



# Responses of Bloom-Forming *Heterosigma akashiwo* to Allelochemical Linoleic Acid: Growth Inhibition, Oxidative Stress and Apoptosis

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Algal blooms have been occurring in many regions worldwide, and allelochemicals are important algacides used to control harmful algal blooms (HABs). The allelopathic effects of linoleic acid (LA) on the harmful raphidophyte *Heterosigma akashiwo* were studied, and the possible mechanisms were investigated through analyses of population growth dynamics, cellular ultrastructure and the physiological levels of *H. akashiwo*. The results showed that the inhibitory effect of LA on *H. akashiwo* cells increased with an increasing LA concentration. The levels of ROS and MDA were significantly elevated, indicating oxidative stress and lipid peroxidation due to LA exposure. At the same time, LA also activated the antioxidant system, including superoxide dismutase (SOD), catalase (CAT), and POD, and non-enzymatic antioxidants such as reduced AsA and glutathione (GSH). Transmission electron microscopy (TEM) revealed that the morphology of the algal cells was impaired in an LA-dependent manner. Annexin V-FITC/PI double staining and flow cytometric analysis revealed that LA exposure decreased the cellular mitochondrial membrane potential (MMP), increased the rate of apoptosis. LA modulated bcl-2/bax homeostasis and increased the expressions of cytochrome c and caspases-3 and -9, proving that LA induced cell death via the mitochondria-mediated apoptotic pathway. It was suggested that LA had allelopathic effects on *H. akashiwo*, inducing physiological and morphological changes and finally triggering the apoptosis of *H. akashiwo*. All of these results showed that LA might have the potential as an algacide to control harmful algae.

**Keywords:** harmful algal blooms (HABs), allelochemical, *Heterosigma akashiwo*, linoleic acid, oxidative damage

## INTRODUCTION

Harmful algal blooms (HABs) as a kind of marine disaster (Anderson et al., 2008, 2012), have devastating impacts on fisheries, tourism, public health and ecosystems worldwide. In recent decades, increases in the severity, frequency and biogeographic range of these phenomena have occurred in many parts of the world (Anderson et al., 2012; Pichierri et al., 2017; Wells et al., 2015).

The motile raphidophyte *Heterosigma akashiwo* is a globally distributed HAB-forming species (Smayda, 1998). As a eurythermic species, *H. akashiwo* can adapt well to the environment and has been found in red tide outbreaks in various countries such as the United States, Japan, and China (Khan et al., 1997; Rensel et al., 2010; Yang et al., 2021). Many blooms of *H. akashiwo* have been reported to be ichthyotoxic, resulting in the death of wild and farmed fish in coastal areas (Keppler et al., 2005; Ge et al., 2010). Relevant studies show that *H. akashiwo* may become the dominant species of red tide under the background of global climate change (Fu et al., 2008; Baek et al., 2017; Smit et al., 2021). In view of the harmfulness of *H. akashiwo* and the occurrence trend of algal bloom, controlling and eliminating *H. akashiwo* blooms has become an urgent worldwide problem.

Numerous regulating methods are currently applied to solve the problem of HAB mitigation, including traditional physical, chemical and biological technologies (Broekman et al., 2010; Chen and Pan, 2012; Mohamed et al., 2014), but secondary pollution, high costs or impractical problems are common (Zhang et al., 2013; Pal et al., 2020). Due to no secondary pollution, allelochemicals are considered to be environmentally friendly and effective biological algicides (Backer et al., 2015; Harke et al., 2016; Eladel et al., 2019). To date, many allelochemicals identified from aquatic plants or algae display strong algicidal activity. For instance, the allelochemical ethyl 2-methylacetoacetate (EMA) isolated from *Phragmites communis* Tris showed strong inhibitory activity against the growth of *Microcystis aeruginosa* and *Chlorella pyrenoidosa* (Li and Hu, 2005). Oh et al. (2010) isolated 5,8,11,14,17-eicosapentaenoic acid (EPA) from the extract of *Corallina pilulifera*, which had algicidal activity against the toxic dinoflagellate *Cochlodinium polykrikoides*. The inhibitory effect of allelochemicals is selective, which may be associated with the inhibitory mechanism. As biological algicides, allelochemicals have an influence on the physiological and biochemical properties of algae (Xue et al., 2018). Previous research indicated that allelochemicals might induce oxidative stress in algae, resulting in changes in cellular morphology, protein levels, antioxidant activities and cellular functions (Yu et al., 2019). The oxidation and antioxidation of organisms themselves are generally in dynamic equilibrium (Wrzaczek et al., 2013). When algal cells are exposed to various noxious stimuli, the quantity of reactive oxygen species (ROS) and free radicals increases, and their antioxidant defense systems can effectively scavenge these free radicals. However, once the concentration of allelochemicals is high enough to disrupt the balance, it may lead to oxidative damage or even the death of algal cells (Tan et al., 2019).

In our previous studies, linoleic acid (LA) was successfully extracted from *Sargassum thunbergii* and *Corallina pilulifera*, and it was found that LA significantly inhibited the population growth of *Skeletonema costatum* and *Karenia mikimotoi*, showing strong algicidal activity (Wang et al., 2012; Han et al., 2018). Although allelochemicals LA have been isolated from a variety of aquatic plants (Nakai et al., 2005; Alamsjah et al., 2008), the sensitivities of its allelopathic effects also vary

among the different phytoplankton groups (Xue et al., 2018), and the mechanisms associated with their ability to inhibit targeted phytoplankton growth are also unknown. To date, the data on the physiological and morphological responses of algae to LA stress are very limited. Therefore, in the present study, the allelopathic effects of LA on the harmful microalga *H. akashiwo*, including growth inhibition, oxidant stress, ultrastructural changes and apoptosis, were explored. These physiological analyses could help elucidate the LA functional mechanisms associated with its inhibitory effects on algal growth.

## MATERIALS AND METHODS

### Algal Culture and Chemicals

*H. akashiwo* cultures were provided by the Algal Center of the Ocean University of China. The cultures were inoculated in f/2 medium (Guillard, 1975) prepared with sterile seawater. The algae were cultured at 25°C with cool white fluorescent light and 12 h:12 h light-dark cycle conditions. Flasks were alternately disposed in the culture chamber, and the flasks were shaken twice a day to prevent wall growth and algal sedimentation. All follow-up experiments used exponential-phase algae at an initial cell density of  $1 \times 10^4$  cells/mL.

LA (purity > 99%, CAS number: 60-33-3) and acetone (purity  $\geq$  99.9%, CAS number: 67-64-1) were purchased from Sigma-Aldrich (St. Louis, MO, United States), and the LA was dissolved in acetone to prepare a stock solution.

### Algal Growth Inhibition Assays

LA dissolved in acetone was added to the algal cultures at exponential phase at final concentrations of 100, 300, 500, 700, and 900  $\mu$ g/L. Two controls were established in the experiments: a blank control (with sterile seawater) and solvent control (only containing  $\leq$  0.2% acetone). All of the above concentrations were applied in a growth inhibition assay for *H. akashiwo*. Every experiment was conducted in triplicate. The exposure experiments were performed for 72 h, and the algal cell density was measured under an optical microscope at 0, 24, 48 and 72 h postexposure with a hemocytometer. The inhibition rate was calculated according to OECD 201 (OECD, 1984).

### Transmission Electron Microscopy Observations

Transmission Electron Microscopy (TEM) (JEM-1200EX, JEOL, Japan) was used to observe the cellular ultrastructure of the algae after 300, 500, and 700  $\mu$ g/L LA exposure. After 48 h of exposure, the control and LA-treated cells were collected and fixed using a solution comprising 3.5% glutaraldehyde in phosphate-buffered saline (PBS, 0.1 mol/L, pH 7.2) and set at 4°C for 2 days. Then, the cells were postfixed in 1% osmic acid and washed 3 times with PBS. All treated samples were dehydrated in graded concentrations of ethanol, and then ultrathin sections were made for TEM observation.

## Determinations of Reactive Oxygen Species and Lipid Peroxidation

Determination of malondialdehyde (MDA) contents and the main ROS components, including superoxide anion ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $\bullet OH$ ). After 48 h of exposure to LA, the control and LA-treated cells were collected by centrifugation ( $950 \times g$ ) for 10 min at  $4^\circ C$ . The harvested algal cells were suspended in PBS (0.1 mol/L, pH 7.2) and immediately disrupted by ultrasonication (JY92-2D, Ningbo Scientiz Biotechnological Co., Ltd., China) for 2 min with a 1 s pause after each 1 s pulse in an ice bath. Then, the homogenate was centrifuged ( $13,500 \times g$ ,  $4^\circ C$ ) for 10 min to remove the cellular debris, and the supernatant was used to determine the intracellular  $O_2^{\bullet-}$ ,  $H_2O_2$  and  $\bullet OH$  levels and the MDA contents according to the manufacturer's instructions provided with the corresponding kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All of the parameters mentioned above were investigated on the basis of the total soluble protein, which was determined by the Bradford method (Bradford, 1976).

Detection of the total ROS levels. The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA, Beyotime Biotechnology Inc., Shanghai, China) was used to detect the intracellular total ROS levels. The control and LA-treated cells were separately centrifuged and collected and then resuspended in PBS (0.1 mol/L, pH 7.2). The samples were mixed with DCFH-DA and incubated at  $37^\circ C$  in the dark for 30 min. After staining, the cells were resuspended in PBS, and their fluorescence intensity was detected by flow cytometry (FCM, Beckman Coulter, Fullerton, CA, United States) in the FL1 channel.

## Enzymatic Antioxidant and Non-enzymatic Antioxidant Assays

Determination of the activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), and the contents of antioxidants, including ascorbic acid (AsA) and glutathione (GSH), in the algal cells was conducted after exposure to LA (300, 500, and 700  $\mu g/L$ ) for 48 h. In brief, the control and LA-treated cells were sampled at 48 h, and the supernatants were extracted according to the method described in section "Enzymatic Antioxidant and Nonenzymatic Antioxidant Assays." SOD activity was determined by the photochemical reduction of nitro blue tetrazolium (NBT) at 550 nm according to the method of Beauchamp and Fridovich (1971). CAT activity was assayed with a spectrophotometric method at 240 nm according to the method of Rao et al. (1996). POD activity was assayed with a guaiacol method at 470 nm according to the method of Rao et al. (1996). Similarly, the supernatant was used to determine the AsA and GSH contents according to the manufacturer's instructions provided with the corresponding kits (Nanjing Jiancheng Bioengineering Ins., Nanjing, China). The activity or the content of antioxidants were all calculated per mg of protein.

## Mitochondrial Membrane Potential Assays

The Mitochondrial Membrane Potential (MMP) ( $\Delta\psi_m$ ) was determined using the probe 5,5',6,6'-tetrachloro-1,1',3,3'-

tetraethylbenzimidazole carbocyanide iodide (JC-1, Beyotime Biotechnology Inc., Shanghai, China). Based on the growth inhibition assay, we established one LA-treated group (500  $\mu g/L$ ), one blank control and one DMSO control. After exposure to LA for 48 h, the algal cells were harvested, washed with PBS (0.1 mol/L, pH 7.2) and centrifuged at  $950 \times g$  for 10 min. The supernatants were discarded, and the microalgae cells were stained with JC-1 probes and incubated at  $37^\circ C$  in the dark for 20 min. Then resuspended cells in PBS, and the fluorescence was detected by FCM in the FL-1 and FL-3 channels for the monomers and J-aggregates. Simultaneously, the stained cells were photographed using fluorescence microscopy (Nikon, Tokyo, Japan).

## Analysis of the Key Components of Apoptosis

The LA concentration was set as described for the MMP experiment. Cells in the blank control, acetone control and LA-treated groups (500  $\mu g/L$ ) were centrifuged ( $950 \times g$  for 10 min) and harvested after 48 h of exposure.

### Apoptosis Analysis

The samples were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions for the apoptosis detection kit (Beyotime Institute of Biotechnology of Shanghai, China). The stained cells were incubated for 20 min at  $25^\circ C$  in the dark. Then, the samples were detected by FCM in the FL-1 and FL-3 channels for FITC fluorescence and PI fluorescence.

### Detection of the Release of Cytochrome c

The samples were analyzed according to the manufacturer's instructions with a Cytochrome c assay kit (Nanjing Jiancheng Bioengineering Ins., Nanjing, China). The absorbance values were detected by a Synergy H1 microporous reader (BioTek, America) at 450 nm.

### Detection of the Ratio of Bax to Bcl-2

The samples were analyzed according to the manufacturer's instructions for the Bcl-2 protein assay kit and Bcl-2 associated x protein (Bax) assay kit (Nanjing Jiancheng Bioengineering Ins., Nanjing, China). The absorbance values were detected by a Synergy H1 microporous reader (BioTek, America) at 450 nm.

### Detection of the Caspase-3, -9 Activities

The samples were analyzed according to the manufacturer's instructions of a caspase-3 activity assay kit and caspase-9 activity assay kit (Beyotime Biotechnology Inc., Shanghai, China). The absorbance values were detected by a Synergy H1 microporous reader (BioTek America) at 405 nm.

## Data Analysis

Data analysis was performed using SPSS v. 24.0, and the data were presented as the mean  $\pm$  standard deviation (SD). All experiments were independently repeated 3 times. The significance of each parameter was tested using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. For all analyses, differences were considered statistically

significant when  $p < 0.05$ . The correlation was analyzed using a Pearson test at  $p < 0.05$ .

## RESULTS

### Effect of Linoleic Acid on the Growth of *Heterosigma akashiwo*

**Figure 1A** illustrates the ability of LA to inhibit the growth of *H. akashiwo*. Growth promotion of algal cells treated with LA was detected at low concentrations (100  $\mu\text{g/L}$ ). When exposed to LA in the concentration range of 300–900  $\mu\text{g/L}$  for 72 h, the cell density of *H. akashiwo* decreased in an LA-dependent manner. Moreover, the inhibition rate of algal cells significantly increased with an increasing LA concentration (**Figure 1B**), and the inhibition rate reached 97.3% when exposed to 900  $\mu\text{g/L}$ , which reached the highest value. These results indicated the inhibitory effects on the growth of *H. akashiwo* induced by LA exposure.

### Effects of Cellular Ultrastructure in *Heterosigma akashiwo* Cells Following Linoleic Acid Exposure

TEM analysis revealed alterations in the internal ultrastructure of the *H. akashiwo* cells. In the blank control and acetone control (**Figures 2A,B**), the algal cells had an intact periplasm membrane, and the cell organelles, including chloroplasts and mitochondria, were uniformly distributed and maintained a normal ultrastructure. Specifically, the mitochondria had intact bilayer membranes and abundant cristae. However, after LA exposure for 48 h, the cells appeared distinctly damaged (**Figures 2C–E**). Compared with the control, damage to the mitochondria of *H. akashiwo* was observed at 300  $\mu\text{g/L}$ , which was mainly manifested as a loss of mitochondrial inclusions, damage to the bilayer membrane, and blurring of the internal cristae (**Figure 2C**). Massive cell vacuolization appeared in the 500  $\mu\text{g/L}$  LA-treated algal cells (**Figure 2D**). High concentrations of LA (700  $\mu\text{g/L}$ ) induced periplasm membrane fracture. Moreover, algal cell organelles such as chloroplasts and mitochondria disintegrated (**Figure 2E**).

### Effects of Linoleic Acid on Reactive Oxygen Species Production and Malondialdehyde Content in *Heterosigma akashiwo*

In the present study, exposure to LA resulted in a remarkable increase in oxidative stress in the treated groups with respect to the control, causing a substantial increase ( $p < 0.05$ ) in the intracellular ROS levels ( $\text{O}_2^{\cdot-}$ ,  $\bullet\text{OH}$ ,  $\text{H}_2\text{O}_2$  and total ROS). The  $\text{O}_2^{\cdot-}$  levels sharply increased in the 500  $\mu\text{g/L}$  group (**Figure 3A**), 4.8 times higher than the control ( $p < 0.05$ ). In addition, the levels of  $\bullet\text{OH}$ ,  $\text{H}_2\text{O}_2$  and total ROS were all increased in an LA-dependent manner (**Figures 3B–D**). Compared with the control, as the exposure concentration of LA increased, the MDA levels increased significantly and reached a maximum in the

700  $\mu\text{g/L}$  group ( $p < 0.05$ ), which was 3.1 times that of the control (**Figure 3E**).

### Antioxidant System Response of *Heterosigma akashiwo* Cells to Linoleic Acid Exposure

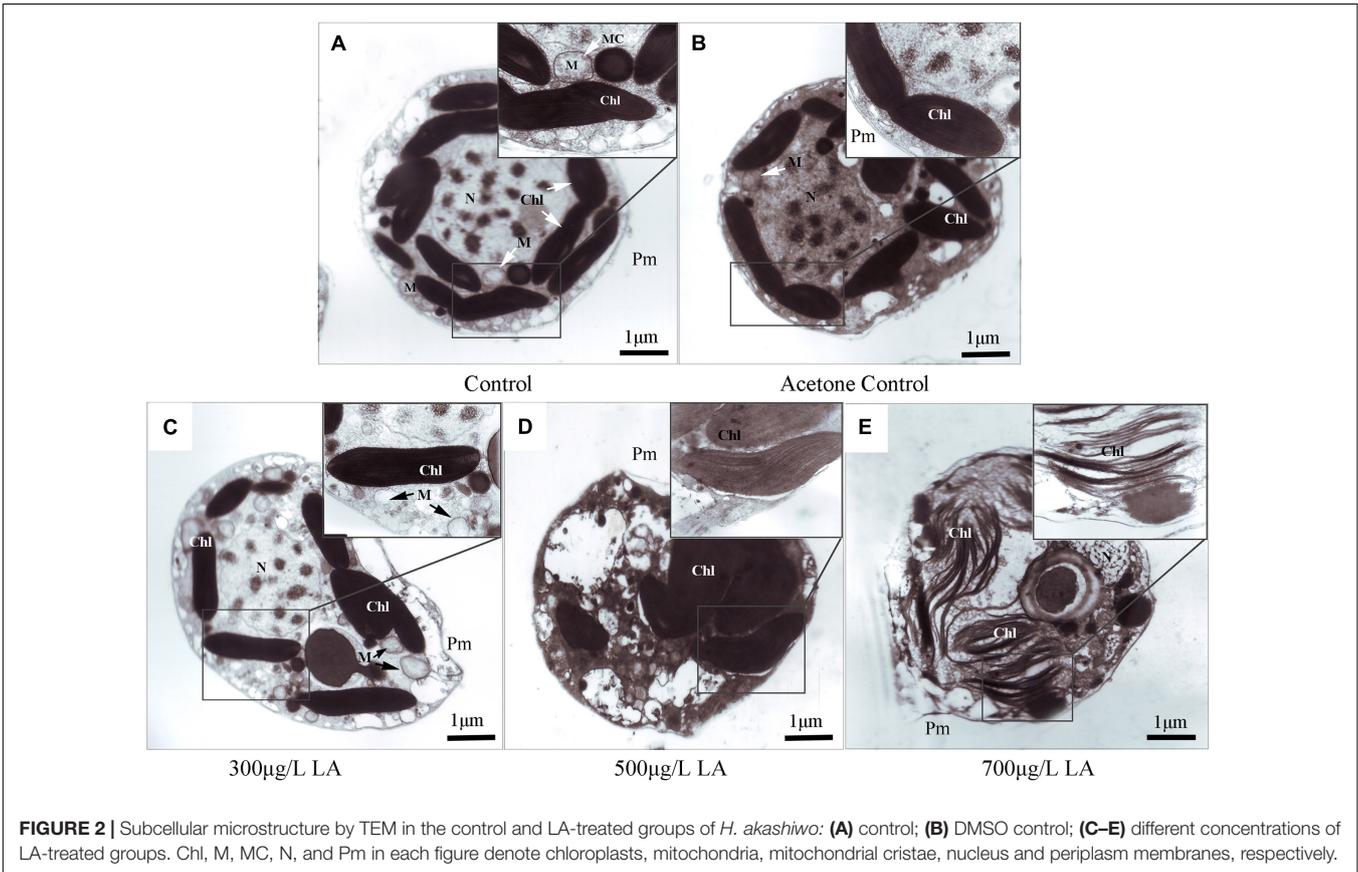
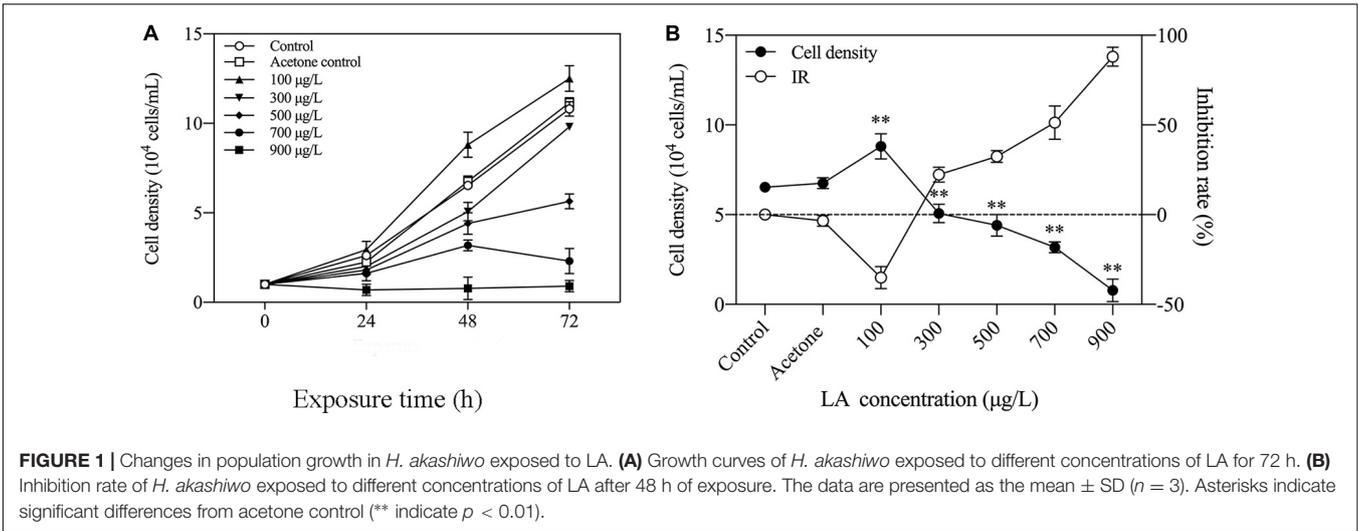
The SOD activity of the LA-treated group increased to the highest value at 300  $\mu\text{g/L}$  (**Figure 4A**), which was obviously higher than that of the control group ( $p < 0.05$ ), but remarkably decreased SOD activity was observed in the higher-dose LA-treated groups. With the increase in the activity in the LA-treated group, the CAT and POD activities showed different trends than SOD, and both reached the highest value in the 500  $\mu\text{g/L}$  group (**Figures 4B,C**), while their activity decreased slightly at 900  $\mu\text{g/L}$  but was still higher than that in the control ( $p < 0.05$ ). The AsA content in the LA-treated groups increased dramatically at 300  $\mu\text{g/L}$  and remained significantly higher than in the control with increasing concentrations of LA (**Figure 4D**). As shown in **Figure 3E**, a significant elevation in GSH content was observed after treatment with LA compared with the control, and the 500  $\mu\text{g/L}$  group had the highest value.

### Analysis of the Key Components of Linoleic Acid-Induced *Heterosigma akashiwo* Cell Apoptosis

The results of the Annexin V/PI analysis demonstrated that the number of apoptotic cells significantly increased in the LA-treated group compared with the control group (**Figure 5A**). Moreover, the apoptotic rate in the LA-treated group was markedly higher than that in the control group, with an approximately 4.25-fold increase (**Figure 5B**). These results suggest that LA at the concentration tested had a significant apoptotic effect on *H. akashiwo* cells.

After the cells were stained with JC-1, stronger green fluorescence was observed in cells treated with LA than in controls (**Figure 6A**). The percentage of JC-1 monomers in the LA-treated group also significantly increased ( $p < 0.05$ ), approximately  $67.7 \pm 0.13\%$  higher than the control (**Figure 6B**). These results indicated that MMP was reduced, and the mitochondrial membrane was damaged in *H. akashiwo* cells.

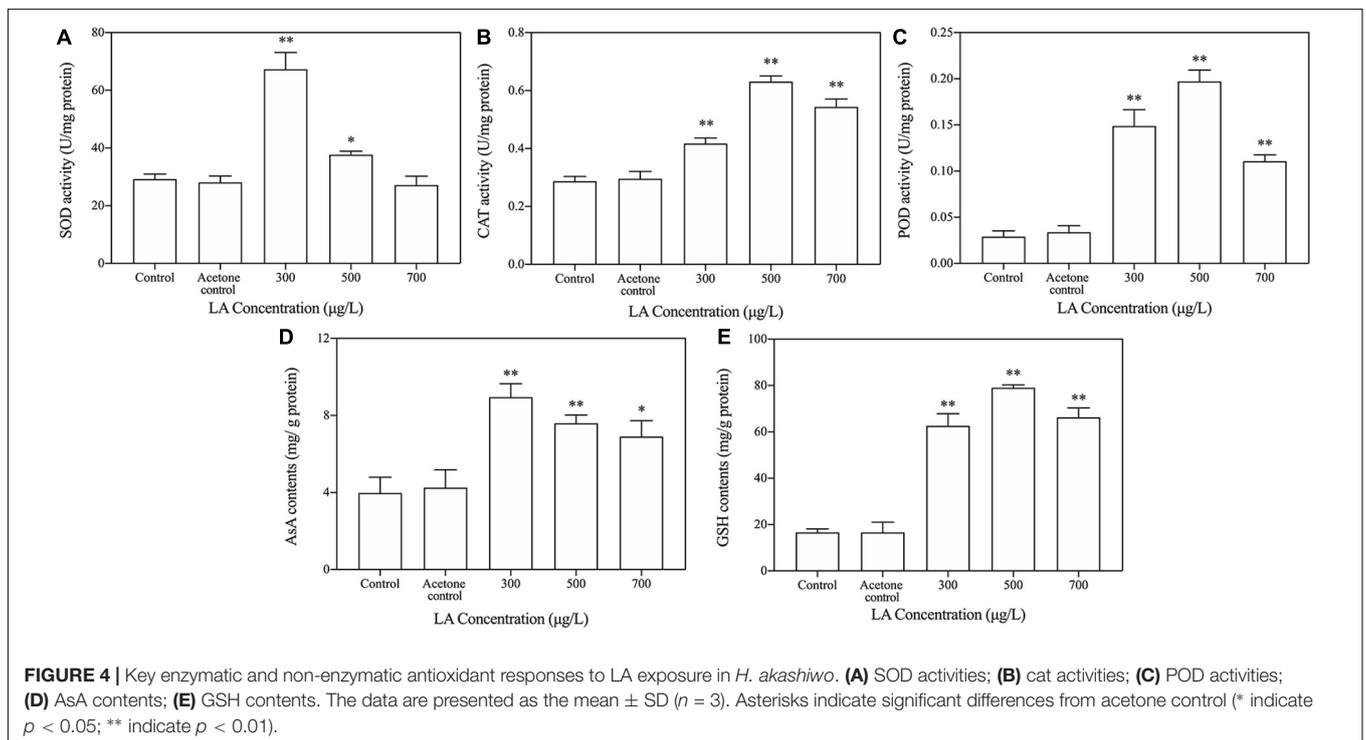
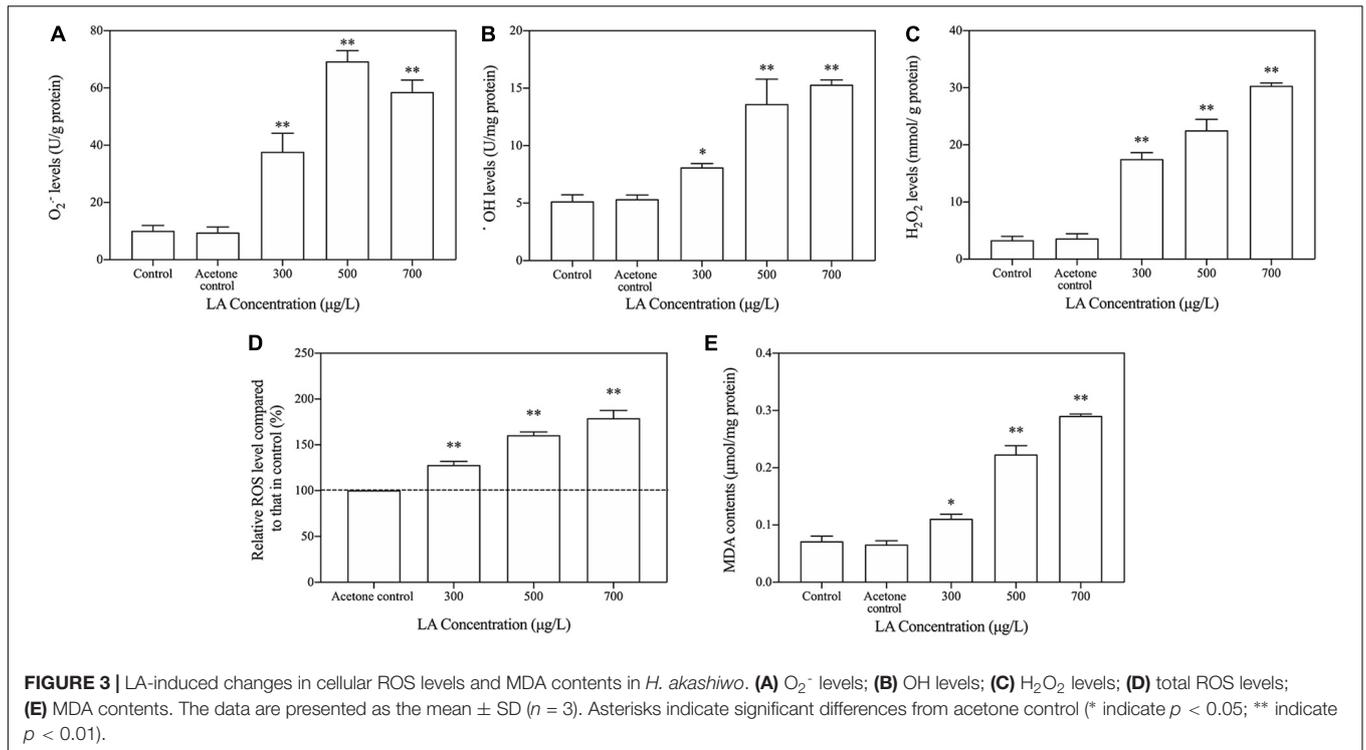
The key components of the apoptosis pathway, i.e., cytochrome *c*, Bcl-2 protein family, caspase-3, and caspase-9, were detected. The results showed that the levels of cytochrome *c* were significantly increased when exposed to LA ( $p < 0.05$ ), which was approximately  $72.6 \pm 0.14\%$  higher than that of the control (**Figure 6C**). The activity of caspase-3 was dramatically increased, by approximately  $52.1 \pm 0.7\%$ , in the LA-treated group. Similar significant increases were observed for caspase-9 in the LA-treated group compared to the control (**Figure 6D**). The Bax to Bcl-2 ratios were simultaneously analyzed, and the results showed that the ratios of Bax to Bcl-2 were elevated in the LA-treated group compared with the control (**Figure 6E**). These results suggest that LA may impair mitochondrial function and induce apoptosis through a mitochondrial-dependent pathway.



## DISCUSSION

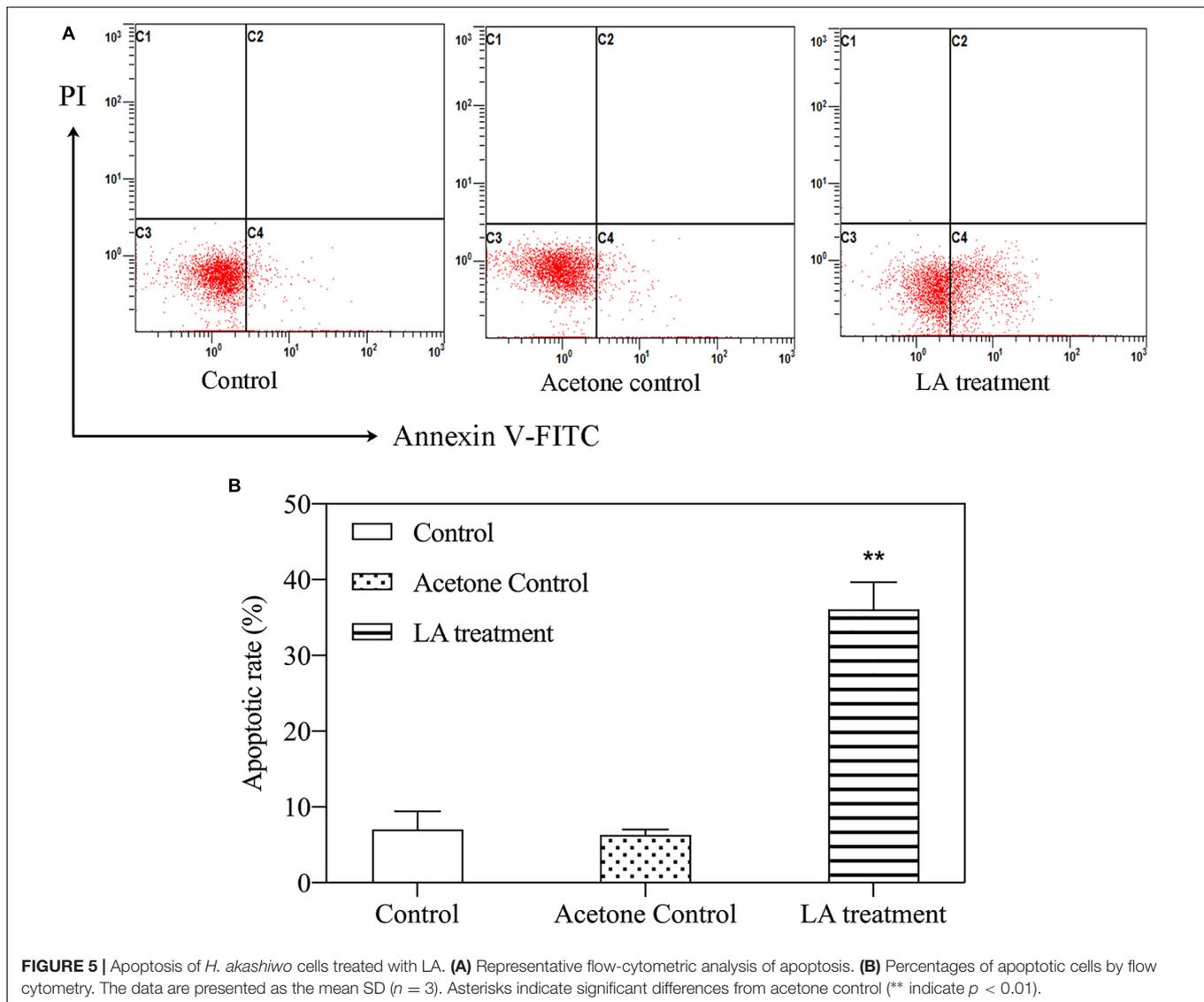
The serious environmental and economic problems induced by HABs have led to great social concern (Lundgren et al., 2013; Lou and Hu, 2014). Although various methods have been proposed to control harmful algal growth, secondary pollution, high cost or impracticability constrain their application (Anderson, 1997; Hoko and Makado, 2011). The discovery of allelopathy provides

a new dimension to solve this problem (Nakai et al., 2001). In aquatic ecosystems, allelopathic inhibition of microalgae by macrophytes is one of the main research fields in allelopathy (Qian et al., 2009). Fatty acids are widely found in seaweeds, and some fatty acids isolated from seaweed, including LA, tetradecanoic (TDA) and hexadecanoic acids (HAD), have been reported to have algae-inhibiting activity. In many studies, LA has shown the properties of high extraction and strong inhibition of



microalgae (Hijano et al., 2021; Sun et al., 2021), which suggested that the number of the unsaturated double bond and the length of the carbon chain fatty acid are important structural element involved in the algal inhibition (Nakai et al., 2005; Zhang et al., 2009). Currently, there have been several studies on the modes

of action of LA. To the best of our knowledge, most studies have focused on freshwater algae, such as *Chlorella pyrenoidosa*, *Selenastrum capricornutum*, and *Microcystis aeruginosa* (Kamaya et al., 2003; DellaGreca et al., 2010; Song et al., 2017), but the potential mechanisms of LA against marine HABs have not been

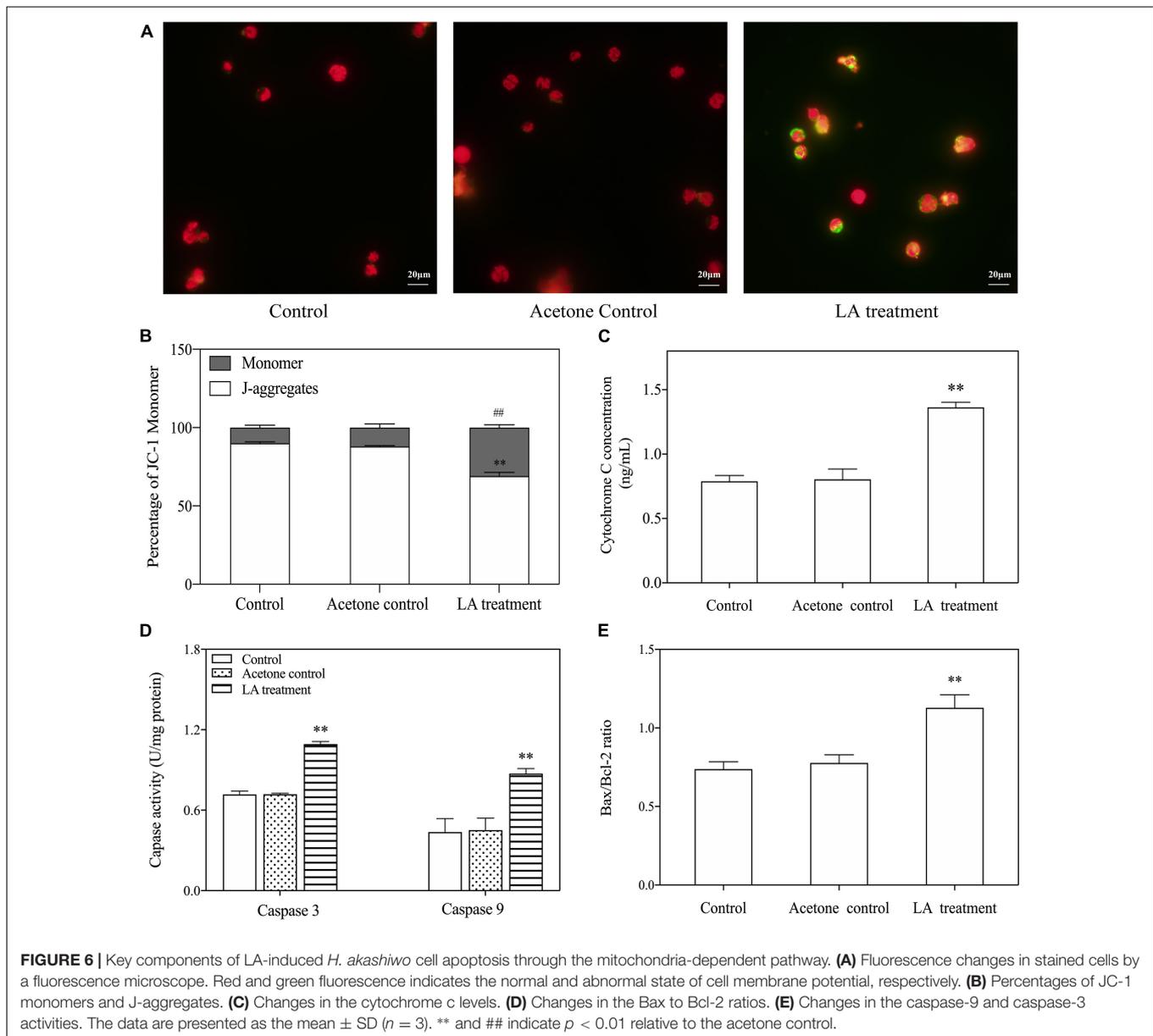


fully elucidated. Hence, the investigation of the mode of action of LA against *H. akashiwo* in this study might help to further explore why inhibition occurs and the role of LA in the field of algal bloom control.

Our results indicated that the inhibitory effect of LA on *H. akashiwo* increased with an increasing concentration. The inhibition rate is a common parameter used to quantify allopathic effects (Nakai et al., 1999). Obviously, the inhibition rate was increased in an LA concentration-dependent manner (Figure 1B). It should be noted that while the *H. akashiwo* cells were significantly inhibited by LA, the effect in the marine microalgae was complex, with stimulation of algal growth at a relatively low LA concentration (100  $\mu\text{g/L}$ ) and a significant inhibition at the higher concentrations tested. LA had a concentration effect of “promotion at a low concentration and inhibition at a high concentration” on *H. akashiwo*, which is consistent with previous results (Calabrese, 2008; Qian et al., 2019). The reason for this effect might be that a low concentration

of LA could increase the permeability of the algal cell membrane by increasing the enzyme activity of algal cells (Karasyova et al., 2007). LA (above 300  $\mu\text{g/L}$ ) significantly inhibited the growth of algae, and the reason for this might be that when the allelochemicals contained in LA exceeded the acceptable range of algal cells, the algal antioxidant enzyme system and cell membrane were increasingly damaged (Mecina et al., 2017). Inhibition of growth could be induced by damage to the cell membranes and organelles of the algal (Gu et al., 2017). After LA exposure for 48 h, the cells appeared to be distinctly damaged, including cell vacuolization and disintegration of algal cell organelles such as the chloroplast and mitochondria (Figures 2D,E). Consequently, damage to algal organelles may inhibit the growth of algae, with damage to chloroplasts and mitochondria in particular leading to oxidative stress.

LA causes oxidative stress, which produces a large amount of ROS by changing intracellular antioxidant enzymes activities and inducing lipid peroxidation (Song et al., 2017; Han et al., 2018;



Guo et al., 2015; Qian et al., 2018). In this study, the total ROS level increased, and the levels of different ROS species include  $O_2^{\bullet-}$ ,  $H_2O_2$  and OH simultaneously increased in algal cells after LA exposure (Figures 3A–D), indicating that LA induces excessive ROS in *H. akashiwo* cells. To scavenge ROS and avoid oxidative damage, algal cells possess a set of cellular defense systems mediated *via* enzymes and non-enzymatic antioxidants (Morales et al., 2004; Yang et al., 2013). SOD plays a central role in the defense against oxidative stress and catalyzes the dismutation of  $O_2^{\bullet-}$  to  $H_2O_2$  (del Río et al., 2018). Analogous to SOD, CAT and POD are also important enzymes with the ability to catalyze the decomposition of hydrogen peroxide in resistance to oxidation damage caused by ROS (Xia et al., 2015; Hua et al., 2018). In the present study, SOD was induced by LA in the early stages to resist stress, which is consistent with previous research

(Hou et al., 2019). Then, the activities of SOD decreased with an increasing LA concentration, and the contents of POD and CAT also went through a process of induction and suppression, which indicated that LA could abate or impair the antioxidant defense system and therefore exhibited a strong algal inhibiting ability.

AsA and GSH also play a preponderant role in the cell redox balance and metabolic regulation. In our study, the sharp increases in AsA and GSH at 300  $\mu\text{g/L}$  LA hinted that the production of  $H_2O_2$  was elevated and that AsA and GSH were actively involved in the antioxidant response to oxidative stress when exposed to LA at low concentrations (Chen et al., 2015). However, the ROS increased dramatically, and oxidative stress was induced as the antioxidant defense system was insufficient to scavenge all ROS, triggering defense system collapse. To clarify whether oxidative stress caused

oxidative harm to *H. akashiwo*, membrane lipid peroxidation was evaluated. MDA was commonly used as a biomarker to estimate oxidative damage in cells (Hua et al., 2018; Liu et al., 2019). Markedly increases in MDA content combined with the changes in cellular ultrastructure, indicated that LA might have induced lipid peroxidation and damaged the *H. akashiwo* cell membranes.

The overproduction of ROS is an important factor involved in cellular apoptosis (Nobel et al., 2000; Calamita et al., 2005). In the present study, flow cytometry and morphological changes indicated that apoptosis occurred quickly after LA exposure in *H. akashiwo* (Figure 5). Indeed, as the location where most intracellular ROS are produced, mitochondria are at a cross-point in the signaling pathways involving ROS, especially those concerning apoptosis (Jones, 2000). In addition, MMP is involved in a number of cellular functions and plays a central role in apoptosis by integrating death signals (Brenner and Kroemer, 2000; Lee et al., 2004). In our experiment, MMP in the algae decreased when exposed to LA (Figures 6A,B), which indicated that LA might lead to a significant decrease in MMP and trigger apoptosis in algae. In the present study, as the key components in triggering and regulating apoptosis by the mitochondrial pathway, the cytochrome *c*, Bcl-2 protein family, caspase-9 and caspase-3 showed significant changes following LA exposure. As an essential component of the mitochondrial respiratory chain, cytochrome *c* released from mitochondria to the cytosol has been demonstrated to be a crucial step in the activation of apoptosis (Moriya et al., 2000; Redza-Dutordoir and Averill-Bates, 2016). In addition, previous studies have shown that the Bcl-2 protein family, which regulates mitochondrial function, is related to apoptosis sensitivity. Among these proteins, the Bax to Bcl-2 ratio is vital to induce permeabilization of the outer mitochondrial membrane and the release of cytochrome *c*, leading to the activation of caspases (Bras et al., 2005; Moll et al., 2005). Therefore, we considered that the excessive production of ROS induced by LA might be the key factor in the collapse of the MMP, followed by disturbances in the Bax to Bcl-2 ratio, concomitant with cytochrome *c* combining with caspase-9 after its release from the mitochondria, and thus activating downstream caspase-3 to initiate apoptosis.

## CONCLUSION

Through the analysis of these physiological indexes, the inhibitory mechanism of LA on *H. akashiwo* growth was

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explored. The results demonstrated that the decrease in algal density was directly related to the activity of the antioxidant system of algal cells. LA-induced overproduction of ROS caused oxidative stress in *H. akashiwo* cells, with lipid peroxidation, compromising membrane integrity and seriously impairing mitochondrial function, resulting in growth inhibition. In addition, upon further increases in ROS generation, cytochrome *c* was released concomitantly with activation of caspase-3 and a loss of mitochondrial membrane potential, eventually leading to apoptosis through the mitochondrial pathway. These results show that LA may be an eco-friendly alternative for use in the control of harmful blooms in aquatic ecosystems.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

RW conceived and supervised the project. QL and RW conceived and designed the experiments. QL performed the experiments and analyzed the data, and wrote the manuscript. The final manuscript, figures, and tables were revised by RW with input from QL. Both authors participated in the discussions of the results and the preparation of the manuscript and contributed to the article and approved the submitted version.

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