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Physiological response and morphological changes of *Heterosigma akashiwo* to an algicidal compound prodigiosin

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ABSTRACT

Harmful algal blooms (HABs) occur all over the world, producing severely negative effects on human life as well as on marine ecosystems. The algicidal compound, prodigiosin, secreted by algicidal bacteria *Hahella* sp. KA22 can lyse the harmful alga *Heterosigma akashiwo*. This study is aimed to investigate the algicidal mechanism of prodigiosin against *H. akashiwo* by detecting physiological and morphological responses of *H. akashiwo* to presence of prodigiosin. The results indicated that prodigiosin showed strong algicidal effects on *H. akashiwo* at the concentration of 3 μg/mL. Chlorophyll *a* and protein levels of the microalgae decreased significantly while malonaldehyde levels increased at this concentration. Contents of ascorbic acid and activities of superoxide dismutase and peroxidase increased fast with the quick decrease of the reactive oxygen species (ROS). For the 3 μg/mL prodigiosin treatment group, transcription of genes related to photosynthesis and respiration were significantly inhibited at 12 h while respiration related genes increased at 24 h. Collectively, the results indicated that prodigiosin could kill the microalgae by inducing ROS overproduction which could destroy the cell integrity and change the antioxidant system levels and functional gene expression. Our results demonstrated that prodigiosin is an effective algicide for the control of harmful algae.

1. Introduction

The contamination of aquatic environments has become an increasingly serious phenomenon along with rapid development of industry and agriculture. Harmful algal blooms (HABs) are one of the products of the marine pollution and eutrophication that cause severe negative effects on human health as well as on marine ecosystems (Berdalet et al., 2016; Medlin, 2013; Anderson et al., 2012). *Heterosigma akashiwo*, a harmful bloom-forming species of Raphidophyceae, causes substantial economic losses and its toxins are responsible for mass fish mortality (Baek et al., 2013; Khan et al., 1997).

A number of physical and chemical methods (e.g., clay and copper sulfate) have been adapted over time to control and manage HABs (Na et al., 1996; Sengco et al., 2001; Rounsefell and Evans, 1958; Lee et al., 2013). Due to secondary pollution, high costs of these methods and inhibition of non-HAB phytoplankton communities, physical and synthetic chemical methods are difficult to use for controlling HABs (Ni et al., 2012). For example, clay or yellow loess being used to remove HABs-causing microalgae by sedimentation could occasionally affect the bottom-dwelling organisms and cause ecological/environmental problems through dispersal of a large quantity of clay into a limited area of sea floor. Synthetic chemical algicides could also cause ecological changes, since these reagents were not easily biodegraded and could accumulate in marine organisms over long periods (Park et al., 2011). Recently, the option of exploring biological methods has attracted increased attention (Guo et al., 2016). Different functional bacteria, actinomycetes and viruses have been reported to exhibit algicidal functions (Yoshinaga et al., 1997; Su et al., 2007; Nakashima et al., 2006; Brussaard et al., 2007; Cai et al., 2011; Li et al., 2014a). Algal-lysing microorganisms kill algal cells mainly by secreting active extracellular substances such as proteins, amino acids, fatty acid, antibiotics, and pigments (Wang et al., 2012; Li et al., 2014b; Wen et al., 2011; Yang et al., 2013; Fei et al., 2013). The culture supernatant of *Pseudomonas chlororaphis* JK12 was found to exhibit strong algicidal activity while washed JK12 cells showed no obvious activity. This indicated that strain JK12 attacked microalgal cells by secreting extracellular algicidal metabolites (Kim et al., 2018). The metabolites of a bacterium strain *Ponticoccus* sp. PD-2 was found to be capable of killing the HABs causing by microalgae *Prorocentrum donghaiense*, *Phaeocystis globose* and *Alexandrium tamarense* with the algicidal activity of more than 60% (Chi et al., 2017). Three compounds produced by *Bacillus* sp. strain B1, urocanic acid (uro), L-histidine (his) and N-acetylhistamine

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(ace) showed dramatic algicide activity against *Phaeocystis globosa* (Zhuang et al., 2018).

Prodigiosin (PG) is a red pigment which can be produced by various microorganisms including *Serratia, Streptomyces*, *Vibrio*, *Hahella, Zooshikella*, and *Pseudoalteromonas* (Danevčič et al., 2016). It is sensitive to light and soluble in chloroform, methanol, acetonitrile and dimethylsulfoxide (DMSO) (Shaikh, 2016). Prodigiosin is receiving increasing attention due to its antineoplastic, antibacterial, antimalarial, antifungal, immunosuppressive, algal-lysing and antiviral activity (Nakashima et al., 2006; Zhou et al., 2015; Jimtha et al., 2017; Nada et al., 2014). It showed selective anticancer activity against over 60 cancer cell lines with a very less or no effect on normal cells (Zhou et al., 2015; Kamble and Hiwarale, 2012). Some studies found that prodigiosin were closely associated with PCD (Tenconi et al., 2018; Darshan and Manonmani, 2016). A prodigiosin concentration of 1 μg/L could result in to a 90% *Cochlodinium polykrikoides* cell mortality within 1 h (Jeong et al., 2005). Prodigiosin produced by strain MS-02-063 (γ-*proteobacterium)* showed effective algicidal activity on *Heterosigma akashiwo*, *Heterocapsa circularisquama*, *Gymnodinium impudicum* and *Alexandrium tamarense* (Nakashima et al., 2006). It was also found that prodigiosin had algicidal activities against *Cochlodinium polykrikoides*, *Gyrodinium impudicum*, *Chattonella* sp. and *Heterosigma akashiwo* (Dockyu et al., 2008). In our previous study, prodigiosin showed particularly strong algicidal activity against *P. globosa*, *H. akashiwo*, *P. donghaiense*, while showed no algicidal activity against the dinoflagellates specie *Scrippsiella trochoidea* and a diatom species *Skeletonema costatum* (Zhang et al., 2016). But these studies mainly focused on the algicidal bacteria, and only measured the host range of the algicidal bacteria. The algae-lysing mechanisms underlying algicidal compounds produced by bacteria have not been fully defined. These compounds might induce oxidative stress to algae, resulting in changes in cellular morphology, protein levels, antioxidase activities and DNA and RNA degradation, thus inducing algal cell death(Park et al., 2011; Cho, 2012; Hu et al., 2015; Zhang et al., 2017). When harmful algae was exposed to algal-lysing bacteria, the algal cells exhibited plasmolysis, organelles disrupted and cell membranes ruptured, followed by algae cell lysis (Kong et al., 2013). Oxidative stress from algicidal compounds could destroy photosynthesis and produce surplus ROS, which could destroy unsaturated fatty acids and cellular protein (Apel and Hirt, 2004). Surplus ROS could be scavenged by a series of antioxidases that includes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) (Shri et al., 2009), and ascorbic acid (AsA) (Ahmad et al., 2010). The algal-lysing effect could destroy organelles such as chloroplast and mitochondria, then influenced gene expression. Changes in the gene expression of *prx, fabZ*, *psbA*, *grpE* and *recA* genes in *Microcystis aeruginosa* under algicide stress, were reported (Shao et al., 2009). A previous study by our group reported alterations in the expression of *psbA* and *psbD* genes in *A. tamarense* in response to supernatant of marine algicidal bacteria DHQ25 (Zhang et al., 2014).

An understanding of the changes that occur in algae under the stress of algicidal compounds secreted by bacteria is useful in understanding the lytic mechanism of these compounds. To-date, such data on physiological and morphological responses of the algae is very limited. To illustrate the mechanism of prodigiosin on *H. akashiwo*, we investigated (1) morphological changes of *H. akashiwo* under transmission electron microscopy (TEM) and scanning electron microscopy (SEM); (2) the activity of antioxidant enzymes; and (3) gene expression changes of photosynthetic and respiratory systems in *H. akashiwo* measured using real-time PCR technology.

2. Materials and methods

2.1. Algal culture preparation and algicidal activity of prodigiosin

Algae culturing conditions. *H. akashiwo* cultures were supplied by the State Key Laboratory of Marine Environmental Science (Xiamen University, Fujian Province, China). The cultures were inoculated in 20 ml sterile f/2 medium prepared with natural seawater (Guillard, 1975) in 50 ml conical flask with a final concentration of 1×10^5 cells/mL. The algal cultures were incubated at 20 ± 1 °C, under a 12 h:12 h light–dark cycle, with 50 μmol photons $m^{-2}s^{-1}$ light intensity.

Prodigiosin concentrations and test-solutions preparation. Prodigiosin dissolved in DMSO was added to algal cultures at exponential phase, at a final concentration of 1, 3 or 5 μg/mL. A control with addition of only DMSO but not prodigiosin was setup to detect the effects of DMSO on algal cells. Algal cells were collected at 12 h, 24 h, 36 h, 48 h, 72 h, 96 h by centrifugation at 3500 rpm for all the following physiological and molecular tests.

Analysis of chlorophyll *a* (chl *a*). The growth of the microalgal growth were evaluated by measuring chl *a* content in liquid cultures, since good linear relationships were observed between the chl *a* concentration and cellular concentrations of *H. akashiwo* (Zhao et al., 2018). To calculate the chl *a* content, 20 mL of algal cells collected by centrifugation and washed by PBS (phosphate buffer saline, 0.1 M, pH 7.4). 5 mL of 90% ethanol was added to cell pellets and chl *a* was then extracted at 4 °C. Extract solutions were centrifuged at 12,000 × *g* for 5 min, and the absorbance of the supernatant was measured at 664 nm and 645 nm wavelengths. Chl *a* content was calculated using the following formula modified from Guan et al (Guan et al., 2014):

Chl *a* (mg/L) = 12.7*A664-2.69*A645

2.2. Analysis of algicidal effects of prodigiosin on algae growth by scanning and transmission electron microscopy

Algal cultures treated with prodigiosin at different treatment time points (6 h, 12 h, 24 h, 36 h, 48 h, 72 h) were fixed in 2.5% glutaraldehyde solution and washed by PBS. Fixed cells were attached onto microscope slides and dehydrated with a series of graded ethanol (30, 50, 70, 90, 95 and 100%). After air-dried and coated with gold, alga cells were viewed using a scanning electron microscope (JSM6390, JEOL, Japan).

Collection and fixation of TEM samples was the same as described above. Cells were post-fixed in 1% OsO_4, and dehydrated in a graded with a series of graded acetone and embedded in araldite resin. Ultrathin section (60–80 nm) were stained with 3% acetic acid uranium-citric acid and viewed using a transmission electron microscopy (JEM2100HC, JEOL, Japan).

2.3. Assays of protein and lipid peroxidation

After being washed by using PBS, cell disruption collected at different time points was ultrasonicated in an ice water mixture with 5 s' intervals for 100 times under the voltage of 100 W by using an Ultraphonic Cell Diruption System (NingBo Scientiz Biotechnological Co., Ltd, China). Algal debris was discarded after centrifugation at 12,000 × *g* for 10 min at 4 °C. The supernatant was used to measure the protein and malondialdehyde (MDA) contents according to manufacturer's instructions of the Protein and Microscale Malondialdehyde (MDA) assay kit (Nanjing Jiancheng Bioengineering Institute, China) as following. For protein concentration assay, coomassie brilliant blue solution were added into all the blank groups (ddH_2O), standard group (standard solution in the Kit) and test group (sample). After being mixed and sat quietly for 10 min, solutions from all three groups were measured at 595 nm. MDA content was measured with a modified thiobarbituric acid–malondialdehyde (TBA–MDA) assay as described by Song et al. (Song et al. (2014)) by using the spectrometer (UV-1800PC, MAPADA, China).

protein content = $(A_{595\ sample}\text{-}A_{595\ blank})/(A_{595\ standards}\text{-}A_{595\ blank}) \times$ standards content;

MDA content = ($A_{532\ sample}$-$A_{532\ control}$)/($A_{532\ standards}$- $A_{532\ blank}$) × standards content ÷ protein content.

2.4. Assays of antioxidant system

The algal cell collection procedure was the same with one for protein detection, as described in section 2.3. The AsA content was measured by the method described by Hong et al. (Yu et al., 2009) by using the spectrometer (UV-1800PC, MAPADA, China). SOD activity were measured by the xanthine oxidase method according to the manufacturer's instructions of the Total Superoxide Dismutase (T-sod) assay kit (Nanjing Jiancheng Bioengineering Institute, China) as following. Briefly, aliquots (400 μl) of sample solution were mixed with 75 mmol/L K-phosphate buffer (pH 7.8), 0.1 mmol/L EDTA, 0.1 mmol/L hydroxylamine hydrochloride, 75 mmol/L xanthine, and 0.037U/L xanthine oxidase. A control group was setup with replacing sample solution by the same volume of ddH_2O. The mixtures were incubated in water bath at 37 °C for 15 min. 1 ml of the chromogenic agent was then added into the mixture and stayed for 10 min. The absorbance of the mixture ($A_{550\ sample}$ for sample and $A_{550\ control}$ for control) was then measured at OD_{550nm}. The SOD activity was then calculated as the equation as following.

SOD activity = ($A_{550\ control}$-$A_{550\ sample}$)/$A_{550\ control}$ ÷ 50% × (reaction volume/sample volume) ÷ protein content;

The POD activity was measured by using the modified guaiacol colorimetric method according to the manufacturer's instructions of the Peroxidase assay kit (Nanjing Jiancheng Bioengineering Institute, China) as following. The reaction mixture (2 ml) was composed by 0.3 mol/L phosphate buffer (pH 6.5), 45 mmol/L guaiacol, 22.5 mmol/L H_2O_2, and 100 μl sample/ddH_2O (control). After being incubated in water bath at 37 °C for 15 min, 1 ml chromogenic agent was added into the mixtures and centrifuges at 3500 *g* for 10 min. The absorbance of the supernatant ($A_{420\ sample}$ for sample and $A_{420\ control}$ for control) was then measured at OD_{420nm} for calculating POD activity by using the equation as following.

POD activity = ($A_{420\ sample}$-$A_{420\ control}$)/(12 × 1 cm) × (reaction volume/sample volume) ÷ 30 min ÷ protein content × 1000.

2.5. RNA extraction, reverse transcription and real-time PCR analysis

Total RNA was extracted from 50 mL of control and treated algal cultures grown to 12 h and 24 h using RNAiso kit (TaKaRa Company, Dalian, China). Reverse transcription and real-time PCR were performed by using the cDNA Synthesis SuperMix kit and qPCR SuperMix kit (TransGen Biotech Company, Beijing, China). Quantitative real-time polymerase chain reaction (PCR) was performed under the following conditions: denaturation at 90 °C for 30 s, then 40 cycles at 94 °C for 5 s, 50 °C for 15 s and 72 °C for 10 s. The expression of five genes (Table 1) in *H. akashiwo* in response to prodigiosin-induced stress were determined. The housekeeping gene 18S rRNA gene was used to normalize changes in gene expression. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the relative gene expression of the treatment groups.

2.6. Statistics

In this study, each experimental set were conducted in triplicates. All data were evaluated using one-way analysis of variance followed by the least significant difference test, with $p < 0.01$ and $p < 0.05$ (Origin 8.5 for Windows).

Paired correlations between the three antioxidant parameters and gene expressed levels were estimated by calculating Spearman coefficient in R software. In addition, the Euclidean distances among different samples were calculated with antioxidant parameters and gene expressed levels, respectively. The Mantel test was subsequently employed to test the matrix correlations of the two dissimilarity matrix to assess the whole relevance.

Table 1
Primer sequences of target genes.

Gene name	Sequence (5′-3′)
18S rRNA	F: CAGGTCCAGACATAGTGAG
	R: CAGAAGCATTACCGACAC
*psb*A	F: ATCTTCCAATACGCTTCA
	R: TGTTGATTACACGACCTT
*psb*D	F: TTACCCATTAGGTCAAGC
	R: GCACATAATAAAGCACCAC
cob	F: GATTACTTTGGGCATCAG
	R: AGCGGAGAATAGGTTAGT
*cox*1	F: CCAGACATGGCTTTCCC
	R: CCGACGGTCCTGAGTGA
*dna*K	F: TGGTGCTGCTGTTCAAGG
	R: GGAATTGTTGTATTACGAGGAA

3. Results

3.1. Algicidal activity of prodigiosin on H. akashiwo

Chl *a* content is a critical parameter when the cell are not countable (Tian et al., 2012). Many algalytic compounds could decrease both cell density and chl *a* content. The transient increase in chl *a* levels indicated a possible self-protective response by algal cells. The results from this study showed that the inhibitory effect of 1 μg/mL treatment was not significant, whereas groups exposed to 3 μg/mL or 5 μg/mL showed significant inhibitory effects after 72 h with the liquid color becoming lighter and clearer (Supplementary Figure S1). However, with the prolongation of treatment, the removal rates of chl *a* levels were reduced to 92% and 89% in the 3 μg/mL and 5 μg/mL groups at 96 h, respectively. The final concentration of 3 μg/mL was considered to have achieved an ideal algicidal effect and was chosen as the ideal concentration for the following algicidal experiments (Fig. 1).

TEM analysis revealed the alterations of internal ultrastructure of *H. akashiwo* cells, which showed loss of cell integrity (Fig. 2). Compared to normal cells from the control treatment (Fig. 2 A–B), organelles from the treatment groups showed greater morphological differences. In control cells, the membrane, plastid, mitochondria, nucleus and Golgi complex were clearly visible, and plastid thylakoids were regularly arranged. After 6 h, the internal cell structure became vacuolized and part of the plasmalemma was ruptured with some of the plastids released from the cells (Fig. 2 C). Although there were abundant cell contents at 12 h and 24 h, the thylakoids in the plastids were not clearly visible (Fig. 2 D–E). After 48 h, cell inclusions were all released and only a shell remained (Fig. 2 G–H). SEM revealed the process of membrane alteration whereby the cells became swollen, and the surface became smooth after the 6 h treatment (Fig. 3 B), while the cells clearly ruptured at 12 h and 24 h (Fig. 3 C–D). An empty shell remained after 48 h confirming the results from the TEM data.

3.2. Effect of prodigiosin on proteins and lipids

Proteins, nucleic acids and lipids are vital substances for cell life. Changes in these biological macromolecular substances reflect the physiological status of cell. In the control group, protein levels increased slowly due to cell growth, whereas in the 1 μg/mL group, protein levels were reduced significantly compared to the control within 72 h, indicating that cell growth was inhibited until the 96 h timepoint. In the 3 μg/mL group, protein levels decreased gradually, suggesting that algal cells were significantly affected by prodigiosin with intracellular substance outflow, which resulted in cell death

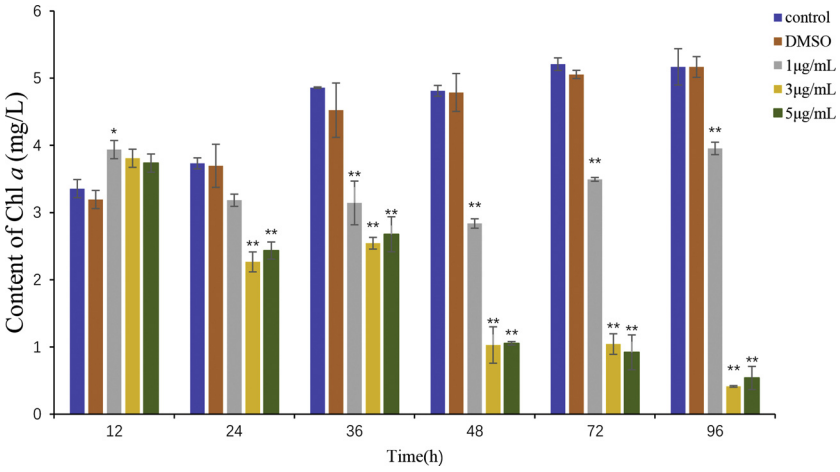

Fig. 1. The changes in chlorophyl *a* of *H. akashiwo* treated with prodigiosin at different concentrations. CK, a control without addition of DMSO and prodigiosin; DMSO, a control with addition of only DMSO but not prodigiosin; 1 μg/mL, 3 μg/mL, 5 μg/mL, the concentrations of DMSO dissolved prodigiosin added to algal cultures. All error bars indicated the SE of three biological replicates. $*p < 0.05$; $**p < 0.01$.

(Fig. 4a).

MDA is produced by lipid peroxidation when oxygen free radicals attack the unsaturated fatty acid. MDA levels in the 1 μg/mL group were much higher than the control at 72 h (Fig. 4b). In the 3 μg/mL group, MDA levels were lower than the control group at 12 h and 24 h, but as the exposure time was increased, MDA levels increased significantly and remained high. MDA levels of the 3 μg/mL group at 36 h, 48 h, 72 h and 96 h were 3.32, 3.94, 2.62 and 6.49 times that of the control, respectively.

3.3. Response of the antioxidant system

Antioxidant activities were assayed to investigate cell protective responses against prodigiosin (Fig. 5). The SOD activity of the 1 μg/mL group increased to its highest volume (281 U/mg prot) at 72 h (Fig. 5a). In the 3 μg/mL group, SOD activity levels were higher than the control after 12 h. POD activity showed a different trend to SOD (Fig. 5b) with increasing activity levels in both treatment groups, with the highest levels at 36 h (14.35 U/mg prot) in the 1 μg/mL group, and at 12 h (14.67 U/mg prot) in the 3 μg/mL group. AsA levels in both treatment groups increased significantly over a short period of time (within 12 h) and remained significantly higher than the control until the 24 h timepoint (Fig. 5c, Supplementary Table S1).

3.4. Effect of prodigiosin on gene expression

The expression of the *psb*A gene was almost unaffected by prodigiosin with a final concentration of 1 μg/mL (Fig. 6a), whereas the expression of the *psb*D gene was 1.21 times more than the control at 12 h (Fig. 6b). The expression of *psb*A and *psb*D genes in the 3 μg/mL group was significantly lower than the control at 12 h. The values for the expression of the two genes were only 22% and 14% of those in control. The expression of these two genes recovered but was still lower than the control to levels of 0.82 and 0.52 times that of the control at 24 h, respectively (Fig. 6A–B).

The expression of *cob* and *cox*1 genes in the 1 μg/mL group was increased significantly at 12 h to levels of 3.20 and 2.77 times that of the control, respectively (Fig. 6C–D). Although they decreased at 24 h, the values were significantly higher than the control. The 3 μg/mL group showed an opposite effect to the 1 μg/mL group, with decreases in levels to 0.42 and 0.43 times of the control at 12 h, respectively. Gene expression then increased to levels much higher than the control. (Fig. 6C–D).

The expression of *dna*K gene treated with 1 μg/mL prodigiosin was upregulated (1.2- and 1.3-fold compared to the control at 12 h and 24 h, respectively), but in the 3 μg/mL group, the degree of upregulation was 2.6- and 9.2-fold compared to the control group, respectively (Fig. 6e).

4. Discussion

In this study, we performed experiments to explore the algicidal mechanisms of prodigiosin against *H. akashiwo*. It was evident that the algicidal activity was concentration-dependent, with 3 μg/mL of prodigiosin resulting in approximately 90% algal mortality by 96 h (Fig. 1), suggesting the potential of prodigiosin as an algal-lysing compound. Similar results were also observed with more than 86.9% algal mortality after 60 h of exposure to algicidal bacteria (Li et al., 2014c). With greater mortality, cell structures were destroyed severely at 48 h and integrated cell membrane and organelles were not observed (Fig. 2G and Fig. 3E). Kong *et al.* (Kong et al., 2013) also observed this process of organelle deformation and dissolution, cell membrane rupture and intracellular content release of *Microcystis auruginosa* against *Streptomyces* sp. HJC-D1.

When microalgae are under environmental stress which are often adversely affect the microalgal growth (Chen et al., 2017), a series of physiological responses are activated to reduce impacts of stress and initiate cell repair. ROS, which are products of cellular processes such as photosynthesis and respiration, include superoxide anion radicals (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) that are removed by antioxidant systems. Excessive ROS may cause damage to cell structures and disrupt physiological processes (Apel and Hirt, 2004; Jacob, 1995; Krieger-Liszkay, 2005; Yang et al., 2010). The inhibition of photosynthesis and lipid peroxidation are all results of ROS damage. The continuous increase in MDA levels after treatment with 3 μg/mL of prodigiosin confirmed that there was significant irreversible oxidative damage to algal cells (Fig. 4b). In order to curb environmental stresses, various antioxidases (such as SOD and POD) and non-enzymatic antioxidants (AsA) were activated to remove intracellular ROS. SOD transforms $O_2^{-}\cdot$ to H_2O_2, which is less toxic, then POD transforms H_2O_2 to non-toxic substances to protect cells by avoiding oxidation (Matés and Sánchezjiménez, 1999; Hegedüs et al., 2001). AsA can also scavenge $O_2^{-}\cdot$ and H_2O_2 to reduce damage from lipid peroxidation (Deutsch, 1998). In this study, SOD activity increased from 24 h and maintained in a high level. The rapid increase in AsA levels at 12 h indicated that AsA plays an important role at the initial stages of cell development (Fig. 5c). *H. akashiwo* has a more active antioxidant system because ROS levels are relatively high in normal cells (Twiner and Trick, 2000). POD may scavenge H_2O_2 by catalyzing them into oxidizing phenols and amines. The rapid increase in POD activity at 12 h indicates that there may be substantial H_2O_2 produced in the 3 μg/mL group (Fig. 5b). However, the activity of the 1 μg/mL group at 36 h implied that algal cells may have produced large amounts of H_2O_2 at that time, and AsA could not have effectively scavenged them (Fig. 5b). The MDA levels of *Anabaena flos-aquae* increased significantly after one day of treatment with 0.5 mg/mL of the algal-lysing bacteria *Bacillus*

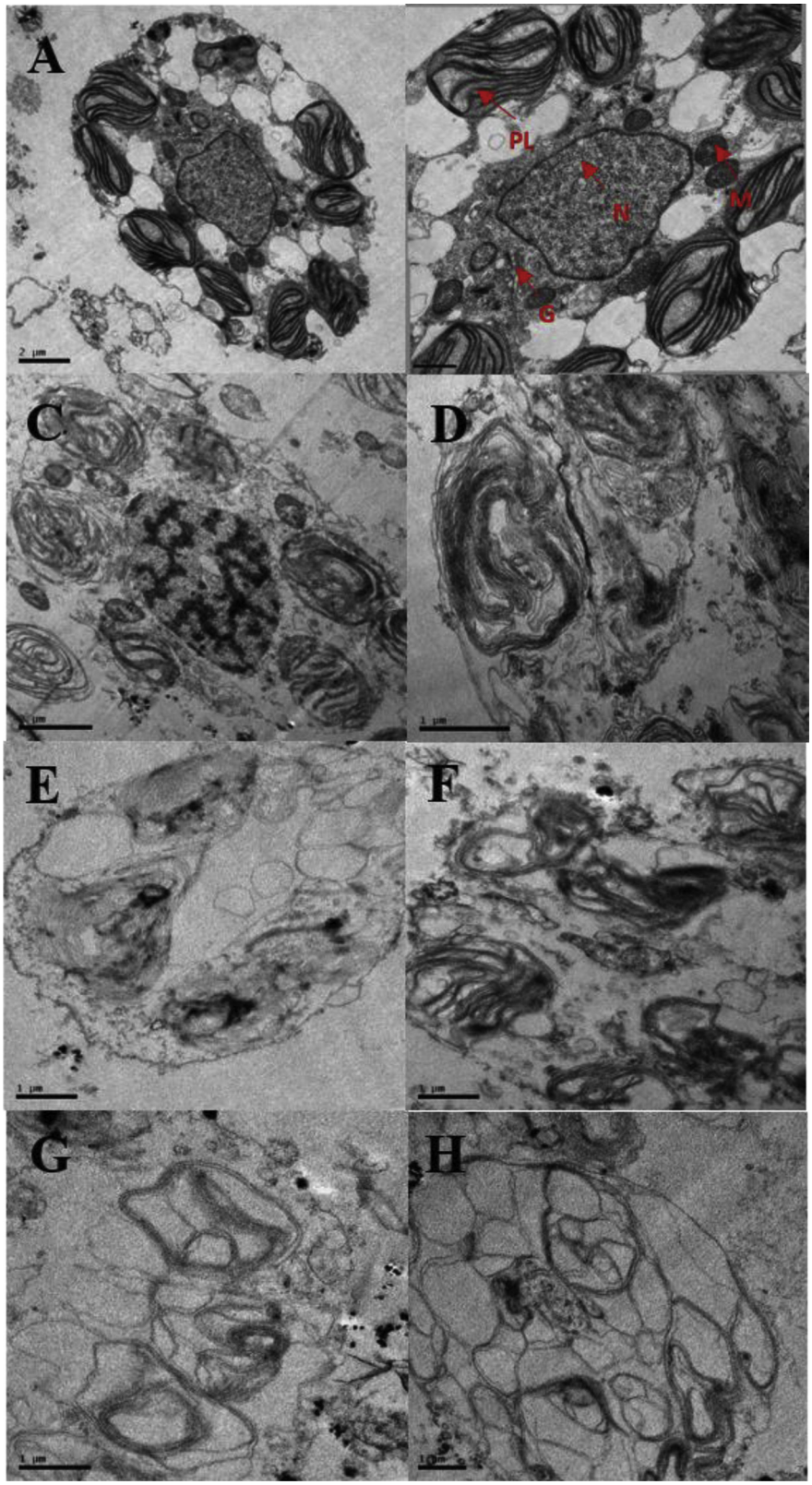

Fig. 2. Changes in the internal structure of *H. akashiwo* cells under transmission electron microscopy treated with 3 μg/mL of prodigosin. A and B were control treatments without addition of DMSO dissolved prodigosin, while C–H were algal cells treated by DMSO dissolved prodigosin for 6 h, 12 h, 24 h, 36 h, 48 h and 72 h, respectively; N: nucleus, M: mitochondrion, G: golgi complex, P: plastid.

cereus strain L7, whereas SOD, POD and CAT activity increased within a short period of time (Zhao et al., 2012).

Plastids are a type of chloroplast that contains a thylakoid membrane system for light harvesting (Allen and Forsberg, 2001). The thylakoid membrane of *H. akashiwo* in this study did not form a regular and clear arrangement after 24 h treatment with 3 μg/mL prodigiosin. The structural damage was not only accompanied by changes in enzyme activity but also by changes in gene expression. *Psb*A and *psb*D genes

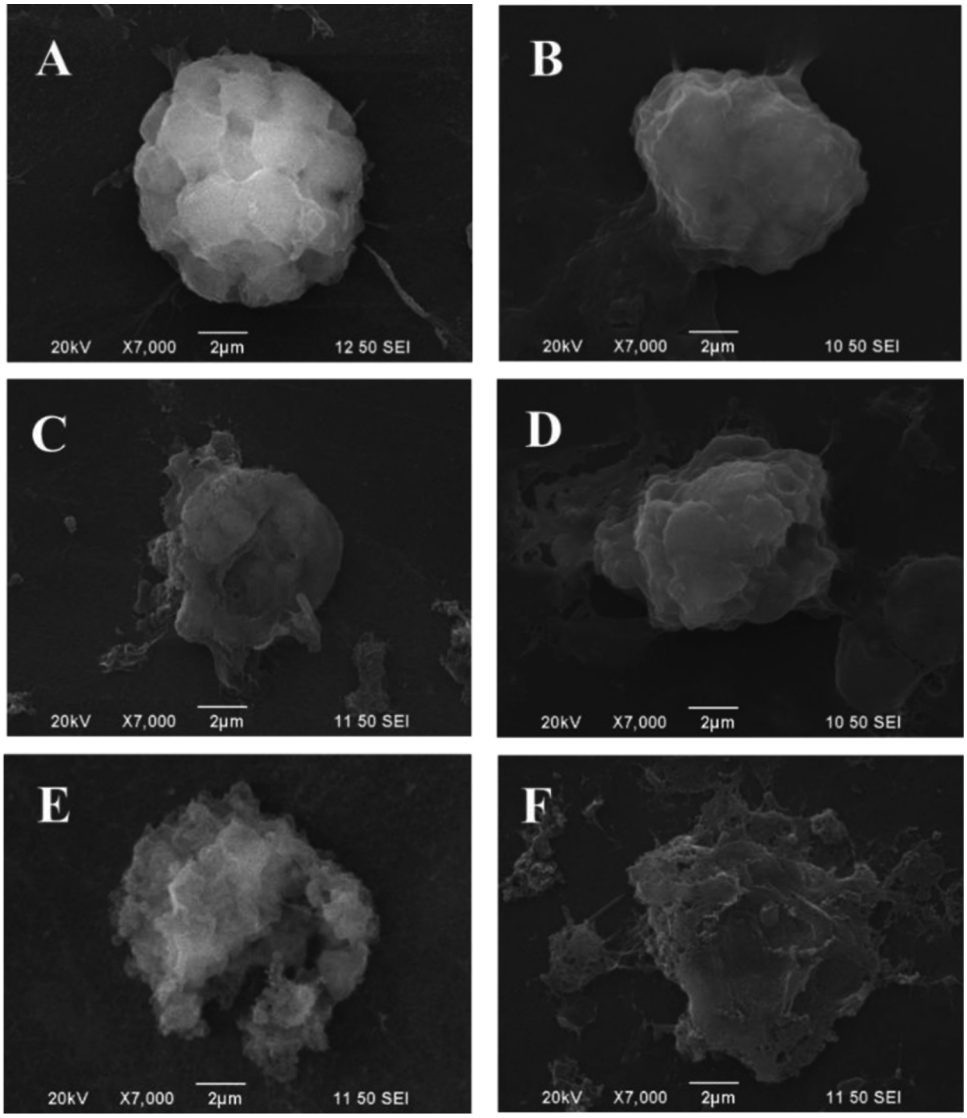

Fig. 3. Changes in *H. akashiwo* cells under scanning electron microscopy treated with 3 μg/mL of DMSO dissolved prodigosin. A was control treatments without addition of DMSO dissolved prodigosin, while B-H were algal cells treated by DMSO dissolved prodigosin for 6 h, 12 h, 24 h, 48 h and 72 h, respectively.

encode the D1 and D2 proteins, which serve as the reaction center of PSII (Erickson et al., 1986). As D1 protein is easily damaged by environmental stress, the degradation of damaged D1 protein and synthesis of new D1 protein is an important equilibrium process responding to environmental stress (Yamamoto et al., 2008; Yokthongwattana et al., 2001). The decrease in gene transcription is consistent with a block of electron transport and generation of ROS. Zhang *et al.* (Zhang et al., 2014, 2013) reported that when a high concentration of BS01 and DHQ25 bacterial supernatant was used to treat *Alexandrium tamarense*, the transcriptional abundance of *psb*A decreased. We concluded that algal-lysing compounds may damage the D1 protein severely and inhibit the self-repair of the photosystem. *Cob* and *cox*1 genes encode the mitochondrial cytochrome b and cytochrome c oxidase subunit I, which are essential for the function of Complexes III and IV of respiration. It has been reported that environmental stress can induce excess ROS from the respiratory chain (Yang et al., 2010). The transcription of these two genes directly affects the intracellular energy supply and thus affects most physiological processes (Feller et al., 2008). The increased transcription level at the 1 μg/mL concentration, at both 12 h and 24 h, indicated that the damaged respiratory chain was repaired by the synthesis of these two components. The decreased transcription level of the 3 μg/mL concentration at 12 h indicated that algal cells were severely damaged, and the rate of degradation was faster than synthesis and repair in a short period of time. However, the transcription levels were higher than the control group at 24 h, suggesting that the synthesis of these two components were responsible for the respiratory chain repair and ATP generation. A similar result was reported by Li *et al.* (Li et al., 2014c) whereby with 6 h exposure of the 0.5% treatment group, the expression of *cob* and *cox* genes were significantly inhibited, while in groups of higher concentrations (1.0% and 2.0%), gene expression was increased compared to the control. DnaK protein, expressed as a molecular chaperone to promote proper protein folding and the resistance to environment stress, is encoded by the *dna*K gene (Scaramuzzi et al., 1992). The significant upregulation of *dna*K in the 3 μg/mL group may indicate that algal cells were severely damaged after treatment with prodigiosin. Previous studies have also shown that the protein DnaK can stabilize the PSII complex in the repair process and assist in the replacement of damaged D1 protein (Yokthongwattana et al., 2001). Therefore, we concluded that the lower concentration of prodigiosin induced a rapid degree of self-repair of PSII because of the minor damage to the cells. When algae cells were treated with a high concentration of prodigiosin, cells were damaged so severely that they could not complete the self-repair process.

The correlation between three antioxidant parameters and gene

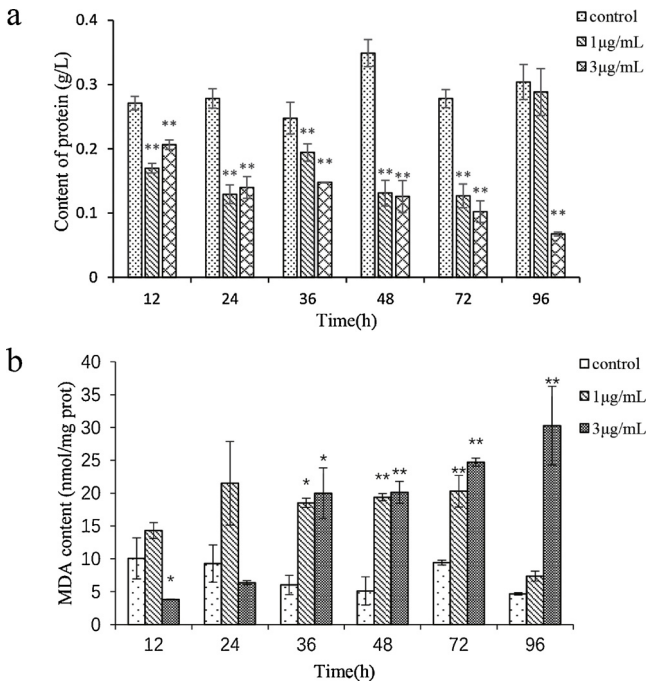

Fig. 4. The changes in total protein content (a) and MDA levels (b) of algal cells after different treatments. control, a control experiment without addition of DMSO and prodigiosin; 1 μg/mL, 3 μg/mL, the concentrations of DMSO dissolved prodigiosin added to algal cultures. All error bars indicated the SE of three biological replicates. $*p < 0.05$; $**p < 0.01$.

expressed levels were analyzed based on Mantel test. Unfortunately, even high values of correlation coefficient were detected, most of P-values were found to be greater than 0.05 (Supplementary Figure S2.), which indicated that correlation between most of antioxidant parameters and gene expressed levels were unreliable. The demonstrated that both antioxidants and gene expressed levels of *psbA*, *psbD*, *cob*, *cox1*, and *dnaK* could respond to the stress of prodigiosin, but no obvious correlation was detected.

Based on our analysis, a possible mechanism of *H. akashiwo* death under prodigiosin was proposed. Prodigiosin inhibited the photosynthesis of the microalgal cells, blocked the electron transport, and promoted excessive ROS generation. The excessive ROS could cause severe oxidative damage to the algal cell, resulting the cell death. This was consistent with the proposed algicidal mechanism in a previous study investigating the mechanism of prodigiosin against *Phaeocystis globosa* (Zhang et al., 2017). But changes of respiration was not explored in that study. Environmental stress was proved to be capable of inducing excess ROS from the respiratory chain (Yang et al., 2010). The results in this study indicated that production of excessive ROS could be caused by the blocked electron transport of photosynthesis and respiration.

Some algicidal compounds produced by bacteria had been identified. For instance, Cyclo-(Gly-Pro) and hydroquinone produced by the strain *Stenotrophomonas* sp. F6 exerted strong algicidal effect against *Microcystis aeruginosa* 9110 and *Synechococcus* sp. BN60 with a median effective concentration value of 0.96 and 5.6 mg L^{-1} (Lin et al., 2016). 1-acetyl-β-carboline, an algicidal compound secreted by *Brachybacterium* sp. YS-3 showed a wide spectrum of algicidal activity which could inhibited the growth of *Alexandrium catenella* (97% inhibation), *Prorocentrum micans* (88% inhibation), *S. trochoidea* (76% inhibation) (Yun et al., 2015). The bacterium strain *Pseudoalteromonas piscicida* also could produce an algicidal compound, 2-heptyl-4-quinolone (HHQ) which could induce mortality in three strains of *Emiliania huxleyi* (Harvey et al., 2016). In this study, prodigiosin showed strong algicidal effects on *H. akashiwo* at the final concentration of 3 μg/mL. Compared with traditional physical and chemical methods, bioactive compounds showed little negative effects on non-target algal species, fish or other aquatic life (Pokrzywinski et al., 2012). In addition, it was reported that prodigiosin possessed the characteristic of rapid photodegradation in 36 h in natural conditions (Zhang et al., 2016; Someya et al., 2004). This characteristic was in favor of decreasing impacts on other aquatic organism and the environment which was a common restrictive factor when bacterial cells were used. In terms of cost for using algicidal compounds, the high efficiency of microbial fermentation was helpful

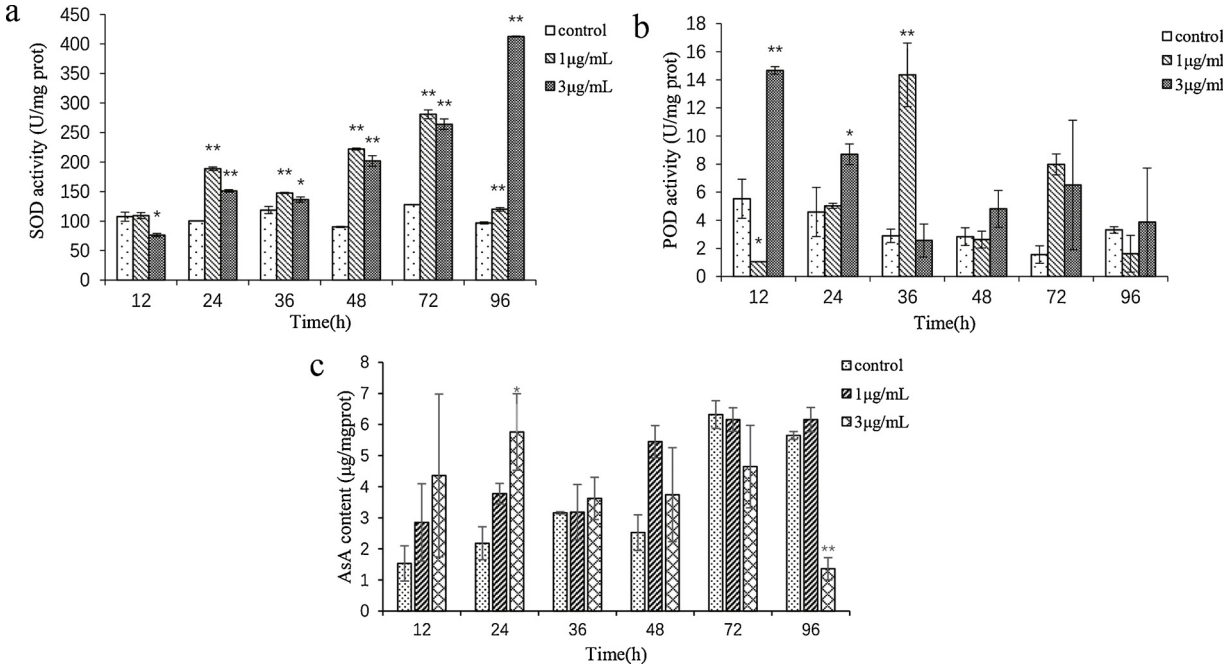

Fig. 5. The changes in SOD (a), POD (b), and AsA (c) levels in *H. akashiwo* cells treated with prodigiosin at different timepoints. control, a control experiment without addition of DMSO and prodigiosin; 1 μg/mL, 3 μg/mL, the concentration of DMSO dissolved prodigiosin added to algal cultures. Error bars indicated the SE of three biological replicates. $*p < 0.05$; $**p < 0.01$.

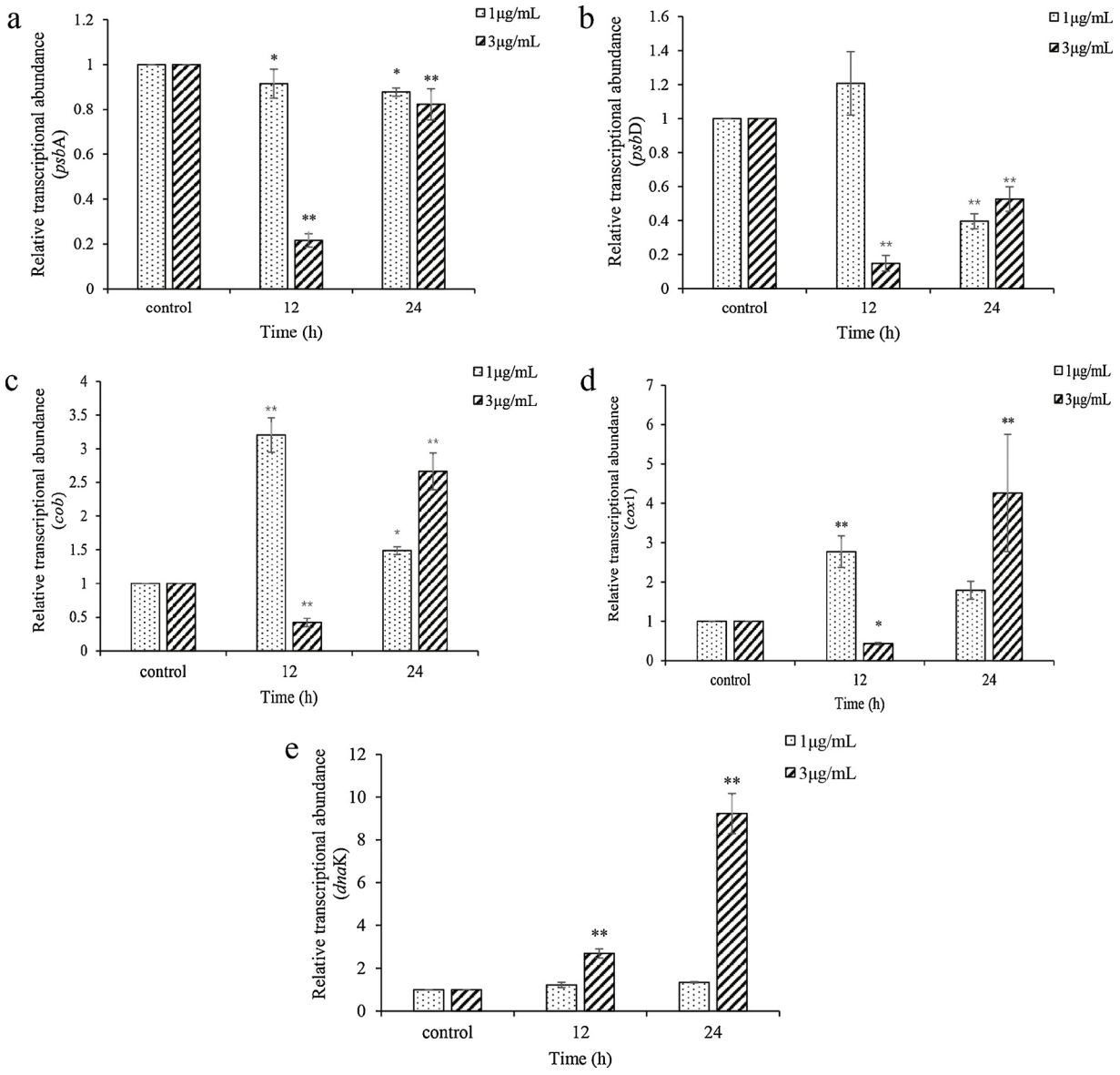

Fig. 6. Relative transcriptional abundance of *psb*A (a), *psb*D (b), *cob* (c), *cox*1 (d), *dna*K (e) after treatment with prodigiosin at 12 h and 24 h. control, a control experiment without addition of DMSO and prodigiosin; 1 μg/mL, 3 μg/mL, the concentration of DMSO dissolved prodigiosin added to algal cultures. Error bars indicated the SE of three biological replicates. $*p < 0.05$; $**p < 0.01$.

to obtain a large amount of prodigiosin with relatively low cost. Based on these three factors, prodigiosin could be regarded as a potential algicide for controlling red tide.

5. Conclusion

This study showed that the algal-lysing compound prodigiosin altered the cellular morphology, antioxidant systems, macromolecular and chl *a* levels, and gene expression of *H. akashiwo*. The prodigiosin treatment resulted in the overproduction of ROS in algal cells. These ROS then destroyed the cellular structure, activated antioxidant systems and affected photosynthesis and respiration, resulting in the alteration of gene expression and, ultimately, algal cell death. Our results indicated that prodigiosin may function as a biological control of HABs.

Ethical statement

This article does not contain any studies with human and animals performed.

Author contribution

S. Z., W. Z. and H. W. designed the experiment; S. Z. and W. Z. performed the experiment; S. Z. and W. Z. collected and analyzed the data; S. Z., and H. W. wrote the draft of the article; S. Z., W. Z. and H. W. revised the draft of the article.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.121530.

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