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# Cellular responses of the dinoflagellate *Prorocentrum donghaiense* Lu to phosphate limitation and chronological ageing

**XIAOZHOU HUANG, BANGQIN HUANG*, JIXIN CHEN AND XIN LIU**

KEY LABORATORY OF COASTAL AND WETLAND ECOSYSTEMS, MINISTRY OF EDUCATION/FUJIAN PROVINCIAL KEY LABORATORY OF COASTAL ECOLOGY AND ENVIRONMENTAL STUDIES, XIAMEN UNIVERSITY

*CORRESPONDING AUTHOR: College of the Environment and Ecology, Xiamen University, Xiamen, Fujian, China. bqhuang@xmu.edu.cn

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*Prorocentrum donghaiense* Lu is a typical harmful algal species in the East China Sea, which has already had significant influences on ecosystems as well as caused economic losses over the past two decades. This study focused on cellular responses to phosphate limitation and chronological ageing for *P. donghaiense*. Results showed that reactive oxygen species (ROS) production, caspase-like activity and a metacaspase gene expression were significantly higher in the phosphate deplete treatment than in the phosphate replete treatment while algal cell death was not associated with caspase-like activity, and the test alga still grew with low photosynthetic efficiency in the phosphate-limited culture. Our results indicate that ROS production played multiple roles in the chronological ageing and regulation stress signalling or/and cell death in *P. donghaiense*. Metacaspase and caspase-like enzymes not only played a programmed cell death (PCD)-specific role (caused cell death) but also linked with chronological ageing of cultures and acclimation (survival) for phosphate limitation. It also demonstrated that classical hallmarks of PCD (such as cleavage of caspase substrates) for metazoa might be not suitable for characterization of PCD pathways in phytoplankton. This study provides a novel insight into why *P. donghaiense* blooms can persist for a long period with high abundance.

KEYWORDS: metacaspases; programmed cell death; reactive oxygen species; caspase-like activity; phosphate limitation; *Prorocentrum donghaiense* Lu; harmful algal bloom

## INTRODUCTION

Since the 1990s, *Prorocentrum donghaiense* Lu has frequently caused large-scale harmful algal blooms (thousands of $km^2$) in the Yangtze River (Changjiang) Estuary and the adjacent East China Sea (ECS) (Lu and Goebel, 2001; Qi and Wang, 2003; Lu *et al.*, 2005). The abundance of

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*P. donghaiense* is very high during blooms, which can reach $3.6 \times 10^8$ cells $L^{-1}$, and be maintained for a long time (Hu *et al.*, 2012). The blooms of *P. donghaiense* have had serious impacts on ecosystem health, and caused economic losses in fisheries and aquaculture (Zhang *et al.*, 2006; Liu *et al.*, 2013). Consequently, it is important to understand why *P. donghaiense* can rapidly develop into blooms and persist for long periods at high concentration and also how to terminate them.

Various field and laboratory studies of *P. donghaiense* in terms of its taxonomy, life history, ecology, physiology and the formation mechanism of its blooms have been conducted (Lu *et al.*, 2003; Qi and Wang, 2003; Zhang *et al.*, 2006; Li *et al.*, 2009; Liu *et al.*, 2013). Increasing nutrient input to the ECS is thought to be the major driver for these more frequent and persistent algal blooms (Anderson *et al.*, 2002; Zhou *et al.*, 2003; Glibert and Burkholder, 2006). Generally, nutrient supply is not balanced, and an excess of one nutrient can alter the balance of the N/P ratio and result in nutrient limitation (Dortch and Whitledge, 1992; Justic *et al.*, 1995). Concentration of phosphate is relatively low compared with that of nitrate, and phosphorus has been considered to be a main limiting nutrient for phytoplankton growth in the ECS, in particular, when blooms occur (Harrison *et al.*, 1990; Wang *et al.*, 2003; Huang *et al.*, 2007). Nevertheless, *P. donghaiense* can sustain their growth after phosphate depletion. Ou *et al.* (Ou *et al.*, 2008) considered that *P. donghaiense* with higher affinities for dissolved organic phosphorus (DOP) can make better use of the DOP than *Skeletonema costatum* under phosphate-limited conditions. However, there is no universally accepted mechanism to explain why blooms of *P. donghaiense* are maintained for a long time and then terminate.

Caspases, a family of cysteine aspartate-specific proteases, are key metazoan enzymes involved in the initiation and execution of programmed cell death (PCD) through the cleavage of various essential proteins in response to pro-apoptotic signals (Thornberry and Lazebnik, 1998). Caspase homologues, paracaspases and metacaspases are identified in diverse organisms, such as higher plants, animals, slime moulds, fungi, bacteria and unicellular protists including phytoplankton (Uren *et al.*, 2000), suggesting that they may represent an ancestral core of executioners that led to the emergence of the cell death machinery (Bidle and Bender, 2008). Analyses of completed genome sequences of prokaryotic and eukaryotic phytoplankton reveal the widespread presence of metacaspases (Bidle and Falkowski, 2004), and metacaspases have received increased attention in phytoplankton ecology (Franklin *et al.*, 2006). Despite this, the roles of metacaspases are still an open question in phytoplankton.

During the last decade, most of the studies of the roles of metacaspases in phytoplankton have focused on PCD regulation. PCD is identified in a wide range of prokaryotic and eukaryotic unicellular phytoplankton and it is triggered by various environmental stresses such as nutrient deprivation, oxidative stress, heat shock, excessive salt concentrations and virus infection (Bidle and Falkowski, 2004; Jauzein and Erdner, 2013). The hypotheses defining benefits of PCD at the population scale include that PCD can remove damaged and/or ageing cells, release nutrients to complement the nutritional needs of other non-PCD algae, exchange nutrients in the microbial loop and maximize the biological fitness of the community (Bidle and Falkowski, 2004; Franklin *et al.*, 2006; Orellana *et al.*, 2013). PCD is an important feature of algal ecophysiology, and it can explain high lysis rates in phytoplankton independently of viral attack. It is an important contributor to the microbial food web, and effectively short-circuit carbon exports to the deep ocean and stimulates upper-ocean biogeochemical cycling (Bidle and Falkowski, 2004; Bidle *et al.*, 2007; Ray *et al.*, 2014). The accumulation of reactive oxygen species (ROS) is proposed as both an initiator and a by-product of the PCD process in phytoplankton (Zuppini *et al.*, 2007; Johnson *et al.*, 2014). Cell death is induced by means of activation of the caspase-like cascades with high concentration of ROS, which often occurs when the cell has suffered an injury or some sort of stress (Cohen, 1997).

However, recent studies reveal that PCD regulation is just one of the multifaceted roles of caspase-like enzymes or/and metacaspases, which can also regulate other biological processes (Tsiatsiani *et al.*, 2011). Adverse environmental conditions involving variations in physicochemical conditions are particularly challenging for phytoplankton. Environmental stress can affect cellular functions through alteration of metabolism and disrupt cell homeostasis, and may result in entrance into quiescence, encystment or death through necrotic or autocatalytic pathways (Bidle and Falkowski, 2004; Bravo *et al.*, 2010; Jauzein and Erdner, 2013). Some studies have shown that metacaspases are also required for cell cycle regulation, clearance of protein aggregates, management of endoplasmic reticulum stress and cell division in plants, fungi and protozoa (reviewed in Tsiatsiani *et al.*, 2011). Thamatrakoln *et al.* (Thamatrakoln *et al.*, 2012) first suggested that death-related genes (metacaspases) played a non-death role in *Thalassiosira pseudonana*, showing metacaspase involvement in stress (Fe-limitation) acclimation. Jauzein and Erdner (Jauzein and Erdner, 2013) also observed that the functions for caspase-like proteases in the dinoflagellate *Alexandrium tamarense* are not only induction of PCD but also the roles in survival pathways and cell cycle progression. However, non-death

roles of metacaspases in phytoplankton have received much less attention than their involvement in PCD pathways (Jauzein and Erdner, 2013).

A gene of caspase-like (metacaspase) function in *P. donghaiense* has been identified and shows preliminarily that the caspase-like (metacaspase) enzymes may mediate PCD in the cellular senescence of *P. donghaiense* (Zhang *et al.*, 2006; Yang *et al.*, 2008). However, the mechanism and roles of caspase-like enzymes and metacaspase in *P. donghaiense* are largely unexplored. Consequently, the aim of our study was to gain insight into stress-related pathways in *P. donghaiense* during the chronological ageing process and under the stress of phosphate deprivation, and to elucidate the multifunction of caspase-like enzymes. In this study, we specifically examined ROS production, caspase-like activity and expression of one metacaspase gene in response to phosphate limitation. The study of potential roles of caspase-like enzymes and metacaspase of *P. donghaiense* not only can provide new insights into intracellular and molecular regulation and transduction of stress-related pathways, but also widens our knowledge of the potential non-death roles in phytoplankton and yields new information on the persistence of *P. donghaiense* blooms.

# METHOD

## Culture and sampling

*Prorocentrum donghaiense* Lu was obtained from the Center for the Collection of Marine Algae of Xiamen University, China. Cultures were grown in f/2 medium (36.2 μM phosphate) at 20 ± 2°C under a 14:10 light: dark regime at 100 μmol photons $m^{-2}$ $s^{-1}$. Cells were harvested by gentle vacuum filtration onto 25-mm diameter, 2-μm pore-size, polycarbonate filters (TTTP ; Millipore) once the stationary phase was reached. They were washed once in filtered (0.22-μm pore-size) autoclaved seawater in order to remove residual medium, then re-suspended in the seawater. Cells were used to inoculate triplicate 3 L cultures of either replete f/2 medium (P replete or +P) or f/2 medium without phosphate addition (P deplete or −P) at a cell concentration of $1 \times 10^4$ cells $mL^{-1}$. All the seawater used was collected from the Taiwan Strait, with a low concentration of soluble reactive phosphate (SRP, only 0.15 ± 0.01 μM), and was filtered onto 0.22 μm pore-size filters, and autoclaved. All glassware was soaked in acid (5% HCl) for 24 h, thoroughly rinsed in deionized water and autoclaved prior to the experiment. Samples were taken at the same time (09:00−10:00) every other day, and the optimum photochemical quantum yield of photosystem II (PSII) fluorescence ($F_V/F_M$; 15-min dark acclimation) was determined using PHYTO-PAM equipped with a PHYTO-ED measuring head (Heinz Walz GmbH).

## Growth rate and cell viability

The abundance of *P. donghaiense* was measured in independent triplicate cultures using an Accuri C6 (Becton-Dickinson) flow cytometer fitted with a 488-nm laser. Milli-Q water was used as a sheath fluid, and 10000−20000 cells were counted. The event rate was between 100 and 400 cells $s^{-1}$ to avoid coincidence. High-green fluorescent beads (1 μm diameter) were added to the samples as an internal standard for the quantification of cell concentration. Bead concentration was calculated by filtering replicated aliquots onto black Nucleopore filters and counting the number of beads under an epifluorescence microscope (Nikon i90).

The abundance of living cells (viability) was analysed using a cell membrane permeability test (cell digestion assay, Darzynkiewicz *et al.*, 1994) as modified for phytoplankton cells by Agustí and Sánchez (Agustí and Sánchez, 2002). Briefly, 200 μL DNAse I (400 μg $mL^{-1}$, Sigma-Aldrich) in Hanks' balanced salts (HBSS, pH 7.4, Sigma-Aldrich) was added to 1 mL samples and then they were incubated at 37°C for 15 min. Then, 200 μL Trypsin (Sigma-Aldrich) 1% in HBSS was added and they were incubated at 37°C for 30 min. At the end of the incubation, the samples were placed on ice to stop enzyme reaction, and the abundance of cells remaining after the assay was quantified using an Accuri C6 flow cytometer, as described above. The cells remaining after the enzymatic treatment represented living or viable cells with intact membranes, whereas dead ones, with compromised membranes, were digested out by the enzymatic cocktail and were undetectable with the flow cytometer.

The growth rate was calculated as follows:

$$\text{Growth rate } (\mu) = \frac{\ln \mathcal{N}_2 - \ln \mathcal{N}_1}{\ln 2(t_2 - t_1)}$$

where $\mathcal{N}_1$ and $\mathcal{N}_2$ are the biomass at Time 1 ($t_1$) and Time 2 ($t_2$) (Levasseur *et al.*, 1993), respectively.

## Soluble reactive phosphorus and bulk alkaline phosphatase activity

SRP, which is operationally thought to represent the phosphate concentration, was determined in duplicate using the molybdenum blue colorimetric method of Murphy and Riley (Murphy and Riley, 1962). The determination limit of this method is 0.1 μM.

Bulk alkaline phosphatase activity (bulk APA) was determined using fluorometry with 4-methylumbelliferyl phosphate (MUP, Sigma) as the substrate (Hoppe, 1983).

MUP with a final concentration of 150 μM was added to each sample in the dark and incubated at 37°C for 1 h. At the end of the incubation, $HgCl_2$ with a final concentration of 16 mM was added to stop the enzyme reaction. Fluorescence measurements were carried out using a Varian Cary Eclipse, with the excitation wavelength set at 365 nm and the emission wavelength at 450 nm.

## Reactive oxygen species

Intracellular ROS production was measured using the fluorescent probe carboxy-$H_2$DCFDA [5-(and 6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate; Invitrogen], and a working solution prepared in 100% ethanol (Royall and Ischiropoulos, 1993). Cells were collected using centrifugation (10000 g, 10 min, 4°C) and washed once with phosphate-buffered saline (PBS, pH 7.4). Then the cells were re-suspended in PBS containing the probe at a final concentration of 10 μM and incubated for 20 min at room temperature and in the dark. The percentage of positively stained (green fluorescence) cells was determined using the flow cytometer as described above.

## Caspase activity

Cellular caspase-like activation within a *P. donghaiense* population was independently assessed by direct staining with VAD-FMK-FITC (CaspACE; Promega), which freely diffuses into the cells and irreversibly binds to activated caspases, thus serving as an *in vivo* marker (Madeo *et al.*, 2002). Cells were pelleted using centrifugation (10000 g, 10 min, 4°C), washed once with PBS, and re-suspended in PBS before the addition of VAD-FMK-FITC (final concentration, 20 μM). The cells were then stained for 20 min at room temperature in the dark, after which they were pelleted again, washed once with PBS, fixed with 2% (vol/vol) paraformaldehyde/PBS and stored at 4°C until analysis (within 1 week) (Vardi *et al.*, 2012) with a flow cytometer as described above.

Caspase-3-like activity also was measured using the CaspACE™ Assay System, Colorimetric (Promega, Gurtu *et al.*, 1997). Cells were harvested using centrifugation (10000 g, 10 min, 4°C), washed in ice-cold PBS and re-centrifuged (10000 g, 10 min, 4°C). They were then re-suspended in Cell Lysis Buffer on ice, and the cells lysed using freeze-thaw. They were then incubated on ice for 15 min, with repeated freeze-thaw cycles as necessary to ensure complete cell lysis. The cell lysates were centrifuged at 10000 g for 10 min at 4°C to precipitate the cellular debris and the supernatant fraction was collected. The protein concentration of the supernatant was determined using a Pierce BCA Protein Assay Kit (Thermo). Clear lysates were used for measuring caspase-3 activity following the manufacturer's protocol. The supernatant (with 50–100 μg total protein) was transferred into triplicate 96-well plates containing the caspase-3 substrate (Ac-DEVD-pNA), and a kinetic analysis was performed for substrate cleavage (excitation, 400 nm; emission, 505 nm) over a 2-h period with measurements taken at 10-min intervals using a GENios-DNA plate reader (Tecan) and the cleavage rates were normalized to protein content.

## Metacaspase gene expression determined using real-time quantitative PCR analysis

Cells were collected onto 25-mm-diameter, 2-μm pore-size polycarbonate filters placed in a 2-mL Eppendorf tube, snap-frozen in liquid nitrogen and stored at −80°C until processing. The total RNA of *P. donghaiense* was extracted using a Trizol kit (Invitrogen Life Technologies), and the concentration and purity was determined using a NanoDrop ND-2000c Spectrophotometer (Thermo Scientific). Then the RNA was reverse-transcribed using a *TransScript*® All-in-one First-Strand cDNA Synthesis SuperMix (TransGen Biotech) or qPCR Kit following the manufacturer's recommended procedure. The cDNA samples obtained were used as templates in real-time quantitative PCR (RT-qPCR). The primers, those that just amplified one of *P. donghaiense* metacaspases, were synthesized following Yang *et al.* (Yang *et al.*, 2008), and the primers of β-actin, which served as a housekeeping gene and a normalizer for RT-qPCR analyses of RNA expression, were also used (Table I). RT-qPCR progression was monitored using the intercalating dye SYBR-Green I on a Rotor-Gene 3000 (Qiagen) following the standard curve method. A serial dilution of cDNA in distilled water was amplified to construct standard curves and the standard curves of metacaspase and β-actin in the present study gave PCR efficiencies of 94 and 99%, respectively. Melting curve analysis of the amplification products was also performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The Relative Expression Software Tool (REST 2008©-version 2) was used to calculate the relative expression.

*Table I: Sequences of forward (F) and reverse (R) primers used for RT-PCR in* Prorocentrum donghaiense *Lu*

| Target | Primer Sequence (5′–3′) | Reference |
|---|---|---|
| Metacaspase | F: CAT CTG GCT GTC TTT CTG G | Yang *et al.* (2008) |
| | R: CGA GCT G GG CAG AGA ACA TG | Yang *et al.* (2008) |
| β-Actin | F: CGG CGG TTC CAT CCT TTC CTC T | Yang *et al.* (2008) |
| | R: CCG CTG CCG TCG ATT GCA TG | Yang *et al.* (2008) |

### Data processing

Results are reported as mean ± S.E. Correlation analysis involving linear regression was done using the statistical software, SPSS Version 16.0 (SPSS, Inc., Chicago, IL, USA) for Windows. Significant differences were analysed with a *t*-test using SPSS, except for mRNA expression, which was analyzed using the Pair-Wise Fixed Reallocation Randomization Test© (Pfaffl *et al.*, 2002). In all cases, a value of $P < 0.05$ was used to indicate significant differences/correlation and $P < 0.005$ was used to indicate highly significant differences/correlation.

## RESULTS

### Algal growth and phosphate limitation

Growth curves were significantly different in the two treatments. Cultures of the P deplete treatment reached the relatively low maximum cell abundance of $3.9 \pm 0.17 \times 10^4$ cells $mL^{-1}$ after rapid growth over the first 5 days and persisted in the stationary phase for the later period (Days 5–15) (Fig. 1). However, the P replete treatment showed more extensive and sustained growth, reaching a peak abundance of $8.7 \pm 0.52 \times 10^4$ cells $mL^{-1}$ at Day 9. After this point, the algal abundance declined by 35.2% to $5.63 \pm 0.28 \times 10^4$ cells $mL^{-1}$ at Day 15 (Fig. 1). The algal abundance of the replete treatment was significantly higher than that of the P deplete treatment ($P < 0.05$, paired-samples *t*-test). The growth rate ($\mu$) of the replete treatment gradually declined, from a maximum growth rate of 0.20 $day^{-1}$ to minus 0.06 $day^{-1}$ during culture (Fig. 1). It is noteworthy that the growth rate of the P deplete treatment was very low after Day 7 of culture, but it was higher than the replete treatment during the last period of culture (Days 11–15).

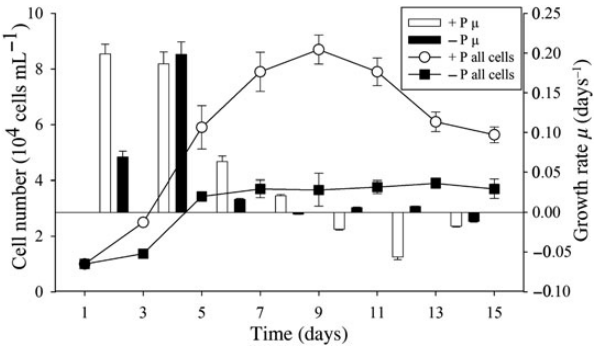


**Fig. 1.** Growth curves and growth rate ($\mu$) of *Prorocentrum donghaiense* Lu during 15-day batch culture with phosphate replete treatment (+P) and phosphate deplete treatment (−P). Error bars represent the standard deviation of the triplicate cultures. *x*-Axis, left *y*-axis and right *y*-axis represent time (days), cell number (cells $mL^{-1}$) and growth rate ($day^{-1}$), respectively.

In general, the percentage of living cells decreased and >90% cells were viable over the experimental time under both culture conditions (Fig. 2). In the P replete treatment, the percentage of living cells became significantly lower than 100% from Day 7 to Day 15, whereas in the P deplete treatment, the percentage of living cells became significantly lower than 100% until Day 11 ($P < 0.05$, Student's *t*-test, Fig. 2). For the first 7 days, the percentage of living cells of the P deplete treatment showed no significant difference with that of the P replete treatment, whereas the percentage of living cells of the P deplete treatment was significantly higher than that of the P replete treatment ($P < 0.05$, paired-samples *t*-test, Fig. 2).

The optimum quantum yield of PSII ($F_V/F_M$), which was monitored as a measure of photosynthetic health, was used as an indicator of physiological status. The $F_V/F_M$ of the P deplete treatment was significantly lower ($P < 0.05$, paired-samples *t*-test) than that of the P replete treatment throughout the experiment (Fig. 3). Both cultures started with $F_V/F_M$ of ~0.6, indicating that PSII photochemistry was healthy in both cultures before the start of the experiment (Fig. 3). In the P deplete treatment, $F_V/F_M$ gradually declined from Day 5 to Day 15, dropping by 39% to $0.37 \pm 0.01$ at Day 15 (Fig. 3). In the P replete treatment, cells maintained an $F_V/F_M$ of ~0.6 for the first 7 days, after which it dropped to $0.5 \pm 0.02$ at Day 15 (Fig. 3).

Results showed that SRP was very low in the P deplete treatment, and the concentration was below the determination limit except on the first day of culture (Fig. 4), whereas SRP was still very high ($18.60 \pm 0.52$ μM) at the end of culture in the P replete treatment (Fig. 4). The total bulk APA was low during the first 11 days of culture, and then increased gradually at a later period of culture for the P replete treatment, with highest values of $4.17 \pm 1.18$ f mol $h^{-1}$ $cell^{-1}$ at Day 15, whereas the total bulk APA increased gradually at the beginning of culture and was significantly higher for the P deplete than for the P replete treatment (Fig. 4, $P < 0.05$, paired-samples *t*-test).

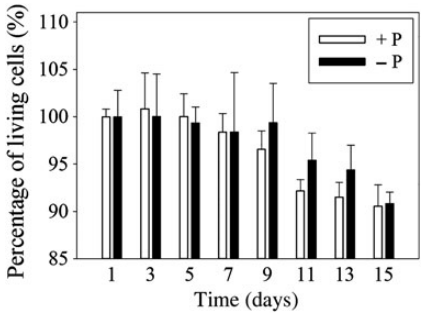


**Fig. 2.** Percentage of living cells of *Prorocentrum donghaiense* Lu during a 15-day batch culture with phosphate replete treatment (+P) and phosphate deplete treatment (−P). Error bars represent the standard deviation of triplicate cultures.

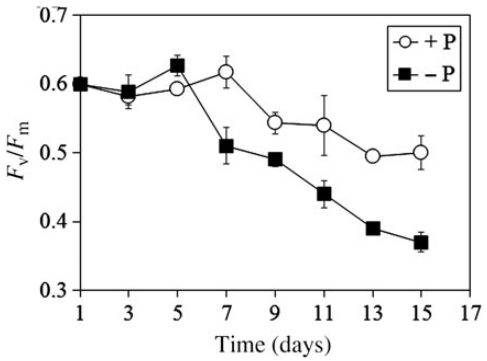


**Fig. 3.** Optimal quantum yield of photosystem II fluorescence ($F_V/F_M$) for *Prorocentrum donghaiense* Lu during a 15-day time course in phosphate replete treatment (+P) and phosphate deplete treatment (−P). Error bars represent the standard deviation of triplicate cultures.

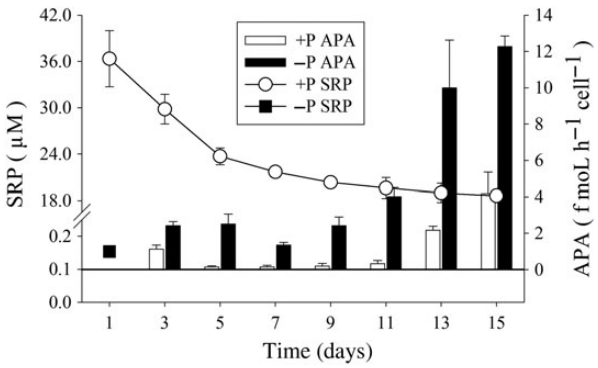


**Fig. 4.** Variations of soluble reactive phosphorus (SRP) and total alkaline phosphatase activities (APA) of *Prorocentrum donghaiense* Lu during 15-day batch culture with phosphate replete treatment (+P) and phosphate deplete treatment (−P). Error bars represent the standard deviation of triplicate cultures. *x*-Axis, left *y*-axis and right *y*-axis represent time (days), SRP (μM) and APA (f mol h$^{-1}$ cell$^{-1}$), respectively.

On the basis of the algal growth curves, $F_V/F_M$, the SRP concentration and the total bulk APA during the incubation period, we believe that the test species was under phosphate limitation (P-limited) after Day 7 of the P deplete treatment.

## Reactive oxygen species

ROS accumulated in *P. donghaiense* cells throughout the experiment (Fig. 5). The percentage of algal cells, which were positively stained in the P deplete treatment, increased from 17.23 ± 3.41 to 97.95 ± 10.84% and was significantly higher than that of the P replete treatment ($P < 0.005$, paired-samples *t*-test), which increased from 3.84 ± 1.14 to 61.67 ± 4.67% (Fig. 5).

## Caspase activity

The percentage of CaspACE-stained cells showed a similar trend as did the ROS-positive cells. In the P

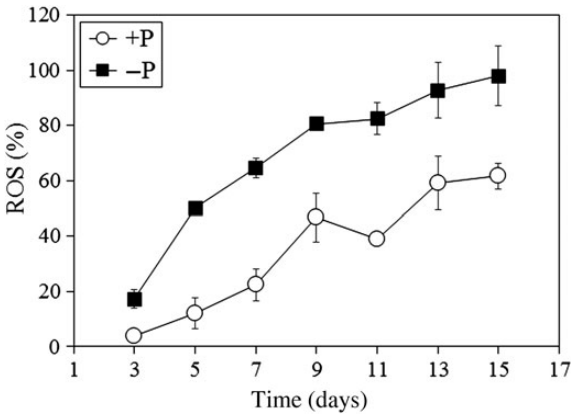


**Fig. 5.** The percentage of positively ROS-stained *Prorocentrum donghaiense* Lu cells determined using the flow cytometer during a 15-day batch culture with phosphate replete treatment (+P) and phosphate deplete treatment (−P). Error bars represent the standard deviation of triplicate cultures.

replete treatment, this increased from 4.17 ± 0.25 to 59.16 ± 2.66%, whereas in the P deplete treatment, it was relatively small (3.75 ± 0.24%) on Day 3 and then dramatically increased over time, up to 91.01 ± 4.65% (Fig. 6a). It showed a highly significant positive linear correlation (both $P$ and $P' < 0.05$) with the percentage of ROS in positive cells ($R$, $P$, circles and thin line represent +P; $R'$, $P'$, black squares and dashed line represent −P; Fig. 6b).

In the P replete treatment, healthy actively growing cultures showed very little Ac-DEVD-pNA cleavage in the exponential phase, and caspase-3-like activity increased from 199 ± 8.5 to 1730 ± 290 relative fluorescence units (RFU) mg protein$^{-1}$ h$^{-1}$. In the P deplete treatment, caspase activity, which was consistently enhanced, was significantly higher than in the P replete treatment at each sampling time, ranging from 1.92- to 3.62-fold, with activity peaking at Day 13 (3656 ± 324 RFU mg protein$^{-1}$ h$^{-1}$), and the caspase-3-like activity at Day 13 increased by 6.67-fold that on Day 3 (Fig. 6c).

## Metacaspase gene expression

In the P replete culture, there was no significant change during Days 5–9 compared with the reference (Day 3 of the P replete treatment), and then the metacaspase mRNA increased significantly during Days 11, 13 and 15 (2.50, 2.46 and 2.59-fold, respectively) compared with the reference (Fig. 7, all $P < 0.05$). For the P deplete experiment, metacaspase mRNA expression was triggered and increased highly significantly compared with that of the reference ($P < 0.005$ on Days 7, 9 and 11, 3.11, 3.33 and 3.30-fold, respectively). It then decreased slightly with a significant difference ($P < 0.05$) on Days 13 and 15

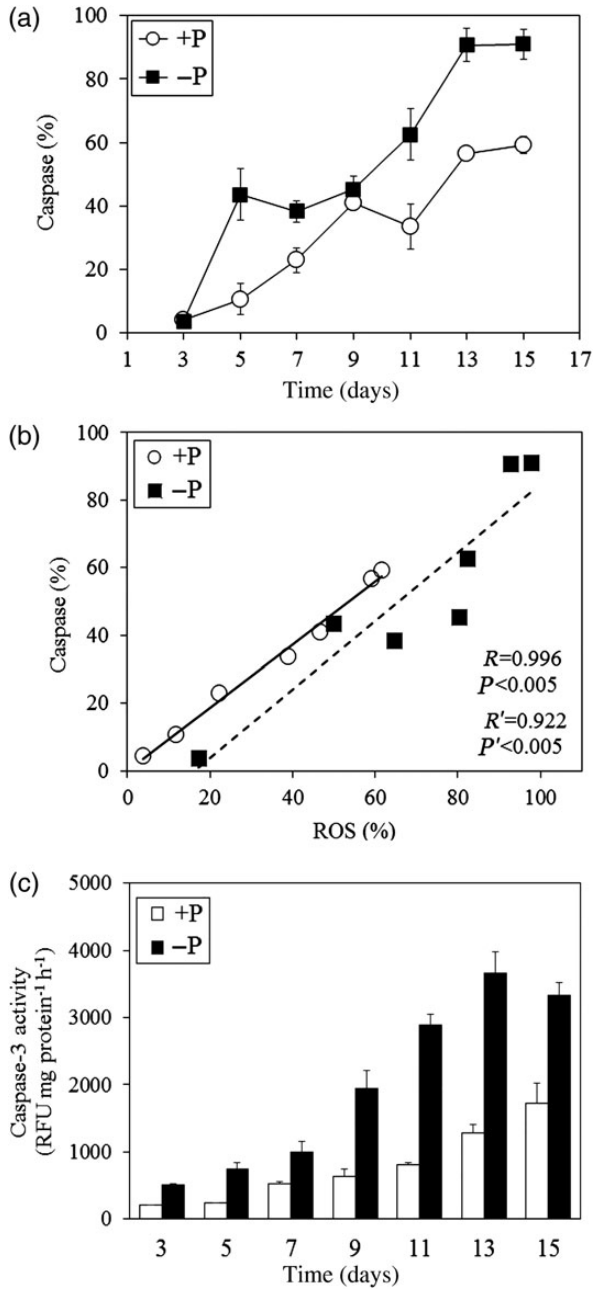


**Fig. 6.** *In vivo* detection of caspase activity (CaspACE) using the flow cytometer (**a**) and time courses of caspase-3 activity expressed (RFU mg protein$^{-1}$ h$^{-1}$, **c**) for *Prorocentrum donghaiense* Lu during a 15-day batch culture with phosphate replete treatment (+P) and phosphate deplete treatment (−P). Linear regression and correlation analysis for the percentage of caspase-positive cells and percentage of ROS-stained cells (**b**). *R*, *P*, circles and thin line represent +P; *R*′, *P*′, black squares and dashed line represent −P; error bars represent the standard deviation of triplicate cultures.

(2.93- and 2.82-fold) compared with that of the reference (Fig. 7). Expression of the metacaspase mRNA of the P deplete treatment was significantly higher than that of the replete treatments during Days 7−11 ($P < 0.05$).

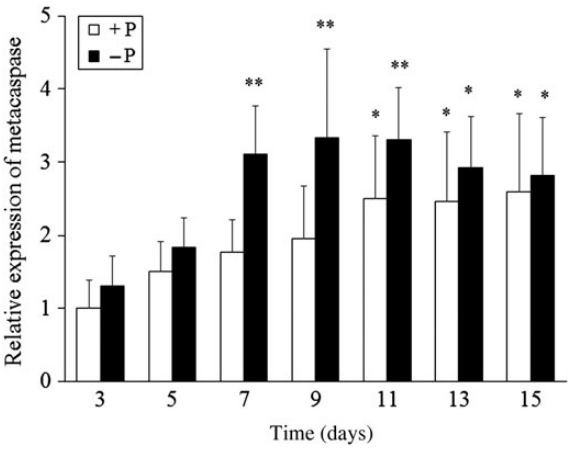


**Fig. 7.** Relative mRNA expression of metacaspase of the *Prorocentrum donghaiense* Lu during a 15-day batch culture with phosphate replete treatment (+P) and deplete treatment (−P). Values were normalized against β-actin. The data are expressed as mean ± SE ($n = 3$). Asterisks indicate that the values are significantly different from that of the control group. (*$P < 0.05$ and **$P < 0.005$).

## DISCUSSION

Phosphorus is considered to be a main limiting nutrient for phytoplankton growth in the ECS, in particular, when blooms occur (Harrison *et al.*, 1990; Wang *et al.*, 2003; Huang *et al.*, 2007). In our study, when *P. donghaiense* was subjected to phosphate starvation, the total bulk APA increased dramatically from Day 9 to 15 and APA was significantly higher than that of the P replete treatment, indicating phosphate stress was induced in the P deplete treatment. The growth rate decreased to $<0.025$ day$^{-1}$ after Day 7 and the numbers of *P. donghaiense* did not show any obvious change during Days 5−15. This evidence suggests the existence of phosphate limitation of algal cells during Days 9−15. The optimum quantum yield of PSII ($F_V/F_M$) for the P deplete treatment was significantly lower than that for the P replete treatment, suggesting that phosphate limitation triggered physiological stress. Algal abundance in the P deplete treatment was lower at each respective sampling time compared with that in the P replete treatment, which further confirmed the phosphate limitation. The cell number in the P deplete treatment showed no obvious change from Days 5 to 15. This was consistent with Ou *et al.* (Ou *et al.*, 2008) who note that the cell numbers of *P. donghaiense* in media with $<0.8$ μM phosphate remain at a relatively steady value in the stationary phase.

PCD is now well established in a variety of diverse phytoplankton lineages including algal bloom species. Previous studies document that PCD is present in *P. donghaiense* (Zhang *et al.*, 2006; Yang *et al.*, 2008). Most studies reporting ROS production in phytoplankton have focused on implication of ROS in PCD pathways (Ding *et al.*, 2012;

Thamatrakoln *et al.*, 2012; Zuppini *et al.*, 2010). Overproduction of ROS is a widely known cellular response to abiotic and biotic stress and it can irreversibly alter DNA, proteins, lipids and carbohydrates and may ultimately result in cell death (Darehshouri *et al.*, 2008; Jacobson, 1996; Lesser, 2012). When *P. donghaiense* was under phosphate limitation, rapid ROS generation was observed. There was a significant negative linear correlation between the percentage of ROS-positive cells and the $F_V/F_M$ (Fig. 8), signifying uncoupled electron flow through PSII ultimately leading to the production of ROS (Berman-Frank *et al.*, 2004; Wolfe-Simon *et al.*, 2006). Similar dynamics reported that ROS production is attributed to compromised photophysiology in *Emiliania huxyleyi* infected by viruses (Evans *et al.*, 2006; Vardi *et al.*, 2012), and ROS production shows a dependence on photosynthetic stress (Vardi *et al.*, 2012).

Some studies have reported that abiotic and biotic stress induced dramatic reductions in photosynthetic efficiency, and then caused elevated production of ROS, ultimately leading to cell death (Berman-Frank *et al.*, 2004; Vardi *et al.*, 2007; Bidle and Bender, 2008). Nevertheless, Lesser (Lesser, 2012) suggests ROS are not only agents of cellular stress/damage, but also can mediate stress signalling in various organisms. In our study, there were no major cell death events, and the percentage of dead cells was significantly lower than that of ROS-positive cells, especially in the P deplete treatment. This suggests that a majority of ROS production played potential roles in mediating stress signalling rather than induction of death for *P. donghaiense*. Jauzein and Erdner (Jauzein and Erdner, 2013) have indicated that ROS participate in the specific purpose of signalling to formation of pellicle cysts or to death, supporting the hypothesis that ROS can keep a delicate balance between roles in signalling and destruction (Suzuki and Mittler, 2006).

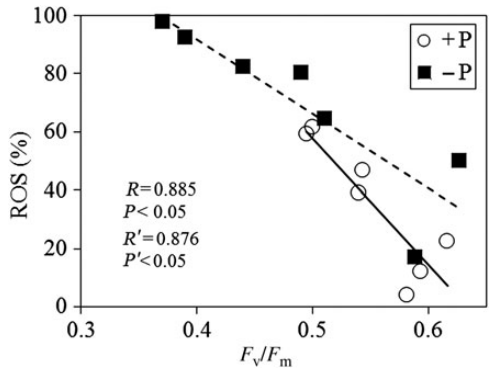


**Fig. 8.** Linear regression and correlation analysis for $F_V/F_M$ and the percentage of ROS-positive cells. *R*, *P*, circles and thin line represent +P; *R*′, *P*′, black squares and dashed line represent −P; error bars represent the standard deviation for triplicate cultures.

Therefore, ROS appear to be playing multiples roles in the biology of chronological ageing and regulation stress signalling or/and cell death in *P. donghaiense*, as in many other organisms (Lesser, 2012).

High levels of caspase-like activity are observed in association with ROS production in many phytoplankton species (Zuppini *et al.*, 2007; Segovia and Berges, 2009; Johnson *et al.*, 2014). Our results also show that P-limited cells with high ROS-positive were also characterized by elevated caspase-like activity based on the cleavage of caspase-3 substrate (Ac-DEVD-pNA) and *in vivo* staining with a fluorescent caspase marker (VAD-FMK-FITC). The percentage of CaspACE-positive cells was significantly higher than that of dead cells, especially in the P deplete treatment and stationary phase, indicating that many viable cells were also characterized by caspase-like activity. Additionally, the caspase-3 activity was higher in stationary phase than that in logarithmic phase under both culture conditions (P deplete treatment and P replete treatment). This agrees with the results of Johnson *et al.* (Johnson *et al.*, 2014), who reported that greater caspase-like activity was induced during stationary phase than logarithmic phase. All these data together suggest that caspase-like enzymes are not solely involved in death (PCD execution) but appear to be linked with chronological ageing processes of *P. donghaiense* cultures.

Although there no caspases have been identified in phytoplankton, metacaspases (caspase orthologues) are widespread in a range of unicellular algae (Bidle and Falkowski, 2004). One metacaspase (caspase-like) gene of *P. donghaiense* has been identified in generated expression sequence tag libraries and shows preliminarily that the expression of the metacaspase gene may participate in PCD (Zhang *et al.*, 2006; Yang *et al.*, 2008). In this context, mRNA expression of the metacaspase using RT-qPCR was concurrent with elevated caspase-like activity. In the P deplete treatment, cell number did not significantly change on Days 5–15 with the lower photosynthetic efficiency, and the percentage of living cells in the P deplete treatment was significantly higher than in the replete treatment on Days 9–15. Furthermore, the growth rate in the P deplete treatment was higher than that in the P replete treatment on Days 11–15. At the same time, elevated caspase-like activity and elevated mRNA expression of the metacaspase in the P deplete treatment were observed, suggesting that the cysteine proteases (metacaspases and caspase-like enzymes) possibly had an adaptive role in cell stress and involved in cell survival in phosphate-limited environments for *P. donghaiense*. The maintenance of a low death rate in actively growing populations supports the role of housekeeping functions for caspase-like proteases, which support non-death roles including involvement in survival pathways and cell proliferation (Segovia *et al.*, 2003; Jauzein

and Erdner, 2013). Indeed, several publications have now demonstrated that metacaspases may participate in other metabolic processes (such as, signal transduction cascade, stress acclimation, regulation of protein aggregates) rather than being responsible for PCD execution in some unicellular organism (Richie *et al.*, 2007; Bidle and Bender, 2008; Lee *et al.*, 2010). Laun *et al.* (Laun *et al.*, 2005) suggest that apoptotic-proteases in yeast play roles in non-death-related cellular processes through differential regulation. Thamatrakoln *et al.* (Thamatrakoln *et al.*, 2012) report that death-related genes play a non-death role in phytoplankton, and imply that the up-regulated PCD-related genes in *Thalassiosira pseudonana* participate in Fe-stress acclimation to Fe-limitation. Jauzein and Erdner (Jauzein and Erdner, 2013) also observe that the functions for caspase-like proteases in the dinoflagellate *Alexandrium tamarense* are not only indicative of PCD but also the roles in survival pathways and cell cycle progression.

In the P replete treatment, cell number was relatively high but it gradually decreased correlated with the reduction in the photosynthetic efficiency on Days 9−15, and the percentage of CaspACE-stained cells was relatively low compared with that in the P deplete treatment, but the value of the percentage of CaspACE-stained cells in the P replete treatment was still very high. Furthermore, the percentage of living cells in the P replete treatment was significantly lower than 100% from Days 7−15 and the percentage of dead cells was higher than that in the P deplete treatment. In the process of cell population decline in the replete treatment, the SRP was still high (∼18 μM); therefore, the cause of death was not phosphate deficiency, and might be another essential nutrient deficiency, virus infection or something else. However, the percentage of CaspACE-stained cells was significantly higher than that of dead cells. The data presented here also indicate that the PCD-related proteases or/and metacaspases played PCD-specific roles (caused death) and non-death functions in the replete treatment, and the death-related proportion was larger compared with that in the P deplete treatment. Therefore, caspase-like enzymes or/and metacaspases appear to control the algal population in response to environmental cues. When the cell abundance was low, more caspase-like enzymes or/and metacaspases likely played the role of stress acclimation to sustain the populations during nutrient deficiency, whereas more caspase-like enzymes or/and metacaspases were associated with death in the population with high abundance of cells. This observation is consistent with the research of Thamatrakoln *et al.* (Thamatrakoln *et al.*, 2012), which reported that there was a delicate balance between pro-survival and pro-apoptotic factors in the *Thalassiosira pseudonana* cell decision between survival and death.

Little is still known about the enzymes responsible for caspase-like activities in phytoplankton (Johnson *et al.*, 2014), and metacaspases have received too much attention as the putative enzymes responsible for the caspase-like activities over the last decade. Actually, the induction expression of metacaspases is not always concomitant with an increase in caspase-like activity for phytoplankton cells. Our results have shown that the expression of metacaspase using RT-PCR peaked on Day 9, and then decreased, whereas the percentage of CaspACE-stained cells and caspase-3 activity continued to rise after Day 9, indicating that metacaspase expression is not related to caspase-like activity. Johnson *et al.* (Johnson *et al.*, 2014) have shown that metacaspases are not responsible for the caspase-like activity in *Karenia brevis*. Several publications have demonstrated that metacaspases are highly specific for arginine and lysine and lack aspartic (Asp) specificity (reviewed in Tsiatsiani *et al.*, 2011). Therefore, caspase-specific substrates with Asp are not indicative of metacaspase catalytic activity (Tsiatsiani *et al.*, 2011). It has demonstrated caspase-specific substrates cannot be cleaved by plant metacaspases (Vercammen *et al.*, 2007; He *et al.*, 2008). Our results suggest that metacaspase and caspase-like enzymes may play dual roles in *P. donghaiense*, that is, in a PCD-specific role (cause death) and a non-death role including potential involvement in stress acclimation (survival), cell division and chronological ageing processes. However, there were only a few deaths (<10%), caspase-like enzymes and metacaspases being more likely to participate in other functions rather than death. Further investigation is necessary to study the potential functions of caspase-like enzymes and metacaspases.

## CONCLUSIONS

This study focused on *Prorocentrum donghaiense* cellular responses to phosphate limitation and chronological ageing, using efficiency of PSII, production of ROS, caspase-like activity and expression of a metacaspase gene, to assess the roles of metacaspase and caspase-like enzymes. Our results indicated that ROS production plays multiples roles in the chronological ageing and regulation stress signalling or/and cell death in *P. donghaiense*. Caspase-like activities and metacaspase gene expression were not associated with PCD pathways, showing that metacaspase and caspase-like enzymes not only play a PCD-specific role to cause cell death (this death part was very few), but also link with chronological ageing of cultures and acclimation (survival) for phosphate limitation. This non-death role of metacaspase and caspase-like enzymes might provide a novel insight into why *P. donghaiense* blooms can persist

for a long period with high abundance in the East China Sea. Although our findings provide a novel mechanistic context for *P. donghaiense* bloom dynamics, more extensive investigations are required to identify the specific gene(s) of metacaspase and caspase-like enzymes, and understand how they activate, regulate and execute PCD in response to a variety of stressors.

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## REFERENCES

Agustí, S. and Sánchez, C. M. (2002) Cell viability in natural phytoplankton communities quantified by a membrane permeability probe. *Limnol. Oceanogr.*, **47**, 818–828.

Anderson, D. M., Glibert, P. M. and Burkholder, J. M. (2002) Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. *Estuaries*, **25**, 704–726.

Berman-Frank, I., Bidle, K. D., Haramaty, L. and Falkowski, P. G. (2004) The demise of the marine cyanobacterium, *Trichodesmium* spp., via an autocatalyzed cell death pathway. *Limnol. Oceanogr.*, **49**, 997–1005.

Bidle, K. D. and Bender, S. J. (2008) Iron starvation and culture age activate metacaspases and programmed cell death in the marine diatom *Thalassiosira pseudonana*. *Eukaryot. Cell*, **7**, 223–236.

Bidle, K. D. and Falkowski, P. G. (2004) Cell death in planktonic photosynthetic microorganisms. *Nat. Rev. Microbiol.*, **2**, 643–655.

Bidle, K. D., Haramaty, L., Barcelos-Ramos, J. and Falkowski, P. (2007) Viral activation and recruitment of metacaspases in the unicellular coccolithophorid, *Emiliania huxleyi*. *Proc. Natl Acad. Sci. USA*, **104**, 6049–6054.

Bravo, I., Figueroa, R. I., Garces, E., Fraga, S. and Massanet, A. (2010) The intricacies of dinoflagellate pellicle cysts: the example of *Alexandrium minutum* cysts from a bloom-recurrent area (Bay of Baiona, NW Spain). *Deep Sea Res. II*, **57**, 166–174.

Cohen, G. M. (1997) Caspases: the executioners of apoptosis. *Biochem. J.*, **326**, 1–16.

Darehshouri, A., Affenzeller, M. and Lütz, M. U. (2008) Cell death upon $H_2O_2$ induction in the unicellular green alga *Micrasterias*. *Plant Biol.*, **10**, 732–745.

Darzynkiewicz, Z., Li, X. and Gong, J. (1994) Assays of cell viability: discrimination of cells dying by apoptosis. In Darzynkiewicz, Z., Robinson, J. P. and Crissman, H. A. (eds), *Methods in Cell Biology*. Academic Press, San Diego, pp. 5–38.

Ding, Y., Gan, N. Q., Li, J., Sedmak, B. and Song, L. R. (2012) Hydrogen peroxide induces apoptotic-like cell death in *Microcystis aeruginosa* (Chroococcales, Cyanobacteria) in a dose dependent manner. *Phycologia*, **51**, 567–575.

Dortch, Q. and Whitledge, T. (1992) Does nitrogen or silicon limit phytoplankton production in the Mississippi River plume and nearby regions? *Cont. Shelf Res.*, **12**, 1293–1309.

Evans, C., Malin, G., Mills, G. P. and Wilson, W. H. (2006) Viral infection of *Emiliania huxyleyi* (Prymnesiophyceae) leads to elevated production of reactive oxygen species. *J. Phycol.*, **42**, 1040–1047.

Franklin, D. J., Brussaard, C. P. D. and Berges, J. A. (2006) What is the role and nature of programmed cell death in phytoplankton ecology? *Eur. J. Phycol.*, **41**, 1–14.

Glibert, P. M. and Burkholder, J. M. (2006) The complex relationships between increasing fertilization of the earth, coastal eutrophication and proliferation of harmful algal blooms. In Graneli, E. and Turner, J. (eds), *Ecology of Harmful Algae*. Springer-Verlag, Berlin, pp. 341–354.

Gurtu, V., Kain, S. R. and Zhang, G. (1997) Fluorometric and colorimetric detection of caspase activity associated with apoptosis. *Anal. Biochem.*, **251**, 98–102.

Harrison, P. J., Hu, M. H., Yang, Y. P. and Lu, X. (1990) Phosphate limitation in estuarine and coastal waters of China. *J. Exp. Mar. Biol. Ecol.*, **140**, 79–87.

He, R., Drury, G. E., Rotari, V. I., Gordon, A., Willer, M., Farzaneh, T., Woltering, E. J. and Gallois, P. (2008) Metacaspase-8 modulates programmed cell death induced by ultraviolet light and $H_2O_2$ in *Arabidopsis*. *J. Biol. Chem.*, **283**, 774–783.

Hoppe, H. G. (1983) Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. *Mar. Ecol. Prog. Ser.*, **11**, 299–308.

Huang, B. Q., Ou, L. J., Wang, X. L., Huo, W. Y., Li, R. X., Hong, H. S., Zhu, M. Y. and Qi, Y. Z. (2007) Alkaline phosphatase activity of phytoplankton in East China Sea coastal waters with frequent harmful algal bloom occurrences. *Aquat. Microb. Ecol.*, **49**, 195–206.

Hu, Z. X., Mulholland, M. R., Duan, S. S. and Xu, N. (2012) Effects of nitrogen supply and its composition on the growth of *Prorocentrum donghaiense*. *Harmful Algae*, **13**, 72–82.

Jacobson, M. D. (1996) Reactive oxygen species and programmed cell death. *Trends Biochem. Sci.*, **21**, 83–86.

Jauzein, C. and Erdner, D. L. (2013) Stress-related responses in *Alexandrium tamarense* cells exposed to environmental changes. *J. Eukaryot. Microbiol.*, **60**, 526–538.

Johnson, J. G., Janech, M. G. and Dolah, F. M. V. (2014) Caspase-like activity during aging and cell death in the toxic dinoflagellate *Karenia brevis*. *Harmful Algae*, **31**, 41–53.

Justic, D., Rabalais, N. N., Turner, E. R. and Dortch, Q. (1995) Changes in nutrient structure of river-dominated coastal waters: stoichiometric nutrient balance and its consequences. *Estuar. Coast Shelf Sci.*, **40**, 339–356.

Laun, P., Ramachandran, L., Jarolim, S., Herker, E., Liang, P., Wang, J. X., Weinberger, M., Burhans, D. T. *et al.* (2005) A comparison of the aging and apoptotic transcriptome of *Saccharomyces cerevisiae*. *FEMS Yeast Res.*, **5**, 1261–1272.

Lee, R. E. C., Brunette, S., Puente, L. G. and Megeney, L. A. (2010) Metacaspase Yca1 is required for clearance of insoluble protein aggregates. *Proc. Natl Acad. Sci. USA.*, **107**, 13348–13353.

Lesser, M. P. (2012). Oxidative stress in tropical marine ecosystems. In Abele, D., Vázquez-Medina, J. P. and Zenteno-Savín, T. (eds.), *Oxidative Stress in Aquatic Ecosystems. Vol. 1*. Blackwell Publishing Ltd, Malden, pp. 9–19.

Levasseur, M., Thompson, P. A. and Harrison, P. J. (1993) Physiological acclimation of marine phytoplankton to different nitrogen sources. *J. Phycol.*, **29**, 587–595.

Li, J., Glibert, P. M., Zhou, M. J., Lu, S. H. and Lu, D. D. (2009) Relationships between nitrogen and phosphorus forms and ratios and the development of dinoflagellate blooms in the East China Sea. *Mar. Ecol. Prog. Ser.*, **383**, 11–26.

Liu, L. S., Zhuo, J., Zheng, B. H., Cai, W. Q., Lin, K. X. and Tang, J. L. (2013) Temporal and spatial distribution of red tide outbreaks in the Yangtze River Estuary and adjacent waters, China. *Mar. Pollut. Bull.*, **72**, 213–221.

Lu, D. D. and Goebel, J. (2001) Five red tide species in genus Prorocentrum including the description of *Prorocentrum donghaiense* Lu Sp. Nov. from the East China Sea. *Chin. J. Oceanol. Limnol.*, **19**, 337–344.

Lu, D. D., Goebel, J., Qi, Y. Z., Zou, J. Z., Han, X. T., Gao, Y. H. and Li, Y. G. (2005) Morphological and genetic study of *Prorocentrum donghaiense* Lu from the East China Sea, and comparison with some related *Prorocentrum* species. *Harmful Algae*, **4**, 493–505.

Lu, D. D., Qi, Y. Z., Jeanette, G., Zou, J. Z. and Gao, Y. H. (2003) Redescription of *Prorocentrum donghaiense* Lu and comparison with relevant *Prorocentrum* species. *Chin. J. Appl. Ecol.*, **14**, 1060–1064 (In Chinese).

Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K. *et al.* (2002) A caspase-related protease regulates apoptosis in yeast. *Mol. Cell*, **9**, 911–917.

Murphy, J. and Riley, J. P. (1962) A modified single solute method for determination of phosphate in natural waters. *Anal. Chem. Acta*, **27**, 31–36.

Orellana, M. V., Pang, W. L., Durand, P. M., Whitehead, K. and Baliga, N. S. (2013) A role for programmed cell death in the microbial loop. *PLoS ONE*, **8**, e62595.

Ou, L. J., Wang, D., Huang, B. Q., Hong, H. S., Qi, Y. Z. and Lu, S. H. (2008) Comparative study of phosphorus strategies of three typical harmful algae in Chinese coastal waters. *J. Plankton Res.*, **30**, 1007–1017.

Pfaffl, M. W., Graham, W. H. and Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.*, **30**, 1–10.

Qi, Y. Z. and Wang, Y. (2003) What the *Prorocentrum* species should be? A review on identification of a *Prorocentrum* species from the East China Sea. *Chin. J. Appl. Ecol.*, **14**, 1188–1190 (In Chinese).

Ray, J. L., Haramaty, L., Thyrhaug, R., Fredricks, H. F., Benjamin, A. S., Mooy, V., Larsen, A., Bidle, K. D. *et al.* (2014) Virus infection of *Haptolina ericina* and *Phaeocystis pouchetii* implicates evolutionary conservation of programmed cell death induction in marine haptophyte–virus interactions. *J. Plankton Res.*, **36**, 943–955.

Richie, D. L., Miley, M. D., Bhabhra, R., Robson, G. D., Rhodes, J. C. and Askew, D. S. (2007) The *Aspergillus fumigatus* metacaspases CasA and CasB facilitate growth under conditions of endoplasmic reticulum stress. *Mol. Microbiol.*, **63**, 591–604.

Royall, J. A. and Ischiropoulos, H. (1993) Evaluation of 2′, 7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular $H_2O_2$ in cultured endothelial cells. *Arch. Biochem. Biophys.*, **302**, 348–355.

Segovia, M. and Berges, J. A. (2009) Inhibition of metacaspase activities prevents the appearance of reactive oxygen species and dark-induced apoptosis in the unicellular chlorophyte *Dunaliella tertiolecta*. *J. Phycol.*, **45**, 1116–1126.

Segovia, M., Haramaty, L., Berges, J. A. and Falkowski, P. G. (2003) Cell death in the unicellular chlorophyte *Dunaliella tertiolecta*. A hypothesis on the evolution of apoptosis in higher plants and metazoans. *Plant Physiol.*, **132**, 99–105.

Suzuki, N. and Mittler, R. (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiol. Plantarum.*, **126**, 45–51.

Thamatrakoln, K., Korenovska, O., Niheu, A. K. and Bidle, K. D. (2012) Whole-genome expression analysis reveals a role for death-related genes in stress acclimation of the diatom *Thalassiosira pseudonana*. *Environ. Microbiol.*, **14**, 67–81.

Thornberry, N. A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science*, **281**, 1312–1316.

Tsiatsiani, L., Van Breusegem, F., Gallois, P., Zavialov, A., Lam, E. and Bozhkov, P. V. (2011) Metacaspases. *Cell Death Differ.*, **18**, 1279–1288.

Uren, A. G., O'Rourke, K., Pisabarro, M. T., Seshagiri, S., Koonin, E. V. and Dixit, V. M. (2000) Identification of paracaspases and metacaspases: two ancient families of metacaspase proteins, one of which plays a key role in MALT lymphoma. *Mol. Cell.*, **6**, 961–967.

Vardi, A., Eisenstadt, D., Murik, O., Berman-Frank, I., Zohary, T., Levine, A. and Kaplan, A. (2007) Synchronisation of cell death in a dinoflagellate population is mediated by an excreted thiol protease. *Environ. Microbiol.*, **9**, 360–369.

Vardi, A., Haramaty, L., Benjamin, A. S., Mooy, V., Fredricks, H. F., Kimmance, S. A., Larsen, A. and Bidle, K. D. (2012) Host-virus dynamics and subcellular controls of cell fate in a natural coccolithophore population. *PNAS*, **109**, 19327–19332.

Vercammen, D., Declercq, W., Vandenabeele, P. and Van Breusegem, F. (2007) Are metacaspases caspases? *J. Cell Biol.*, **179**, 375–380.

Wang, B. D., Wang, X. L. and Zhan, R. (2003) Nutrient conditions in the Yellow Sea and the East China Sea. *Estuar. Coast. Shelf Sci.*, **58**, 127–136.

Wolfe-Simon, F., Starovoytov, V., Reinfelder, J. R., Schofield, O. and Falkowski, P. G. (2006) Localization and role of manganese superoxide dismutase in marine diatoms. *Plant Phys.*, **142**, 1701–1709.

Yang, G., Liu, Y. and Li, B. (2008) Death of *Prorocentrum donghaiense* may be a programmed autolysis. *J. Ocean. Univ. Chin.*, **7**, 276–280.

Zhang, X., Yang, G., Liu, Y., Yu, W., Pan, K., Li, R. X. and Zhu, M. (2006) Induction of programed cell death in aging *Prorocentrum donghaiense* cells as was evidenced preliminarily by the identification of associated transcripts. *Acta Biol. Hung.*, **57**, 473–483.

Zhou, M., Yan, T. and Zou, J. (2003) Preliminary analysis of the characteristics of red tide areas in Changjiang River estuary and its adjacent sea. *Chin. J. Appl. Ecol.*, **14**, 1031–1038 (In Chinese).

Zuppini, A., Andreoli, C. and Baldan, B. (2007) Heat stress: an inducer of programmed cell death in Chlorella saccharophila. *Plant Cell Physiol.*, **48**, 1000–1009.

Zuppini, A., Gerotto, C. and Baldan, B. (2010) Programmed cell death and adaptation: two different types of abiotic stress response in a unicellular chlorophyte. *Plant Cell Physiol.*, **51**, 884–895.