCLR initially was observed. The level of intracellular MCLR was as low as 2% after 30-min contact time (Fig. 6c). The extracellular MCLR continuously increased and finally achieved to 18.0 μg/L at 30 min, and this value was 133% higher than the highest level of extracellular MCLR at 1.2 mg/L of chlorine. After that, the extracellular MCLR rapidly decreased to 5.5 μg/L at 60 min, and more gradual degradation was observed in the remaining period. This might be attributed to the release of other intracellular organics from algae cells, and these

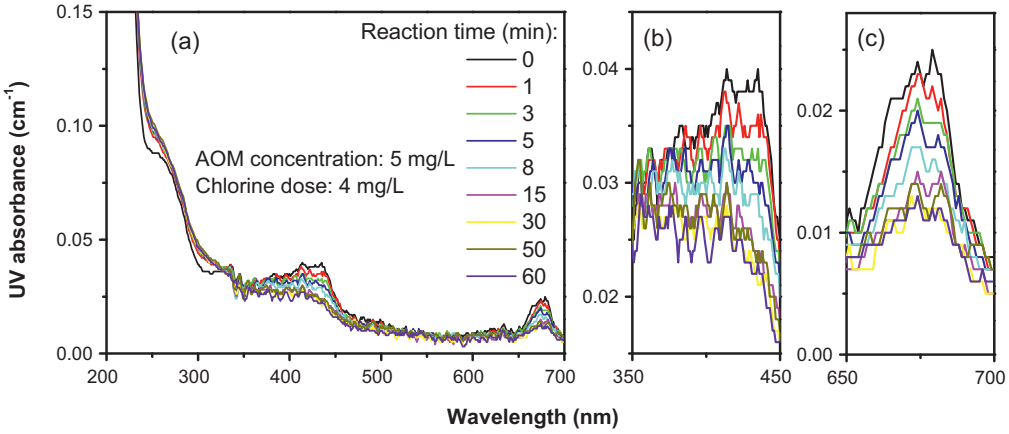

Fig. 5. Degradation of AOM by chlorine. AOM concentration: 5 mg/L DOC. Cl_2 dose: 4 mg/L.

species competed with MCLR towards chlorine. This competition is also responsible for the much slower releasing rate of intracellular MCLR after 30 min. In support of this suggestion, cyanobacteria, such as *M. aeruginosa*, has been shown to produce many other cyclic peptides with chemical structure similar to microcystins [32] and some of these peptides have been shown to exhibit greater reactivity towards chlorine than the microcystins [14].

The release and degradation behaviors of intracellular and extracellular MCLR at 2.0 mg/L of chlorine were similar to that at 1.6 mg/L. The concentrations of intracellular MCLR decreased more rapidly to as low as 1.5 μg/Lafter10 min. Correspondingly, the elevated chlorine dose to 2.0 mg/L substantially advanced the critical time to the appearance of maximum level of extracellular MCLR (1 min) on the time response curve, and increased, the level to as high as 23.5 μg/L accordingly (Fig. 6d).

3.4. Kinetics analysis

Table 1 shows the results of kinetics analysis which was conducted based on the effect of chlorination on Cl_2 decay, OD_{680} decrease, K^+ release, and toxin release. At all chlorine doses, the release of K^+ was faster than that of organics, especially during the initial 10 min. This suggested that chlorine inactivated *M. aeruginosa* cells by membrane-crossing rather than massive damage to cells membrane. If chlorine caused massive deformation to cell membrane, there would be large cracks or holes on the membrane and thus there would be no difference between the releasing rates of K^+ whose form is ion and organics whose structures are much larger. The ionic K^+ tended to cross the spots that have been damaged by chlorine, whereas the release of organics such as MCLR and Chlorophyll-*a* through these spots was to lower extent.

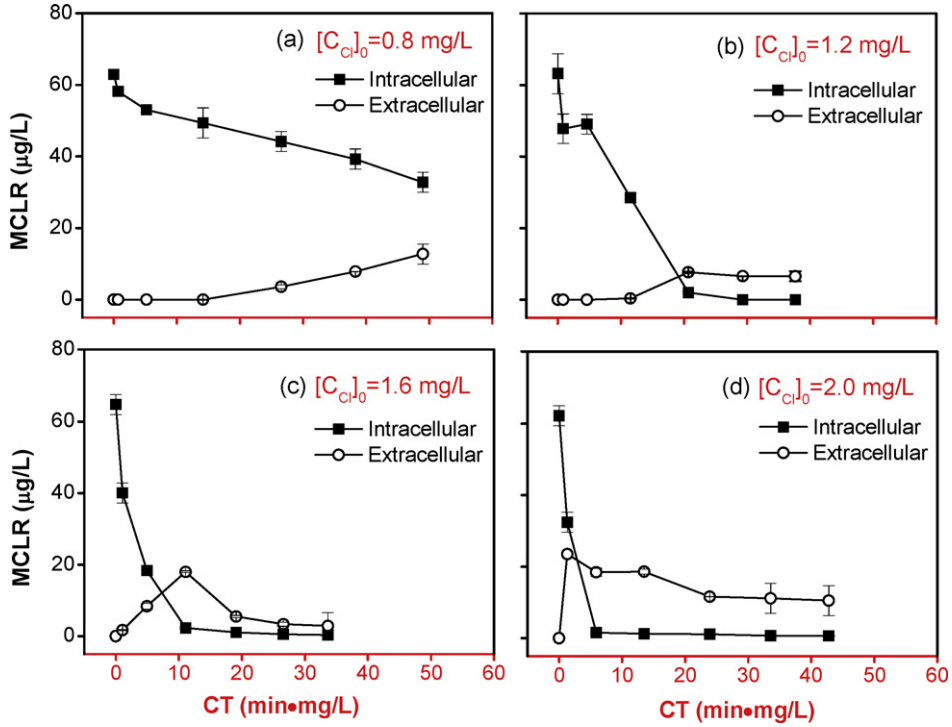

Fig. 6. Effect of chlorine dose and chlorination time on the intracellular and extracellular MCLR. Chlorine dose (mg/L): (a) 0.8, (b) 1.2, (c) 1.6 and (d) 2.0.

Table 1
Variation rate of several parameters during chlorination process at different doses of chlorine.

	Variation rate (%/min)							
	1 mg/L Cl_2		1.2 mg/L Cl_2		1.6 mg/L Cl_2		2.0 mg/L Cl_2	
	0–10 min	10–120 min	0–10 min	10–120 min	0–10 min	10–120 min	0–10 min	10–120 min
Cl_2 decay	4.07	0.15	6.89	0.08	7.94	0.06	8.00	0.05
K^+ release	7.16	0.18	8.14	0.13	9.30	0	9.57	0
OD_{680} decrease	0.25	0.16	0.46	0.19	0.95	0.20	1.50	0.20
Toxin release	1.57	0.29	2.25	0.70	7.16	0.25	9.75	0.01

Table 2
Regression result of chlorine consumption as dependent variable Y (mg/L).[a]

Independent variable	Factor	Regression coefficient
X_1	K^+ release (%)	0.794
X_2	Released toxin (%)	0.127
X_3	Degraded toxin (%)	0.150

[a] Regression constant: −17.178; R^2: 0.897; F: 58.372; Sig.: 0.000.

There have been concerns of the pre-chlorination application due to the massive increase in the level of soluble organics including toxins [11]. As K^+ is associated with the inactivation of algae cells which facilitates the algae removal, the difference between the releasing rates of K^+ and intracellular organics in this study indicates that the enhanced removal of algae might be achieved before the substantial release of IOM.

To model the effect of chlorination on the levels of intracellular and extracellular toxins in *M. aeruginosa*-burden water, regression analysis was conducted with results in this study. After being dosed into algae suspension, there were mainly two pathways for the decay of chlorine: (1) penetrating the cell membrane and inactivating algae cells to result in the release of intracellular metabolites; and (2) degrading organics including MCLR. Therefore, the consumption of chlorine can be expressed as follows (Eq. (2)):

$$\begin{aligned} &P_{Cl_2\ \text{consumption}}(t) \\ &= f\,[P_{K^+\ \text{release}}(t),\quad P_{\text{released MCLR}}(t),\quad P_{\text{degraded MCLR}}(t)] \\ &= a \times P_{K^+\ \text{release}}(t) + b \times P_{\text{released MCLR}}(t) \\ &+ c \times P_{\text{degraded MCLR}}(t) + d \end{aligned} \tag{2}$$

where t is the chlorination time; $P_{Cl_2\ \text{consumption}}(t)$, $P_{K^+\ \text{release}}(t)$, $P_{\text{released MCLR}}(t)$, and $P_{\text{degraded MCLR}}(t)$ indicate the percentage of consumed chlorine (%), released K^+ (%), released MCLR (%), and degraded MCLR (%) at time t, respectively; a, b, c and d are coefficients. This model was fitted to results at each dose of chlorine in this study and the correlation coefficient (R^2) was determined to be 0.897. Regression results are shown in Table 2. This model indicates that the application of chlorine as a pre-treatment for algae removal should be very careful due to the chlorine-induced release of intracellular organics including toxins. The good relationship between chlorine consumption (%), extracellular and degraded toxin (%), and cell lysis (%) may enable the optimization of the chlorination process and control the toxin pollution.

4. Conclusions

(1) Chlorination damages cell membranes and contributes to the release of intracellular substances such as toxins, K^+, and chlorophyll-*a*, although SEM images show slight change of cell morphology.
(2) The release and degradation behaviors of toxins during chlorination are dependent on pH, chlorine doses and contact time. The levels of extracellular MCLR increase initially and then decrease steadily with prolonged contact time, owing to the faster rate of MCLR release than its being degraded thereafter. The critical contact time to appear the maximum level of extracellular MCLR varies at different doses of chlorine.
(3) In pre-chlorine process, the doses and dosing points of chlorine may be optimized to achieve the inactivation of algae cells and the control of toxin levels simultaneously.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2012.03.030.

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