Vol. 24: 109–115, 2015
doi: 10.3354/ab00643

AQUATIC BIOLOGY
Aquat Biol

Published online December 3

Physiological and biochemical responses of a marine diatom *Phaeodactylum tricornutum* exposed to 1-octyl-3-methylimidazolium bromide

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ABSTRACT: The marine diatom *Phaeodactylum tricornutum* is an important basal resource in the marine food chain and is used as a standard test organism in toxicological studies. In this study, *in vivo* experiments were performed to analyze the effects of 1-octyl-3-methylimidazolium bromide ([C_8mim]Br) on the growth, photosynthetic activity, and antioxidant enzymes of *P. tricornutum* using 96 h growth tests in a batch-culture system. The results showed that [C_8mim]Br significantly inhibited the growth of *P. tricornutum*, with a 96 h EC_{50} of 8.89 mg l^{-1}, and likely restricts PSII electron transfer and light use efficiency of the diatom. With increasing [C_8mim]Br concentrations, the soluble protein content in the diatom increased by 35.9, 58.5, 123.3, 197.7, and 207.0% in 5, 10, 20, 40, and 80 l^{-1} [C_8mim]Br treatments relative to the controls, respectively. Concentrations of [C_8mim]Br ≤10 mg l^{-1} caused a slight increase of superoxide dismutase (SOD) activity (from 16.37 to 23.04 U g^{-1} protein) in the diatom, but inhibited its activity at concentrations above 10 mg l^{-1}. These observations indicate that moderate [C_8mim]Br stress (about 10 mg l^{-1}) likely stimulates the synthesis of proteins and free radical quenching. The general increase in malondialdehyde (MDA) content suggests that the physiological effects of [C_8mim]Br were caused by free radical generation. Thus, potential risks exist if [C_8mim]Br is accidentally released into the aquatic environment.

KEY WORDS: Ionic liquid · *Phaeodactylum tricornutum* · 1-octyl-3-methylimidazolium bromide · Photosynthetic activity · Antioxidant enzymes

INTRODUCTION

Ionic liquids (ILs), composed of a bulky organic cation and organic or inorganic anions, are environmentally friendly replacements for industrial volatile organic compounds because of their relatively low melting point (Pham et al. 2010). The main advantage of ILs is the significantly lower risk of industrial exposure and solvent loss to the environment. However, ILs are very stable and soluble in water (Bruzzone et al. 2011), which may potentially lead to water pollution and related risks once the ILs enter an aquatic environment (Latała et al. 2005, Zhao et al. 2007, Cho et al. 2008, Ventura et al. 2010, Cvjetko Bubalo et al. 2014). Recent studies have documented that ILs have toxic effects on aquatic organisms such as algae (Latała et al. 2009, 2010, Das & Roy 2014), cladocerans (Couling et al. 2006, Luo et al. 2008, Pretti et al. 2009, Ventura et al. 2010), mussels (Costello et al. 2009), and fish (Pretti et al. 2009, Cvjetko Bubalo et al. 2014). The effects of ILs on aquatic organisms consist of 'alkyl side chain' effects (an increase in antimicrobial activity with the elongation of the alkyl chain) and the 'cut-off' effect (beyond a given chain length, the effects cannot increase any further) (Ventura et al. 2012).

Diatoms are important primary producers in aquatic ecosystems, contributing ca. 20–25% to

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global primary productivity (Mann 1999, Montsant et al. 2005). Thus, diatoms have an important role in sustaining a healthy ecosystem. If they are adversely affected by a toxicant, the surrounding organisms may also be affected (either directly or indirectly) due to lack of food sources. Although some studies concerning the toxicity of ILs to diatoms have been reported (Latała et al. 2009, Ma et al. 2010, Samorì et al. 2011), to our knowledge, little work has been done regarding the toxic mechanism.

In this study, 1-octyl-3-methylimidazolium bromide ([C_8mim]Br) was selected as the IL to be tested for the following reasons: (1) it is one of the representative imidazolium ILs (Luo et al. 2008, Yu et al. 2008, Ma et al. 2010, Li et al. 2012); (2) it is easily synthesized and widely used in the chemical industry (Bonhôte et al. 1996); (3) it has been commonly used in previous studies, with toxicity levels between that of [C_6mim]Br and [C_{10}mim]Br (Luo et al. 2008, Yu et al. 2008, Ma et al. 2010, Li et al. 2012). Our objectives were to evaluate the effects of [C_8mim]Br on the growth, photosynthetic activity, soluble protein content, antioxidant enzyme activity, and degree of lipid peroxidation on *Phaeodactylum tricornutum*. To our knowledge, this study is the first to report the toxic mechanism of imidazolium-based ILs on marine diatoms.

MATERIALS AND METHODS

Test chemicals and solutions

The IL [C_8mim]Br (CAS number: 61545-99-1, purity >99.9%) was purchased from Chengjie Chemical; its chemical structure is shown in Fig. 1. Other chemicals used in this experiment were obtained from Sinopharm Chemical Reagent. Stock solutions were prepared in distilled water at a concentration of 5 g l^{-1}. Test solutions were obtained by diluting the stock solution in f/2 medium (Guillard & Ryther 1962). Concentrations used for the test solutions were 0, 5, 10, 20, 40, and 80 mg l^{-1}.

Fig. 1. Chemical structure of 1-octyl-3-methylimidazolium bromide ([C_8mim]Br)

Test organism

The unicellular diatom *Phaeodactylum tricornutum* was obtained from the State Key Laboratory of Marine Environmental Science (Xiamen University), and grown photoautotrophically in 500 ml Erlenmeyer flasks containing 200 ml of f/2 medium. The inoculum was pre-cultured aseptically in 250 ml Erlenmeyer flasks with 100 ml of f/2 medium. The flasks were placed in a 20°C incubator (Jiangnan Instrument Factory), and illuminated from 2 sides by vertical cool white fluorescent lamps placed parallel to the flasks with a 12 h light:12 h dark photoperiod and a light density of 40 µmol photons m^{-2} s^{-1}.

Experimental setup

After pre-cultivation for 7 d, the microalgal inoculum reached the exponential growth phase with a cell density of 2.2×10^7 cells ml^{-1}. A total of 5 ml of the microalgal inoculum was collected by centrifugation (4000 × *g*, 4°C, 15 min). The collected microalgal cells were washed twice with sterile seawater and then inoculated into the growth medium with an initial cell density of 1.1×10^6 cells ml^{-1}.

In the growth inhibition experiments, cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml of f/2 medium with different [C_8mim]Br concentrations (0, 5, 10, 20, 40, and 80 mg l^{-1}), each in triplicate. The cultivation conditions were as described above.

Microalgal growth analysis

Microalgal cell density was determined spectrophotometrically at 625 nm using a multi-mode microplate reader (SpectraMax M5; Molecular Devices). The relationship between microalgal cell density (D, cells ml^{-1}) and optical density of the microalgal culture at 625 nm (OD_{625}) was determined experimentally, and is shown in the following equation:

$$D = (6.7685 \times OD_{625} + 0.0145) \times 10^7, \quad (R = 0.9897) \tag{1}$$

The concentration for 50% of maximal effect (EC_{50}) values of [C_8mim]Br against *P. tricornutum* were determined using PASW Statistics 18 software (SPSS) according to Deng et al. (2012).

Measurements of photosynthetic parameters

The photosynthetic parameters of *P. tricornutum* exposed to different [C_8mim]Br concentrations (0, 5, 10, 20, 40, and 80 mg l^{-1}) at different times (0, 12, 24, 48, 72 and 96 h) were measured by pulse-amplitude modulation (PAM) fluorometry (Phyto-PAM; Heinz Walz). Prior to measurements, samples were kept in the dark for 15 min. Then, the maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m), potential activity of PSII (F_v/F_0), maximum relative electron transport rate ($rETR_{max}$), and light use efficiency (α) were determined and calculated according to Schreiber (1998).

Chlorophyll *a* concentration measurements

To determine chlorophyll *a* (chl *a*) concentration, triplicates of 5 ml well-blended cultures were centrifuged at 4000 × *g* for 15 min to remove the supernatants. The pellets were then homogenized with 5 ml of HPLC-grade methanol for pigment extraction. The mixtures were vigorously shaken with a Vortex and placed in a refrigerator in the dark at 4°C for 24 h. The methanol-extracted samples were then centrifuged at 10 000 × *g* for 5 min to remove the pellet, and the supernatants were transferred into 96-well plates (Corning Incorporated Life Sciences) and measured for chlorophyll at 750, 665, and 652 nm using a multimode microplate reader. All absorbance values were corrected using HPLC-grade methanol as a control. Concentrations of chl *a* were calculated following Porra (2002); to determine chl *a* in µg ml^{-1} (C_a):

$$C_a = 16.29 \times (A_{665} - A_{750}) - 8.54 \times (A_{652} - A_{750}) \quad (2)$$

where A_{652}, A_{665} and A_{750} are the absorbance values at 652, 665 and 750 nm, respectively. The final value of chl *a* content was expressed as micrograms of chl *a* per 10^7 cells (µg × 10^7 $cell^{-1}$) (C'_a). This was calculated using:

$$C'_a = \frac{C_a \times V_{MeOH}}{D \times V_{sample}} \quad (3)$$

where V_{MeOH} is the methanol volume (5 ml), V_{sample} is the volume of sample (5 ml) and D is the microalgal cell density (cells ml^{-1}).

Biochemical analysis

Protein determination

After 96 h of [C_8mim]Br exposure, 40 ml of well-blended cultures were harvested by centrifugation (4000 × *g*, 15 min, 4°C). The harvested microalgae were placed in 1.5 ml of extraction buffer containing 0.05 M sodium phosphate buffer (pH 7.8), and immediately lysed by sonication (Scientz Biotechnology) for 10 min with a repeating duty cycle of 5 s in an ice bath. The cellular homogenate was centrifuged at 12 000 × *g* for 10 min at 4°C, and the liquid supernatant was stored at −70°C for protein determination and enzyme assay.

Total soluble protein content was measured using the Bradford method with bovine serum albumin as standard (Bradford 1976). Results were expressed as micrograms of protein per 10^7 cells (µg × 10^7 $cell^{-1}$).

Superoxide dismutase and malondialdehyde determination

Superoxide dismutase (SOD) and malondialdehyde (MDA) assay kits were purchased from Jiancheng Bioengineering Institute. SOD and MDA were extracted and determined from the supernatant liquids (above) according to the manufacturer's instructions and Wang & Zheng (2008). The results of SOD activity and MDA content are given as units of enzyme activity per microgram of total soluble protein (U g^{-1} protein) and nanomole per 10^7 cells (nmol 10^7 $cell^{-1}$), respectively.

Statistical analysis

All results are presented as mean ± SD. Statistical analysis was performed using PASW Statistics 18 software. A 1-way ANOVA was used to establish differences among treatments, with a significance level set at α = 5%.

RESULTS AND DISCUSSION

Growth of *Phaeodactylum tricornutum* after exposure to [C_8mim]Br

The effects of [C_8mim]Br on cell density and growth of *P. tricornutum* are shown in Fig. 2. Although *P. tricornutum* presented positive growth in all [C_8mim]Br treatments, the cell densities in 5, 10, 20, 40, and 80 mg l^{-1} [C_8mim]Br treatments were 64.1, 49.5, 43.4, 25.8, and 24.0% of that in the controls after 96 h exposure, respectively. Growth inhibition also increased with increasing exposure time from 0 to 96 h (e.g. an increase in growth inhibition

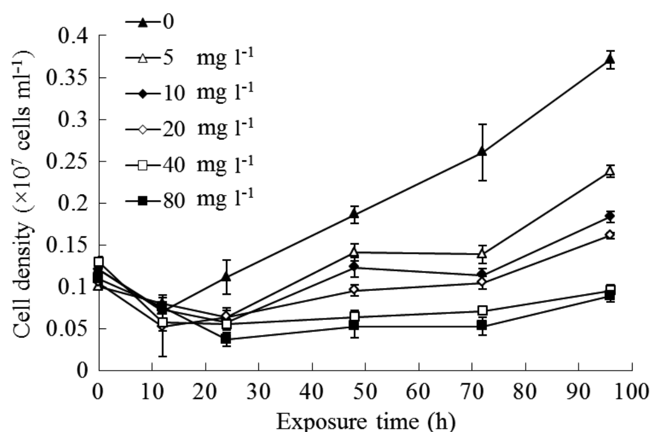

Fig. 2. Growth curves of *Phaeodactylum tricornutum* under different [C_8mim]Br concentrations (0 to 80 mg l^{-1}) during 96 h exposure. Points: means of 3 replicates; error bars: SD

from 0 to 76.0% in 80 mg l^{-1} [C_8mim]Br treatments) and increasing [C_8mim]Br concentration from 5 to 80 mg l^{-1} (e.g. an incremental growth inhibition from 35.9 to 76.0% at 96 h exposure). A similar phenomenon has also been described for *Scenedesmus obliquus* after treatment with [C_8mim]Br (Ma et al. 2010). Our results were easily visible due to the color change of the microalgal cells from brown to white (Fig. 3). Therefore, we conclude that potential risks do exist if [C_8mim]Br is accidentally released into the aquatic environment.

In this study, the EC_{50} values of [C_8mim]Br against *P. tricornutum* were determined and calculated by Marquardt's algorithm method (Deng et al. 2012), and our results compared to data on [C_8mim]Br toxicity to microalgae in previously published literature (Table 1).The differences in EC_{50} values — spanning several orders of magnitude — between algal taxa may help to explain the mechanism of IL toxicity (Kulacki & Lamberti 2008, Samorì et al. 2011). One clue to this mechanism may be related to the cell wall structures of different microalgal species, since the cell wall plays an important role in the transport of materials in and out of the cell (Kulacki & Lamberti 2008, Samorì et al. 2011). However, additional studies are required in order to fully investigate the effect of these cell wall structural differences, particularly with respect to IL toxicity.

Table 1. Comparison of the toxicity of [C_8mim]Br to microalgae. EC_{50}: concentration for 50% of maximal effect; nr: not reported

Microalgae	Duration (h)	EC_{50} (mg l^{-1})	Reference
Freshwater species			
Chlamydomonas reinhardtii	96	50.69	Kulacki & Lamberti (2008)
Chlorella ellipsoidea	96	6.37	Ma et al. (2010)
Selenastrum capricornutum	96	7.24–15.11	Cho et al. (2007)
Scenedesmus obliquus	96	0.34	Ma et al. (2010)
Scenedesmus quadricauda	96	0.005	Kulacki & Lamberti (2008)
Marine species			
Phaeodactylum tricornutum	96	8.89	This study
Oocystis submarina	288	nr	Latała et al. (2005)
Cyclotella meneghiniana	288	nr	Latała et al. (2005)

Photosynthetic activity of *P. tricornutum* after exposure to [C_8mim]Br

The changes in photosynthetic activity of *P. tricornutum* exposed to [C_8mim]Br are shown in Fig. 4. After 24 h exposure, F_v/F_m and F_v/F_0 showed no significant differences at [C_8mim]Br concentrations of ≤20 mg l^{-1} ($p > 0.05$), but there were slight decreases (73.8 and 65.6% of that in controls, respectively) in the 40 mg l^{-1} treatments, and a sharp decrease (27.6 and 18.2%, respectively) in the 80 mg l^{-1} treatments. However, in the controls, F_v/F_m and F_v/F_0 increased sharply from 24 to 48 h exposure, and then remained constant (about 0.56 and 1.32, respectively) after 48 h exposure. The F_v/F_m and F_v/F_0 in the 5, 10, 20, 40, and 80 mg l^{-1} treatments were 88.1, 75.4, 70.7, 51.1, 17.6%, and 72.9, 54.1, 48.8, 29.9, 7.6% that of the controls after 96 h exposure, respec-

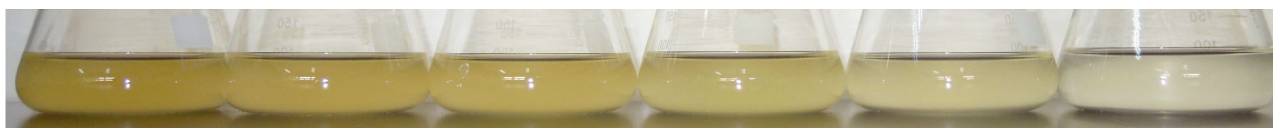

Fig. 3. Change in morphology of *Phaeodactylum tricornutum* with different [C_8mim]Br treatments: from left to right: control, 5, 10, 20, 40, and 80 mg l^{-1}

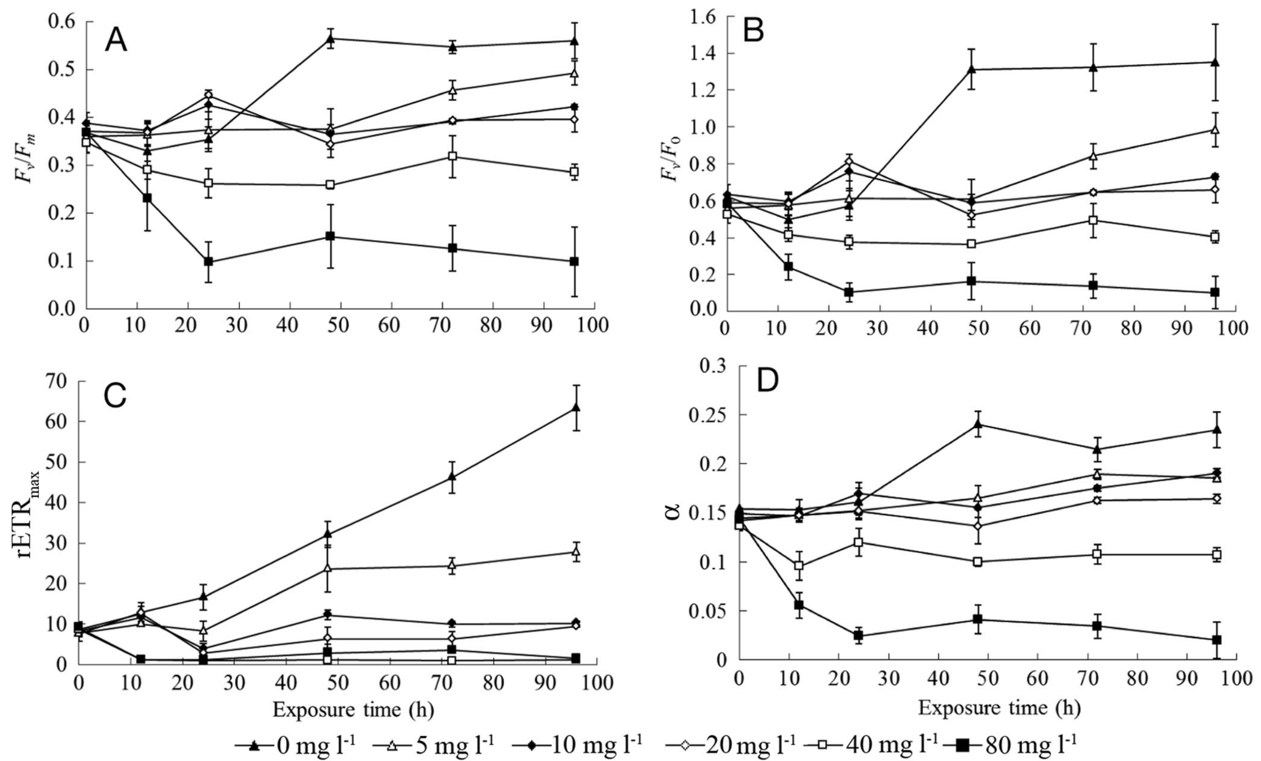

Fig. 4. Photosynthetic activity changes in *Phaeodactylum tricornutum* under different levels of [C_8mim]Br stress during 96 h of exposure, showing changes in (A) maximum photochemical efficiency of PSII (F_v/F_m), (B) potential activity of PSII (F_v/F_0), (C) maximum relative electron transport rate (rETR$_{max}$), and (D) light use efficiency (α). Points: means of 3 replicates; error bars: SD

tively (Fig. 4A,B). This reduction in F_v/F_m and F_v/F_0 may lead to a decrease in conversion efficiency of primary light energy. Another photosynthetic parameter, rETR, mainly reflects the electron transport status of the PSII reaction center (Kitajima & Butler 1975). Fig. 4C shows that rETR$_{max}$ increased sharply from 7.7 to 63.4 in the controls after 96 h cultivation, but there were almost no increases in any of the [C_8mim]Br treatments. The rETR$_{max}$ decreased by 56.2, 83.8, 85.2, 98.0, and 97.6% in 5, 10, 20, 40, and 80 mg l^{-1} [C_8mim]Br treatments relative to the controls at 96 h exposure, respectively. Reduction of rETR$_{max}$ indicates that the photosynthetic electron transfer of *P. tricornutum* was hindered by [C_8mim]Br. In addition, α (representing light use efficiency) was not significantly different in [C_8mim]Br treatments of ≤20 mg l^{-1} before 24 h exposure ($p > 0.05$). But in the 40 and 80 mg l^{-1} [C_8mim]Br treatments, there were decreases to 74.4 and 15.3% of that in the controls, respectively, at 24 h exposure. At that point, α increased in the controls and remained constant in the [C_8mim]Br treatments after 48 h exposure. At 96 h exposure, the α decreased by 21.0, 18.8, 30.0, 54.3, and 91.3% in 5, 10, 20, 40, and 80 mg l^{-1} [C_8mim]Br treatments relative to the controls, respectively (Fig. 4D). Thus, we conclude that [C_8mim]Br likely hinders photosynthetic electron transfer and restrict light use efficiency leading to growth inhibition.

Chlorophyll in *P. tricornutum* after exposure to [C_8mim]Br

As antenna pigments, chlorophyll can transfer photons to the reaction center (P680) in PSII of microalgae, and this change will affect the microalgal photosynthetic activity (Kalaji & Guo 2008). The change in chl *a* in *P. tricornutum* under [C_8mim]Br stress is illustrated in Fig. 5. It shows that chl *a* concentrations did not change significantly in [C_8mim]Br treatments of ≤20 mg l^{-1} ($p > 0.05$), but a marked decrease (44.9 and 41.2% of that in the controls) was observed in the treatments with 40 and 80 mg l^{-1} [C_8mim]Br, respectively. According to Couling et al. (2006), the alkyl chain possessed by [C_8mim]Br may be incorporated into the polar head groups of the phospholipid bilayer, which would result in the disruption of membrane-bound proteins and the structural integrality of chloroplasts.

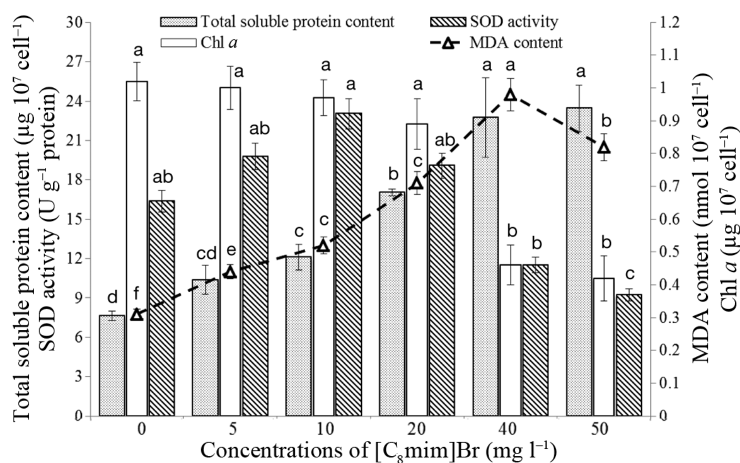

Fig. 5. Mean (±SD) values (n = 3) of chl *a* content, total soluble protein content, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in *Phaeodactylum tricornutum* after 96 h of exposure to different levels of [C_8mim]Br. Points: means of 3 replicates; error bars: SD; different lowercase letters: datasets that are significantly different from each other ($p < 0.05$)

Soluble protein content, SOD activity and MDA content of *P. tricornutum* after exposure to [C_8mim]Br

Fig. 5 shows that soluble protein content increased by 35.9, 58.5, 123.3, 197.7, and 207.0% in the 5, 10, 20, 40, and 80 mg l^{-1} [C_8mim]Br treatments relative to the controls, respectively. Enzymes, including antioxidant and biotransformation enzymes, are important components in soluble proteins, and may serve as an active defense mechanism to protect cells from [C_8mim]Br stress (Kumar et al. 2008). Thus, we suggest that [C_8mim]Br could increase soluble protein synthesis in the diatom cells.

SOD is the most important enzyme in reactive oxygen species (ROS) scavenging, and can catalyze the dismutation of the highly reactive superoxide anion to H_2O_2 (Blokhina et al. 2003). In the present study, [C_8mim]Br concentrations ≤10 mg l^{-1} caused a slight increase (from 16.37 to 23.04 U g^{-1} protein) in SOD activity in the diatoms, with the maximum SOD activity of 23.04 U g^{-1} protein obtained in the 10 mg l^{-1} [C_8mim]Br treatments (Fig. 5). SOD activity may also be enhanced in different organisms following exposure to IL stresses (Yu et al. 2009, Li et al. 2012, Zhang et al. 2013). However, SOD activity in the diatom decreased from 23.04 to 9.24 U g^{-1} protein when [C_8mim]Br concentrations increased from 10 to 80 mg l^{-1} (Fig. 5). The inactivation of antioxidant enzymes may result in high lipid peroxidation and low photosynthetic pigments, thereby inhibiting microalgal cell growth.

When microalgae are exposed to various abiotic stresses, ROS production increases in the microalgal cells. Lipid peroxidation often occurs in microalgal cells when ROS is excessive, and an end-product of lipid peroxidation (i.e. MDA) is detected (Apel & Hirt 2004). In this study, MDA content in the diatom significantly increased by 40.0, 66.1, 129.7, and 215.7% with increasing [C_8mim]Br concentrations from 5 to 40 mg l^{-1}. The maximum MDA content (0.98 nmol 10^7 cell^{-1}) was obtained in 40 mg l^{-1} [C_8mim]Br treatments (Fig. 5). But the MDA content decreased to 0.82 nmol 10^7 cell^{-1} in the diatom exposed to 80 mg l^{-1} [C_8mim]Br (Fig. 5). We infer that free radicals such as ROS were generated in the diatom under [C_8mim]Br stress, which gave rise to MDA. More MDA would damage the diatom cells because MDA may readily interact with several functional groups of molecules, such as proteins, lipoproteins, and DNA (Maes et al. 2006).

CONCLUSIONS

This is the first report on the toxic mechanism of [C_8mim]Br on a marine diatom. In this study, the growth of *Phaeodactylum tricornutum* was significantly inhibited by [C_8mim]Br, which inhibited the chlorophyll synthesis, hindered photosynthetic electron transfer and restricted light use efficiency of this diatom. Simultaneously, remarkable physiological and biochemical responses were observed in the diatom. The increase of protein content and SOD activity at low concentrations may be viewed as an active defense against moderate [C_8mim]Br stress by free radical quenching. The general increase in MDA level suggests that physiological effects of [C_8mim]Br were likely caused by free radical generation.

Acknowledgements. The manuscript was supported by the National Natural Science Foundation of China (No. 31200381), China Postdoctoral Science Foundation funded project (No. 2013M531370, 2014T70532), Key Laboratory for Ecological Environment in Coastal Areas, State Oceanic Administration (201209).

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Editorial responsibility: Kedong Yin, Brisbane, Queensland, Australia

Submitted: May 20, 2015; Accepted: November 4, 2015
Proofs received from author(s): November 27, 2015