Effects of an allelochemical in *Phaeodactylum tricornutum* filtrate on *Heterosigma akashiwo*: morphological, physiological and growth effects

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Abstract: The effects of an allelochemical extracted from the culture filtrate of diatom *Phaeodactylum tricornutum* on the raphidophyte *Heterosigma akashiwo* were investigated using a series of morphological, physiological and biochemical characters. Growth experiments showed that *H. akashiwo* was significantly inhibited immediately after exposure to the allelochemical, with many cells rapidly dying and lysing based on microscopic observation. The effects of the allelochemical on the surviving cells were explored using Scanning Electron Microscopy (SEM) and Flow cytometry (FCM), the latter by examination of a suite of physiological parameters (membrane integrity, esterase activity, chlorophyll-*a* content, membrane potential). The results demonstrate that the membrane of *H. akashiwo* was attacked by the allelochemical directly, causing cell membrane breakage and loss of integrity. Esterase activity was the most sensitive indicator of the impacts of the allelochemical. Membrane

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potential and chlorophyll-*a* content both showed significant decreases following exposure of the *Heterosigma* cells to high concentrations of the allelochemical for 5 and 6 days. Both were affected, but the membrane potential response was more gradual compared to other effects. The cell size of *H. akashiwo* did not change compared with the control group. The surviving cells were able to continue to grow and in a few days, re-establish a successful culture, even in the presence of residual allelochemical, suggesting either development of cellular resistance, or the degradation of the chemical.

Key words: allelochemical; *Phaeodactylum tricornutum*; *Heterosigma akashiwo*; Flow cytometry; physiological characters

1. Introduction

Harmful algal blooms (HABs) occur frequently in marine coastal areas and freshwater ecosystems worldwide, causing serious consequences on the environment, aquaculture industries and human health (Anderson, 1997; Horner et al., 1997; Anderson et al., 2012; Dorantes-Aranda et al., 2015). One of the factors thought to be important in phytoplankton competition for resources and community dynamics is allelopathy - the release of secondary metabolites into an organism's surroundings, thereby affecting the growth or viability of co-occurring organisms (Rice, 1984; Legrand et al., 2003; Roy et al., 2006; Yang et al., 2014).

The production of allelochemicals among dinoflagellates, diatoms, chrysophytes and cyanobacteria has been reported in many marine systems (e.g., Sukenik et al., 2002; Gross, 2003; Legrand et al., 2003; Irfanullah and Moss, 2005; Granéli et al., 2012), and allelopathic interference has been proposed as an important mechanism to stabilize the clear-water states for macrophytes dominating in shallow lakes (Hilt and Gross, 2008; Wang et al., 2016 a). The mechanisms through which allelochemicals are released by phytoplankton and impact on other phytoplankton remain unclear in many marine

ecosystems. Allelochemicals influence multiple cell functions including cell division, metabolism, photosynthesis, respiration, and enzyme activity (Duke, 2003; Singh and Thapar, 2003; Belz and Hurle, 2004). For example, *Chlorella vulgaris* cell membrane was detached from the cell wall after exposure to N-phenyl-2-naphthylamine (Qian et al., 2009), an allelochemical isolated from root exudates of water hyacinth (*Eichhornis crassipes*) (Sun et al., 1993). The inhibition of photosynthesis (especially photosystem II) and the inhibition of enzyme activities (e.g., alkaline phosphatase) were also identified as common modes of actions for allelochemicals (Gross et al., 1996; Körner and Nicklisch, 2002; Zhu et al., 2010; Wang et al., 2016 a). Enzyme activity is increasingly measured in microalgae (e.g., peroxidases, b-galactosidases, esterases) with a rapid and sensitive endpoint (Peterson and Stauber 1996; Blaise and Ménard 1998; Franklin et al., 2001; Eigemann et al., 2013). Ethyl 2-methyl acetoacetate (EMA) isolated from *Phragmites communis* had impacts on respiration and photosynthesis of *Microcystis aeruginosa* (Li et al., 2007).

One of the challenges in this field of investigation is in characterizing allelochemical effects beyond simple growth rate reductions. In recent years, flow cytometry (FCM) has emerged as a rapid and highly efficient analytical method to measure these types of changes in microalgae (e.g., Xiao et al., 2010; 2011; 2014). The application of FCM provides a convenient diagnostic approach for understanding and quantifying allelopathic interactions (Rioboo et al., 2009). Here we use FCM method, together with scanning electron microscopy (SEM) to characterize the morphological, physiological and growth effects of an allelochemical produced by the diatom *Phaeodactylum tricornutum* on the raphidiphyte *Heterosigma akashiwo.*

Heterosigma akashiwo is a common, highly successful bloom - forming species responsible for many fish-killing blooms throughout the world, typically in nearly mono-specific blooms at cell

densities that lead to the appearance of discolored water (e.g., red tides) (Smayda et al. 1998). The production of chemical compounds (allelochemicals) by *H. akashiwo* is well established as a strategy to inhibit the growth of other species of co-occurring microalgae (Yamasaki et al., 2007; Qiu et al., 2012). In our study, *Phaeodactylum tricornutum* has hard siliceous walls with a strong resistance to certain types of allelochemicals, particularly to those that act at the cell surface. We successfully demonstrate that *Phaeodactylum* has evolved an ability to release allelochemicals that can dramatically affect organisms like *Heterosigma* (Wang et al., 2016 b), whereas the compounds released by *Heterosigma* do not appreciably affect *Phaeodactylum*, even though they have been shown to inhibit other diatoms. A putative allelochemical (a type of glycinamide compound) was isolated from the filtrate of *P. tricornutum* (Wang et al., 2016 b), however, details of the growth inhibition and mechanism of action are lacking. In the present study, we document responses of *H. akashiwo* cells to the allelochemical present in *P. tricornutum* filtrate. These results provide new insight into the mechanism of allelochemicals in marine ecosystems.

2. Materials and methods

2.1 Algal culture and isolation of allelochemical from P. tricornutum filtrate

Phaeodactylum tricornutum and *Heterosigma akashiwo* were obtained from the Algal Center of Key Laboratory of Marine Chemistry Theory and Technology, Ocean University of China. The microalgae were cultivated in f/2 medium (Guillard, 1975) prepared using autoclaved seawater (filtered through 0.45 μm Millipore membranes) from Jiaozhou Bay of China. Cultures were grown at (20 ± 1) °C with a 12/12-h light/dark cycle. Illumination was provided by cool white filament lamps at 70 μmol $m^{-2}s^{-1}$. All glassware was acid-soaked, cleaned with milli-Q water, and autoclaved. Cultures were gently shaken twice manually every day to avoid wall growth and prevent the sedimentation of algae.

A 27-L culture was maintained in a transparent polyethylene container until late exponential phase. The filtrate was obtained by centrifugation (1814.4×g, 15 min). A small number of cells from the pellet were observed under the microscope (Leica DM4000B, Germany) after centrifugation to demonstrate that the cells remained intact. The supernatant was then filtered through a 0.22 μm membrane and the filtrate extracted with ethyl acetate three times in succession. The extracts were pooled and evaporated to dryness using a rotary vacuum evaporator (Beijing Bo Kang Laboratory Instruments Medical Co., Ltd.) under reduced pressure at 40 °C. The extract was diluted to 10 mL with DMSO and stored at 4 °C.

The ethyl acetate extract from the filtrate of *Phaeodactylum tricornutum* was purified using HPLC with 99 μL injection volume (repeated 10 times) according to the elution times of chromatographic peaks. Nine isolated fractions were dried under N_2 and the residues diluted with 1.0 mL DMSO respectively. The putative allelochemical was obtained from fraction Ⅵ (Wang et al., 2016 b). The isolated allelochemical was dissolved in 1 mL DMSO to the same concentration of crude ethyl acetate extract before the HPLC separation, and this was then used for all bioassays in the present study.

2.2 Sample preparation for SEM

Heterosigma akashiwo was cultured for 4 d with an initial cell density of 1.0×10^4 cells mL^{-1}. 37 μL DMSO solution was then added into the culture medium of *H. akashiwo.* After 4 d of exposure to the DMSO solution, algal cells were collected by centrifugation (1814.4×g, 10 min) and fixed overnight with 2.5% glutaraldehyde at 4 °C. Samples were washed with 0.1 mol L^{-1} phosphate buffer solution (PBS, pH =7.4) and centrifuged (1814.4×g, 10 min) three times, then the supernatant was discarded. Algal cells were fixed with 1% osmium tetroxide at 4 °C for 1 h, washed by 0.1 mol L^{-1} PBS (pH=7.4), then centrifuged three times, discarding the supernatant. Algal samples were dehydrated with 30%,

50%, 70%, 80%, 90%, 95% and 100% alcohol solutions for 20 min. Samples were then fixed in tert butyl alcohol and freeze-dried for final SEM (Hitachi, Japan) observation after dehydration.

2.3 Flow cytometric measurements

Flow cytometry was conducted with a BD Accuri C6 flow cytometer (Becton Dickinson, USA) equipped with a blue and red laser (488 nm emission), two light scatter detectors, and four fluorescence detectors with optical filters, including FL1 530/15 nm; FL2 585/20 nm; FL3>670 nm and FL4 675/12.5 nm. The program C Flow Plus from Becton Dickinson was used to collect and analyze signals.

All added concentrations of allelochemical were divided into three dose levels (high, medium, low) in the following experiments (Table 1). When the medium dose of DMSO solution was added into a 100-mL culture of *Heterosigma akashiwo*, the added concentration was approximate equivalent to the maximum concentration of allelochemical from the filtrate of *Phaeodactylum tricornutum*. Low and high doses of DMSO solution were equivalent to 0.5 and 3 times the maximum concentration of allelochemical from the *P. tricornutum* filtrate. DMSO without allelochemical were added to the culture of *H. akashiwo* as a control. The growth of *H. akashiwo* was monitored by counting cell numbers directly using both light microscopy and FCM.

Chlorophyll-*a* content provides information about a cell's capacity for absorption, transmission and consumption of energy for photosynthesis. 1.0 mL of each *Heterosigma* culture was filtered through a 40 micron mesh to remove large particles. The cells were then re-suspended in 1.5 mL centrifuge tubes for FCM analysis. Chlorophyll-*a* was detected using the FCM's FL3 detector and mean fluorescence intensity per cell was calculated.

Propidium Iodide (PI) was used to verify cell viability as it can combine with DNA and produce pink

fluorescence when the algal cell membrane is broken. The cell staining was performed by treating each 0.5 mL algal suspension with 0.455 mL PI (0.14 mg mL^{-1}, working solution dissolved in Milli-Q water) and incubating for 15 min at room temperature. The fluorescence intensity was detected using the FL2 channel of the FCM.

Fluorescein diacetate (FDA) was used to assess esterase activity (Franklin et al., 2001). The cell staining was performed by treating 1mL algal suspensions with 20 μL FDA (0.5 mg mL^{-1}, working solution dissolved in acetone) and incubating for 15 min at room temperature. Green fluorescence was detected using the FL1 channel of the FCM.

3,3'-dihexyloxacarbocyanineine iodide (DiOC6(3)) was used to estimate membrane potential. The cell staining was performed by treating 1 mL algal suspensions with 25 μL DiOC6(3) solution (11.52 μg mL^{-1}, working solution dissolved in DMSO) and incubating for 10 min at room temperature. Fluorescence was measured using the FL1 channel on the FCM.

The forward-angle light scatter signal (FSC) was also measured as an indicator of cell size or cell volume, as the signal intensity is linearly related to the square of the cell diameter or cross sectional area (Cunningham and Buonnacorsi, 1992, Wang et al., 2016 a).

All samples were kept on the ice under dark conditions before the FCM measurements. FCM data were interpreted as the mean fluorescence intensity (MFI).

2.4 Data analysis

To estimate the effect of the isolated allelochemical released by *Phaeodactylum tricornutum* on *Heterosigma akashiwo*, inhibition rate *IR* was calculated using Equation. 1 as follows (Sun and Ning, 2005):

$$IR(\%) = (1 - T/C) \times 100\% \quad (1)$$

where T and C represent the cell density of treatments and control, respectively.

One-way ANOVA analysis was used to test for significant differences in effects among different treatments by SPSS 19. Mean values and standard deviations were calculated from replicates for each treatment (n=3), and the significance level p was set at < 0.05.

3. Results and discussion

3.1. Algal growth inhibition and morphology

The growth of *Heterosigma akashiwo* was affected by different doses (A: high concentration; B: medium concentration; C: low concentration) of *Phaeodactylum tricornutum* allelochemical by day 6 or sooner. Cell densities were determined by FCM and direct microscope counting, and the two methods showed a good correlation (R^2=0.98; data not shown). As other physiological characters were also determined by FCM, analyses of the inhibition rates of different treatments were based on FCM measurements (Fig.1).

3.1.1 Cell mortality

Compared to the control group, *Heterosigma akashiwo* cell density decreased significantly ($p < 0.05$) as almost 80% of the cells disappeared on day 1 and the percentage of surviving cells remained in the 10-30% range on days 3, 5 and 6 when treated with the highest concentration of the *Phaeodactylum tricornutum* allelochemical. The inhibition rate was about 20-60% throughout the 6-day culture time for the treatment with the medium concentration. Approximately 20% of the *H. akashiwo* cells disappeared in low concentration treatment group. The cell density of each treatment thus decreased with increasing concentrations of allelochemical. The allelochemical clearly had a strong disruptive effect on *H. akashiwo* growth, inducing significant cell mortality and lysis (Fig.1). Inhibition rate is a common parameter used to quantify allopathic effects (e.g., Nakai et al., 1999). Obviously, the acute

lethal effect directly killed many *H. akashiwo* cells and left only a small number of survivors at the high allelochemical concentrations. The FCM analysis then revealed the physiological condition, and future growth of those survivors in each treatment.

3.1.2 Cell Size and Morphology

Changes in cell size were explored using the ratio of the mean FSC signal in the treated cultures to that of the controls. No significant change of cell size was observed in the treated cells ($p<0.05$; Fig.2). In a similar finding, the size of *Microcystis aeruginosa* did not change when exposed to the allelochemical ferulic acid (FA) at concentration less than 0.7 mM (Wang et al., 2016 a).

SEM images were used to observe the effect of the allelochemical on the morphology of. *Heterosigma akashiwo* cells exposed to the medium dose of DMSO solution for 4 days. The healthy cells of *H. akashiwo* in the control group were intact and agglomerated as shown in Fig.3A. There is no rigid cell wall around the *H. akashiwo* cell, as the outermost layer of the cell is a naked membrane, which may facilitate agglomeration during the process of sample preparation prior to SEM observation (Guo, 1994). In the treated cells, the outer membrane of *H. akashiwo* was damaged, with multiple holes of different sizes and shapes apparent on many cells (Fig.3B). Some cells were in very bad condition, and would likely die with such damage (Fig.3C, D, E). The effect on the membrane was presumably caused by the allelochemical isolated from *Phaeodactylum tricornutum* filtrate. Further examination of this effect was explored using probes for membrane integrity and other physiological parameters.

3.2 Effects on photosynthetic activities

The mean in vivo chlorophyll-*a* fluorescence of *Heterosigma akashiwo* cells was measured after exposure to different concentrations of the *Phaeodactylum tricornutum* allelochemical. There was no apparent change in the low and medium dosage treatments compared to the control group (Fig.4). The

chlorophyll-*a* content decreased by 10% after 1 and 3 days of exposure to the high concentration of allelochemical, and by 20% after days 5 and 6.

Photosynthesis is the central physiological process for primary producers in marine systems, and thus has been widely reported as an important target of allelochemicals (Körner and Nicklisch, 2002). Li et al (2007) found the allelochemical EMA produced by *Phragmites communis* decreased the content of chlorophyll-*a* for *Microcystis aeruginosa*. The decrease in chlorophyll-*a* content in the present study confirmed inhibition of photosynthetic activity of *Heterosigma akashiwo* cells by the allelochemical extracted from *Phaeodactylum tricornutum* filtrate, although the effects were only observed with the highest exposures.

3.3 Influence on membrane integrity and potential

Intact cell membranes are necessary for maintaining normal cellular functions. Damaged cell membranes will thus affect cell survival and growth. Cell membrane integrity was quantified as the percentage of viable cells in the different concentrations of allelochemical treatments revealed by PI-staining. Vital dyes such as PI are normally excluded from the inside of healthy cells, but freely cross the membrane and enter the cell to stain internal components (like DNA) if the membranes are damaged. After a short-term exposure (day 3), the percentage of cells with intact membranes in different treatments was lower than the control group ($p < 0.05$). After longer duration exposures (days 5 and 6), membrane integrity of the medium and low concentration treatments was not significantly different from the control group. However, high allelochemical exposures decreased the percentage of intact *Heterosigma akashiwo* cells by about 10-18% throughout the 6-day culture time ($p < 0.05$). The percentage of viable *H. akashiwo* cells was about 3-5% lower after short-term allelochemical exposure to the low and medium concentration treatments (day 3), with the effect disappearing on days 5 and 6.

This transient effect presumably reflects the growth and division of surviving cells (Fig.5), and perhaps also demonstrates that the survivors had some inherited resistance to the allelochemical.

Many allelochemicals have been found to reduce algal cell membrane integrity, thereby leading to the leakage of cell constituents (e.g., proteins, nucleic acids and inorganic ions), enhancing proton influx (Johnston et al., 2003; Campos et al., 2009) and finally causing catastrophic cell membrane damage. Ethyl 2-methyl acetoacetate (EMA) was found to oxidize the major fatty acids of cyanobacterial cell membranes and cause leakage of Ca^{2+}, K^{+} and Mg^{2+} (Hong et al., 2008). Likewise, when ferulic acid (FA) was added to a culture of *Microcystis aeruginosa*, the percentage of damaged cells increased with increasing concentration (Wang et al., 2016 a). No intact cells were found when *M. aeruginosa* was exposed to 3.47 mM FA at 96 h. Based on evidence from SEM and the membrane integrity dyes used here, it is clear that damage of cell membranes is one reason for the inhibitory effects of the *Phaeodactylum tricornutum* allelochemical on *Heterosigma akashiwo*.

The fluorescence of DiOC6(3) was also used to assess the change of membrane potential due to the allelochemical. This measures the difference in potential between the interior and exterior of a biological cell. The mean fluorescence intensity of DIOC6(3) is shown in Fig.6. It did not respond rapidly to the different doses of allelochemical treatments used here. The mean fluorescence intensity of DIOC6(3) showed no difference from the control group after 3 d of exposure, but did eventually decrease by 40% on day 5 for the three treatment groups. The inhibitory effect became even more evident with high allelochemical concentration on day 6, with fluorescence suppressed by 70% compared to the control. However, the DIOC6(3) intensity increased and showed no difference from the control group on day 6 at medium and low concentrations of the allelochemical. This result was not in accordance with the rapid change in membrane potential observed for *Microcystis aeruginosa*

exposed to FA after only 8 h, highlighting the dependence of the effect on the sensitivity of the target cell and the nature of the allelochemical (Wang et al., 2016 a). More studies of the effects of allelochemicals on membrane potential should reveal more details of the mechanisms of the inhibitory response.

3.4 Inhibition on esterase activity

Esterase is a type of hydrolase enzyme that exists in many organisms and plays an important role in many biological functions. An esterase activity assay using fluorescein diacetate (FDA) has been proposed as a rapid endpoint to evaluate the toxicity of environmental pollutants on algal species (Regel et al., 2002; Hadjoudja et al., 2009). As shown in Fig.7, FDA fluorescence was significantly inhibited by allelochemical exposure even with the lowest concentration treatment and in the earliest stages of the experiment (days 1 and 3), following a dose-dependent pattern. The fluorescence of FDA for the three treatments all showed a pronounced decrease of 35-50% compared to the control group on day 1. It increased subsequently, but still remained lower than the control group, reaching a 10-20% decrease on day 3. However, esterase activity of each treatment group showed no difference compared to the control group on days 5 and 6. This suggests that *Heterosigma akashiwo* was vulnerable during the initial period of exposure, but that subsequent divisions of the surviving cells led to a population that had physiologically adapted to the allelochemical through time, perhaps though some type of resistance among daughter cells.

Esterase activity of *Heterosigma akashiwo* cell was the most sensitive and rapid response to the allelochemical of *Peterosigma tricornutum* compared to the other physiological measures. Correlation analysis showed that the growth of *H. akashiwo* had a positive relationship with the integrity of the cell membrane ($r = 0.812$, $p < 0.001$), and the activity of esterase also exhibited a positive correlation with

the integrity of the cell membrane, especially with the medium and high concentrations treatments (r=0.746，p<0.05；r=0.791，p<0.05).

Allelopathy is clearly an important factor in competition among phytoplankton in marine ecosystems (e.g., Legrand et al., 2003), however, the exact nature of compounds involved in the inhibition process and the inhibitory mechanisms are still unclear. The allelopathic effect of *Prymnesium parvum*, which produces toxins with haemolytic, ichthyotoxic and cytotoxic properties, caused changes in the plankton community structure, resulting in a decrease in both chlorophyll a and carbon uptake (Fistarol et al., 2003). *Alexandrium tamarense* also produces potent allelochemicals comprising a suite of large non-proteinaceous and probably non-polysaccharide compounds between 7 kDa and 15 kDa with lytic activity against a wide variety of marine microorganisms (Ma et al., 2011).

Allelopathy has the potential to be used as the basis of an effective control or bloom mitigation strategy to inhibit algal growth in natural blooms, particularly those that are harmful and where bloom suppression has benefits to society or to ecosystems. There is, however, a need for further study to advance this concept and evaluate its logistical feasibility and environmental suitability. Until recently, the most successful application of the use of naturally-produced chemicals in harmful algal bloom (HAB) control involves allelochemicals released from barley straw as a bloom suppression strategy for freshwater HABs (Xiao et al., 2014). Phenolic compounds in barley straw are thought to be the main inhibitor of algal growth (Terlizzi et al., 2002; Xiao et al., 2014; Huang et al., 2015). Iredale et al (2012) showed that microbial degradation of the barley straw releases hydrogen peroxide as well as inhibitory products from the lignin. Unfortunately, barley straw would have limited use against HABs in coastal marine environments due to the continual exchange of seawater with tides, etc., and the relatively limited number of algal species that are sensitive (Terlizzi et al. 2002; Hagström et al. 2010).

Hu and Hong (2008) reviewed the potential application of allelopathy from aquatic plants (macroalgae) on microalgae, and Shao et al (2013) reviewed the use of several other biologically-derived substances that have negative impacts on algal (mainly phenols, quinones, alkaloids, organic acids, amino acids, and terpenes). Those authors also discussed the reasons for the slow progress in the use of allelochemicals in HAB control, including difficulties in obtaining sufficient material, relatively low sensitivity of targeted HAB species, ecological and public health concerns, and the potential release of toxins as a result of cell lysis during the treatment. Clearly, the practical application of allelochemicals in bloom control needs further study and careful field evaluation.

4. Conclusions

Heterosigma akashiwo growth was significantly suppressed by the allelochemical contained in the ethyl acetate extract of *Phaeodactylum tricornutum*. Many *H. akashiwo* cells rapidly died and disappeared from the medium, as observed by the light microscope and the FCM. A possible mechanism for this effect was revealed by SEM imagery, which showed numerous holes with different shapes and sizes on the outer cell. FCM was applied with vital stains to examine physiological parameters in the surviving cells. Although those cells were still intact, or semi-intact, the FCM analyses could reveal the nature of the damage that had been experienced. The allelochemical released by *P. tricornutum* was found to influence *H. akashiwo* mainly by decreasing the esterase activity and the integrity of the cell membrane, thereby releasing cytoplasm and other cellular constituents. Esterase activity was the most useful and sensitive parameter to evaluate the influence of the *P. tricornutum* allelochemical on *H. akashiwo*. Membrane potential and the content of chlorophyll-*a* were also affected, but the membrane potential response increased through time, in contrast to other parameters,

which generally followed an opposite trend. More studies of the mechanisms underlying the response of membrane potential are needed.

In summary, a *Phaeodactylum* allelochemical caused catastrophic damage to exposed *Heterosigma* cells, leading to cell lysis and death, but surviving cells were also impacted, showing effects that reflect damage to membrane integrity and some biochemical properties such as esterase activity. Nevertheless, surviving cells can continue to grow and in a few days, re-establish a successful culture, even in the presence of residual allelochemical, suggesting either development of cellular resistance, or the biodegradation of the chemical.

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Figure captions

Figure. 1 Inhibition rate on *H. akashiwo* growth during six-day exposure to different concentrations of an allelochemical isolated from *P. tricornutum* filtrate. A: high dose; B: medium dose; C: low dose. Data are presented as mean ± standard deviation (n=3). Dose concentrations given in Table 1.

Figure. 2 Influence of different concentrations of allelochemical isolated from *P. tricornutum* filtrate on the size of *H. akashiwo* cells during six-days of exposure. A: high dose; B: medium dose; C: low dose; D: Control. Dose concentrations given in Table 1. Data are presented as mean ± standard deviation (n=3). Similar lowercase letters indicate no significant difference ($p < 0.05$).

Figure. 3 SEM micrographs of *H. akashiwo* cells. A: Control group; B, C, D, E, cells treated with the allelochemical isolated from *P. tricornutum* filtrate.

Figure. 4 Influence of six-day exposure to different concentrations of allelochemical isolated from *P. tricornutum* filtrate on the chlorophyll-*a* content of *H. akashiwo* cells. A: high dose; B: medium dose; C: low dose; D: Control. Dose concentrations given in Table 1. Data are presented as mean ± standard deviation (n=3). Similar lowercase letters indicate no significant difference ($p < 0.05$).

Figure. 5 Influence of six-day exposure to different concentrations of allelochemical isolated from *P. tricornutum* filtrate on the membrane integrity of *H. akashiwo* cells. A: high dose; B: medium dose; C: low dose; D: Control. Dose concentrations given in Table 1. Data are presented as mean ± standard deviation (n=3). Similar lowercase letters indicate no significant difference ($p < 0.05$).

Figure. 6 Influence of six-day exposure to different concentrations of allelochemical isolated from *P. tricornutum* filtrate on the membrane potential of *H. akashiwo* cells. A: high dose; B: medium dose; C: low dose; D: Control. Dose concentrations given in Table 1. Data are presented as mean ± standard deviation (n=3). Similar lowercase letters indicate no significant difference ($p < 0.05$).

Figure. 7 Influence of six-day exposure to different concentrations of allelochemical isolated from *P. tricornutum* filtrate on the esterase activity of *H. akashiwo* cells. A: high dose; B: medium dose; C: low dose; D: Control. Dose concentrations given in Table 1. Data are presented as mean ± standard deviation (n=3). Similar lowercase letters indicate no significant difference ($p < 0.05$).

Table

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Table 1. The addition of three levels of allelochemical of *P. tricornutum* for FCM measurements

	Control	High dose	Medium dose	Low dose
f/2 medium(mL)	100	100	100	100
Fractions (μL)	111 DMSO	111	37	18.5

High dose: About 3 times of the maximum concentration of allelochemical of *P. tricornutum.*

Medium dose: The approximate maximum concentration of allelochemical of *P. tricornutum.*

Low dose: About half of the maximum concentration of allelochemical of *P. tricornutum.*

Control: 111 μL DMSO

Figure.1

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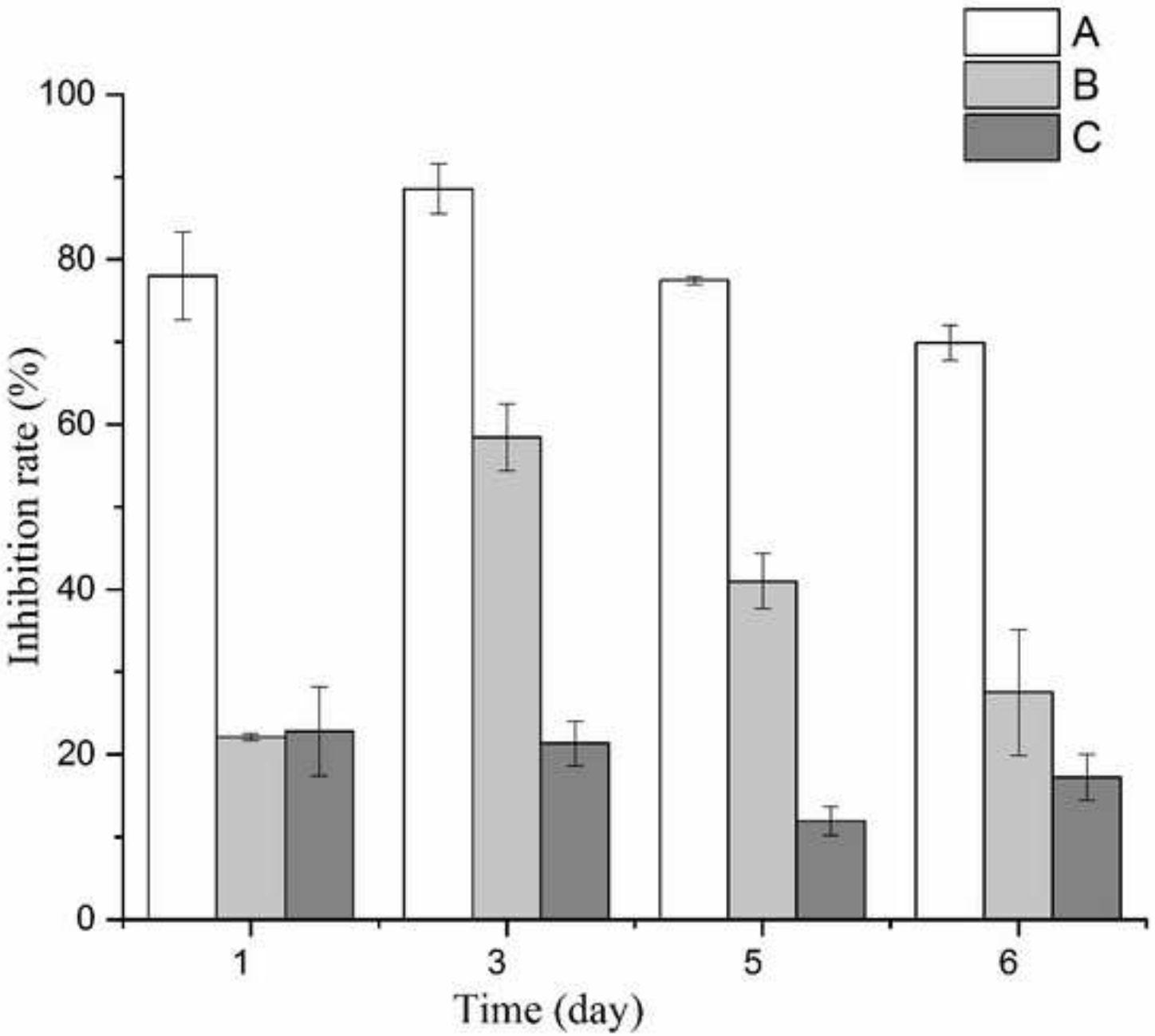

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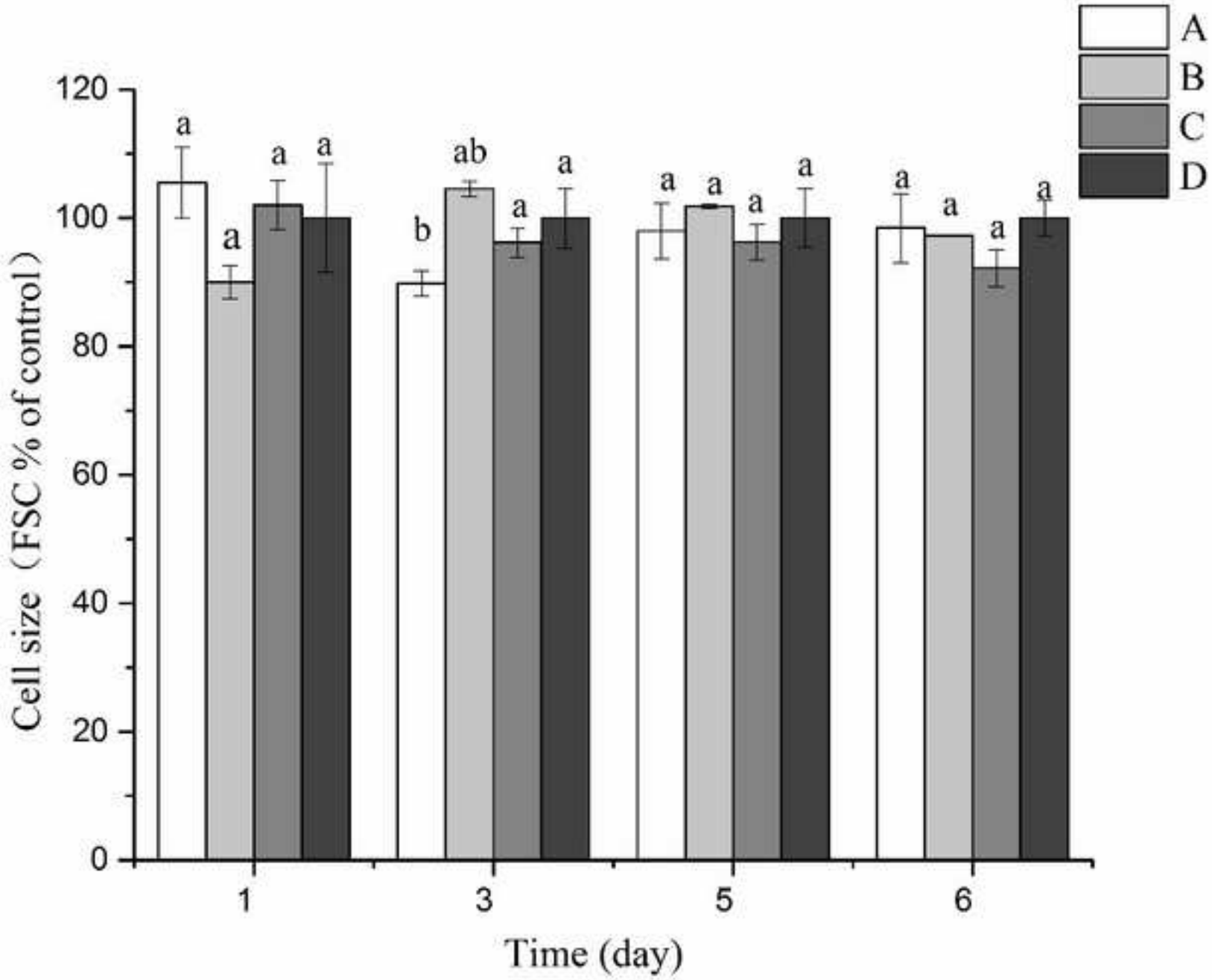

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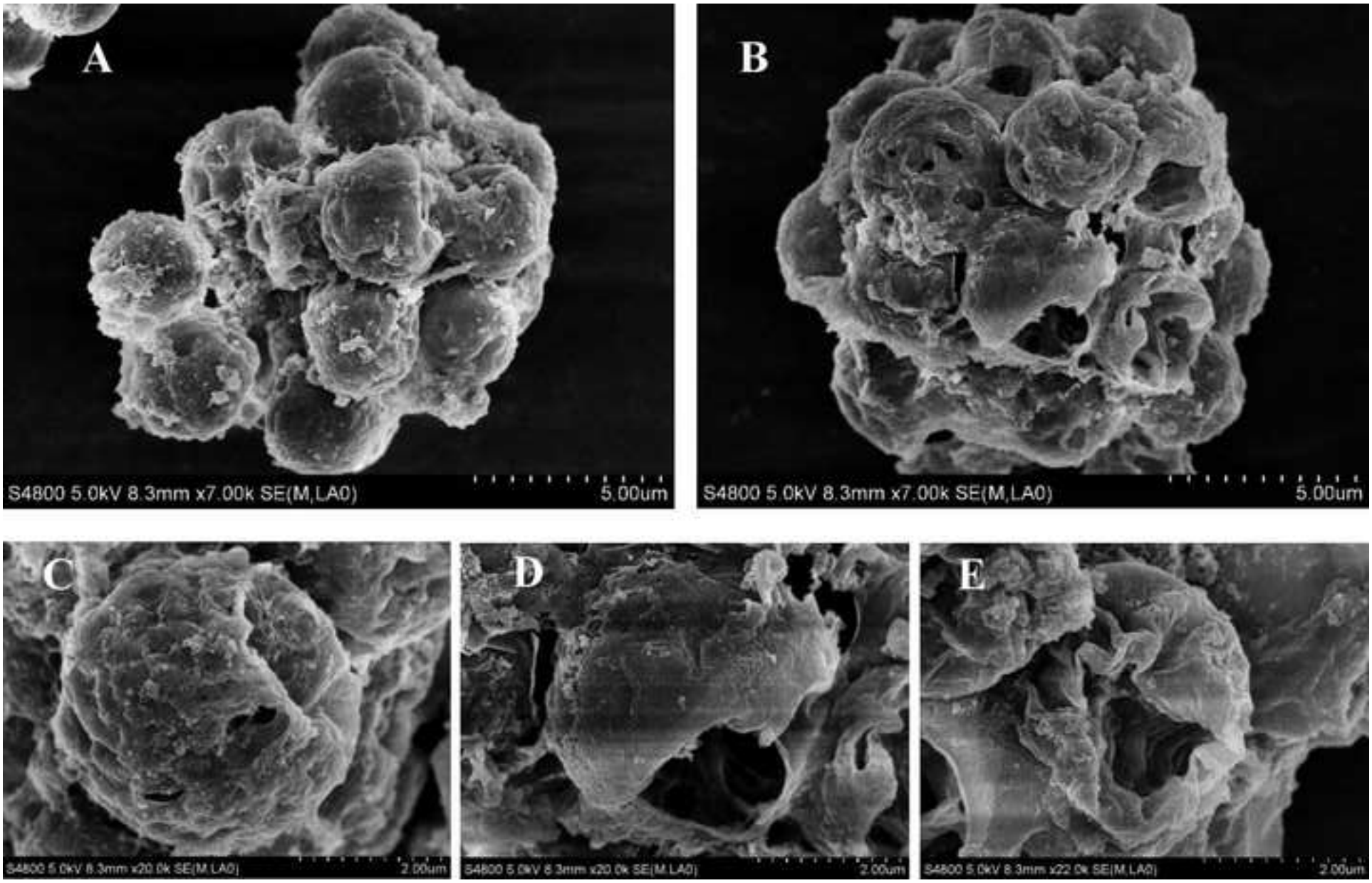

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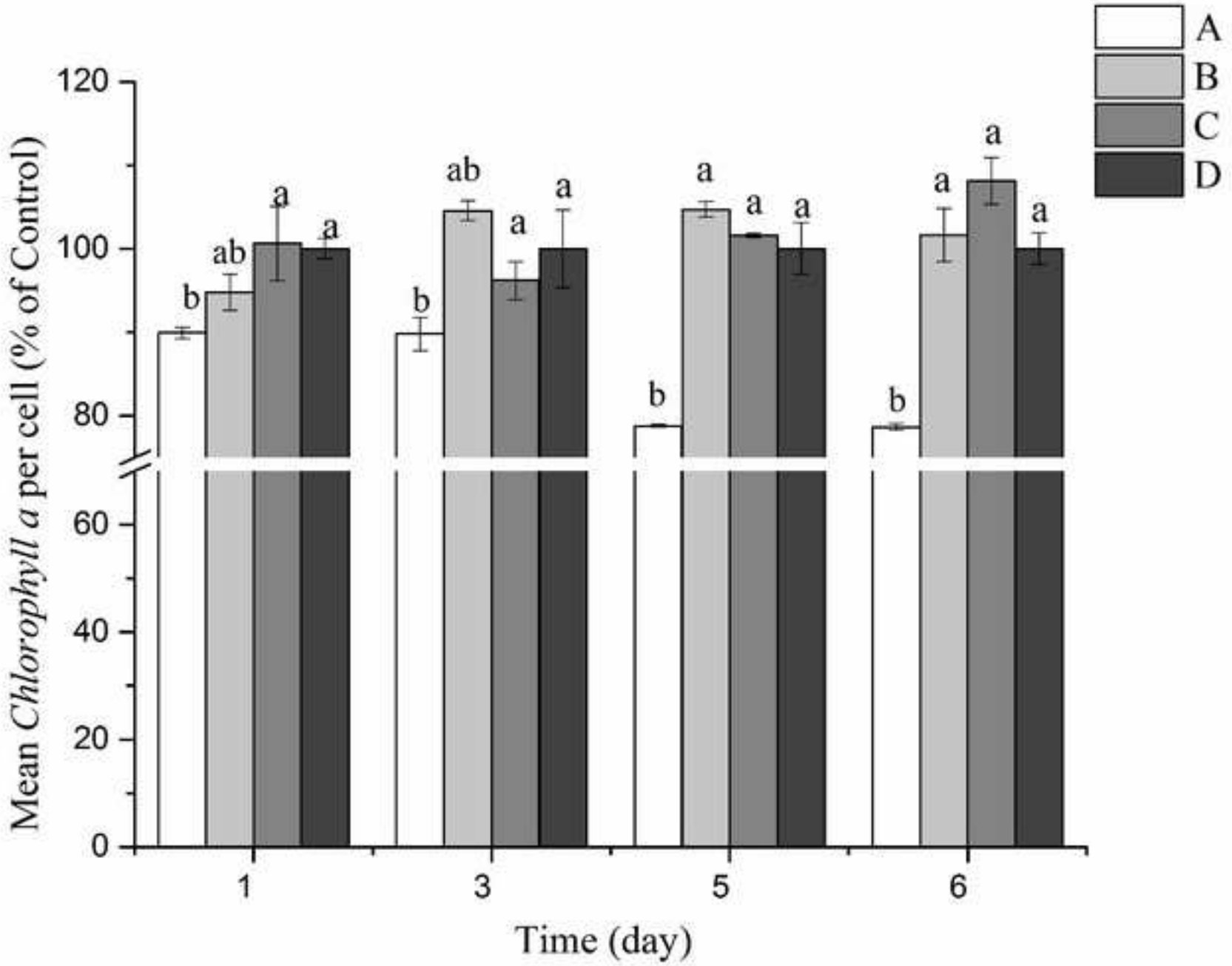

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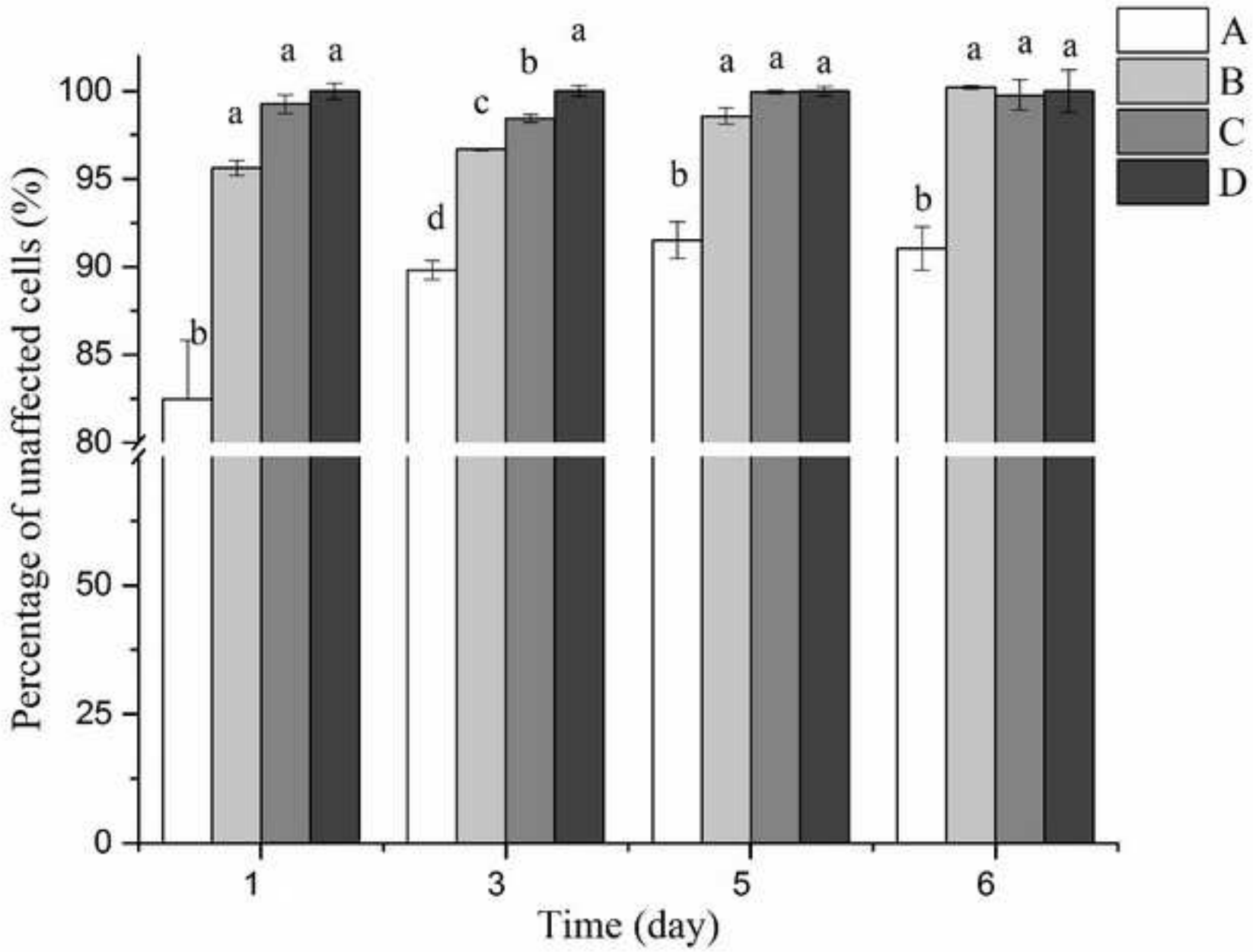

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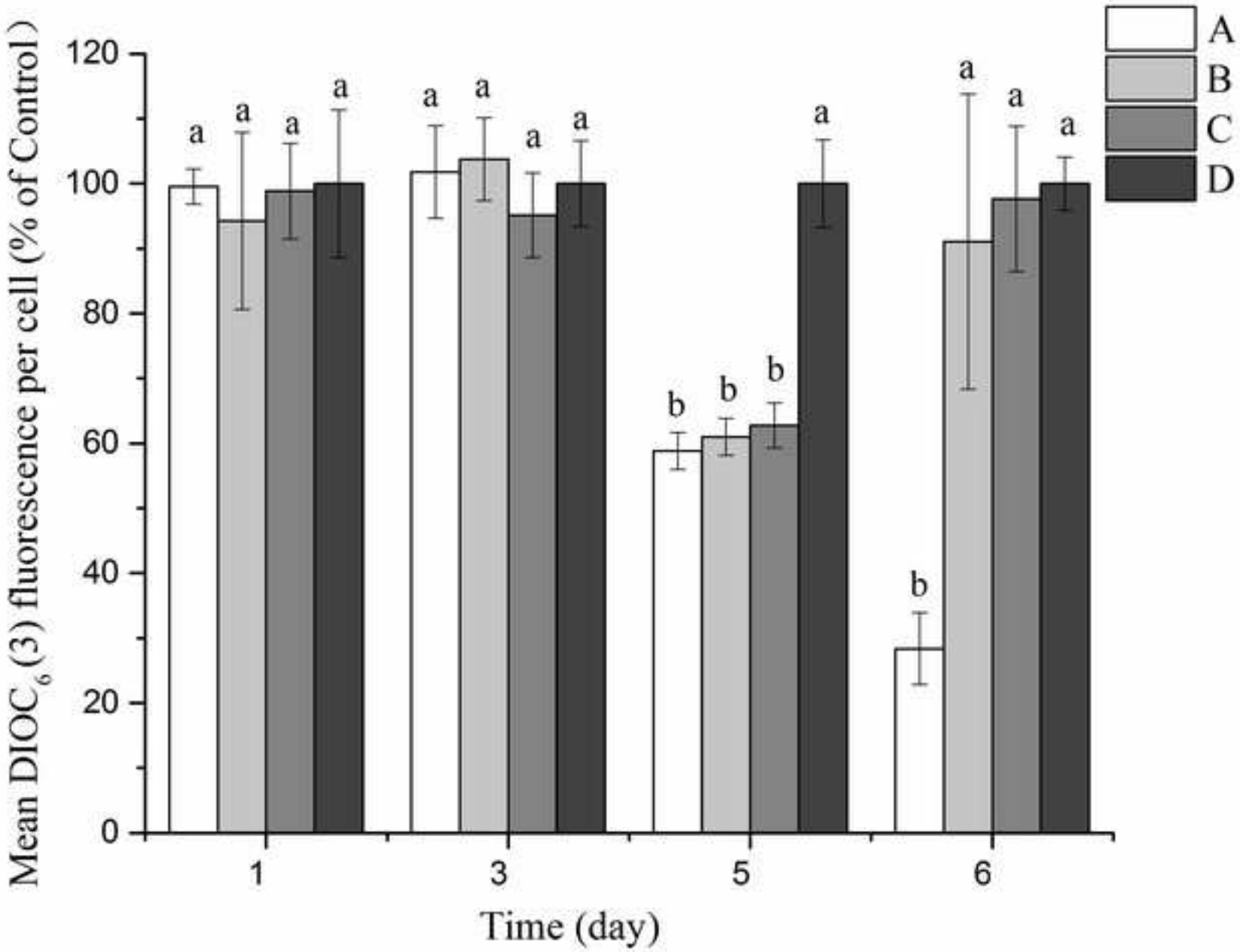

Figure.7

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