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Kinetics of cell lysis for *Microcystis aeruginosa* and *Nitzschia palea* in the exposure to β-cyclocitral

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ABSTRACT

The effect of an algal metabolite, β-cyclocitral, on the cell integrity of two cyanobacteria and one diatom was investigated. The cyanobacteria, *Microcystis aeruginosa* PCC 7005 and PCC 7820, and the diatom, *Nitzschia palea*, were exposed to various concentrations of β-cyclocitral. Scanning electron microscope (SEM) results indicate that the cells of tested species were greatly altered after being exposed to β-cyclocitral. A flow cytometer coupled with the SYTOX stain and chlorophyll-a auto-fluorescence was used to quantify the effect of β-cyclocitral on cell integrity for the tested cyanobacteria and diatom. Kinetic experiments show that about 5–10 mg L^{-1} of β-cyclocitral for the two *M. aeruginosa* strains and a much lower concentration, 0.1–0.5 mg L^{-1}, for *N. palea* were needed to cause 15–20% of cells to rupture. When the β-cyclocitral concentration was increased to 200–1000 mg L^{-1} for *M. aeruginosa* and 5–10 mg L^{-1} for *N. palea*, almost all the cells ruptured between 8 and 24 h. A first-order kinetic model is able to describe the data of cell integrity over time. The extracted rate constant values well correlate with the applied β-cyclocitral dosages. The obtained kinetic parameters may be used to estimate β-cyclocitral dosage and contact time required for the control of cyanobacteria and diatoms in water bodies.

1. Introduction

Freshwater cyanobacteria and algae may cause many problems in drinking water systems. Common problems include the production of toxins [1], undesirable taste and odor (T&O) in water sources [2,3], the clogging of filters [4], and the formation of disinfection byproducts (DBPs) [5] in water treatment processes. The control of cyanobacteria and algae in sources of water, such as drinking water reservoirs, can facilitate water treatment and safeguard drinking water quality.

A wide range of control measures has been proposed for the control of cyanobacteria and algae, including chemical algaecides, which have been widely used in reservoirs and rivers in the last century [7,8]. Copper sulfate used to be the most common chemical for controlling algal bloom in reservoirs. However, copper ions may accumulate in water and sediment, impacting the environment and ecology. Therefore, the use of copper sulfate is prohibited for drinking water reservoirs in most countries, and it can only be used under critical situations in the USA and Australia [9]. Oxidants, such as ozone, chlorine, and permanganate, are mostly used for controlling cyanobacteria and algae and associated metabolites in drinking water treatment processes [10–13] to improve coagulation, flotation, and/or the removal of metabolites and cells [14]. When these oxidants are applied to drinking water sources, the cyanobacterial and diatom cells become rapidly damaged, causing a sudden release of metabolites and cellular material into the water. In addition, DBPs and DBP precursors may form in the source water [15]. The elevated organic loading in the water may also make subsequent water treatment processes difficult [16,17].

A group of environmentally friendly chemicals, such as those produced by cyanobacteria (β-cyclocitral, geosmin, and 2-MIB), has been studied for their effect on the cell lysis of *Microcystis* and *Anabaena* [18,19]. These chemicals were also examined for their inhibition of the growth of a green alga, *Chlorella pyrenoidosa* [20]. Among the chemicals tested, β-cyclocitral was able to cause color changes of the tested cyanobacteria. The chemical β-cyclocitral (2,2,6-trimethyl-1-cyclohexene-1-carboxaldehyde) is produced naturally. It is the product of an oxidative cleavage reaction of β-carotene, catalyzed by β-carotene oxygenases bound on the *Microcystis* cell membrane under aerobic conditions [21]. Although β-cyclocitral has been shown to be anticyanobacterial, the studies were mostly based on color change [19], absorbance measured with a spectrophotometer, or scanning electron microscope (SEM) observation [22]. In addition, the effect of β-cyclocitral on other algae, such as diatoms, has not been reported. Therefore, a quantitative analysis is needed to better understand the effect of

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β-cyclocitral on cell integrity for cyanobacteria and diatoms. The objectives of the present study are: (1) to develop a method to quantify cell integrity before and after β-cyclocitral addition, (2) to analyze the kinetics of cell rupture under various β-cyclocitral concentrations, and (3) to evaluate the kinetic data with appropriate models.

2. Experimental method

2.1. Cell cultures

Two cyanobacteria (*Microcystis aeruginosa* PCC 7005 and PCC 7820) and one diatom (*Nitzschia palea*) were selected in this study. *M. aeruginosa* PCC 7005 and PCC 7820 were obtained from the Pasteur Culture Collection of Cyanobacteria, France, and diatom *N. palea* was isolated from Cheng Kung Pond, located on the campus of National Cheng Kung University, Tainan, Taiwan. The cyanobacteria strains were cultured in BG-11 media [23] and the diatom strain was cultured in a specially prepared medium (referred as diatom medium) [24]. The two *M. aeruginosa* strains and the *N. palea* strain were incubated at 25 °C and 20 °C, respectively, under a constant light flux with a light/dark cycle of 12 h/12 h. After having reached the exponential growth phase, usually in 7–10 days, the strains were used for experiments.

2.2. Cell counting

Cell counting for the two *M. aeruginosa* strains and the *N. palea* strain was conducted using a microscope with a Sedgwick Rafter chamber (Graticules Ltd., UK). The procedures used are the same as those recommended by Apha et al. (2000). Before cell counting, 1 mL of a sample was placed into the Sedgwick Rafter chamber. After being allowed to settle for 10 min, the samples were counted at 200× magnification.

2.3. Experiments of cell rupture by β-cyclocitral

To investigate the effect of β-cyclocitral on the cell integrity of *M. aeruginosa* and *N. palea*, batch type experiments were conducted. In the experiments, 100-mL glass flasks were used as reactors. The reactors were maintained at room temperature (25 ± 2 °C) and contained Teflon-coated magnetic stirrers. Before the reaction, the cultured cyanobacteria and diatom were diluted with BG-11 medium and diatom medium, respectively, to achieve an initial cell density of 2×10^5 cells mL^{-1}. Then β-cyclocitral was spiked into the reactors at 5, 10, 50, 100, 200, and 1000 mg L^{-1} for *M. aeruginosa*, and at 0.05, 0.5, 5, 50, 200, and 1000 mg L^{-1} for *N. palea*. The pH was maintained at 8.4–8.7 during the reaction. Samples were collected at predetermined time intervals for the analysis of cell integrity.

2.4. Analysis of cell integrity

A flow cytometer (FCM) based method was employed for determining cell integrity of the tested cyanobacteria and diatom species. The method was used by Daly et al. [25] and Lin et al. [13] for measuring the cell integrity of *M. aeruginosa* during chlorine oxidation. Detailed procedures can be found in the above two studies; only a brief description is given here. An FCM (FACS Calibur™, Becton Dickinson, USA) with one of its argon lasers emitting at a fixed wavelength of 488 nm was used for all the FCM measurements. A standard green fluorescence detector (FL1, 530 nm) was used to detect cells stained with SYTOX green nucleic acid stain (Invitrogen, USA) and fluorescein diacetate (FDA) (Invitrogen, USA), and a red fluorescence detector (FL3, 650 nm) was used to detect the auto-fluorescence from chlorophyll in the cells. A software package (WinMDI 2.9) was used to collect and analyze the data. SYTOX can permeate ruptured cells and stain the nucleic acid, and FDA can stain the membrane of intact cells. These two dyes were used to determine the integrity cells, with SYTOX used as the major dye and FDA used for confirming the results obtained using SYTOX. Therefore, only SYTOX data are shown. A development time of 7 min was used for both stains, with a concentration of 0.1 μM for SYTOX and 40 μM for FDA. The flow rates of algal cells were controlled at 100–400 cells s^{-1} for the analysis. Data were collected until the combined number of events recorded in the intact and ruptured regions reached 1000 or the analysis time reached 2 min. Before the analysis, calibration curves were made with various ratios of ruptured and integral cells. The results show that the calibration curves are linear ($R^2 = 0.996$) and that the observations correlate closely with the prepared samples, indicating that the dyes employed can differentiate ruptured and integral cells.

2.5. Observation of cell surface

To observe the surface of algal cells, a scanning electron microscope (SEM, S-3000N, Hitachi, Japan) was used. Before analysis, a 0.2-μm nylon filter (Min-Yu, Taiwan) was used to filter the cyanobacterial and diatom cells from the samples. The cell-laden filters were soaked in a mixed solution of 2.5% glutaraldehyde (analytical grade, Merck, Germany) and phosphate buffered saline (PBS buffer, Merck, Germany) (1:100) at 4 °C for 8–10 h, washed twice with a mixed solution of PBS buffer and 5% sucrose (analytical grade, Merck, Germany) (1:1) at 4 °C for 15 min, soaked in a mixed solution of 1% osmium (Merck, Germany) and PBS buffer (1:100) at room temperature for 1 h, and then washed twice with a mixed solution of PBS buffer and 5% sucrose (1:1) at 4 °C for 15 min. The formations of the cells were thus fixed on the filter membrane. After fixation, dehydration of the samples was performed with a sequential ethanol-in-water (50%, 70%, 80%, 90%, 95%, and 100%, Merck, Germany) extraction; the samples were then soaked in isoamyl acetate (Merck, Germany) for 20 min. Finally, the samples were dried using a critical point dryer (HCP-2, Hitachi, Japan), and then coated with gold (ion sputter, E-1010, Hitachi, Japan) for analysis.

3. Results and discussion

3.1. Change of surface morphology of cells

To understand the effect of β-cyclocitral on the surface morphology of the cyanobacterial and diatom cells, SEM micrographs were taken before and after the addition of β-cyclocitral. Fig. 1 shows the morphology for the surface of *M. aeruginosa* PCC 7005 (Fig. 1(a)), PCC 7820 (Fig. 1(b)), and *N. palea* (Fig. 1(c)) before and after the spiking of β-cyclocitral. Before reaction, all three types of cell look very intact and smooth (eee (A) in Fig. 1(a–c)). However, after the addition of 500 mg L^{-1} of β-cyclocitral, the surface morphologies of the three types of cell changed. After reaction for 1.5 h, both *M. aeruginosa* strains were deformed and the surfaces became less smooth (Fig. 1(a)-B and (b)-B). After further contact time, both *M. aeruginosa* strains shrank in size and deflated (Fig. 1(a)-C and D and (b)-C and D). Similar SEM micrographs were obtained by Ozaki et al. [22] and Lin et al. [12] for *Microcystis* cells exposed to various algal metabolites and for the cells of *M. aeruginosa* after chlorination. For *N. palea*, cracks formed on the surface at 1.5 h (Fig. 1(c)-B). After further contact time, a clear separation of epitheca and hypotheca was observed for the cells (Fig. 1(c)-C and D).

3.2. Change of fluorescence and cell integrity

To determine the cell integrity, calibration curves were made with various ratios of ruptured and integral cells. Fig. 2 shows typical results. The calibration curve appears to be linear and the

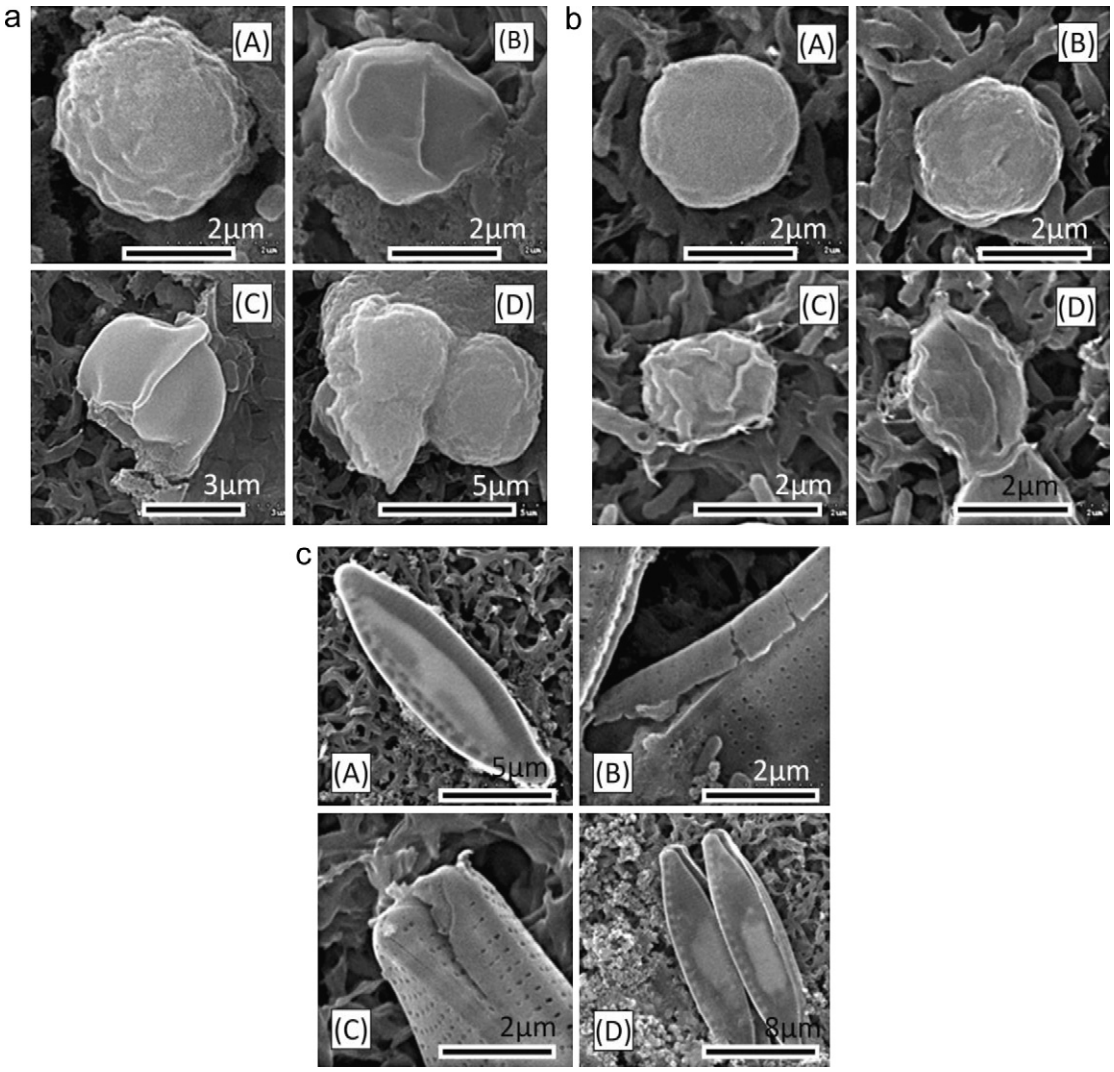

Fig. 1. Surface morphologies of (a) *Microcystis aeruginosa* PCC 7005, (b) *Microcystis aeruginosa* PCC 7820, and (c) *Nitzschia palea* before and after the addition of 500 mg L^{-1} β-cyclocitral: (A) before β-cyclocitral addition, (B) after 1.5 h, (C) after 5 h, and (D) after 18 h.

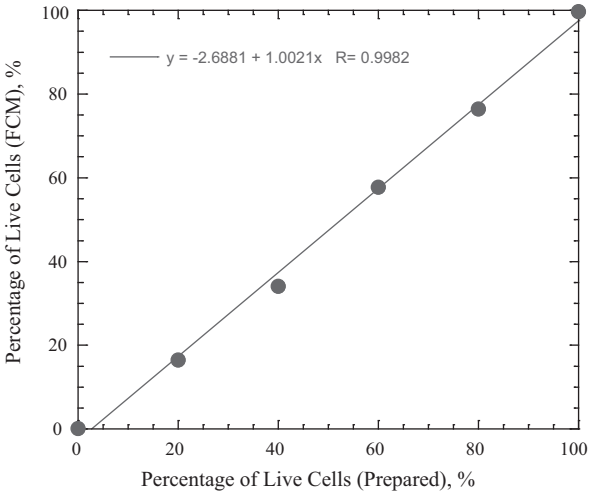

Fig. 2. Correlation of cell integrity between prepared samples and observed data for flow cytometry measurement of *Microcystis aeruginosa* PCC 7005.

observations were close to those for prepared samples, indicating that the dye employed can differentiate ruptured and integral cells.

Fig. 3 shows the change of cell fluorescence for *M. aeruginosa* 7005 before and after the addition of β-cyclocitral at various concentrations for 8 h. In the figure, two-dimensional plots are used to show fluorescence intensities produced by chlorophyll-a (FL3 in red) and those produced by the dye SYTOX (FL1 in green) stained on nucleic acids.

This type of two-dimensional fluorescence intensity plot is similar to that presented by Brussaard et al. [26]. According to the intensities of FL1 and FL3, each datum point is attributed to one of the three subpopulations shown in the figures. The live portion represents integral cells, for which FL3 (chlorophyll-a) intensity is strong and FL1 (SYTOX) intensity is low. The dead portion represents cells that were ruptured, meaning that the SYTOX dye stained the nucleic acids within the cells, which exhibit strong FL1 and FL3 intensities. For the third portion, low chlorophyll-a fluorescence intensities were obtained, which is indicative of the degradation of chlorophyll [26]. In addition, the population with low FL1 fluorescence intensities probably had already lost most of its nucleic acid due to partial cell lysis or DNA breakdown. Therefore, the dead and low chlorophyll-a populations are considered to be ruptured cells for the analysis.

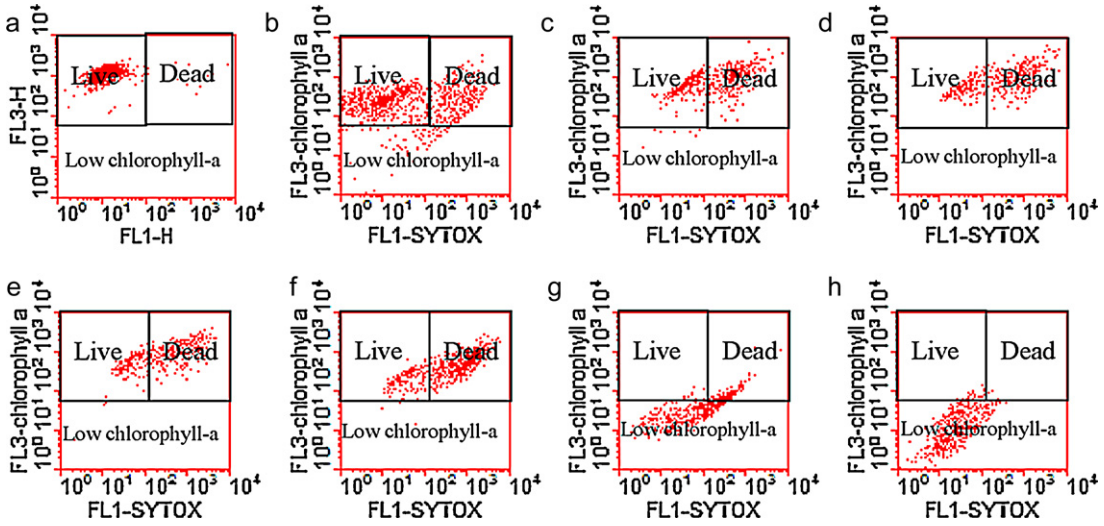

Fig. 3. Two-dimensional fluorescence results for *Microcystis aeruginosa* PCC 7005 spiked with β-cyclocitral at 5–1000 mg L^{-1}: (a) before spiking, (b) controlled sample after 8 h of reaction, and samples spiked with (c) 5 mg L^{-1}, (d) 10 mg L^{-1}, (e) 50 mg L^{-1}, (f) 100 mg L^{-1}, (g) 200 mg L^{-1}, and (h) 1000 mg L^{-1} of β-cyclocitral after 8 h of reaction.

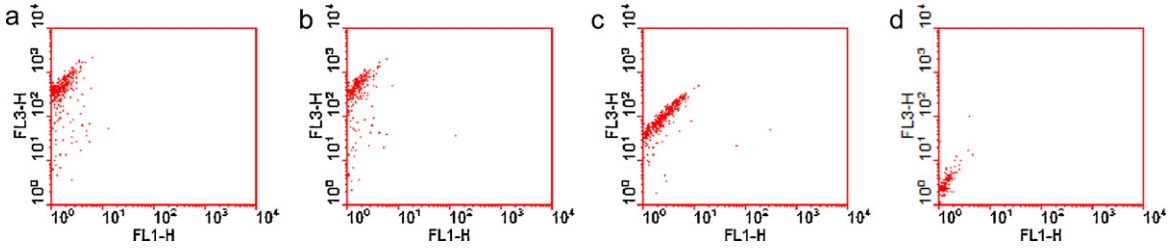

Fig. 4. Changes of chlorophyll-a fluorescence for *Microcystis aeruginosa* PCC 7005: (a) not spiked, and samples spiked with (b) 10 mg L^{-1}, (c) 200 mg L^{-1}, and (d) 1000 mg L^{-1} of β-cyclocitral after 3 h of reaction.

To confirm the shift of red fluorescence to lower intensities, in one set of experiments for the reaction of β-cyclocitral with *M. aeruginosa* cells, no SYTOX was added in the FCM analysis, and only auto-fluorescence of chlorophyll-a was measured. Fig. 4 shows the change of fluorescence intensities after 3 h of reaction. As expected, low FL1 (green) fluorescence intensities were observed for all the samples. However, the intensities of FL3 (chlorophyll-a) were initially high and then substantially decreased with increasing applied β-cyclocitral concentration. This is in accordance with Fig. 3, where the population with lower chlorophyll-a fluorescence increased with increasing β-cyclocitral concentration.

3.3. Effect of β-cyclocitral on cell integrity

Figs. 3 and 5 show the effect of β-cyclocitral on the cell integrity of *M. aeruginosa* 7005 and *N. palea* after 8 h of reaction, respectively.

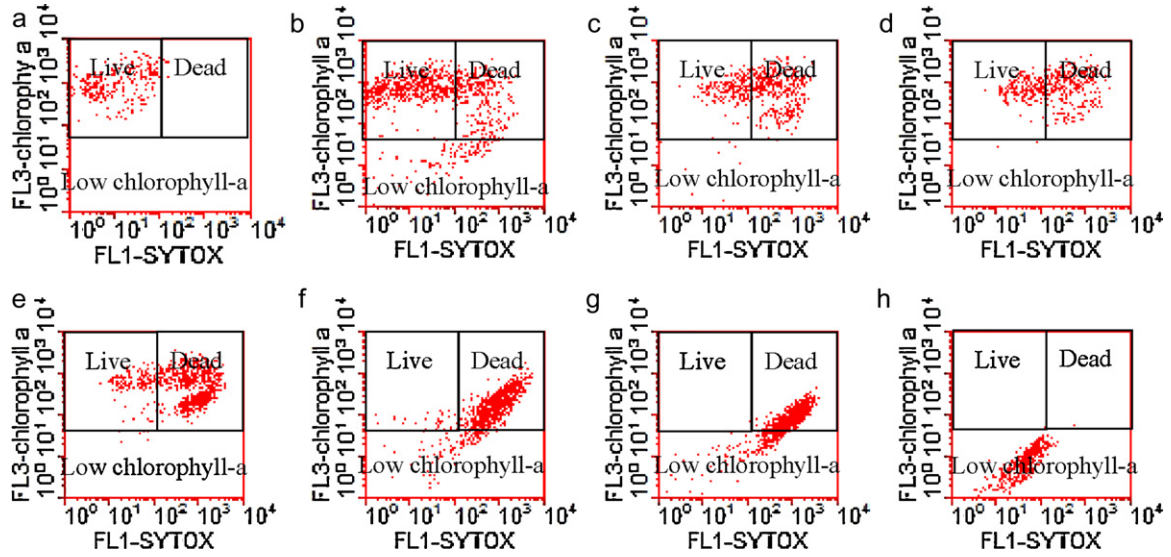

Fig. 5. Two-dimensional fluorescence results for *Nitzschia* sp. spiked with β-cyclocitral at 0.05–1000 mg L^{-1}: (a) before spiking, (b) controlled sample after 8 h of reaction, and samples spiked with (c) 0.05 mg L^{-1}, (d) 0.5 mg L^{-1}, (e) 5 mg L^{-1}, (f) 50 mg L^{-1}, (g) 200 mg L^{-1}, and (h) 1000 mg L^{-1} of β-cyclocitral after 8 h of reaction.

Before the application of β-cyclocitral, all the cells are expected to be integral. This is confirmed in Figs. 3(a) and 5(a), which show results for cells before chemicals were applied. Therefore, all the datum points are concentrated in the live portion. Figs. 3(b) and 5(b) show results for the control experiment. Since the cultures were transferred to deionized water and reacted for 8 h for *M. aeruginosa* and *N. palea*, respectively, the cells may have suffered stress due to a change of environment. Therefore, a small portion of the population (~35% for *M. aeruginosa*, and 15% for *N. palea*) is in the dead zone after 8 h of reaction.

Compared with the controls, cells exposed to β-cyclocitral show lower ratios of live cells. Fig. 3(c)–(f) clearly shows that as β-cyclocitral concentration increased, the cells gradually shifted to the high intensity region of FL1 fluorescence. This observation indicates that β-cyclocitral ruptured the cells, allowing SYTOX dye to stain the nucleic acids within the cells. The difference between controls and cells spiked with β-cyclocitral only appear to be significant when β-cyclocitral concentrations were larger than 5 mg L^{-1}. When the β-cyclocitral concentration was increased to >200 mg L^{-1}, a significant portion of the *M. aeruginosa* cells were identified in the low chlorophyll-a portion, indicating the destruction of chlorophyll-a and/or nucleic acids in the cells. The trends of cell rupture for the other *M. aeruginosa* strain, PCC 7820, were very similar to those for *M. aeruginosa* 7005. Therefore, no two-dimensional fluorescence intensity plots are shown here. However, the data are included in Section 3.4.

Similar results were also observed for *N. palea*, except that much lower β-cyclocitral concentrations are required to damage the cells. The ratios of live cells also decreased with increasing β-cyclocitral concentration. However, only 0.05 mg L^{-1} is needed to see the effect for *N. palea* as compared to 5 mg L^{-1} required for *M. aeruginosa*. At a dosage of >50 mg L^{-1}, a portion of cells was detected with low chlorophyll-a and/or SYTOX fluorescence intensities. The concentration required to degrade chlorophyll-a and/or nucleic acid is also significantly lower than that required for *M. aeruginosa* (200 mg L^{-1}).

3.4. Kinetics of cell rupture

To quantify the effect of β-cyclocitral on the cell integrity of the three tested types of cell, the two-dimensional fluorescence data were further analyzed for the kinetics of cell rupture. In analyzing the data, the controlled samples (samples without spiking of β-cyclocitral) were used as the reference. The cell integrity of the samples is expressed as:

$$\frac{C}{C_0} = \frac{N_L/(N_D + N_C)}{N_{L_0}/(N_{D_0} + N_{C_0})} \quad (1)$$

where N_L, N_D, and N_C are the numbers of live cells, dead cells, and low chlorophyll-a cells obtained from the two-dimensional fluorescence plot, respectively. The subscript 0 represents data from reference samples.

Fig. 6 shows the kinetics of cell rupture for *M. aeruginosa* PCC 7005, PCC 7820, and *N. palea* exposed to various β-cyclocitral concentrations. About 5–10 mg L^{-1} of β-cyclocitral was found to cause 15–20% of cells to rupture for both *M. aeruginosa* strains. However, for the diatom (*N. palea*), a much lower concentration, 0.1–0.5 mg L^{-1}, was needed to induce a similar degree of cell damage. When the β-cyclocitral concentration was increased to 200–1000 mg L^{-1} for *M. aeruginosa* and 5–10 mg L^{-1} for *N. palea*, almost all the cells ruptured within 8–24 h. For all the experimental combinations, the reactions took 5–8 h to reach steady ratios of cell damage.

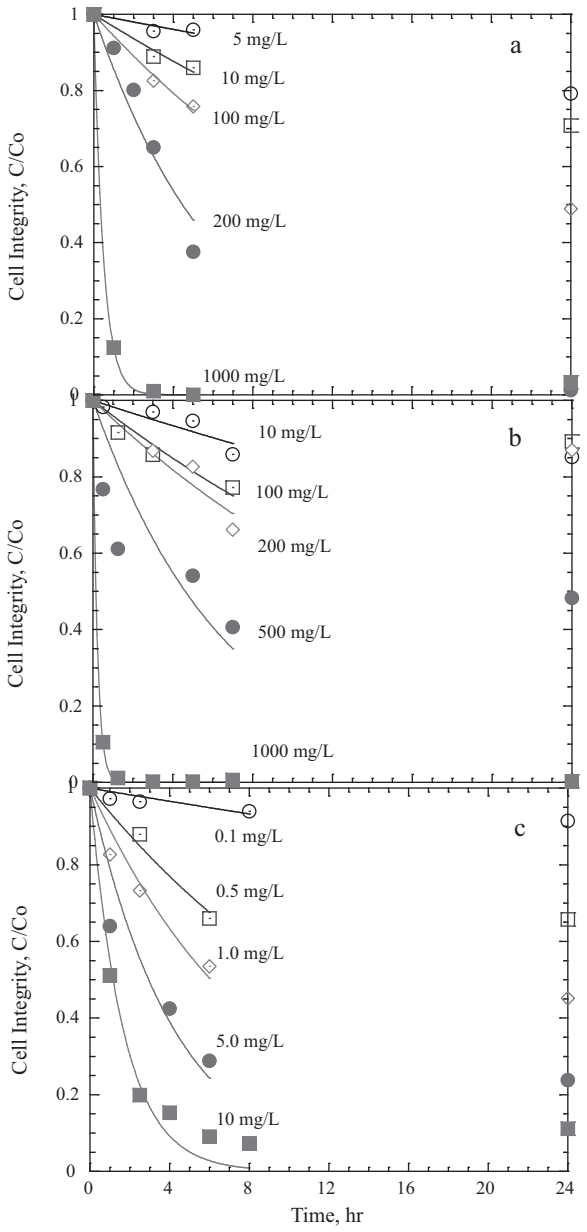

Fig. 6. Kinetics of cell rupture for (a) *M. aeruginosa* PCC 7005, (b) *M. aeruginosa* PCC 7820, and (c) *Nitzschia palea* under various β-cyclocitral dosages, where symbols represent experimental data and lines represent fitted models.

The following first-order kinetic model was employed for the analysis of cell integrity over time:

$$\frac{C}{C_0} = \exp(-kt) \quad (2)$$

where C/C_0 is the ratio of cell integrity (%) at reaction time t (h), and k is the rate constant (h^{-1}).

Since the reaction mostly reached steady state after 8 h, only the data before 8 h were evaluated. As shown in Fig. 6, for all the cases tested, the model fit the data relatively well (with R^2 mostly >0.90), suggesting that the model describes the kinetics of cell rupture found in this study. Table 1 summarizes the extracted rate constants (k, h^{-1}) for the model. The k value increases with β-cyclocitral concentration. Fig. 7(a) plots the correlation between rate constant and applied β-cyclocitral dosage for the two *M. aeruginosa* strains. The figure shows that the k values are very similar at similar applied β-cyclocitral concentrations. In particular, the k values correlate

Table 1
The fitted kinetic parameters of cell rupture for the tested cyanobacteria and diatom.

Algae/ cyanobacteria	β-Cyclocitral conc. (mg L^{-1})	Number of data	k (h^{-1})	R^2
Microcystis aeruginosa PCC 7005	5	3	0.01	0.76
	10	3	0.033	0.96
	100	3	0.058	0.99
	200	5	0.16	0.94
	1000	4	2.08	0.99
Microcystis aeruginosa PCC 7820	10	5	0.017	0.83
	100	4	0.041	0.92
	200	4	0.050	0.93
	500	5	0.15	0.61
	1000	6	4.49	0.99
Nitzschia palea	0.1	4	0.009	0.66
	0.5	3	0.065	0.98
	1.0	4	0.12	0.95
	5.0	4	0.24	0.91
	10	6	0.60	0.98

reasonably well with β-cyclocitral dosages at concentrations below ~500 mg L^{-1}. For 1000 mg L^{-1}, both *M. aeruginosa* strains exhibited much faster kinetics. Fig. 7(b) shows that k values for *N. palea* correlate excellently with β-cyclocitral dosages for all the concentrations tested (R^2 = 0.97). In addition, a much steeper slope was extracted for *N. palea* than that for *M. aeruginosa*, suggesting that much lower β-cyclocitral concentrations are needed for *N. palea* to achieve the same degree of cell rupture. The linear relationship between k values and the concentration of β-cyclocitral for all three cases tested may be interpreted as the kinetics of cell rupture being a second order processes, one for cell integrity and one for β-cyclocitral. However, more experimental data are needed to confirm this.

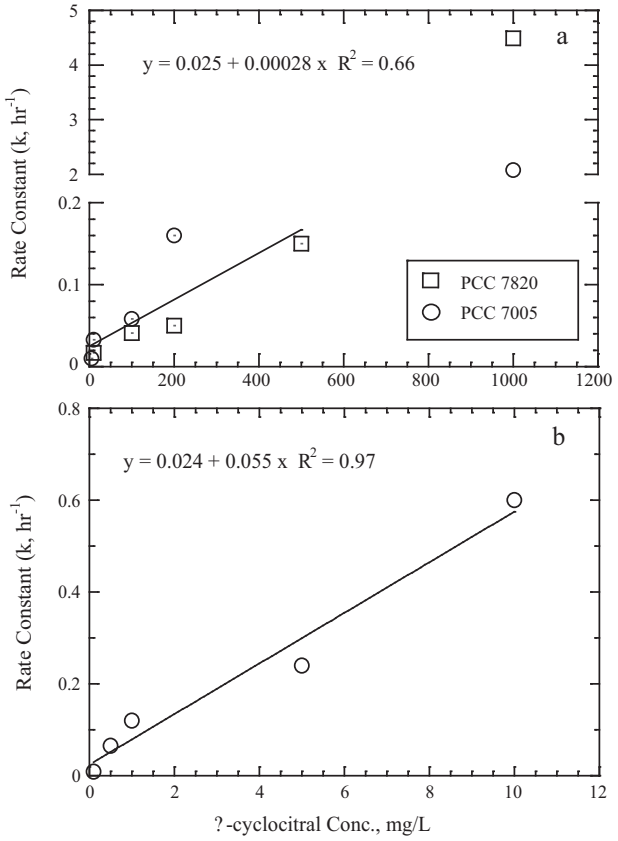

Fig. 7. Rate constants for (a) two *M. aeruginosa* strains, and (b) *Nitzschia palea* under various β-cyclocitral dosages.

4. Conclusion

SEM micrographs show that the cells of two *M. aeruginosa* strains and *N. palea* are greatly affected by exposure to β-cyclocitral. An FCM coupled with the SYTOX stain and chlorophyll-a autofluorescence was used to characterize three subpopulations with different degrees of cell integrity for two *M. aeruginosa* strains and *N. palea*. The effect of β-cyclocitral on cell integrity was determined for the tested cyanobacteria and diatom. Kinetic experiments show that about 5–10 mg L^{-1} of β-cyclocitral is needed to cause 15–20% of cells to rupture for the two *M. aeruginosa* strains. However, a much lower concentration, 0.1–0.5 mg L^{-1}, was needed for the diatom (*N. palea*) to induce a similar degree of cell damage. When the β-cyclocitral concentration was increased to 200–1000 mg L^{-1} for *M. aeruginosa* and 5–10 mg L^{-1} for *N. palea*, almost all the cells ruptured within 24 h. A first-order kinetic model describes the data of cell integrity over time. The extracted k values well correlate with applied β-cyclocitral dosage. The obtained kinetic parameters may be used to estimate β-cyclocitral dosage and contact time required for the control of cyanobacteria and algae in water bodies.

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