Effect of chlorination on the cell integrity of two noxious cyanobacteria and their releases of odorants

Tsair-Fuh Lin, De-Wei Chang, Shao-Kai Lien, Yun-Shen Tseng, Yi-Ting Chiu and Yi-Shen Wang

ABSTRACT

The effect of chlorination on cell integrity and release of metabolites of two noxious cyanobacteria, a β-cyclocitral producer—*Microcystis aeruginosa*—and a geosmin producer—*Anabaena circinalis*—was investigated. Photos from a scanning electron microscope revealed that *Microcystis* cell surfaces were deformed after chlorination, and filamentary *Anabaena* cells were ruptured at the junction of vegetative cell units. Chlorination experiments indicated that both cyanobacteria are susceptible to attack by chlorine. A first-order decay model was used to simulate the cell-rupture kinetics during chlorination. Using laboratory cultures, the observed rate constants are 670–1,100 $M^{-1} s^{-1}$ for *M. aeruginosa*, which are 1.3–5.0 times as large as those for *A. circinalis*. For unfiltered *Microcystis*-laden reservoir waters, a broader range of rate constants, 70–590 $M^{-1} s^{-1}$, were obtained. The rate constant was reduced in more eutrophic water, due probably to the competition of chlorine with other cyanobacteria in water. Geosmin was rapidly released into water immediately after the *Anabaena* cells were ruptured. However, a portion of geosmin remained cell-bound. For *Microcystis*, the total β-cyclocitral concentration decreased quickly during chlorination in most cases. It is speculated that the enzymes leading to the formation of β-cyclocitral were inhibited by chlorine, thus causing a sudden reduction of β-cyclocitral in the system.

Key words | *Anabaena*, β-cyclocitral, cell rupture, chlorination, geosmin, *Microcystis*

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INTRODUCTION

The presence of off-flavour chemicals in drinking water has received increasing attention during the last few decades (Suffet *et al.* 1995; Watson 2003; Tung *et al.* 2008; Yang *et al.* 2008). Although the causes of water taste and odour (T&O) are complicated, a group of microorganisms, mainly cyanobacteria, are known to be responsible for many episodes in water sources (Suffet *et al.* 1995; Izaguirre & Taylor 2004). Among the odour-producing cyanobacteria, *Microcystis*, a producer of wooden-odour β-cyclocitral (2,2,6-trimethyl-1-cyclohexene-1-carboxaldehyde) (Juttner & Hoflacher 1985; Jones & Korth 1995; Young *et al.* 1999), and *Anabaena*, a producer of earthy-odour geosmin (*trans*-1, 10-dimethyl-*trans*-9-decalol) (Juttner & Watson 2007), are two common genera detected in many eutrophic lakes and reservoirs around the world. The two odorants are detectable at trace concentration in water, 4–10 $ng l^{-1}$ for geosmin (Watson *et al.* 2000) and 500 $ng l^{-1}$ for β-cyclocitral (Young *et al.* 1999).

Removal of cyanobacteria (blue-green algae) and cell-bound odorants can be achieved with a train of conventional treatments: coagulation and flocculation, clarification either by a dissolved air flotation or sedimentation, and sand filtration. The treatment processes were found to have a minimal effect on the cell integrity of cyanobacteria and to cause no release of intracellular metabolites (Chow *et al.* 1999). To improve the removal of algae during the

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coagulation and separation process, a pretreatment step using oxidants such as ozone, chlorine or permanganate is commonly employed in many instances (Plummer & Edzwald 2002; Chen & Yeh 2005; Henderson *et al.* 2008). For example, in many waterworks in Taiwan, a pre-chlorination step is a standard practice. This practice is also considered as a remedy for inhibiting the growth of algae in sand filters. However, the oxidation-based pre-treatment may lyse cyanobacteria cells and cause the release of intracellular metabolites (Ashitani *et al.* 1988; Peterson *et al.* 1995; Tung *et al.* 2004).

The effect of chlorine on the cell integrity has been investigated in many studies. For example, Ashitani *et al.* (1988) observed an increase of 2-MIB and geosmin concentrations in water following prechlorination at a water treatment plant. Peterson *et al.* (1995) and Tung *et al.* (2004) indicated that cyanobacterial cells might be lysed in the presence of chlorine, causing the release of geosmin and dissolved organic carbon. Once the cell-bound odorants were released into water, it would be more difficult to remove them in conventional treatment processes. Chlorination and conventional treatment processes are generally not effective to destroy and/or remove geosmin (Lalezary *et al.* 1986; Duguet *et al.* 1995). Unlike the earthy odorant geosmin, β-cyclocitral has been studied to a much lesser extent. Only one study reported the oxidation of dissolved β-cyclocitral. Dietrich *et al.* (1995) showed that chlorine at 1 and 3 mg l^{-1} dosage had very little effect on odour characteristics in water. The results were based entirely on a sensory evaluation, called the flavour-profile analysis, and no chemical analysis was performed.

While chlorination is still considered as a common practice in many waterworks for the pretreatment of eutrophic raw water, the kinetics for the rupture of cyanobacteria cells during chlorination is not well understood. In addition, to our knowledge, the formation and release of β-cyclocitral in the chlorination process has never been reported. Therefore, the objective of this study is to understand the effect of chlorination on the cell integrity and odorant release of two noxious cyanobacteria, including a β-cyclocitral producer–*Microcystis aeruginosa*–and a geosmin producer–*Anabaena circinalis*. The kinetics on cell rupture caused by chlorination and the subsequent formation and/or release of odorants were studied using laboratory cultures in batch experiments. For comparison, similar chlorination experiments using *Microcystis*-laden reservoir waters were also conducted. The data on cell rupture were evaluated with a kinetic model.

MATERIALS AND METHODS

Cell cultures

In this study, two cyanobacteria were selected: *Microcystis aeruginosa PCC 7820* obtained from Pasteur Culture Collection of Cyanobacteria, France, and *Anabaena circinalis* from Australian Water Quality Centre, Australia. The former is a producer of both microcystin-LR and β-cyclocitral (a wooden odorant) (Young *et al.* 1999) and the latter is a geosmin producer. Both strains were cultured in ASM media (Rippka 1988) at 25°C under constant light flux.

The raw water from Tai-Hu Reservoir (THR), Kin-Men Island, Taiwan, was used for laboratory experiments. The average water quality is listed in Table 1. For chlorination experiments, the ASM cultures were incubated in the filtered, sterilized THR raw water, with addition of nutrients similar to those in ASM media. After reaching a stationary phase ($\sim 10^6$ cells ml^{-1}), the samples were then used for the experiments.

Table 1 | Water quality of the four studied source waters

Reservoir	Total organic carbon (mg l^{-1})	NH_3-N (mg l^{-1})	Total phosphorus (μg l^{-1})	Chlorophyll-a (μg l^{-1})	Alkalinity (mg l^{-1} as $CaCO_3$)	Turbidity (NTU)
Tai-Hu	9.6 ± 3.2	0.24 ± 0.23	94.6 ± 18.0	61.7 ± 26.5	78.0 ± 11.6	17.0 ± 7.8
Rong-Hu	14.6 ± 4.9	0.09 ± 0.05	80.0 ± 20.3	61.9 ± 20.3	83.5 ± 13.2	18.1 ± 7.3
Lan-Tan	1.8 ± 0.3	0.03 ± 0.01	16.4 ± 5.9	3.7 ± 2.8	111.8 ± 6.2	3.2 ± 2.4
Ren-Yi-Tan	1.7 ± 0.6	0.04 ± 0.2	18.3 ± 10.3	2.8 ± 1.8	116.9 ± 9.8	5.1 ± 4.1

Source: Data collected by Taiwan Environmental Protection Administration seasonally during 2007 and 2008.

To mimic the field situation, similar chlorination of unfiltered *Microcystis* spp.-laden source waters was also conducted. Each of the four selected reservoirs serves as the source water of a nearby waterworks, and all the waterworks employ a prechlorination step in their treatment trains. Therefore, these waters were used to simulate the prechlorination process in the waterworks. The average water quality from 2007 to 2008 for the four source waters are listed in Table 1. In summary, THR and Rong-Hu Reservoir (RHR), both located in Kin-Men Island, are eutrophic with high concentrations of chlorophyll-a, nutrients and total organic carbon (TOC). Two other reservoirs, Lan-Tan Reservoir (LTR) and Ren-Yi-Tan Reservoir (RYTR), are less polluted and contain much lower levels of nutrients and TOC. These two reservoirs are located next to each other in Cha-Yi, Central Taiwan. Both are off-channel reservoirs with the same source water. Therefore, their water qualities are very similar.

Determination of cell integrity

The integrity and rupture of cyanobacteria cells were determined by a flow cytometer (FCM) (FACSCalibur™, Becton Dickinson, USA) for *M. aeruginosa* in laboratory cultures and by an epifluorescence microscope (EFM) (BX51, Olympus, Japan) for *A. circinalis* in laboratory cultures and *M. aeruginosa* in reservoir water samples. An argon laser emitting at a fixed wavelength of 488 nm was used for all the FCM measurements. The standard green fluorescent detector (FL1, 530 nm) was used to detect the cells stained with fluorescein diacetate (FDA) (Invitrogen, USA) and SYTOX (Invitrogen, USA), while the red fluorescent detector (FL3, 650 nm) was used to detect the auto-fluorescence of chlorophyll in the cells. WinMDI 2.8 software was used to collect and analyse the data. FDA, which stains only the membrane of intact cells, and SYTOX, which permeates ruptured cells and stains nucleic acids, were both used to determine the percentage of intact to ruptured cells in a sample using the method of Regel *et al.* (2002) and Daly *et al.* (2007), respectively. A development time of 7 min was used for both stains, with a final concentration of 0.1 μM for SYTOX and 40 μM for FDA. Data were collected until the combined number of events recorded in the intact and ruptured regions reached 5,000. When measuring *Anabaena* in EFM, similar FDA and SYTOX concentrations were used and the development time was 20 min. The samples were measured with an exposure time of 20 ms.

Chlorination experiments

A batch-type reaction was used for chlorination experiments. In the experiments, one-litre glass vessels were adopted as reactors. The reactors were maintained at 25 ± 1°C, with a Teflon-coated magnetic stirrer placed in the reactor. For chlorination of laboratory cultures, the dose of sodium hypochlorite (GR grade, Riedel-de Haën, Germany) was controlled at between 0.5 and 4.0 mg l^{-1} (Cl_2 basis), and the pH was at 8.3–8.6. This pH range is similar to the typical values of the four reservoirs in this study. For chlorination of *Microcystis*-laden reservoir waters, the chlorine dose was set to be the same as applied in the corresponding waterworks. The pH values as well as chlorine doses applied for the experiments with reservoir waters are discussed below in the 'Kinetics of cell rupture' section. In the experiments, samples were collected at predetermined time intervals for analyses of the cell integrity, residual chlorine and odorants.

Analysis

A Water Quality Analyzer (Merck, Nova 60, US) was used to determine the concentration of the residual chlorine in water. A portion of the samples (~ 5%) was also analysed following the DPD-FAS Method reported in *Standard Methods* (1998). The difference of detected chlorine concentrations by the two methods was all less than 10%.

Concentrations of geosmin and β-cyclocitral were analysed using the solid-phase microextraction (SPME) coupled with a gas chromatograph (GC, Agilent 6890) and a mass spectrometer (MS, Agilent 5973). A commercially available fibre (30/50 μm DVB/CAR/PDMS (No. 57348-U)) and a manual fibre holder (No. 57330-U), both from Supelco (Bellefonte, Pennsylvania), were used for extraction of the odorants. The odorants trapped in the fibre were then quantified by the GC/MS. The procedure

for analysing the odorants was the same as prescribed in *Standard Methods* 6040D (2000), except for minor adjustments. Detailed analytical procedures on the parameters involved were reported in Lin *et al.* (2002, 2003). Differentiation between odorants in water and those in the cells was determined by separate analyses of filtered and unfiltered samples. A 0.45 μm glass fibre filter (Mixed cellulose ester, MFS, US) was used for the separation.

In some *M. aeruginosa* samples, microcystins were analysed using an enzyme-linked immuno-sorbent assay (ELISA). A commercially available microcystin plate kit (CPP- 023, Beacon, US) was employed together with a microplate reader (MR5000, Channel Islands, US) for the analysis. The kit is able to detect microcystin LR, RR and YR, with a detection limit of 0.1 $\mu g\,l^{-1}$.

The cell enumeration for cyanobacteria was conducted using a microscope equipped with haemocytometers or 1 ml Sedgwick Rafter chamber (Graticules Ltd, UK). Before the cell counting, 10 μl of a sample were injected into the two chambers in the haemocytometer and 1 ml of the sample was injected into the Sedgwick Rafter chamber. After settling for 30 min, the sample was then counted at 200 × magnification and a precision of $<20\%$ was achieved for all the samples.

To visualize the surface of the cyanobacteria, microphotos of the studied strains were taken using a scanning electron microscope (SEM, S-3000N, Hitachi, Japan). Before analysis, the samples were filtered by a 0.2 μm Nylon filter (Min-Yi, Taiwan). The filter was then fixed with a mixed solution of 2.5% glutaraldehyde (Analytical grade, Merck) and phosphate buffered saline (PBS buffer, Merck) (1:100) at 4°C for 1 h, a mixed solution of PBS buffer and 5% sucrose (Analytical grade, Merck) (1:1) at 4°C for 15 min, a mixed solution of 1% osmium (Merck) and PBS buffer (1:100) at room temperature for 1 h, and a mixed solution of PBS buffer and 5% sucrose (1:1) for 15 min. The samples were then dehydrated by a sequential ethanol-in-water (50%, 70%, 80%, 90%, 95% and 100%, Merck, Germany) extraction, and dried by a critical point dryer (HCP-2, Hitachi, Japan). Finally, the samples were then coated with gold (with an ion sputter, E-1010, Hitachi, Japan) for analysis.

RESULTS AND DISCUSSION

Surface morphology of cells before and after chlorination

Figure 1 shows the microphotos taken for the surfaces of *M. aeruginosa* and *A. circinalis* before and after chlorination. As shown in the figure, both strains look very intact and smooth on the surface before oxidation (Figure 1(a) and 1(d)). However, after chlorination, the surface morphology of the two strains changed to different extents. At a low dosage ($1\,mg\,l^{-1}$) and a short contact time (1 min), *M. aeruginosa* (Figure 1(b)) started to deform with an appearance of cellular materials being released around the cell. After longer contact time of 30 min at a higher dosage ($2\,mg\,l^{-1}$), the cell became deflated and shrank to a smaller size (Figure 1(c)). For *A. circinalis*, at a short contact time (1 min) and a low chlorine dosage, the trichome was broken at the junction of two adjacent vegetative cells, making the trichome become shorter (Figure 1(e)).

Chlorination of *M. aerginosa* and formation of β-cyclocitral

Chlorination of *M. aeruginosa*-laden water was conducted at different cell concentrations and chlorine dosages. Figure 2 shows the experimental results from two of the experiments, with cell concentrations at 4.3×10^5 (referred as Case M-L) and $1.1 \times 10^6\,cells\,ml^{-1}$ (referred as Case M-H) and a chlorine dosage at $2\,mg\,l^{-1}$. For both cases, residual chlorine concentrations became steady after about 5 min reaction time. Comparing the two cases, a lower residual chlorine concentration was found for Case M-H (Figure 2(c)). This finding is reasonable, because more cells and associated metabolites present in water would demand more chlorine. As also shown in Figures 1(a) and 1(c), *M. aeruginosa* cells were prone to attack by chlorine. For Case M-L with lower cell concentration, almost all the cells ($>$ 93%) were ruptured within 1 min. In this case, chlorophyll-a (chl-a) was also measured. A sudden decrease of chl-a was observed in 1 min (Figure 2(b)), suggesting that chl-a was released from the cells and reacted with chlorine. For Case M-H, a slower kinetic was observed; about 75% of cells were ruptured within 1 min.

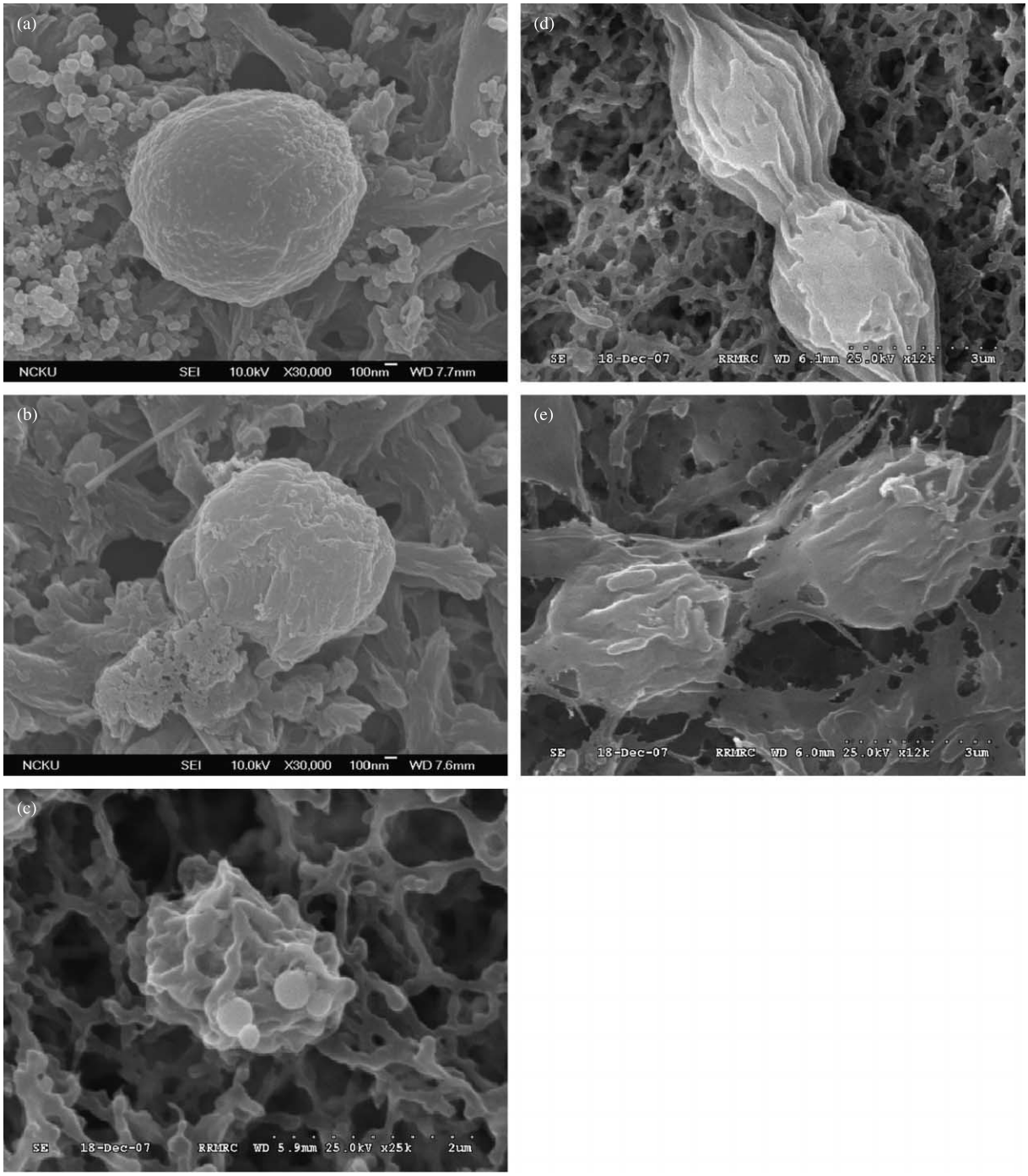

Figure 1 | SEM microphotographs of *Microcystis* (a) before chlorination, (b) after chlorination (1 mg l^{-1} and 1 min), and (c) after chlorination (2 mg l^{-1} and 30 min), and *Anabaena* (d) before and (e) after chlorination (1 mg l^{-1} and 1 min).

The change of β-cyclocitral concentration is presented in Figure 2(b) and (d). Before chlorination, a total concentration of 1,200 and 2,300 ng l^{-1} was observed for the two cases, respectively. About 8% and 10% of the β-cyclocitral was present extra-cellularly. Juttner & Hoflacher (1985) discovered that β-cyclocitral is the product of an oxidative cleavage reaction of β-carotene. The reaction is catalysed by β-carotene oxygenases bound on *Microcystis*

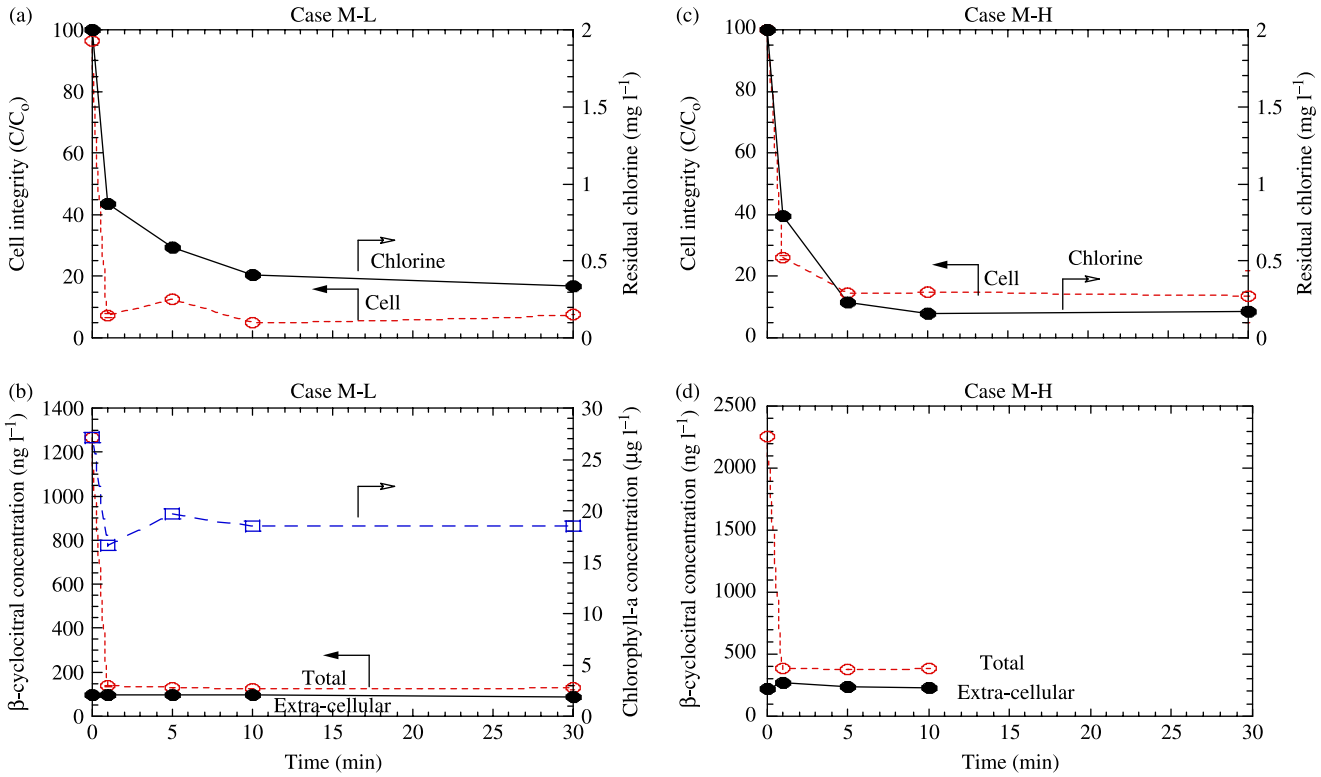

Figure 2 | Residual free chlorine, cell integrity and β-cyclocitral concentration during chlorination of *M. aeruginosa*, where (a) and (b) are under cell concentration = 4.3 × 10^5 cells ml^{-1} and chlorine dosage = 2 mg l^{-1}, and (c) and (d) are under cell concentration = 1.1 × 10^6 cells ml^{-1} and chlorine dosage = 2 mg l^{-1}.

cell membrane under aerobic conditions, and the reaction is activated when the cells are disintegrated. Considering that β-cyclocitral is formed after the cell is ruptured, it is reasonable that most β-cyclocitral is initially associated with *Microcystis* cells. Therefore, a high concentration of β-cyclocitral is observed when the cells are disintegrated during the analysis using SPME method.

Figure 2(b) and (d) also illustrates that, right after chlorination, the total β-cyclocitral concentrations of the samples suddenly decreased by 83–88%. Dietrich *et al.* (1995) examined the effect of chlorine on the flavour profile of β-cyclocitral in water. They found that at 1 and 2 mg l^{-1} of chlorine, the odour intensity of β-cyclocitral samples did not change much, suggesting that β-cyclocitral was resistant to chlorine. To confirm this, chlorination of β-cyclocitral was examined in de-ionized water at different chlorine dosages for one hour; the results are shown in Figure 3. It is clear that chlorine did not oxidize β-cyclocitral, even at as high a chlorine dosage as 10 mg l^{-1}. Therefore, the observation of a sudden decrease of β-cyclocitral concentration after chlorination shown in Figure 2(b) and (d) is not likely a result of the chlorine oxidation. Although no direct evidence is available from this study, a probable reason for the reduction of β-cyclocitral is the damage of

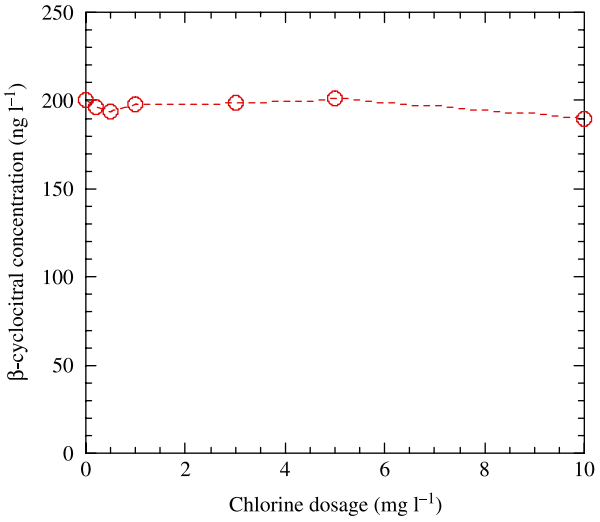

Figure 3 | Effect of chlorine on dissolved β-cyclocitral concentration.

β-carotene oxygenases by chlorine. As pointed out by Juttner & Hoflacher (1985), β-carotene oxygenases are bound on the *Microcystis* cell membrane. Therefore, it is possible that chlorine not only ruptured the cell membrane but also damaged the enzymes on the membrane, inhibiting the formation of β-cyclocitral.

Chlorination of *Microcystis* spp. in THR water

Figures 4 and 5 display the experimental results from chlorination of *Microcystis*-laden THR water at two different runs with *Microcystis* spp. (mainly *M. aeruginosa*) cells, one with 1.3×10^5 cells ml^{-1} (referred to as Case THR-L) and the other with 4.4×10^5 cells ml^{-1} (referred to as Case THR-H). Although the chlorine dosage was high (6 mg l^{-1}) for both cases, the chlorine demand by the water was also

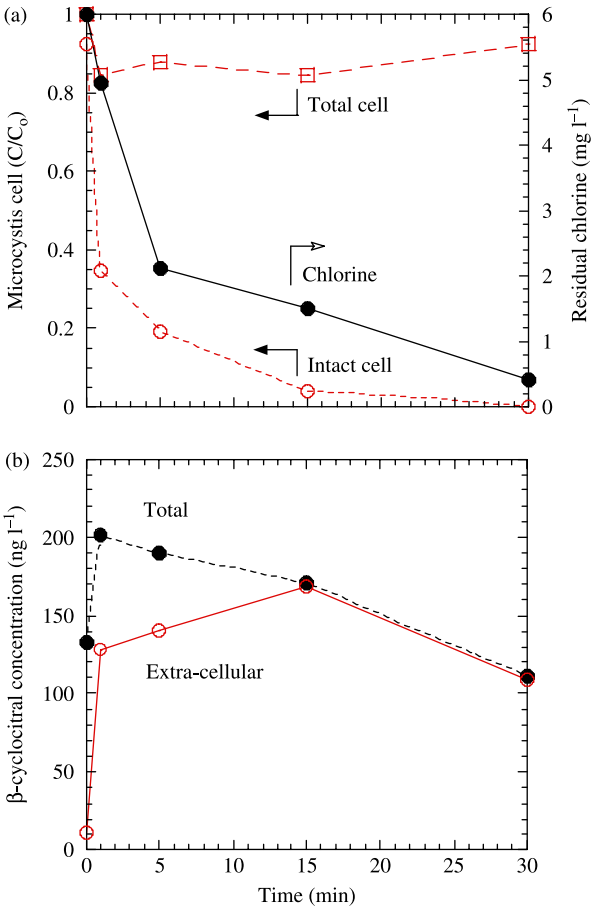

Figure 4 | Residual free chlorine, cell integrity and β-cyclocitral concentration during chlorination of *Microcystis*-laden THR water, where cell concentration = 1.3×10^5 cells ml^{-1} and chlorine dosage = 6 mg l^{-1} (Case THR-L).

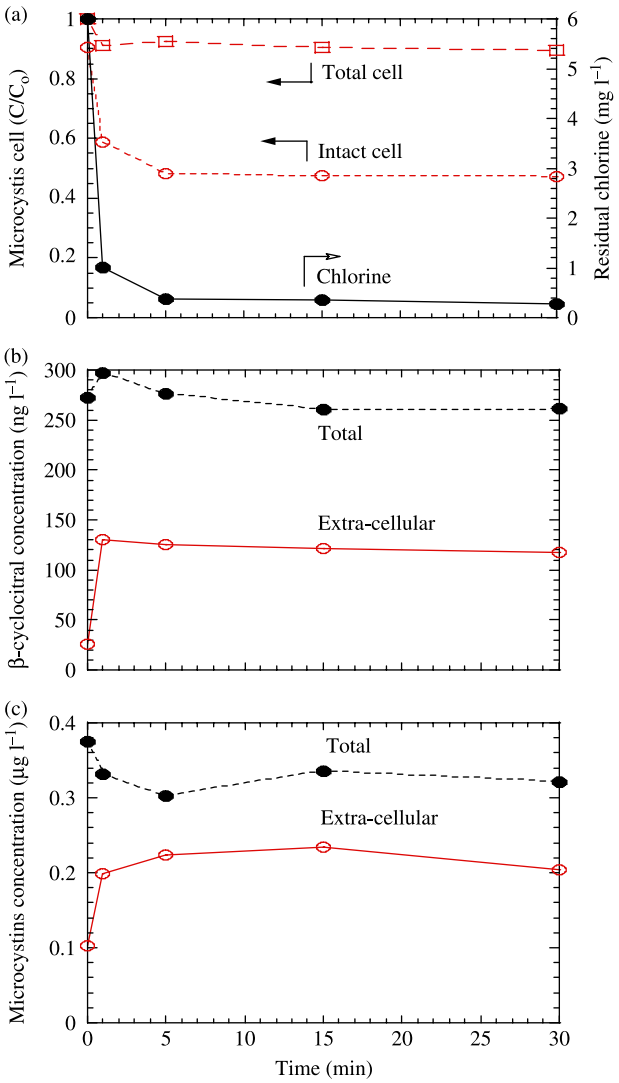

Figure 5 | Residual free chlorine, cell integrity, β-cyclocitral and microcystins concentration during chlorination of *Microcystis*-laden THR water, where cell concentration = 4.4×10^5 cells ml^{-1} and chlorine dosage = 6 mg l^{-1} (Case THR-H).

high. Almost all the chlorine was exhausted within 30 min for Case THR-L (Figure 4(a)), and within 5 min for Case THR-H (Figure 5(a)). The rapid decrease of chlorine in the THR water may be attributed to the high TOC concentration (at ~9.6 mg l^{-1}), high levels of cyanobacterial cells and associated metabolites. In fact, in addition to the *Microcystis* spp., another cyanobacterium, *Cylindrospermopsis raciborskii*, was also observed in Case THR-H with a concentration of 6.1×10^5 cells ml^{-1}. This high concentration of cyanobacteria may be the reason for the rapid depletion of chlorine within 1 min in the experiment (Figure 5(a)).

Figures 4(a) and 5(a) also show the total numbers and fractions of integral *Microcystis* cells. Before chlorination, about 90–92% were considered as intact according to the analysis, suggesting that the majority of the *Microcystis* cells were healthy in THR raw water. In Case THR-L, about 60% of cells were ruptured within 1 min of chlorination, 80% within 5 min, and ~100% within 30 min. In the second case, only 42% of cells were ruptured within 1 min, and about 52% of cells were damaged during the experimental period. The different degree of cell rupture in the two cases may be related to different chlorine demands in a short time. For Case THR-H, almost all the chlorine was exhausted within 5 min, and therefore, no residual chlorine was available to damage the cells. Although a portion of cells was disintegrated in the two cases, the cells were still recognizable in the analysis. As illustrated in Figures 4(a) and 5(a), the total cell numbers only decreased by about 10% during the experimental periods for both cases, suggesting that chlorine in the two cases was able to rupture the cells but not to the extent of complete disintegration.

The changes of β-cyclocitral concentrations are shown in Figures 4(b) and 5(b). In both cases, only <10% of β-cyclocitral were present extra-cellularly before the chlorination. This is similar to those observed in Case M-L and M-H discussed above. Right after the chlorination (at 1 min), β-cyclocitral was released into water, the fractions being similar to the ruptured/total cell ratios. For example, the intracellular β-cyclocitral was at a level of about 36% and 26% for Case THR-L at 1 and 5 min, respectively, which is similar to the observed ratios of intact cells at 35% and 19%, respectively. Similarly, the intracellular β-cyclocitral in Case THR-H was at a level of 59% and 48% at 1 and 5 min, respectively, which is close to the ratios of intact cells at 56% and 55%, respectively. A similar observation is also noted for the microcystin concentration shown in Figure 5(c). At 1 min after the chlorination, about 49% of the microcystins remained cell-bound, and 30–42% were cell-bound after 5 min of reaction. Dissolved microcystins are readily destroyed by free chlorine (Rodriguez *et al.* 2007). The observation in Case THR-H suggests that chlorine was able to rupture a certain portion of cells, but its residual level did not have an enough power to damage β-carotene oxygenases and to destroy the dissolved microcystins. Thus, the total β-cyclocitral and microcystin concentrations remained almost constant within the experimental period. For Case THR-L, another interesting observation is that the total β-cyclocitral concentration increased right after the chlorination but then decreased with time. This is very different from all other experimental observations, in which total β-cyclocitral either decreased (Case M-L and Case M-H) or remained almost constant (Case THR-H). The mechanisms of increasing total β-cyclocitral concentration after chlorination in this case are not clear. Sommerburg *et al.* (2003) investigated the oxidation of β-carotene using sodium hypochlorite and observed that β-cyclocitral is one of the by-products. This may be one of the reasons for the increase of β-cyclocitral, if β-carotene or other β-cyclocitral precursors were present in water. However, more studies are needed to confirm this view.

Chlorination of *Anabaena* and release of geosmin

Figure 6 shows the results from chlorination of *Anabaena*-laden water in the laboratory. The cell concentrations were 6.0×10^5 (referred to as Case A-L) and 1.1×10^6 cells ml^{-1} (referred to as Case A-H), respectively, and chlorine dosages were at 2 and 0.5 mg l^{-1}, respectively. The residual chlorine concentration in Case A-L suddenly reduced by 80% within 1 min of reaction time and reached a constant after about 5 min. In Case A-H, chlorine slowly reduced to about 0.15 mg l^{-1} within the 30 min reaction time. As also shown in Figure 6(a) and (c), *A. circinalis* cells were very fragile to chlorine. For Case A-L, where the cell concentration = 6.6×10^5 cells ml^{-1}, the majority of the cells (~90%) were ruptured within 1 min. Although a small chlorine dosage was applied, in Case A-H, ~ 75% cells were ruptured within 1 min, and >90% cells were ruptured within 30 min of reaction time.

In both Cases A-L and A-H, geosmin was almost all bound with *Anabaena* cells before chlorination. However, right after chlorination, ~85% of the geosmin was rapidly released into water in Case A-L (Figure 6(b)), which is only slightly lower than the ratio of ruptured cells (90%). For longer reaction time, almost all the geosmin was in the dissolved phase. In Case A-H (Figure 6(d)), only 19% and 39% of the cell-bound geosmin were released into

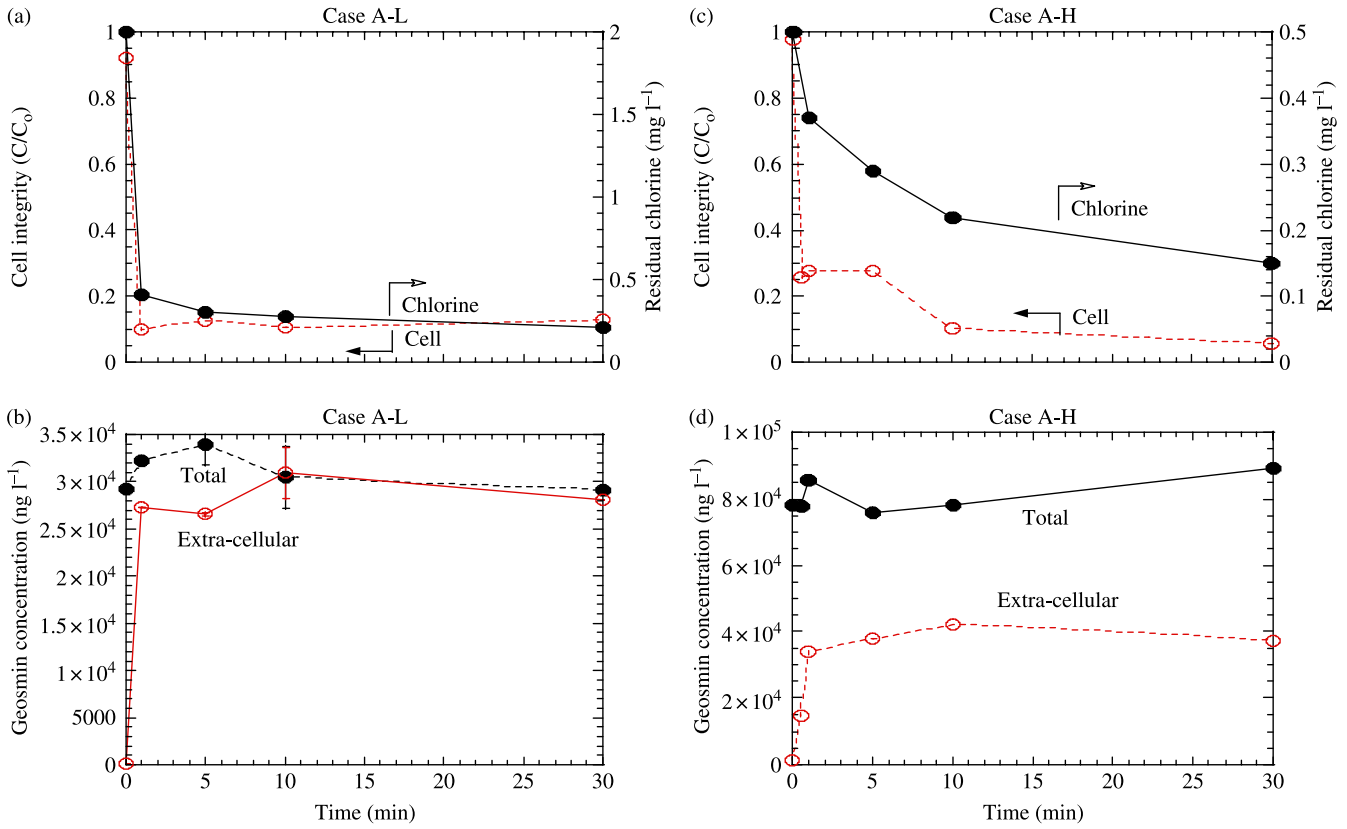

Figure 6 | Residual free chlorine, cell integrity and geosmin concentration during chlorination of *A. circinalis*, where (a) and (b) are under cell concentration = 6.0×10^5 cells ml^{-1} and chlorine dosage = 2 mg l^{-1}, and (c) and (d) are under cell concentration = 1.1×10^6 cells ml^{-1} and chlorine dosage = 0.5 mg l^{-1}.

water after 1 and 5 min of reaction, respectively. About 50% of geosmin was bound with cells after 30 min of reaction. These ratios of cell-bound geosmin are much higher than the ratios of intact cells shown in Figure 6(c). The discrepancy between the ratios of intracellular/total geosmin and intact/total cells may be linked to the extent of cell rupture in this case. In contrast to the finding that all the geosmin was released into water in Case A-L, the low chlorine dosage employed in this experimental case may only be able to cause the damage of *Anabaena* cells. The chlorine dosage applied may not be enough to rupture the cells to a degree enabling the release of all the geosmin. It is possible that a portion of the geosmin may still bind with the damaged cells and were filtered by the 0.45 μm membrane filter, causing the higher concentration of cell-bound geosmin observed in the experiment. In fact, geosmin was observed to bind with thylakoid and cytoplasmic membranes for *Oscillatoria tenuis* (Wu & Juttner 1988). Therefore, a portion of geosmin in partially ruptured cells may remain within the cells during the filtration treatment for analysis.

Kinetics of cell rupture

To further understand the kinetics of the cell rupture, laboratory cultures of *Microcystis* and *Anabaena* were chlorinated at different dosages. The cell concentrations were controlled at 4.3×10^6 and 1.9×10^6 cells ml^{-1} for *Microcystis* and at 3.3×10^6 and 6.0×10^6 cells ml^{-1} for *Anabaena*. The cell integrity and residual chlorine concentrations of the samples were monitored at different times during the experiments. For comparison, chlorination of raw water from the two THR cases, as well as from three other raw waters (RHR, LTR and RYTR), were also analysed for the rupture kinetics of *Microcystis* cells. The experimental procedures for the chlorination of RHR, LTR

and RYTR were the same as those shown in the THR cases, except that the initial chlorine dosages, *Microcystis* cell numbers and raw water quality were different.

The kinetics of cell rupture is modelled using a similar approach to that of Daly *et al.* (2007). In brief, the chlorine decay kinetics was first fitted with a first-order decay model, as shown in Equation (1).

$$C_{Cl} = C_{Cl,1}e^{-k_{Cl}t} + C_{Cl,2} \quad (1)$$

where C_{Cl} is the chlorine concentration ($mg\,l^{-1}$), $C_{Cl,1}$ is the chlorine demand of the sample ($mg\,l^{-1}$), k_{Cl} is the rate constant for chlorine decay (min^{-1}), and $C_{Cl,2}$ is the amount of chlorine not reacted with the sample. The three-parameter model simulated the chlorine decay data very well in this study, with a correlation coefficient (R^2) exceeding >0.96 in each case (data not shown).

Then, the chlorine exposure (Ct, $mg\,l^{-1}$-min) was estimated from an integration of Equation (1) with time (t, min), as shown in Equation (2).

$$C_{Cl}t = \frac{C_{Cl,1}}{k_{Cl}}(e^{k_{Cl}t} - 1) + C_{Cl,2}t \quad (2)$$

Assuming that the rupture kinetics of cyanobacteria cells is similar to that for the disinfection of bacteria cells (Daly *et al.* 2007), a first-order decay model (or a special form of the Chick-Watson equation) may be used to simulate the rupture of cyanobacteria cells.

$$\frac{C}{C_o} = e^{-k(C_{Cl}t)} \quad (3)$$

where C/C_o is the ratio of integral cells, and k is the first-order rate constant.

Table 2 summarizes the rate constants of cell rupture for the laboratory cultures of *Microcystis* and *Anabaena* under chlorination. Although the cell numbers are different by more than twofold, the rate constant (k) for *M. aeruginosa* is different by only 40%, $790\,M^{-1}S^{-1}$ for the 4.3×10^6 cells ml^{-1} case and $1{,}100\,M^{-1}S^{-1}$ for the 1.9×10^6 cells ml^{-1} case. These rate constants are 1.2 and 1.6 times that reported by Daly *et al.* (2007) for *M. aeruginosa* with a concentration of 3.0×10^5 cells ml^{-1}. Considering that water matrix, cell densities and strains of *M. aeruginosa* used were different, these rate constants are similar, within the range of $670–1{,}100\,M^{-1}S^{-1}$. Table 2 also displays the rate constants for the cell rupture of *Anabaena* samples. Compared with those for *Microcystis*, a faster rate of cell rupture was observed with *Anabaena*, by a factor of 1.3 to 5.0. This may imply that *A. circinalis* cells are more prone to rupture during chlorination compared with *M. aeruginosa* cells.

The chlorination rate constants for *Microcystis* cells in unfiltered reservoir waters are presented in Table 3. In the table, the 30 min chlorine demand is also displayed. Only about a $2.0\,mg\,l^{-1}$ chlorine demand is observed for LTR and RYTR, while a chlorine demand of $>5\,mg\,l^{-1}$ is observed for both THR and RHR. This is in accordance with the fact, as summarized in Table 1, that THR and RHR are eutrophic, with high concentrations of chlorophyll-a, nutrients and TOC, whereas LTR and RYTR are less polluted. Therefore, the k values for the five experiments using raw waters may be separated into two groups, one for those from THR and RHR, and the other for LTR and RYTR. For the cases of THR and RHR, the rate constants are within a narrow range, from 70 to $180\,M^{-1}S^{-1}$, which is much smaller than those given in Table 2 for laboratory

Table 2 | Rate constants of cell rupture for chlorination of laboratory cultures

Cell number (cells ml^{-1})	Initial HOCl dosage applied ($mg\,l^{-1}$)	Rate constant k ($M^{-1}S^{-1}$)	Number of datum points/ correlation coefficients (R^2)	Source
Microcystis aeruginosa				
4.3×10^6	1, 3	790	10/0.83	This study
1.9×10^6	1, 2	1,100	9/0.83	This study
3.0×10^5	1–20	670 ± 77	–	Daly *et al.* 2007
Anabaena circinalis				
3.3×10^6	1, 2	1,400	7/0.84	This study
6.0×10^5	1, 2	3,400	7/0.82	This study

Table 3 | Rate constants of cell rupture for chlorination of *Microcystis* in field samples

	Cell number (cells ml^{-1})	pH	Initial Cl_2 dosage applied (mg l^{-1})	Residual Cl_2 @ 30 min (mg l^{-1})	Rate constant k (M^{-1} S^{-1})	Number of datum points/ correlation coefficients (R^2)
Tai-Hu Reservoir	1.3×10^5	10.4	6.0	0.3	180	5/0.96
	4.4×10^5	10.7	6.0	0.4	110	4/0.94
Rong-Hu Reservoir	1.6×10^5	9.7	6.9	0.4	70	5/0.92
Lan-Tan Reservoir	2.1×10^4	8.0	2.3	0.3	590	5/1.00
Ren-Yi-Tan Reservoir	1.6×10^4	7.9	2.5	0.4	430	5/0.99

M. aeruginosa (670–1,100 M^{-1} S^{-1}). The slower kinetics of cell rupture for the unfiltered reservoir waters may be attributed to the presence of other cyanobacteria, particles and other reductive materials, which may also react with chlorine. In fact, in both THR and RHR, *Cylindrospermopsis* is another noted dominant cyanobacteria. Another factor that may cause the slower kinetics for cell rupture is the high pH of the water in THR and RHR. In addition, the *Microcystis* found in the raw water samples were in small colonies with 50–100 cells. Compared with those single cells in the laboratory cultures, the small colonies would also increase the resistance to chlorine for the *Microcystis* cells in the unfiltered water.

The rate constants for LTR and RYTR cases are 590 and 430 M^{-1} S^{-1}, respectively, and are very similar to those for the laboratory cultures (670–1,100 M^{-1}-S^{-1}). The two reservoirs, with a very similar water quality as shown in Table 1, have much lower concentrations of nutrients and TOC compared with those in THR. This is in accordance with the observation that the 30 min chlorine demand was only around 2 mg l^{-1}. In addition, the *Microcystis* cell concentrations in the water were also relatively low, only around 1.6 and 2.1×10^4 cells ml^{-1}. The low organic concentration and low cell concentrations in LTR and RYTR may be the reason that the kinetics for cell rupture are as fast as that found for the laboratory cultures.

CONCLUSIONS

The surface morphology based on SEM microphotos and the intact/ruptured cell ratios based on FCM/EFM methods indicate that *Microcystis* and *Anabaena* cells are very susceptible to attack by chlorine, leading to relatively fast chlorination kinetics. A first-order decay model effectively simulated the kinetics of cell rupture during the chlorination process with laboratory cultures and unfiltered cyanobacteria-laden reservoir water. With laboratory cultures, the measured rate constants are 670–1,100 M^{-1} S^{-1} for *M. aeruginosa* and those for *A. circinalis* are 1.3–5.0 times higher. With unfiltered reservoir waters, the rate constants for rupturing *Microcystis* cells fell in a much broader range. For the reservoirs with low TOC and concentration of cyanobacteria, the kinetics were similar to that for the laboratory cultures. However, with more eutrophic reservoir waters, the kinetics were slower by a factor of 2.4–8.4 than that for the less polluted reservoirs, probably owing to the competition of chlorine with other cyanobacteria and particles in water. A large portion of geosmin was released into water immediately after the *Anabaena* cells were ruptured. Depending on the chlorine dosage and cell concentration, the ratios of cell-bound geosmin may be similar to or much higher than the ratios of intact to total cells. The discrepancy between the ratios of intracellular/total geosmin and intact/total cells may be linked to the extent of the cell lysis. A portion of geosmin remained bound to the damaged cells and was filtered off by the 0.45 μm membrane filter, resulting in a higher level of the observed cell-bound geosmin. For *Microcystis*, the total β-cyclocitral concentration decreased quickly during chlorination, when most cells were ruptured. Experimental results showed that the chlorine dosage at 10 mg l^{-1} with 1 h reaction time was not able to destroy β-cyclocitral. Therefore, it is speculated that β-carotene oxygenases, the enzymes responsible for the formation of β-cyclocitral, were inhibited by chlorine, thus causing a sudden reduction of β-cyclocitral in the system.

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