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Chlorination of *Microcystis aeruginosa*: cell lyses and incomplete degradation of bioorganic substance

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ABSTRACT

The frequent occurrence of blue-green algae bloom in recent years has raised a series of troubles in water treatment processes, and has become a global common concern. We investigated the lethal effect of chlorine, one common chemical algicide, to inactivate *Microcystis aeruginosa* in the process. Multiple methods were employed to investigate the chlorination effect of *M. aeruginosa*, including cells calculation with fluorescent staining, photosynthetic pigment contents determination, organic carbon analyses, and fluorescence excitation–emission matrix analyses. The results indicated that even more significant lethal effect of cells with higher chlorine exposure, and chlorophyll-a could hardly be a competent indicator for cell inactivation. Meanwhile, incompletely degraded characteristic of bioorganic substance was reflected during chlorination. Our results offer insight into the lethal effect of *M. aeruginosa* by chlorination, which will be valuable for understanding both inactivation mechanisms of *M. aeruginosa* and degradation process of bioorganic substance.

Keywords: Microcystis aeruginosa; Chlorination; Fluorescence; Bioorganic substance

1. Introduction

The safety of drinking water should never be an ignored problem and is closely related to the source water from lakes, rivers, and reservoirs in general [1]. However, the frequent occurrence of algae bloom in recent years has caused an environmental crisis due to eutrophication, which has been regarded as a common environmental issue and increasingly gained public attention [1]. Once the source water of algae bloom is pumped into water treatment plants, the algae will penetrate the filter chambers and block them, then raise the turbidity of discharged water quality, and even arouse the deterioration of water quality from the pipe network [2]. In addition, blue-green algae bloom is one of the most common frequent occurrences of algae bloom in the present, and especially due to its harmful microcystis toxins have aroused great effort to treat it [3]. Moreover, some organic matter contained in algal cells can serve as precursors to subsequently form disinfection byproducts (DBPs) during chlorination [4].

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Chlorination has been reported as the common utilization treatment method currently. Previously, many factors about chlorination on algae have been extensively investigated to assess the treatment effect, including pH value, temperature, chlorine dosage, and reaction time [5]. To assess appropriate degree of chlorination, the concept of chlorine exposure (ΔCT value), where consumed dosage of chlorine multiplied by reaction time, was proposed [6]. But during actual process of water treatment in treatment plants, very long reaction time could hardly be carried out in general case. Thus, the certain effective consumed chlorine dosage might dominate mostly in the parameter of ΔCT value.

Among the algae bloom causing water quality issues, the release of algae organic matters (AOM) should be particularly concerned, as it would directly contribute to the yield of variable DBPs when using certain chemical algicide (Cl_2, ClO_2, etc.). Under the different chlorine exposure, chlorination will cause different degraded effects, especially leading to the release of some organic matter, such as carbon-sourced organic or nitrogen-sourced organic, including humic acid-like, fulvic acid-like, soluble microbial byproduct-like matters or protein, may act the precursor role of trihalomethanes (THMs) and N-nitrosodimethylamine (NDMA) [7]. Besides, due to the different distribution of molecular weight (MW) of algae compounds, some compounds like pigments, will be easily degraded, yet humic acid-like matters or fulvic acid-like matters with huge MW might significantly capture the chemical oxidant then cause the competition reaction with other compounds. Therefore, the research specifically on AOM, rather than the whole algae cell, might be more advisable to evaluate the favorable chlorine exposure. Meanwhile, understanding the characteristics of chlorination on the AOM is essential to design appropriate water treatment processes. Excitation–emission matrix (EEM) is one of the spectrum fingerprint techniques, which could be used for the identification to the objects of fluorescence spectra in one complicated system with different multiples of components. Nowadays, EEM has been widely applied on the investigation to water quality [8]. However, to use EEM characterization, the variation of algae removal treatment process was very little.

In this research, *Microcystis aeruginosa* was chosen as the target pollutant as it is one of the most popular species found in freshwater bloom. All of the objectives included in this research were as below: (1) to investigate the effect of chlorination on the inactivation of *M. aeruginosa* cells and the photosynthetic pigments; (2) to investigate the variation of organic carbon and the release of IOM; (3) to distinguish the degradation characteristic of both extracellular dissolved organic matter (EDOM) and intracellular dissolved organic matter (IDOM).

2. Materials and methods

2.1. Materials and reagents

Cyanobacterial species of *M. aeruginosa* (FACHB315) were obtained from the Fresh Algae Culture Collection at the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences (Wuhan, China), which is one of the strains with nontoxin production [9]. The strain was cultivated in the BG11 medium in 1-L glass conical flasks. The flasks were installed with the conditions of 25℃ and the illumination of 2,000 Lux for a cycle of 12-h diurnal period and 12-h darkness period. NaClO was prepared for chlorination and its stock solution was prepared by diluting the bleach to Milli-Q water and stored at 4℃ in darkness. All chemical reagents used in the study were of analytical grade.

2.2. Experimental procedures

M. aeruginosa stock suspension was obtained from the growth exponential phase and diluted with the Milli-Q water to a favorable density (3.54×10^7 cells/mL) for the experiments. The density of algae cells was calculated under the microscope with an algae counting box. The reaction systems were installed with a batch of glass conical flasks (250 mL). Some certain algae suspension, Milli-Q water were prepared in prior, and then some volume (0–5.5 mL) of chlorine stock solutions (200 mg/L) were added to the reaction systems to reach a certain desirable initial level (the algae density of 1.13×10^6 cells/mL) for applying to the experiments. The initial chlorine concentrations were set with 0.3, 0.6, 1.3, 3.4, and 5.5 mg/L, respectively, and the final pH value of solutions varied within 5.5–6.8. The control group was duplicately operated under the same condition without adding chlorine stock solution. To assess appropriate degree of chlorination, the concept of chlorine exposure (ΔCT value) was obtained in this research. After exposure of 30 min, the remained concentration of chlorine in the algae suspensions was 0.1, 0.2, 0.6, 2.3, and 3.9 mg/L, and thus, corresponding ΔCT value was 6, 12, 21, 33, and 48 mg min/L, respectively. Then several drops of saturated $Na_2S_2O_3$ solution were added to neutralize the initial oxidizing agent. After the treatment, some algae suspension (100 mL) was filtered with 0.7-μm GF/F (glass-fiber filter, Whatman, UK) to extract the chlorophyll-a, and then the extraction was isolated

through adding 10 mL of 90% acetone for extracting 10 h. Meanwhile, in order to characterize the variations of IDOM and EDOM of algae suspension after the treatment, some fraction of original suspension (25 mL) was separated through the low vacuum pressure filtration (below 0.30 MPa) with 0.2-μm polycarbonate membranes filter (Millipore, USA) to obtain the EDOM solution. Meanwhile, the polycarbonate membrane filter attached with the algae cells together with its diluted algae suspension by ultrapure water were transferred into a cryovial (5 mL), and then the cryovials were processed with freeze thawing through liquid nitrogen for 5 min. The procedure of freeze thawing was continuously executed for triplicate, and the suspension was obtained. Subsequently, the suspension treated from freeze thawing was diluted to 25 mL with ultrapure water, and then filtered to obtain the IDOM solution.

2.3. Analytical methods

The content of free chlorine was determined by N, Ndiethyl-p-phenylenediamine photometry method using UV–visible absorption spectrophotometer (2,501, Cecil, UK). The morphological observations and the calculation for lethal cells combined with the coloring agent SYTOX® Green (Cambrex Bio Science Rockland, Inc., USA) were carried out using fluorescence microscope (90I, Nikon, Japan) [6]. Through fluorescence microscope, *M. aeruginosa* cells were observed by their auto-fluorescence signals. Organic carbon concentration was measured using a total organic carbon (TOC) analyzer (TOC-L CPH, Shimadzu, Japan). The content of chlorophyll-a and the fluorescence EEM spectra of algae suspensions were measured using fluorescence spectrophotometer (Agilent, Cary Eclipse, USA). The PMT voltage was set at 600 V, and the slits for both excitation and emission were 10 nm with scanning speed at 1,200 nm/min. In order to obtain an overview of the EEM pattern, the spectra with a high intensity (excitation range from 200 nm to 700 nm, and emission range from 250 nm to 750 nm) were scanned with the sampling emission internals of 5 nm and excitation internals of 2 nm. The EEM spectra were corrected by subtracting the signal of a blank Milli-Q water sample analyzed under the same conditions, and were plotted using software SigmaPlot 10.0 with fixed contour lines according to the fluorescence intensity.

3. Results and discussion

3.1. The variation of algae density of M. aeruginosa

As shown in Fig. 1, the live cells of *M. aeruginosa* were decreased with the level of chlorine in the suspension. The fluorescence staining method was conducted for the calculation of lethal cells. The coloring agent used for the calculation was SYTOX® Green, which is one kind of chemical coloring reagents could be wreathed to those lethal cell cytomembranes and be emitted with the green fluorescence signals under the observation of fluorescence microscope. When the suspension was treated with chlorine exposure of 6, 12, and 21 mg min/L, the live cells were only remained as $(36.5 \pm 4) \times 10^4$, $(7 \pm 3) \times 10^4$, and 7.0 ± 2 cells/mL, respectively. Especially, when the chlorine exposure was 21 mg min/L, the lethal rate was closed to 100%, which indicated that the chlorine exposure with 21 mg min/L had been approached to the condition of lethal threshold value in this research. With increasing the chlorine level in the suspensions, the cells of *M. aeruginosa* were completely apoptosis with the chlorine exposure were 33 and 48 mg min/L, respectively.

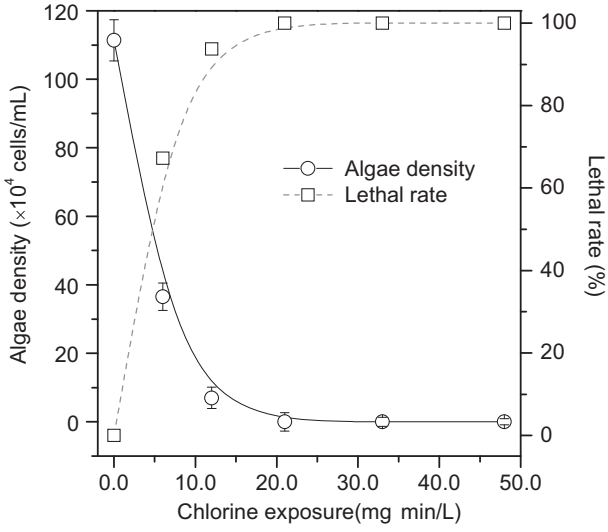

Fig. 1. The lethal effect of *M. aeruginosa* treated by different levels of chlorine. The error bars represent the standard deviation of replicate measurements ($n = 3$). Of note that, lethal rate (%) = (1 − Treated cell density/Control cell density) × 100.

3.2. The variation of chlorophyll-a of M. aeruginosa

As shown in Fig. 2, the chlorophyll-a content of *M. aeruginosa* was also presented with the same decreasing tendency with increasing chlorine exposure in the suspension. After the chlorination treatment, as the damage of the photosynthetic pigments is posterior to the damage of the cytomembrane [10], the damage rate of chlorophyll-a was reflected conspicuously lower than the lethal rate of cells. When chlorine

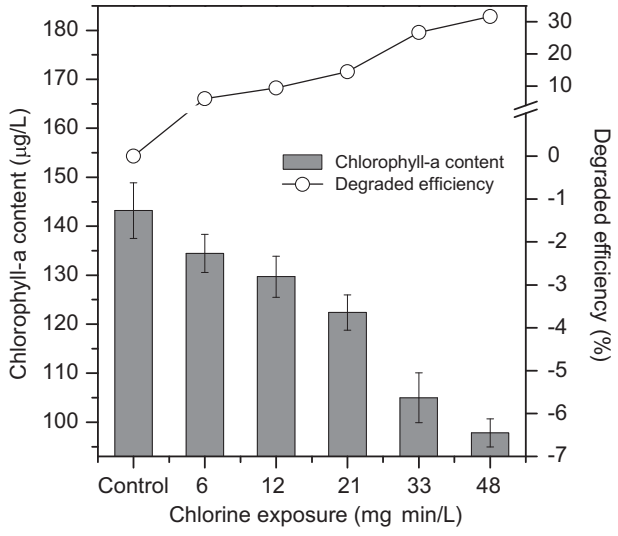

Fig. 2. The damage effect on chlorophyll-a treated by different levels of chlorine. The error bars represent the standard deviation of replicate measurements ($n = 3$). Of note that, damaged efficiency (%) = (1 − Treated content/Control content) × 100.

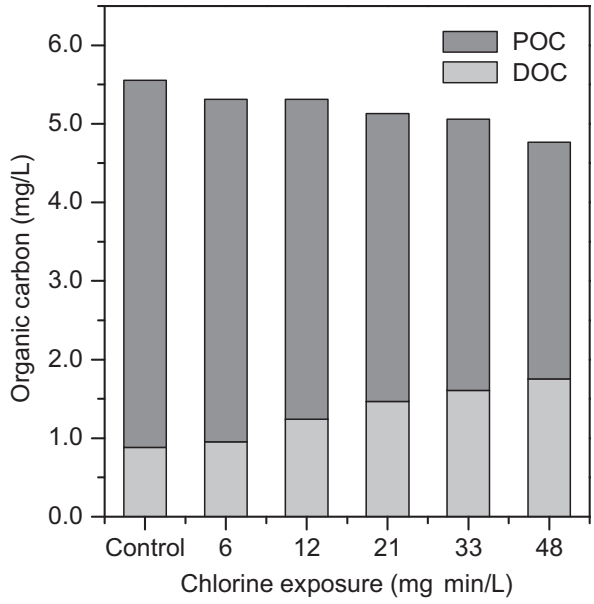

Fig. 3. The variation of organic carbon of *M. aeruginosa* suspension treated by different levels of chlorine (DOC, POC, and TOC are referred to dissolved organic carbon, particulate organic carbon, and total organic carbon, respectively, of note that TOC = POC + DOC).

exposure was 21 mg min/L in the suspension, the content of chlorophyll-a still remained as 122.4 ± 4 μg/L, compared with the content of control groups (143.2 ± 6 μg/L), which indicated that low content of chlorophyll-a had been slightly damaged (as the damaged rate was only 14.5%). Until the chlorine exposure was 48 mg min/L, the chlorophyll-a contained was still 97.8 ± 3 μg/L remained (the damaged rate was only 31.7%), indicating that the photosynthetic pigments could hardly thoroughly decompose in this condition. Although the content of chlorophyll-a had been considered as one of representative indexes of the biomass in some previous researches, the finding in this result suggested that it could hardly be a proper indicator to represent the impairments of chlorination on *M. aeruginosa*. This distinct result was in agreement with the findings by Ou et al. [11].

3.3. *Organic carbon variation of* M. aeruginosa

Fig. 3 presents the variation of organic carbon in algae suspension after chlorination. The decreasing of particulate organic carbon (POC) was resulted from the cell lyses after chlorination; meanwhile, under the chlorine exposure, produced hypochlorous acid by hydrolysis would effectively cause intracellular decomposition, which could be estimated from increased concentration of dissolved organic carbon (DOC) in the suspensions. Until the exposure level of 48 mg min/L, the concentration of POC and DOC were remained with 1.75 and 3.01 mg/L, which had been decreased by 36% and increased by 98%, compared with control group (the concentration of DOC and POC were 0.88 and 4.67 mg/L, respectively). Overall, the TOC concentration of suspensions was decreased with chlorine exposure level, suggesting that some small bioorganic matter in the cell had been oxidized and mineralized into CO_2 and H_2O, additionally, and different chlorine exposure could change the fate of DOC and POC, and thereafter lead to altering the structure and chemical composition of DOM. The phenomena are depended on possible competitive association and reaction happened between DOM and chlorine [6]. Nonetheless, the detailed degradation of DOM happened in intracellular and extracellular could hardly be identified and isolated because of its complex composition. Therefore, to use effective qualitative and quantitative measurements to further determine how the reaction process seemed to be very essential.

3.4. *Bioorganic substance variation of* M. aeruginosa

After the chlorination, different pattern variations of IDOM and EDOM in fluorescence spectra of EEM were obtained to qualitatively characterize the degradation degree (Fig. 4). Red gridline was used to distinguish the seven regions of typical representative

model compounds (shown in Table 1) in EEM. The aromatic protein-like (e.g. tyrosine and tryptophan-like) and soluble microbial byproduct-like compounds were exhibited with the fluorescence property of *M. aeruginosa*, with the peak located at the wavelengths Ex/Em of 230/350 and 280/335 [12]. Meanwhile, the fulvic acid-like and humic acid-like compounds were exhibited with the fluorescence property, located at the wavelengths Ex/Em of 260/450 and 330/450 [13,14]. Besides, the EEM regions located in

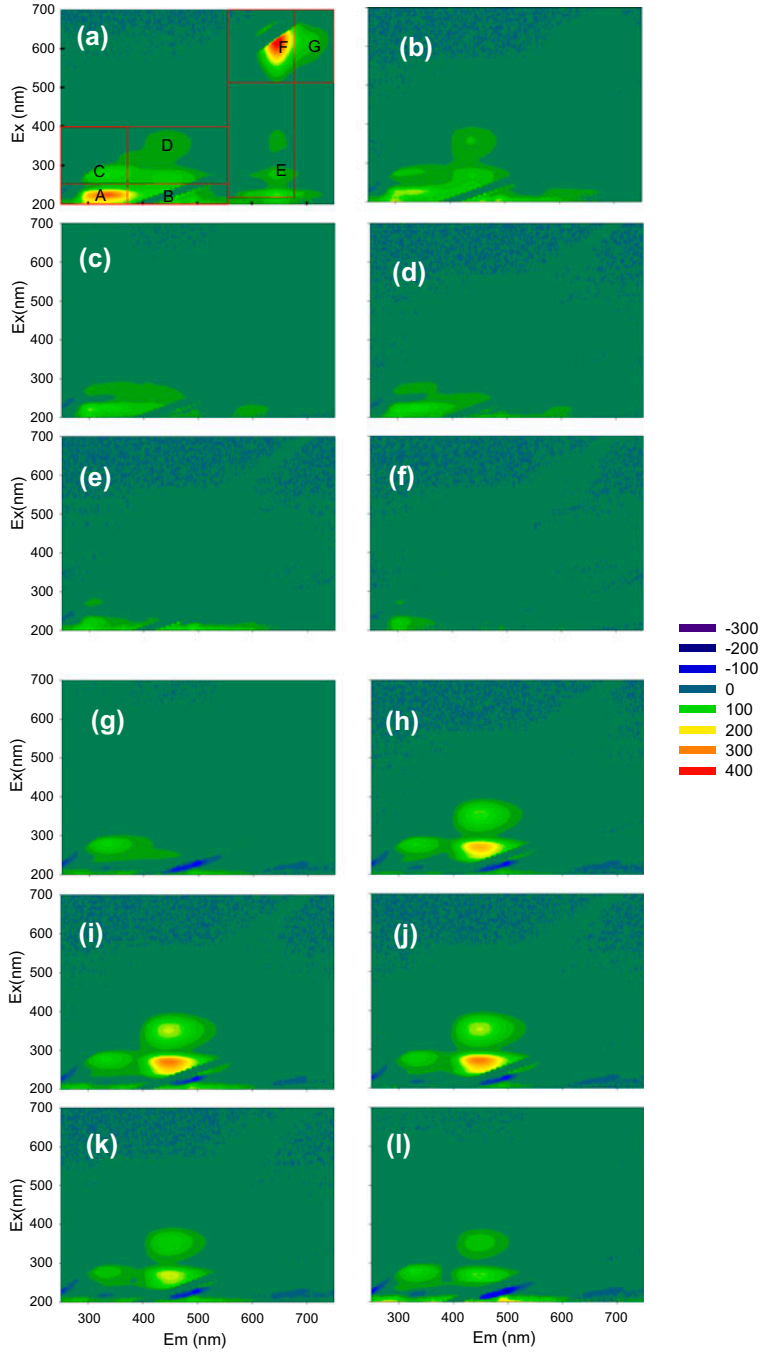

Fig. 4. EEM fluorescence spectra of DOM in suspension treated by different chlorine exposures. Of note that, (a)–(f) and (g)–(l) are referred to the EEM spectrum of IDOM and EDOM under different chlorine exposures (control, 6, 12, 21, 33, and 48 mg min/L, respectively). In addition, the letters from A to G represent typical compounds in relevant regions of excitation wavelength and emission wavelength, respectively, shown in Table 1.

Table 1
FRI regions and typical representative model compounds

EEM region	Wavelength range	Typical representative compounds
A	Ex < 250 nm, Em < 380 nm	Aromatic protein (tyrosine and tryptophan-like) [8]
B	Ex < 250 nm, 380 nm < Em < 550 nm	Fulvic acid-like [8]
C	250 nm < Ex < 400 nm, Em < 380 nm	Soluble microbial byproduct-like [8,15]
D	250 nm < Ex < 400 nm, 380 nm < Em < 550 nm	Humic acid-like [8]
E	210 nm < Ex < 510 nm, 550 nm < Em < 680 nm	By-product deriving from phycocyanin or other pigments [17]
F	510 nm < Ex < 700 nm, 550 nm < Em < 680 nm	Phycocyanin [12]
G	510 nm < Ex < 700 nm, 680 nm < Em < 750 nm	Photosynthetic pigments [12]

250 nm < Ex < 400 nm and Em < 380 nm are mainly referred to the byproducts from the bacterial decomposition and secretion of algae residue [8,15]. In addition, the phycocyanin could emit fluorescence at a peak at the wavelengths Ex/Em of 620/650 [16].

There was obviously weakened fluorescence intensity response from typical representative compounds of IDOM (shown from Fig. 4(a)–(f)). For the control group of IDOM (Fig. 4(a)), the most conspicuous fluorescence intensity responded from the aromatic protein, phycocyanin, and pigments in intracellular EEM spectra. To increase the exposure level, the variation of EEM tended to be even more remarkable, only until to the exposure of 48 mg min/L (Fig. 4(f)), very extremely weak fluorescence intensity responded from aromatic protein-like organics remained could be observed. This result indicated that the intracellular bioorganic substances had been suffered from releasing, oxidation, or degradation after the chlorination.

However, EEMs of EDOM of algae suspensions were exhibited with different variation tendencies. For the control group of EDOM, there was only some spectral data about soluble microbial byproduct-like compound detected in the EEM spectrum. Compared with the control group, even stronger fluorescence intensity was responded from the pattern of EEM (EDOM) when increasing the chlorine exposure level (shown from Fig. 4(g)–(l)), especially for those regions, e.g. Region C (250 nm < Ex < 400 nm, Em < 380 nm), Region B (Ex < 250 nm, 380 nm < Em < 550 nm), and Region D (250 nm < Ex < 400 nm, 380 nm < Em < 550 nm), in responding to representative organic compound of soluble microbial byproduct-like, fulvic acid-like, and humic acid-like compounds, respectively. In contrast, under the conditions of chlorine exposure level ranged from 21 to 48 mg min/L, the response from these bioorganic substances tended to be gradually diminished. Until to the exposure of 48 mg min/L, the response for certain typical compounds (region A, B, and D) could still be detected from EDOM (Fig. 4(l)). This observation indicated the release of IDOM, and some certain of degradation on bioorganic substance after chlorination as well as the chlorination level-dependent effect.

Moreover, in order to quantificationally characterize of the typical representative model compounds, the fluorescence regional integration (FRI), which has been regarded as one of the quantitative methods to the quantificational analyses of EEM spectra in recent years [18], was applied in this research. The FRI results were integrally calculated using Eq. (1) according to previous researches [11,17]. Of particular note that, in the Eq. (1), the parameters of $\Delta\lambda_{ex}$ and $\Delta\lambda_{em}$ are excitation wavelength internal and emission wavelength internal, which are taken as 5 and 2 nm, and the parameter of $I(\lambda_{ex}\lambda_{em})$ is the fluorescence intensity in each excitation emission wavelength pair. The unit of the parameter of Φ_i of FRI result value is AU nm^2.

$$\Phi_i = \sum_{ex}\sum_{em} I(\lambda_{ex}\lambda_{em})\Delta\lambda_{ex}\Delta\lambda_{em} \quad (1)$$

The FRI results were generally in agreement with the EEM variation, shown as Fig. 5. For the IDOM (Fig. 5(a)), the accumulation of integral value from each region (A–G) was presented with decreasing tendency, when chlorine exposure was 48 mg min/L, the level of IDOM was significantly lower than the control group. On the other hand, for the EDOM (Fig. 5(b)), the regions of A, C, and E referred to aromatic protein, soluble microbial byproduct-like compounds, and byproduct deriving from phycocyanin or other pigments were initially performed with increased tendency, but were decreased hereafter with even higher chlorine exposure. While compared with control group, the FRI value of regions F and G in EDOM were nearly declined to zero in other exposure groups (Fig. 5(b)), which indicated that phycocyanin (region F) and pigment (region G) were completely degraded. Additionally, under the chlorine exposure level of 12 mg min/L, the FRI value of regions B and D of EDOM were as 5.0 and 6.3 times as control group, and were reached to the maximum value, indicating

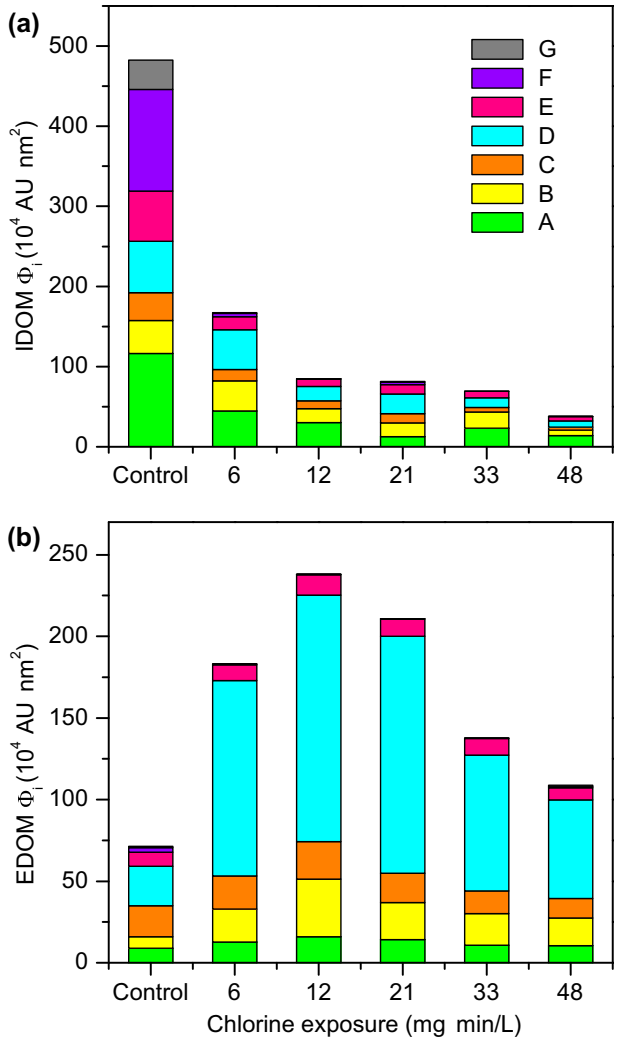

Fig. 5. FRI results of *M. aeruginosa* suspension treated by different chlorine exposures: IDOM (a) and EDOM (b) (Φ_i was calculated by Eq. (1), letters from A to G mean FRI value from the regions shown in Table 1).

that humic acid-like and fulvic acid-like compounds were removed initially and then leak into algae suspension due to cytomembrane damage. During chlorination, it had been revealed that even higher chlorine exposure level could result in more bioorganic substance being released, but simultaneously following with partial degradation of bioorganic substance as well. Nonetheless, although some certain favorable chlorination effect on the bioorganic substance could be acquired, there was still high level of humic acid-like compound remained from the observation of EDOM, combining with the result of less than 15% of TOC removed (Fig. 3), suggesting that an incomplete degradation of bioorganic substance even very significant cell lyses after chlorination. The reason for the phenomena could be explained as the humic acid-like compound with its high MW and complicated chemical construction would capture huge amount of oxidant [19].

3.5. Inactivation and degradation mechanism of chlorination and environmental implication

Under a complex system, the detailed chlorination effect regarding the inactivation of algae cells and the degradation of bioorganic substance have not been well understood. Well, Ma et al. [20] proposed that inactivation of *M. aeruginosa* cell by chlorination was through membrane crossing rather than massive damage to cell membrane. Ou et al. [11] have also validated this deduction through the observation by transmission electron microscope, and the corrosion and wrinkling of the cell walls could be observed after chlorination. Since the exposure of chlorine and its chlorination, it is inevitable to result in the release of algae intracellular organics and the simultaneous reaction with the bioorganic substance as well. The assumption could be demonstrated from EEM with the initial increase of humic acid-like, fulvic acid-like of EDOM, and the disappearance of phycocyanin, pigment of EDOM thereafter. Additionally, for aromatic proteins with unstable chemical functional group, like the weak stability of covalent bond between carbon atom and nitrogen atom is likely to be ruptured [21], which might easily be destroyed by disturbing their aromatic property through ring opening and breaking the aromaticity of an aromatic ring, then even forming the compounds with single chain [21,22]. When exposed in high chlorine exposure level, the abundant chlorine will continue to degrade those big molecular compounds, which could be evidenced from FRI value (F and G) of both EDOM and IDOM have been declined to zero. Besides, Beggs et al. [23] found that the difficult chlorination with some chemical compound with high MW of DOM, e.g. humic acid-like and fulvic acid-like compounds, which was possible to contribute to other formation, like THMs, and even attributed to the competition with other organic. Although the final byproduct had not been determined, the similar behavior occurred in humic acid-like or fulvic acid-like compounds had been verified in this research, as much of them were still remained and could hardly completely degraded in final. Overall, the process of chlorination on *M. aeruginosa* cells and degradation for bioorganic substances could be described as below (shown as Fig. 6): (1) chlorine was penetrated into cell walls and cell membranes, and then caused the release of intracellular metabolites; (2) the chromophores were destroyed, the IDOM was

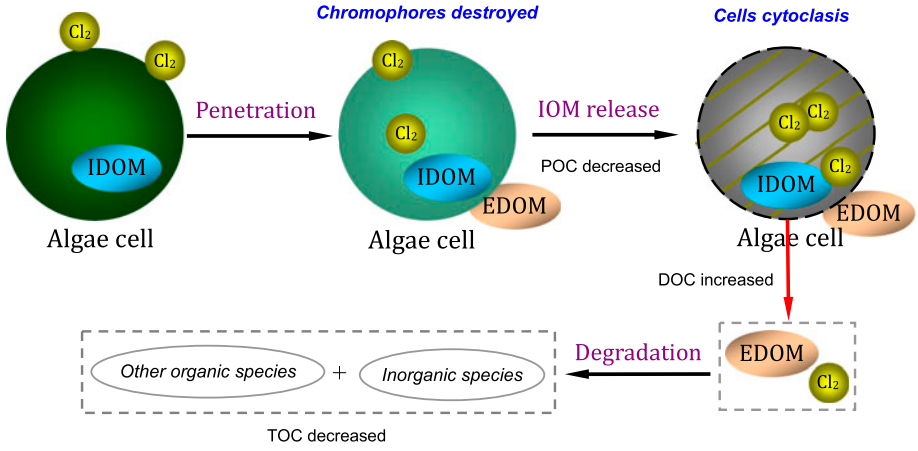

Fig. 6. Presumed inactivation and degradation mechanism by chlorination of *M. aeruginosa*.

then leaked out, and degraded as well; and (3) simultaneously, the organics with higher MW were degraded into those lower MW or other inorganic species.

Meanwhile, according to previous researches, some special disinfect byproducts' (DBPs) formation, like nitrosamines, trihalomethanes, and haloacetic acids, were contributed from the reaction between chlorine and potential organic precursor sourced from AOM, e.g. soluble microbial byproduct-like, fulvic acid-like and humic acid-like compounds [7]. Furthermore, the potential health effects associated with high nitrate level in water would cause potential human carcinogens by combining with amines to form nitrosamines. Thus, the occurrence of nitrates should never be a negligible issue under high chlorination treatment, especially Cl/N molar ratios would directly influence the formation and evolution of odorous products, e.g. N-chlorophenylalanine, phenylacetonitrile, and N-chlorophenylacetaldimine [24]. The results from this study could be also extended to other amino acids, e.g. alanine, leucine [24], due to the incomplete degradation on DOM. They are susceptible to be present in water at higher concentrations when associating with high level of chlorine, and their chloroaldimines will probably be even more odorous and aggravate sensory quality [19,24]. Thus, there is significance on raising public attention on judging requirement for water sensory quality and also enlightening the water operators to establish an optimized water treatment process to restrict AOM (the precursor of DBPs), nitrate, and high chlorine level.

Therefore, to further understand the structure–reactions relationships for different chemical compounds under an individual treatment process seems to be significantly necessary, thus more kinetic researches, precise characterized methods for chlorine-mediated destruction or the distributions of products should be employed for the identification of chlorination. The model established in this research elucidated that the application of chlorination as a feasible pretreatment for blue-green algae bloom water should be paid more attention on the effective chlorination exposure due to the chlorine-induced release of IOM and even potential environmental risk of DBPs and odorous product demonstrated by other researches. The relationship between chlorine exposure and cell lyses, degradation of bioorganic substance would provide the enlightenment to promote the optimization of chlorination effect and control the subsequent possible byproduct with some feasible methods.

4. Conclusions

In this research, the methods including fluorescence staining, EEM, FRI, and other biochemical parameters were employed to investigate the inactivation of *M. aeruginosa* and the degradation on the bioorganic substance from both IDOM and EDOM by chlorination. The results indicated that the remarkable cell lyses, the disagreement of lethal variation of algae cells with the damaged efficiency of chlorophyll-a, and the incomplete degradation on DOM corresponded with low TOC removal after chlorination. Thus, an appropriate method to comprehensively identify the inactivation effect of *M. aeruginosa* should be established. Additionally, the possible route regarding the chlorination effect on the inactivation of algae cells was proposed: through penetration, accompanied with the IOM release, then the further degradation of bioorganic with high MW into low MW. Meanwhile, different degraded characteristic of bioorganic

compounds could be obtained. A significant degraded effect of phycocyanin and photosynthetic pigments could be observed in this research. However, there was still plentiful of humic acid-like and fulvic acid-like compounds remained after treatment, this observation was similar with previous researches and was possible to cause other byproduct.

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