Allelochemical ethyl 2-methyl acetoacetate (EMA) induces oxidative damage and antioxidant responses in Phaeodactylum tricornutum

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1. Introduction

Allelopathy is the release of organic compound by plants or bacterial species that affects other plants or bacterial species, which is regarded as a form of interference competition [1]. Allelochemicals from macrophytes or other organisms that inhibit microalgal growth have gained great interests due to their environmental potential as algaecides to control water blooms or red tide. In the past several decades, many allelochemicals have been identified and studied for controlling microalgal growth. For example, monoterpenic substance secreted from tropical marine red algae inhibit the growth of chlorella fusca [2] and the diterpenoid substances from the aquatic plants inhibit the growth of single-cell green alga Selenastrum capricornutum [3]. Kamaya et al. [4] reported that the growth of S. capricornutum was strongly inhibited by long chain fatty acids. There are still many related reports that can be referenced [5–7]. However, from these reports we have found that more studies are focused on controlling freshwater algal blooms, but not red tide, and the mechanism of allelochemicals inhibition on marine microalgae is still not clear. However, researchers have pointed out that the core of allelochemical study is to understand the action mechanism of these compounds and their behavior in the environment [8].

Previous reports showed that the mechanisms of allelochemicals inhibition on algal growth were mainly in four aspects, destruction of cell structure, alteration of algal photosynthesis, respiration and enzymatic activities [9,10]. So far, only a few allelochemicals have been reported to induce enzymes and non-enzymatic antioxidant systems [11,12]. To scavenge ROS and avoid oxidative damage represented by MDA,1 algal cells possess a set of cellular defense system via the enzymatic and non-enzymatic antioxidant systems.

The antioxidant enzymes include SOD, CAT, GR, GPX, GST and so on. SOD blocks ROS-induced cell damage [13]. CAT and GPX break down H₂O₂ into H₂O [14], avoiding the production of OH−, which is highly reactive, and potential hazard to all biological molecules...
without any known enzymatic mechanism to eliminate it [15]. GR converts GSSG to GSH for removing environmental oxidative stress. GST is an important enzyme to transform harmful substances from environment [16,17]. Non-enzymatic antioxidant system includes GSH system and AsA system, which is another important cellular defense system to remove environmental oxidative stress [18–20]. The relationship between enzymes and non-enzymatic antioxidants is very complicated. Some enzymes involve in the formation of antioxidants, and some non-enzymatic antioxidants involve in the enzymes cycle to scavenge reactive oxygen species, etc. For example, GR and GPX participate in the conversion between GSH and GSSG. AsA cycle requires the involvement of ascorbate peroxidase (APX), while AsA is also involved in enzymes activities including GR, APX and so on. Qian et al. [21] reported that enzymes activities including SOD, CAT and peroxidase (POD) were significantly increased after treated with ROS disrupted the subcellular structure of Chlorella vulgaris. Hong et al. [22,23] also reported that allelochemical EMA inhibited the growth of freshwater algae S. capricornutum and Microcystis aeruginosa and induced cellular antioxidant responses. The significant increase of ROS and MDA contents indicated that algal cells were damaged and cellular SOD, CAT, AsA and GSH systems changed correspondingly to suppress the stress from allelochemical EMA. However, the effect of EMA on marine algae is still unknown, not to mention its mechanism of inhibition on marine algae.

EMA is one of novel allelochemicals isolated from reed (Phragmites communis or Phragmites australis) [24], and it significantly inhibits the growth of freshwater algae including M. aeruginosa and Chlorella pyrenoidosa, and the medium effective concentrations (EC50) of EMA on M. aeruginosa and C. pyrenoidosa are 0.65 and 0.49 mg/L, respectively. The inhibition mechanism of EMA on freshwater microalgae has been studied in recent years [22–24]. EMA is very effective to inhibit freshwater microalgae. Considering that EMA and marine microalgae come from two totally different ecosystems, marine microalgae might have no natural resistance to EMA. EMA could be developed as an effective allelochemical to control red tide. Our present study has demonstrated that EMA significantly inhibits the growth of marine diatom P. tricornutum (unpublished data). To better understand the mechanism of EMA inhibition on the growth of P. tricornutum, we assessed the effect of EMA on physiological levels by measuring cellular viability and inclusions, ROS, oxidant marker (MDA), antioxidant enzymes and non-enzymatic antioxidants.

2. Materials and methods

2.1. Reagents and algal cultures

Ethyl 2-methyl acetocetate (EMA) was purchased from Alfa Aesar Company. Fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), was purchased from Sigma Company. All other chemicals were analytical or higher grades. The unicellular marine diatom P. tricornutum was provided by the Institute of Oceanology, Chinese Academy of Sciences. The microalgae was grown in axenic conditions, in f/2 medium based on autoclaved natural seawater at 20°C and light intensity of 48 μmol photons m⁻² s⁻¹ with a 12:12 h light:dark cycle. All cultures were shaken twice a day and cultured to the exponential phase before inoculation in the following experiments.

2.2. EMA treatment on P. tricornutum

Flasks (250 mL) were prepared and each of them contained 150 mL f/2 algal culture medium. EMA concentrations were designed as follows: 0, 3.5, 7, 10.5, 14 mmol/L. The medium without EMA was taken as the control. The initial algal density was 3.3 × 10⁵ cells/mL. Algal cells were harvested to determine cell viability, cellular inclusions, physiological and biochemical indicators after EMA exposure for 3 and 6 days. All the flasks and culture medium were autoclaved and all experiments were repeated three times.

2.3. Cell viability and cellular inclusions assays

Cell viability was evaluated by 2,3,5-triphenyltetrazolium chloride (TTC) method [25]. With slight modifications, algal cells were harvested by centrifugation at 4000 rpm for 5 min, and washed twice with PBS (50 mM, pH 7.8), stained with an equal volume of 0.2% TTC for 24 h, 30°C. Centrifuged and washed twice with PBS again, extracted with 95% ethanol for 30 min, the supernatant was determined the absorbance of 485 nm to represent cell viability (A485/10⁶ cells).

Cellular protein was detected by the Coomassie brilliant blue G-250 dye-binding method [26], using bovine serum albumin as the standard. The content of carbohydrate was determined according to phenol–sulfuric acid colorimetric methods of Masuko, using glucose as the standard [27].

2.4. ROS and lipid peroxidation assays

Intracellular ROS was detected by using a fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), according to [28], with slight modifications. DCFH-DA (final concentration in the mixture was 5 μM) was added to the cells suspended in 3 mL of 0.1 M PBS (pH 7.8) and the mixture was incubated in an incubator at 20°C in the dark for 1 h. Then the cells were immediately washed three times with PBS (0.1 M, pH 7.8) and finally suspended with 3 mL PBS (0.1 M, pH 7.8). The fluorescence intensity was monitored using a spectrophotometer (LS55, USA) with excitation wavelength at 485 nm and emission wavelength at 525 nm. Lipid peroxidation was measured based on the detection of MDA (a byproduct of lipid peroxidation), using the thiobarbituric acid (TBA) test as described by Shiu and Lee [29]. With some modifications, the EMA-treated and untreated (control) algal cells were harvested from culture medium by centrifugation at 4000 rpm for 5 min, and washed with PBS (50 mM, 7.8), then suspended in 5% trichloroacetic acid (TCA), disrupted by sonication in a 3-s burst for a total of 7 min with a 4-s cooling period after each burst using an ultrasonic disintegrator below 4°C. Cellular debris was removed by centrifugation at 4°C for 10 min at 8000 rpm, the supernatant was used to analyze the content of MDA. The reaction mixture in a total volume of 3 mL containing 1.5 mL of extracts, 1.5 mL 0.67% TBA, was heated in boiling water for 30 min and then quickly cooled. The absorbance of the supernatant was measured at 532 nm after the mixture was centrifuged at 5000 rpm for 15 min. MDA content was determined by subtracting the absorbance value measured at 600 nm.

2.5. Enzymatic activities assays

The control and EMA-treated groups algal cells were harvested by centrifugation at 4°C and 8000 rpm for 10 min, then washed in PBS (50 mM, pH 7.8), again centrifuged as before, and the pellets were resuspended in the same buffer. The supernatant which was prepared in the same manner as MDA extracts was used to analyze the activities of SOD, CAT, GR, GPX and GST.

SOD activity was detected according to [30]. The total volume of reaction mixture was 3 mL, including 1.5 mL PBS (50 mM, pH 7.8), 0.3 mL methionine solution (130 mM), 0.3 mL nitroblue tetrazolium solution (NBT, 750 μM), 0.3 mL Na₂EDTA solution (100 μM),...
0.3 mL riboflavin solution (20 μM), 0.25 mL distilled water and 0.05 mL enzyme extract. SOD could inhibit the photochemical reduction of NBT, the assay utilized the negative controls (full-dark without any photochemical reduction of NBT), positive controls (deficiency of SOD activity, in light with full photochemical reduction of NBT) and treatment groups (in light with SOD inhibition on photochemical reduction of NBT) to measure SOD activity. The 560 nm absorbance was measured after 20 min irradiance of 40–60 μmol photons m⁻² s⁻¹. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the rate of NBT reduction under the assay conditions.

CAT activity was measured in terms of the decomposition of hydrogen peroxide, which was monitored directly by the decrease in absorbance at 240 nm [14]. The reaction mixture of 3 mL contained 50 mM PBS (pH 7.0) 1.8 mL, 0.2% H₂O₂ 1 mL and the enzyme extract 0.2 mL. All data recorded were processed with a linear fitting method to obtain the slope of the fitted line. One unit of CAT was defined as the decrease of absorbance at 240 nm up to 0.01 in 1 min.

GR activity was measured by following the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction medium containing 50 mM Tris–HCl buffer (pH 7.5) (640 μL), 1.0 mM oxidized glutathione (120 μL), 1 mM NADPH (120 μL) and 120 μL enzyme extract in a final volume of 1 mL [31]. One unit of GR activity was defined as the decrease of absorbance at 340 nm up to 0.01 in 1 min.

GPX activity was measured according to [32] using glutathione as substrate. GPX could promote the reaction of H₂O₂ and GSH to H₂O and oxidized glutathione (GSSG). The decrease rate of GSH could be used to express GPX activity. GSH content was assayed according to the formation rate of 5-thio-2-nitrobenzoic acid (TNB) reduced from DTNB with 412 nm absorbance. The determination of AsA included 50 μM PBS (pH 7.0) 1.8 mL, 0.2% H₂O₂ 1 mL and the enzyme extract 0.2 mL. All data recorded were processed with a linear fitting method to obtain the slope of the fitted line. One unit of GPX activity was defined as the amount of enzyme causing 50% inhibition of the rate of NBT reduction under the assay conditions.

The total ascorbic acid (TAsA) contents included reduced AsA and DAsA. AsA contents were determined according to the formation of red chelate between 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline, BP) and ferrous ion reduced from ferric ion by AsA in acid solution. The reaction mixture of AsA assay included 1 mL trichloroacetic acid (TCA, 5%), 1 mL ethanol, 0.5 mL H₂PO₄–ethanol (0.4%), 1 mL BP–ethanol (0.5%), 0.5 mL FeCl₃–ethanol (0.03%), 1 mL enzyme extract and the final volume 5 mL. Then the mixture was left at 30 °C for 90 min and centrifuged at 4000 rpm for 5 min. The supernatant was used to detect the absorbance of 534 nm. The contents of AsA were estimated according to the standard curve. The determination of TAsA contents were by reducing DAsA into AsA with dithiothreitol (DTT) in alkaline solution. The TAsA reaction mixture included 0.5 mL DTT–ethanol (60 mM), 0.5 mL Na₂HPO₄–NaOH and 1 mL enzyme extract, which was left at room temperature for 10 min to make DAsA into AsA, then 20% TCA was added into the mixture. According to the method of AsA, TAsA contents were determined. DAsA contents were determined by subtracting AsA contents from TAsA contents [35].

2.7. Statistics

All data shown in this study were the means ± SE of three independent experiments and were evaluated by using one-way analysis of variance (ANOVA) followed by least significant difference test (LSD), p < 0.01 and p < 0.05 (Origin 7.5 for Windows).

3. Results

3.1. Effects of EMA on cell viability and inclusions

To investigate the effects of EMA on the growth of P. tricornutum, algal incubation was selected to assay the cell viability and cellular protein and carbohydrate contents. The results were shown in Fig. 1. Fig. 1A showed that the cell viability significantly decreased with the increase of EMA treatment concentrations. Cell viabilities were 51.4%, 45.0%, 26.5%, 12% of the control, respectively, when algal cells exposed to 3.5, 5, 10.5, 14 mmol/L EMA for 3 days. After 6 days exposure, cell viabilities were 78.7%, 56.7%, 37.3%, 6.1% of the control group, respectively. The cellular protein and carbohydrate contents. The results were shown in Fig. 1. Fig. 1B. The contents of protein were significantly increased when algal cells were treated for 3 days (p < 0.05 or p < 0.01). And the cellular protein contents in the algal cells exposed to 3.5, 7, 10.5, 14 mmol/L EMA for 3 days. After 6 days exposure, cell viabilities were 78.7%, 56.7%, 37.3%, 6.1% of the control group, respectively. The cellular protein contents increased with the treatment concentrations of EMA (Fig. 1B). The contents of protein were significantly increased when algal cells were treated for 3 days (p < 0.05 or p < 0.01). And the cellular protein contents in the algal cells exposed to 3.5, 7, 10.5, 14 mmol/L were 1.9 times, 3.3 times, 3.8 times, 8.4 times of the control, respectively. At the 6th day treatment, protein contents of 10.5, 14 mmol/L EMA treatment groups were 2.1 times and 2.8 times of the control, respectively. And the cellular carbohydrate contents were obviously increased after algal cells exposure to EMA for 3 days (Fig. 1C). The carbohydrate con-
tents were 1.2 times, 1.4 times, 3.9 times ($p < 0.01$) and 6.6 times ($p < 0.01$) of the control, respectively. When algal cells were treated for 6 days, cellular carbohydrate contents were significantly decreased when cells were exposed to 10.5, 14 mmol/L EMA ($p < 0.01$), while other treatment groups only showed a slight decrease compared to that of the control. The carbohydrate contents of algal cells exposed to 3.5, 7, 10.5, 14 mmol/L EMA were 0.8 times, 0.8 times, 0.2 times ($p < 0.01$) and 0.3 times ($p < 0.05$) of the control, respectively.

3.2. Effects of EMA on ROS and lipid peroxidation levels

ROS and lipid peroxidation levels reflect cellular redox status and cellular composition such as fatty acids in cell membrane (Fig. 2). Fig. 2A showed that ROS levels increased gradually in response to EMA compared to the control. The increase in ROS level was positively correlated with EMA concentration. ROS levels in algal cells after 3 days exposure to 3.5, 7, 10.5, 14 mmol/L were 1.5 times ($p < 0.01$), 2 times ($p < 0.05$), 2.7 times and 3.9 times ($p < 0.01$) of the control, respectively. When algal cells were exposed to EMA of 3.5, 7 mmol/L for 6 days, the ROS levels remained almost no change versus the control, but that increased significantly when cells treated with higher concentrations of EMA (10.5, 14 mmol/L) ($p < 0.01$) and the ROS levels were 3.1 times and 7.0 times higher than that of the control. Under relative low EMA concentrations (3.5, 7, 10.5 mmol/L) exposure, ROS levels on the 3rd day were slightly higher than that on the 6th day. When the EMA concentration reached 14 mmol/L, ROS levels on the 6th day were higher than that on the 3rd day. Lipid peroxidation levels were expressed by MDA contents. MDA contents in algal cells treated with 3.5, 7, 10.5 mmol/L showed almost no change versus the control after either 3 or 6 days exposure. But when exposed to 14 mmol/L EMA, they increased significantly ($p < 0.01$). The results showed that MDA content after 3 days exposure was 6.4-fold higher relative to the control, and after 6 days, 2.7-fold higher (Fig. 2B).

3.3. Effects of EMA on enzymatic activities

Cellular enzymatic activities including SOD, CAT, GR, GSH-PX and GST were determined to investigate the cellular defense response induced by EMA stress (Fig. 3). SOD activity showed a slight decrease compared to the control when algal cells were treated with 3.5 mmol/L EMA for 3 or 6 days, and with the EMA concentration increasing, the activity of SOD increased correspondingly after 3 days exposure. The activity values were 3.1 times ($p < 0.01$), 3.3 times ($p < 0.05$) and 12.5 times ($p < 0.01$) of the control when algal cells were treated with EMA of 7, 10.5, 14 mmol/L, respectively. But on the 6th day, SOD activities increased no more distinctively with the EMA concentration. Different from SOD, CAT activities had almost no change between the treatment and the control when algal cells were treated with 3.5 and 7 mmol/L of EMA for 3 or 6 days. The activities of CAT were 59.2 times and 19.8 times higher than that of control when algal cells were exposed to 14 mmol/L EMA for 3 or 6 days, respectively ($p < 0.01$).

The enzymes of GR, GSH-PX and GST were assayed in the work. On day 3 and day 6, GR activities in algal cells exposed to lower EMA concentrations (3.5, 7, 10.5 mmol/L) were not different from that of control. GR activities exposed to 14 mmol/L of EMA were 6.8 times and 6.2 times versus the control after 3 or 6 days exposure, respectively (Fig. 3C). The effects of EMA on GSH-PX activities...
followed the similar manners to that of SOD (Fig. 3D). After 3 days exposure, GSH-PX activities were increased significantly with EMA concentrations increasing. The values were 1.4 times ($p < 0.05$), 3.8 times ($p < 0.01$), 5.3 times ($p < 0.01$) and 13.6 times ($p < 0.01$) versus the control when the algae were exposed to 3.5, 7, 10.5, 14 mmol/L of EMA, respectively. On day 6, only the higher concentrations of EMA (10.5 and 14 mmol/L) treatment groups remarkably increased the activities of GSH-PX. The values were 6.7 times and 12.4 times in contrast to that of control. GST activities of all treatments were higher than that of control when algal cells were treated for 3 or 6 days. And the activities of GST treated with different EMA concentrations (3.5, 7, 10.5, 14 mmol/L) for 3 days were 5.0 times ($p < 0.05$), 11.8 times ($p < 0.01$), 6.1 times and 34.3 times ($p < 0.01$) higher than the control, respectively. However, GST activities on the 6th day were only slightly increased and remained almost no significant difference versus that of control. All of the values of GST activities on day 3 were higher than that on day 6 during the experiment.

3.4. Effects of EMA on non-enzymatic antioxidants

The effects of EMA on non-enzymatic antioxidants including AsA and GSH pools were quantified in the experiment and the results were shown in Fig. 4. The contents of AsA showed no significant difference from that of controls when algal cells were exposed to lower EMA concentrations (3.5, 7, 10.5 mmol/L) on day 3 or 6 days. Under higher EMA concentration (14 mmol/L) exposure, the values of AsA contents were increased remarkably compared to that of control, which were 2.5 times ($p < 0.05$) and 5.1 times ($p < 0.01$) of the controls on the 3rd and 6th day, respectively. DASA and TASA contents increased drastically with the EMA concentrations after 3 days exposure, and the DASA contents were 1.4 times ($p < 0.05$), 1.7 times ($p < 0.05$), 3.3 times ($p < 0.01$), 6.8 times ($p < 0.01$) higher versus that of control, respectively. Similarly, the TASA contents were 1.4 times ($p < 0.05$), 1.7 times ($p < 0.05$), 3.2 times ($p < 0.01$) and 6.4 times ($p < 0.01$) of the control, respectively. But when algal cells were exposed to lower concentrations of EMA (3.5, 7 mmol/L) for 6 days, the contents showed no significant change compared to that of the control. However, the significant increase occurred at higher EMA concentrations (10.5, 14 mmol/L) exposure, and the contents of DASA were 5.7 times ($p < 0.01$) and 24.5 times ($p < 0.01$) of the control, respectively, and the contents of TASA were 4.8 times ($p < 0.01$) and 18.6 times ($p < 0.01$) versus that of the control. To explore the regeneration rate of AsA, the ratios of AsA/DASA were calculated in Fig. 4D. There was a slight decrease in the ratio of AsA/DASA with the increase of exposure concentrations of EMA on day 3, but the AsA/DASA values of all the treatments were not different significantly from that of the control. And when algal cells were treated with 3.5 mmol/L EMA for 6 days, there was a peak but no obvious difference between the treatment and control, followed by a drop with the increase of EMA exposure concentrations. The AsA/DASA ratios of all the treatments and control in the algal cells were not significantly changed by EMA.

The contents of GSH, GSSG, TG and the ratio of GSH/GSSG in the algal cells exposed to EMA were shown in Fig. 4E–H. GSH contents remained no significant difference between the treatments and the control on day 3. Under lower EMA concentrations (3.5, 7 mmol/L) exposure for 6 days, GSH contents of the treatment groups showed a slight decrease but no significant difference from that of the control. With the increase of EMA concentrations, GSH contents were obviously increased. The values of GSH contents were 4.5 times ($p < 0.01$) and 6.8 times ($p < 0.01$) of the control respectively when algal cells were exposed to higher EMA concentrations (10.5, 14 mmol/L). GSSG contents of the treatments were also not significantly different from that of control at lower EMA concentrations (3.5 and 7 mmol/L) exposure. When algal cells were exposed to 10.5 mmol/L EMA, a significant increase occurred after 3 or 6 days exposure, and GSSG contents were 2.1 times ($p < 0.05$) and 4.8 times ($p < 0.01$) of the control, respectively. When the treatment concentration of EMA reached 14 mmol/L, the highest exposure concentration in the experiment, the highest contents of GSSG in algal cells were obtained and the values were 2.5 times ($p < 0.01$) and 6.8 times ($p < 0.01$) of the control on day 3 and day 6, respectively. The change trend of TG contents was similar to that of GSSG. The only difference between them was that TG contents were not significantly different from the control when cells were treated with 10.5 mmol/L EMA for 3 or 6 days. Then, an obvious increase occurred when algal cells were exposed to the highest EMA concentration (14 mmol/L), and the values of TG contents were 2.1 times ($p < 0.05$) and 6.8 times ($p < 0.01$) of the control on day 3 and day 6, respectively. The ratio of GSH/GSSG which suggested the GSH regeneration rate was shown in Fig. 4H. The ratio of GSH/GSSG on day 3 was higher than that on day 6 when algal cells were exposed to lower EMA concentrations (3.5 and 7 mmol/L). And the ratio in algae treated with 7 mmol/L EMA decreased significantly than that of the control, but there was no significant difference at other lower EMA concentrations. When the EMA concentration reached 7 mmol/L above, the ratio showed a significant decrease with the concentration ($p < 0.05$). All those results were obtained after 3 days treatment. On the 6th day exposure, the ratio of GSH/GSSG firstly decreased obviously at EMA concentration of

Fig. 2. Effects of EMA on ROS and MDA contents of P. tricornutum. All error bars indicate SE of the three replicates. *$p < 0.05$ and **$p < 0.01$ indicate significant differences.

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7 mmol/L ($p < 0.05$), and then increased dramatically at 10.5 mmol/L EMA concentration ($p < 0.01$), while that of the other treatment groups remained no significant difference compared to the control.

4. Discussion

4.1. Cell viability and products

*P. tricornutum* is widely spread around the world and is a good candidate for the study of allelopathy in the ocean [36,37]. Our previous study confirmed that EMA significantly inhibited the growth of marine diatom *P. tricornutum* (unpublished data). To further understand the mechanism of EMA inhibition on marine diatom, algal cell viability, cellular inclusions, ROS, lipid peroxidation products, enzymatic and non-enzymatic antioxidants were determined.

Cell viability represents the ability of cell metabolism and cell numbers in algal population growth [38]. In our study, cell viability was significantly inhibited by EMA. However, with the time extended cell viability was gradually restored, and the viability on day 6 was higher than that on day 3, which indicated that cell viability increased with cell numbers increasing. All these results
demonstrated that EMA weakened cell metabolism and resulted in the reduction of cell vision. Yin et al. [28] also reported that exogenous substance (cyanobacterial toxin) inhibited plant cell viability, and confirmed that the viability of toxin-treated cell was significantly decreased compared to that of the control. However, cell viability was restored after recovery treatment for a period of time.

Cellular protein and carbohydrate are two basic indicators to reflect the physiological state of cells. The increase of cellular protein contents suggested that new protein was synthesized to resist environmental stress. However, with EMA-treated time extending the contents of protein began to decrease in our experiment, which implied that cells gradually adapted to the external environmental stress. Cellular carbohydrates are the main source of biological energy and reflect cellular effective nutrient matters and energy levels. Intracellular changes of carbohydrate contents were different from that of protein. After 3 days exposure, the carbohydrate contents increased significantly with EMA concentrations increasing, which suggested that algal cells had an initiative adaptation to EMA stress. But the following decrease of cellular carbohydrate

Fig. 4. Effects of EMA on the contents of AsA, DAsA, TAsA, AsA/DAsA and GSH, GSSG, TG, GSH/GSSG of P. tricornutum cells. All error bars indicate SE of the three replicates. *p < 0.05 and **p < 0.01 indicate significant differences.
contents showed that carbohydrate consumption continued to provide basic energy and maintain physiological function after 6 days exposure, which was a passive adaptation to EMA stress.

ROS including superoxide anion radicals, hydrogen peroxide and hydroxyl radicals are produced through various metabolic pathways localized in mitochondria, chloroplasts and peroxisomes [39,40]. Algal cells can generate ROS by activating various oxidases and peroxidases in response to environmental stresses [41,42]. Our present work showed that EMA significantly induced ROS formation in algal cells after 3 or 6 days exposure to different EMA concentrations. Under lower concentrations (3.5 and 7 mmol/L), ROS levels on the 3rd day were relative higher than that on the 6th day, which indicated that EMA treatment increased intracellular ROS significantly in a short time. However, with time extended intracellular defense system cleared the excessive ROS, which explained why the ROS contents became lower when algal cells were exposed to EMA for 6 days. On the contrary, ROS levels on the 6th day became slightly higher than that on the 3rd day at the highest concentration (14 mmol/L) of EMA exposure. The phenomenon implied that EMA in high concentration extended the time of algal cells producing redundant ROS, so ROS could not be totally cleared even though algal cells were treated for a longer time of 6 days. The increase of ROS levels suggested that cells were under an oxidative stress and it might cause cell damage, which was consistent with previous report of Hong et al. [22], where ROS level changes were dependent on the initial algal density of *S. capricornutum* exposure to EMA, and the acute increase of ROS might be a potential cause of triggering inhibition on the algal growth. On the other hand, there were also other evidences to support that ROS accumulation might stimulate cell growth or proliferation. For example, Xie et al. [43] reported that ROS stimulated the growth of three marine microalgae. The consistent view was that ROS had the “homersis-like” effect, which showed that the growth or proliferation of cells could be stimulated at lower contents of ROS and inhibited at higher contents of ROS. In this study, we further confirmed that algal cells needed more time to scavenge ROS to avoid oxidative damage at high concentration of EMA exposure and the increase of ROS level inhibited the growth of algae.

MDA is an indicator of lipid peroxidation to reflect cellular oxidative damage. Environmental stress generally causes MDA contents to be increased in cells. Polyethylene glycol increased MDA contents in cucumber leaves [44]. And Zhang et al. [45] reported that significant increases in MDA contents were observed in *M. aeruginosa* cells with greater exposure concentrations of berberine and the prolongation of exposure time. In the current study, lower concentrations of EMA exposure (below 14 mmol/L) slightly increased MDA contents in *P. tricornutum*, and the significant increase in MDA contents was only observed at the highest concentration of EMA exposure (14 mmol/L). These results showed that lower concentrations of EMA did not cause obvious algal cell membrane damage, and the oxidative damage might be reduced by cellular defense system.

4.2. Enzymatic antioxidant system

The endogenous protection systems including antioxidant enzymes and non-enzymatic antioxidants are formed to protect cells from reactive oxygen damage. Our results indicated that algal cellular antioxidant enzymes were triggered in different degrees when cells were exposed to EMA. SOD activities were enhanced significantly on day 3 when exposed to 7 mmol/L or above, but only showed a slight change on the 6th day. It showed that SOD activities were activated in a short time and increased with the EMA concentrations increasing. However, SOD activities were restored to the normal level after a longer time exposure, which implied the EMA-stress in algal cells was relieved. CAT and GR activities in EMA-treated cells were significantly higher than that of the control under the treatment concentration of EMA 14 mmol/L on the 3rd and 6th day, which indicated that CAT and GR also took part in eliminating ROS directly or indirectly. However, when algal cells were exposed to low EMA concentrations (below 14 mmol/L), there was no obvious difference in CAT and GR activities between treatments and control except the CAT activities on the 3rd day under 10.5 mmol/L EMA exposure, which showed significantly higher than that of the control. Both GSH-PX and GST significantly participated in the removing of EMA oxidative stress on *P. tricornutum*, and their activities increased more remarkably than that of the control under all treated-concentrations on day 3, and the variation of enzymes on day 6 was similar to that on day 3 at the higher concentrations exposure (above 10.5 mmol/L EMA). It suggested that GSH-PX and GST might serve as more effective intrinsic defense tools than CAT and GR to resist EMA-induced oxidative damage in algal cells.

Under normal conditions, the production and scavenging of reactive oxygen species are in dynamic equilibrium and the excessive reactive oxygen contents can’t be accumulated in algal cells, so cells grow and develop normally. However, when cells are suffered from drought, low temperature, high temperature, salinity, high light intensity stress and so on, ROS metabolisms of production and scavenging are in severe imbalance, resulting in excessive reactive oxygen accumulated in cells, which cause cells to be damaged. Antioxidant enzymes are the most basic defense systems to
resist environmental stress. It was reported that the addition of silicon increased SOD activity under stress of salt and drought [41,42], and the increase of SOD activity induced the higher tolerance to oxidative stress [46]. Similar patterns of CAT activity had been observed in plant tissues under cadmium toxicity, acid rain and lead stress [47–49]. The antioxidant enzymes including GPX, GR and GST are all related to GSH cycles, which promote the conversion of GSH to remove environmental oxidative stress. GPX activity was enhanced significantly by foliar spraying with Spd or Spm under CdCl$_2$ treatment [50]. GR activity in plant was drastically reduced when treated with lower concentrations of Cd while increased under higher concentrations of Cd and Zn. The report suggested that Zn played an active role in alleviating Cd-induced toxicity through the efficient function of GR and other glutathione-related enzymes. For example, GST showed an increase in Cd treated plant, which accounted for the increased detoxification of stress metabolites because GST was considered as an important cellular detoxifier of metabolites involved in oxidative stress [16]. In present work, all enzymes were involved in resisting the EMA stress in algae.

This was the first report that EMA induced oxidative stress and activated the antioxidant defense system in marine diatom. If algal cells could not prevail over the oxidative stress induced by EMA, the overproduction of ROS would cause lipid peroxidation, which could induce cellular disorder and damage. Finally, cell growth inhibition and death would occur. Our present study demonstrated that EMA enhanced ROS production in $P$. tricornutum and cell growth (unpublished data), viability, inclinations were inhibited because redundant ROS could not be promptly cleared. Cellular enzyme activities obviously increased to relieve oxidative stress. However, with the EMA-treated time extending, all the enzymes activities showed a downward trend, which suggested that cellular oxidative stress was gradually cleared.

### 4.3 Non-enzymatic antioxidant system

AsA and GSH are considered as two kinds of non-enzymatic antioxidants to protect cells against environmental oxidative stress. AsA has a variety of functions in cellular metabolism, such as removal of ROS, regeneration of α-tocopherol and regulation of photosynthesis [51]. AsA also serves as an excellent electron donor, with a wide range of functions, even as a cofactor to some hydroxyl enzymes linking AsA to photo-protective xanthophyll cycle [20]. At the same time, AsA is one of the important redox status molecules participating in the AGC enzymes activities including APX, GR and so on.

In this study, the contents of AsA, DAsA and TAsA were significantly higher than that of control at 14 mmol/L EMA exposure for 3 or 6 days. But AsA contents in other treatment groups were not different from that of control, while DAsA contents were still significantly increased. Accompanied by the increase of DAsA, TAsA contents also increased obviously on the 3rd day. It suggested that algal cellular AsA took part in removing oxidative stress by EMA, making AsA reduce to DAsA. The increase of TAsA contents showed that new AsA was synthesized in algal cells. However, the regeneration of AsA (the ratio of AsA/DAsA) showed no significant difference from that of the control when algal cells were treated for 3 days, which implied that the rate of AsA synthesis were relatively lower than that of consumption. Under the 6th day exposure, the ratio of AsA/DAsA showed an obvious trend from increase to decrease. The reason was that the generation ratio of DAsA was relatively low under lower concentrations of EMA exposure, which resulted in the ratio of AsA/DAsA being increased, and with the increase of EMA concentrations, cells needed consume more AsA to produce more DAsA, which made the ratio decrease. Although the variation of the ratio was induced by EMA stress on algal cells, the values of all treatments were not significantly different from that of the control. Our results showed that the reaction of AsA system might have a lag phenomenon compared with that of antioxidant enzymes reported as above and AsA regeneration might not be quickly and effectively activated to remove EMA stress on $P$. tricornutum, which might mainly depend on the consumption of endogenous AsA during our experiment.

On the contrary, Hong et al. [23] reported that AsA was important for $M$. aeruginosa in defending against oxidative stress imposed by EMA at early exposure, but the protective function will be exhausted with extension of exposure time and increase of exposure dose. The AsA regeneration rate was initiated only in the highest EMA concentration after a short time exposure. Under lower EMA concentrations, the ratio was increased with treat-time extending. In our study, the significant increase or decrease of the ratio was not found in marine diatom when exposed to EMA. There had been reports that AsA, DAsA, AsA/DAsA ratio were crucial for the cell to sense oxidative stress and respond accordingly [52]. However, whether the ratio of AsA/DAsA increased or decreased under environmental stress, they mainly depended on algal species and stress factors. Further study showed that the increase mode of the ratio also varied with species and stress factors. The copper stress on *Enteromorpha compressa* significantly increased the AsA regeneration ratio because of the high levels of DAsA accumulation, but AsA contents remained almost no change in the algae exposed to copper-enriched mine-impacted sites [53]. While for *Dunaliella tertiolecta*, the increase of the ratio was attributed to the increase of AsA and the decrease of DAsA in algae exposed to salinity stress [54].

GSH system is another important non-enzymatic antioxidant defense system in addition to AsA system. GSH removes ROS, alkyl peroxide and protects proteins from the oxidation of protein thiol groups into denaturation [55]. GSH contents in algae increased significantly only when exposed to higher concentrations of EMA (10.5 and 14 mmol/L) on the 6th day in our experiment. Similarly, GSSG contents also increased remarkably compared to that of control when algae were treated with the same concentrations of EMA on the 3rd and 6th day. The ratio of GSH/GSSG which represented the rate of GSH regeneration increased firstly and then decreased with the concentration on day 3. On day 6, the ratio value firstly decreased significantly under 7 mmol/L EMA treatment, then increased remarkably under 10.5 mmol/L EMA, and subsequently, a slight decrease was observed. All the results showed that the accumulation of GSSG resulted in the decrease of the GSH regeneration rate when algal cells were exposed to higher EMA concentrations (10.5 mmol/L above) on day 3. On day 6, GSH were gradually consumed to resist EMA stress, so the regeneration rate of GSH/GSSG decreased. But with the treatment concentration increase, the regeneration of GSH/GSSG was stimulated. The change of regeneration rate might be related to cellular enzyme activity including GR, GPX, GST, etc., and depended on the increase or decrease of their activities. Hong et al. [23] reported that GSH pool and its regeneration rate were affected by EMA, but the sensitivity of GSH regeneration to EMA was lower than that of AsA. However, according to our results, GSH system seemed to be more sensitive to EMA stress than AsA. At least, GSH pool regeneration rate changed significantly with EMA concentration and exposure time, while AsA pool regeneration rate had no significant difference from that of the control during our experiment. AsA and GSH contents had been owed to alleviating the injury by ROS [56]. In our study, although the change of regeneration rate of AsA and GSH pool were not significant in a regular pattern, the increase of AsA and GSH contents still showed that AsA and GSH systems were served as defense systems to resist EMA stress in marine diatom, and the previous reports also indicated that AsA and GSH pool alteration were different with algal species and stress factors [10,23].
The results from this study suggested that EMA inhibited the marine diatom P. tricornutum growth, cell viability and altered cell inclusions. The increase of ROS and MDA contents proved that cellular oxidative stress was induced by EMA, and to remove the EMA stress, cells activated the internal antioxidant enzymes and non-enzyme antioxidants, which suggested that one of the allelopathic mechanisms responsible for the inhibition was related to EMA-induced oxidative damage in P. tricornutum. To our knowledge, no studies have reported the effects of EMA on marine algae as P. tricornutum.

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