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ORIGINAL ARTICLE

Expression of death-related genes and reactive oxygen species production in *Skeletonema tropicum* upon exposure to the polyunsaturated aldehyde octadienal

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ABSTRACT

The effects of 4*E*/*Z*-octadienal (OCTA) on *ScDSP-1* and *ScDSP-2* gene expression and reactive oxygen species (ROS) production were investigated in the marine diatom *Skeletonema tropicum* (formerly *costatum*) using qRTPCR and flow cytometry. *ScDSP-1* and *ScDSP-2* genes have been previously shown to be involved in cell death in ageing cells and in response to photosynthetic stress. OCTA induced a differential, concentration-dependent *DSP* gene expression associated to ROS production, 821.6 and 97.7 folds higher for *ScDSP-1* and *ScDSP-2*, respectively. Among the concentrations tested, only 8 μM OCTA, which caused a reduction of 50% in cell concentrations at 24 h, was able to elicit an expression pattern consistent with a signalling role. Interestingly, only intermediate levels of reactive oxygen species (ROS) (*i.e.*, 1.5±0.1 increase) were observed to be elicited by such concentration. These results suggest that ROS are key components of the molecular cascade triggered by polyunsaturated aldehydes (PUA) and leading to cell death. This could have implications for bloom final stages at sea, where PUA may act as effectors of diatom population dynamics through ROS acting as modulators.

Key words: Diatom; chemical ecology; bloom evolution; Programmed cell death; ROS.

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INTRODUCTION

Marine diatoms are some of the most successful groups of unicellular eukaryotic photosynthetic organisms, forming massive blooms in coastal and open waters and having a key role in global marine primary production (Armbrust, 2009). Elucidating the mechanisms that regulate diatom bloom dynamics and mainly their decay determinants is therefore fundamental to understand nutrient and carbon cycling so as energy flow at the global as well as the local scale.

Programmed cell death (PCD) is an active, self-controlled death mechanism well known in higher organisms which has been also detected in unicellulars (Bidle *et al.*, 2007; Segovia, 2011). However, its occurrence in unicellular organisms is still considered a matter of ongoing debate (Deponte, 2008; Jimenez *et al.*, 2009), and the specific mode of action in which PCD is initiated remains to be elucidated. PCD has been suggested to act in phytoplankton populations to eliminate aged cells and to reduce population size in order to cope with nutrient limitation, for instance, in the final stages of blooms (Bidle and Falkowski, 2004; Vardi *et al.*, 1999, 2006, 2007). After a certain threshold of limitation of environmental factors (mainly nutrients), PCD induces the massive decay of the bloom, sometimes through massive production of resting or resistance stages (Ellegaard *et al.*, 2013; McQuoid *et al.*, 2002). These factors include nitrogen and light limitation (Berges and Falkowski, 1998), iron starvation (Bidle and Bender, 2008; Thamatrakoln *et al.*, 2012), virus infection (Bidle *et al.*, 2007; Vardi *et al.*, 2009), CO_2 limitation (Vardi *et al.*, 1999) and several secondary metabolites (Cervia *et al.*, 2009; Costas *et al.*, 1993). Reactive oxygen species (ROS) are often involved in inducing PCD in reaction to a stress factor in a wide range of organisms and cell lines, from bacteria to mammalians and plants (Andrianasolo *et al.*, 2007; Jones, 2008; Kuwabara *et al.*, 2008; Lam, 2008; Rockenfeller and Madeo, 2008; Scherz-Shouval and Elazar, 2007), mainly at intermediate concentrations. In fact, several cell reactions to ROS strongly depend upon the ROS concentration and may range from production of antioxidant compounds to necrotic cell death (Chandra *et al.*, 2000), or PCD (Costas *et al.*, 1993; Vardi *et al.*, 2009). Apart from ROS concentrations, several variables come into play by modulating and regulating the cell response to ROS. These include the cell type, the stress type, its magnitude and its duration in time (Martindale and Holbrook, 2002).

Different case studies report the co-occurrence of ROS and PCD in marine phytoplankton and suggest a correlation between them (Butow *et al.*, 1997; Segovia and Berges, 2009; Vardi *et al.*, 1999). In the dinoflagellate *Peridinium gatunense*, PCD leading to the collapse

of the algal population is mediated by oxidative stress induced by carbon dioxide (CO_2) limitation (Vardi *et al.*, 1999). Similarly, ROS-mediated cell death was observed at the end of a bloom of the same species, despite an increase in antioxidant activity (Butow *et al.*, 1997). PCD-like cell death concomitant with accumulation of ROS is also reported to be induced by the toxic cyanobacterium *Microcystis* sp. (Vardi *et al.*, 2002), or H_2O_2 (Vardi *et al.*, 2007). In the chlorophyte *Dunaliella tertiolecta,* dark-induced PCD, involving the activity of caspase-like enzymes has been linked to an increase in ROS production (Segovia and Berges, 2009). In the prymnesiophyte *Emiliana huxley* viral infection has been associated to an enhanced production of ROS, possibly leading to PCD (Evans *et al.*, 2006).

Interestingly, the secondary metabolite Euplotin C derived from the marine ciliated protist *Euplotes crassus*, is found to induce PCD in the congeneric *Euplotes vannus*, which does not produce this metabolite (Cervia *et al.*, 2009). This suggests that specific secondary metabolites can play an ecological role in broadening phytoplankton niche size through different mechanisms, including PCD (Cervia *et al.*, 2009).

In the marine diatom *Phaeodactylum tricornutum*, the exposure to diatom-derived secondary metabolites, polyunsaturated aldehydes (PUA), results in an altered expression of metacaspases through the overexpression of a gene associated to NO generation (Vardi *et al.*, 2008). In the marine diatom *Thalassiosira weissflogii*, PUA were shown to induce PCD-like cell death, suggesting for a role in activating the cell death cascade, therefore regulating population dynamics and species succession (Casotti *et al.*, 2005). In *Phaeodactylum tricornutum* it is speculated that a sophisticated stress surveillance system exists, involving NO and Calcium, in response to PUA, which could lead to bloom termination and population-level cell death at specific PUA concentrations (Vardi *et al.*, 2006). Even PUA precursors, the polyunsaturated fatty acids (PUFAs), have been reported to inhibit growth of *Candida albicans* and *Candida dubliniensi* by inducing ROS accumulation and consequent PCD (Thibane *et al.*, 2012).

In the marine diatom *Skeletonema tropicum* (formerly *costatum*) two genes have been identified, coding for two identical Ca-regulated proteins, named *S. costatum* Death-Specific Proteins (*ScDSP-1* and *ScDSP-2*) (Chung *et al.*, 2005), which are both composed of a transmembrane domain and a pair of EF-hand motifs (Luan *et al.*, 2002; Means and Dedman, 1980). Both *ScDSP* gene expressions were shown to be strongly upregulated in aging cells and also during light stress. Their expression is correlated to DNA fragmentation, suggesting for a possible role of the encoded protein in the signal transduction of stress to the cell death machinery (Chung *et al.*, 2005, 2008). In particular, *ScDSP* expression has been related to the blockage of the electron flow between PSII and cytochrome b_6f and it was found to be NO dependent (Chung *et al.*, 2008). Moreover, two *DSP*-like proteins (TpDSP1 and TpDSP2), have been recently identified in the marine diatom *Thalassiosira pseudonana* and have been shown to be upregulated both under iron limitation and starvation, suggesting for a role in Fe-induced stress and consequent death (Thamatrakoln *et al.*, 2012). However, they have also recently been shown to enhance growth under Fe limitation and to be controlled by light intensity (Thamatrakoln *et al.*, 2013).

In this study we report the gene expression of the two death-related genes *ScDSP-1* and *ScDSP-2* in ST exposed to the polyunsaturated aldehyde octadienal, and relate it to ROS production. The overall aim is to relate cell death to chemical stress induced by PUA and to advance hypotheses on bloom decay mechanisms at sea.

METHODS

Experimental setup and cultures

A unialgal culture of *Skeletonema tropicum* Cleve (formerly *costatum*) was grown at 23°C on a 12h-12h light-dark cycle under a photon flux density of 110 µmol quanta m^{-2} s^{-1} (Chung *et al.*, 2005). Natural seawater, amended with f/2 nutrients (Guillard, 1975) was used as medium. For gene expression measurements, *S. tropicum* was grown in 10 L polycarbonate carboys (Nalgene). When the culture reached the late exponential phase of growth (LE, Fig. 1), cells were transferred into 2 L polycarbonate bottles (Nalgene) and treated with different concentrations of 4*E*/*Z*-octadienal (OCTA) or H_2O_2. Samples were collected at 1,2,3,4,5 and 1,2,3,4,12 days since the beginning of the

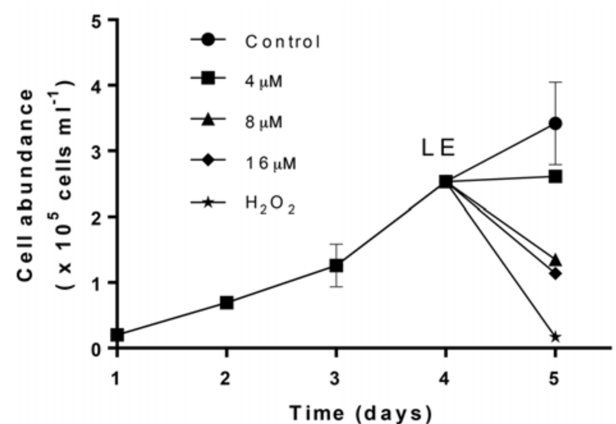

Fig. 1. Effect of OCTA and H_2O_2 on cell concentrations of *S. tropicum*. LE is the time point indicated as Late Exponential growth phase. Data are means of triplicates ±SD. When the bar is not visible, it is smaller than the symbol.

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experiment for cell counts and total RNA extraction, respectively. Cell concentrations were determined by using a Sedgwick-Rafter counting chamber (Hausser Scientific Partnership). At least 200 cells from each sample were counted using a light microscope (BX60; Olympus, Center Valley, USA) at 200× magnification. Growth rates were calculated using the following equation:

$$\mu=\ln\left[(N_1/N_0)/t\right] \quad \text{(eq. 1)}$$

where N_0 and N_1 are cell density at the start and the end of the growth period, and t is the time between measurements (in days). Since no dead *vs* alive cells assay has been used, it is possible that the cells counts reported in the study might include damaged or dying cells.

Gene expression experiments were performed using three OCTA concentrations, namely the concentration which caused a reduction of 50% in cell concentration at 24 h, half this concentration and twice this concentration (4, 8 and 16 μM). For ROS detection, three independent cultures were grown in 1 L glass flasks until the late exponential growth phase. Cells were harvested and directly exposed to the same concentrations of OCTA used for gene expression measurements and analysed through flow cytometry as described below. The controls were dye-loaded samples processed exactly as the other samples except for PUA inoculation.

Each experiment was performed at least twice, but generally three times, *i.e.* the data presented are the results of biological replicates.

Chemicals

The PUA used in this study was 2*E*,4*E*/*Z*-octadienal (OCTA, from Sigma Aldrich Inc. Milan, Italy). This was chosen because it is the main and most commonly produced PUA by diatoms, and, in particular, by *Skeletonema* spp. (Wichard *et al.*, 2005). The concentrations used were chosen accounting for multiples of the concentration causing a reduction of 50% in cell concentrations at 24h, as empirically determined for *S. tropicum* (data not shown) resulting to be 8 μM. The reference value of 8 μM has been chosen also based on Ribalet *et al.*, (2007), where 8 μM represented the EC_{50} for growth in the congeneric species *S. marinoi*. OCTA working solutions were dissolved in HPLC-grade pure Methanol (JT Baker, Phillipsburg, NJ, USA) at room temperature. Since Methanol can be toxic, it was tested on growing cultures and cultures showed no reaction up to 7 microliters per mL. Subsequently, and conservatively, no more than 4 microliters of Methanol plus OCTA were ever inoculated into the cultures. Before incubation, the exact OCTA concentration in the working solution was assessed spectrophotometrically (Hewlett-Packard 8453- Hewlett-Packard Co., Palo Alto, CA, USA) by measuring absorption at 274 nm and a specific molar absorption coefficient of 31,000 (Pippen and Nonaka, 1958). Hydrogen peroxide (H_2O_2, 30 wt. % from Sigma Aldrich, Milan, Italy) was used as positive control, after dilution 1/200 in filtered seawater and added to samples as a 50 μL:1 mL ratio.

The ROS scavenger, 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL, Sigma-Aldrich, Milan, Italy) was used as negative control, so as sodium diethyldithiocarbamate trihydrate (DETC, Sigma-Aldrich), an inhibitor of superoxide dismutase (SOD) at a final concentration of 5 mM and 1 mM, respectively, in filtered sea water (FSW). Samples with DETC were incubated for 120 min prior to PUA addition. The ROS-sensitive dye dihydrorodamine 123 (DHR, Molecular Probes, Leiden, NL) (5 mM stock in DMSO) was used to monitor ROS production at the single-cell level. DHR can be oxidized by different ROS, including hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), to form the fluorescent derivative rhodamine 123 (excitation WL 492-495 nm, emission WL=517-527 nm), and is commonly used to investigate oxidative stress *in vivo* (Ischiropoulos *et al.*, 1999). DHR has been previously used in different microalgal species to measure oxidative burst (Jamers *et al.*, 2009; Vardi *et al.*, 1999). Samples were incubated with both 10 μM DHR and PUA in the dark at room temperature and analysed using flow cytometry (see below).

Flow cytometry

To quantify ROS production *in vivo*, a flow cytometer (FACScalibur, Becton Dickinson, Palo Alto, CA, USA) equipped with a 488-nm laser as excitation source and filtered sea water as sheath was used. A 530/30-nm beam-pass emission filter was used for detection of DHR-derived green fluorescence. Both the sheath fluid and the sample velocity (65 $\mu L min^{-1}$) were kept constant during all the experiments. As a trigger signal, red fluorescence was used with a threshold at channel 52. Red fluorescence was collected through a 650-nm long-pass filter and was also used as a proxy for chlorophyll cell fluorescence. Fluorescent beads (Coulter FlowSet Fluorospheres, Beckman Coulter, Fullerton, CA, USA) were used as internal standard. Green fluorescence values were normalized to the green fluorescence of the beads, and all data were expressed as fold changes of green fluorescence relative to the control values. Data acquisition (10^4 cells on average for each sample) was performed using CellQuest software (Becton-Dikinson). Data analysis was performed using FCS4 Express (De Novo Softwares, Los Angeles, CA, USA).

Total RNA extraction

Two-hundred mL of culture were collected through a filtration system using a 2-μm pore size polycarbonate filter (Nucleopore; Whatman, Maidstone, UK), placed over a

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GF/F glass microfiber filter (Whatman). Filtration never lasted longer than 30 min. Cells were then resuspended in 0.7 mL guanidine isothiocyanate buffer (RLT buffer, Qiagen, Valencia, CA, USA) containing 1% β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA), immediately frozen at -80°C, and stored for a few days. After disrupting the cells by sonication on ice (Sonicator ultrasonic processor XL, Heat System Ultrasonics, Farmingdale, NY, USA), total RNA was extracted using the silica-membrane spin column included in the RNeasy Plant Mini Kit (Qiagen). The residual genomic DNA was removed by an additional treatment with the RNase-Free DNase Set (Qiagen) according to the manufacturer's instruction. Total RNA concentration and purity were determined with a spectrophotometer (ND-100; NanoDrop Technologies, Wilmington, DE, USA) by measuring absorption at 260 and 280 nm.

Reverse transcription and Real time quantitative polymerase chain reaction

One µg of total RNA was treated with DNase I and reverse transcribed into first-strand complementary DNA (cDNA) fragments by using random hexamer oligonucleotides and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The conditions for the retro-transcription were set as in Chung *et al*. (2005): 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Real time quantitative polymerase chain reactions (Q-PCR) were performed using 2× SYBR Green PCR master mix (Applied Biosystems) with 250 nM of each of the forward and reverse primers. The nucleotide sequences of all primer pairs used in the Q-PCRs are indicated in Tab. 1. The GeneAmp 7000 sequence detection system (Applied Biosystems) was used to carry out all the reactions. The threshold cycle at which the fluorescence intensity exceeded a preset threshold was used to calculate the target gene mRNA and 18S rRNA expression levels. The RNA molar ratio of *ScDSP* mRNA and 18S rRNA was calculated using the formula described in Chung *et al.*, (2005), and the data normalized to the control values. The levels of *ScDSP* mRNA expression were expressed as fold changes of mRNA abundance relative to the control. All data reported are means of three technical replicates but the experiments were repeated at least twice.

Statistical analyses

Pairwise comparisons among treatments and control were assessed by Student's *t*-test using the Excel spreadsheet (Microsoft, Redmond, Washington, MA, USA).

RESULTS AND DISCUSSION

Cultures were affected by OCTA exposure. 16 µM OCTA reduced cell concentrations to 51.17% of initial values after 24 h (Fig. 1; Tab.2). H_2O_2 also strongly affected cell concentrations which decreased to 50% as early as 2 h after inoculation and to zero 24 h later (Fig. 1).

ScDSP-1 and *ScDSP-2* gene expression and ROS levels increased in *S. tropicum* cultures exposed to different concentrations of OCTA. A peak in *ScDSP-1* and *ScDSP-2* expression was visible as early as 1 h after exposure and its intensity for cells in LE exposed to the concentration of 8 µM OCTA was ca. 822 and 98 folds that in the control for *ScDSP-1* and *ScDSP-2*, respectively (Fig. 2 a,b). Gene expression decreased afterward and showed no further peak until the end of the experiment. In the same strain, an increase in *ScDSP* expression has been previously reported when cultures entered senescence (Chung *et al.*, 2005) and also in response to a light stress involving the blockage of electron flow at the level of the photosystem II (Chung *et al.*, 2008). The latter also involved NO increase in the gene regulation and involvement of a PCD machinery (Chung *et al.*, 2008). Overall, these results provide evidence that the *DSP* genes have a role in regulating the molecular mechanism of mortality in *S. tropicum* under stress.

In our experiments, gene expression was highest at the concentrations where ROS production was intermediate, suggesting that there exists a threshold for the activation of the two death-related genes investigated. A peak in ROS was observed 20 min after inoculation with OCTA (Fig. 3), followed by a decrease. The intensity of the peak

Tab. 1. Primer pairs used for the qRT PCR reactions, as in Chung *et al.* (2008).

Target gene	Primer name	Nucleotide sequence (5'-end to 3'-end)
ScDSP-1	*ScDSP*-SG-F	GAACA AGCAA ACTGC ACTCG TC
	ScDSP-SG-R	GTCAA GAATG TTGGT CGTCG CG
ScDSP-2	*ScDSP*-SG-F	GAACA AGCAA ACTGC ACTCG TC
	ScDSP2-SG-R	GTAGG CATCT GCTAT TCTTT CTG
18S rRNA	Ske-18S-F	GAATT CCTAG ATATC GCAGT TCATC
	Ske-18S-R	GCTAA TCCAC AATCT CGACT CCTC

Tab. 2. Effect of OCTA on growth rate of *S. tropicum cultures* after 24 h. Initial concentration (t_0, *i.e.*, LE) was 2.53 10^5 cell mL^{-1}.

		Growth rate (day^{-1})
	Concentration (µM)	LE
OCTA°	0	0.30±0.10
	4	0.03±0.08
	8*	-0.63±0.39
	16	-0.80±0.22-
	16H_2O_2	2.70±0.35

LE, Late exponential growth phase (as in Fig. 1); °2*E*,4*E*/*Z*-octadienal; *concentration causing a reduction of 50% in cell concentrations at 24 h (see text). Data are means ±SD, n=3.

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was PUA concentration-dependent, and was highest for 16 µM OCTA (1.9±0.2 increase; P<0.001, n=3). Both the ROS scavenger Tempol and the SOD inhibitor DETC reduced the intensity of the peak, therefore supporting the conclusion that the peak was really due to an increase of ROS. No increase in DHR-derived green fluorescence was observed when methanol only was added to the cultures, excluding an artifact due to the solvent used (*data not shown*). Hence, it is suggested that cell death is triggered in *S. tropicum* only by specific PUA concentrations, which are those causing a reduction of 50% in cell concentrations at 24 h (8 µM OCTA). This is supported by the observation that 8 µM OCTA induced intermediate levels of ROS production (1.5±0.1 increase in LE cultures), while a higher concentration (16 µM OCTA) elicited a considerably lower *ScDSP* expression, even though it induced a higher ROS production (1.9±0.2 increase). This could be related to different molecular signals triggered in the two cases.

In our experiments, while the addition of H_2O_2 to the cultures induced an increased *DSP* gene expression, the levels were not as high as those elicited by OCTA, even though the DHR-ROS derived green fluorescence was the highest observed (7.5±0.9 times the control at 40 min after exposure, *not shown*). This suggests that ROS could be important modulators in the pathway leading to cell death. Even though evidence for the occurrence of apoptotic-like features in *S. tropicum* in response to PUA was not provided in this study, it has been previously reported that PUA are able to induce apoptotic-like cell death in the marine diatom *Thalassiosira weissflogii* (Casotti *et al.*, 2005). In addition, in the marine diatom *Phaeodactylum tricornutum* PUA exposure induced overexpression of a gene associated to NO production (designated as PtNOA), that resulted in an altered expression of superoxide dismutase and an increased metacaspase activity, which are known factors in the stress responses and PCD pathway (Vardi *et al.*, 2008). Indeed, in our OCTA-exposed *S. tropicum* cultures, PUA had a toxic effect drastically affecting growth and inducing cell death.

ROS are generally indicated in the literature as PCD-inducing factors (Aldsworth *et al.*, 1999; Simon *et al.*, 2000). However, we suggest that a difference exists between the intracellular effectors of a PCD-like process (*e.g.*, NO and ROS), and other factors and stimuli that, causing an alteration of NO and ROS, are able to modulate the response leading to cell death. Based on the results reported here, it is proposed that PUA act as such factors, which induce modifications of the intracellular ROS levels, which then result in a differential activation of *ScDSP* genes. The successive reaction depends then on the amount of ROS produced and possibly on the cell physiological and growth status. This scenario is supported by the observation that the increase in *DSP* gene expression in our experiments was not linearly correlated to PUA concentrations. While it is possible that the experimental design was not appropriate to detect sudden peaks occurring at intermediate time points, it is unlikely that these lasted for shorter than 1 h. Indeed, when a further time point at 30 min after inoculation was included, the expression levels were not even comparable to the highest expression measured during the experiments (*data not shown*). Therefore,

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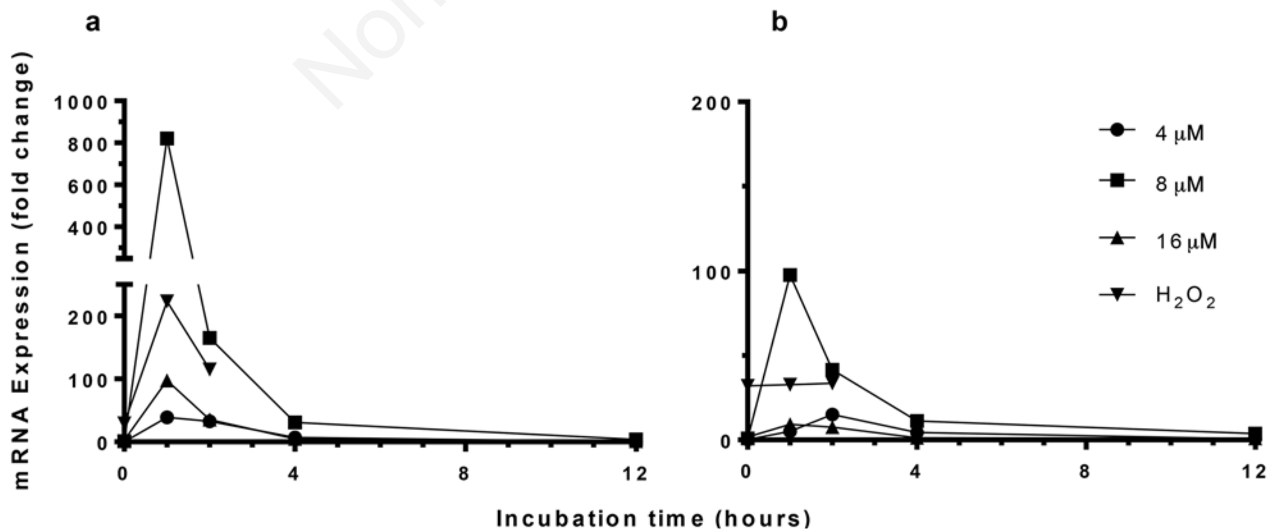

Fig. 2. *ScDSP1* (a) and *ScDSP2* (b) mRNA expression in *S. tropicum* cultures exposed to different OCTA concentrations and H_2O_2 in LE cultures. Data are expressed as mean of fold changes of mRNA abundance (relative to control) from technical triplicates. The experiments were repeated at least twice.

considering that a burst in ROS production always occurred 20 min after exposure to all the different PUA tested, it is possible to conclude that the concentration of 8 μM OCTA represents a threshold value below which the cell activates defences mechanisms to cope with the related oxidative stress. (Chung *et al.*, 2005, 2008).

Based on the results reported here, it is suggested that in the case of PUA-induced chemical stress, *DSP* genes expression is regulated by ROS. Although we cannot exclude a role for NO in the process, data from the congeneric *S. marinoi* show that ROS and not NO have a main role in the response to PUA in this diatom and probably in the congeneric *S. tropicum* (Gallina *et al.*, 2014). In addition, considered that *DSP* is reported to be a Ca^{2+} regulated protein (Chung *et al.*, 2005), it is then possible that in *S. tropicum* ROS are acting as Ca^{2+} mobilizing agents, as also happens in other biological systems (Gonzalez *et al.*, 2002; Mallilankaraman *et al.*, 2011; Wendehenne *et al.*, 2004).

CONCLUSIONS

The reported findings are only preliminary and need to be substantiated by further experiments aimed at proving a clear connection between ROS and DSP expression. In addition, direct data on PCD activation have to be acquired, possibly using the genomic approach used by Lauritano *et al.*, (2015). However, based on these preliminary results and combining them on the vast literature on PUA in diatoms, we propose that in *Skeletonema* spp. the ROS downstream response to PUA activates either a protection pathway (*i.e.*, antioxidant defence) or a cell death cascade either through activation of specific genes or through non-genetically controlled cell death, depending on the PUA used, its concentration, the time of exposure, the concentration of ROS produced and the physiological state of the cell. This has important implications for bloom regulation and population dynamics and induction of cell lysis, as instance, immediately after the peak stage of a diatom bloom, when cell concentrations are highest but environmental factors become limiting and do not support further growth (Vanboekel *et al.*, 1992).

It has been proposed that the evolution of PCD in unicellular organisms as a byproduct of selection might be explained by the possibility that PCD could be a maladaptive stress-induced process that can be selected under certain conditions to benefit only some organisms or part of a population (Nedelcu *et al.*, 2010). As related to this concept, if an intrapopulation signal (*e.g.*, PUA) triggers the production of PCD-inducing signal (*e.g.*, ROS), and if this intrapopulation signal-dependent death is beneficial for a part of the population (for instance, by fitting cell density to the environmental conditions), then PCD could be adaptively selected for and consequently evolve into altruistic adaptation (Nedelcu *et al.*, 2010). This hypothesis needs to be tested *in situ*, where PCD has been shown to exist, for instance in reaction to a viral attack (Bidle *et al.* 2007), supporting its role as population regulatory mechanism, or, triggering the production of survival forms such as cysts or resting stages, using high ROS levels as signals (Vardi *et al.*, 1999), so to assure perpetuation of the species later on, when the environment conditions become favourable again (McQuoid *et al.*, 2002).

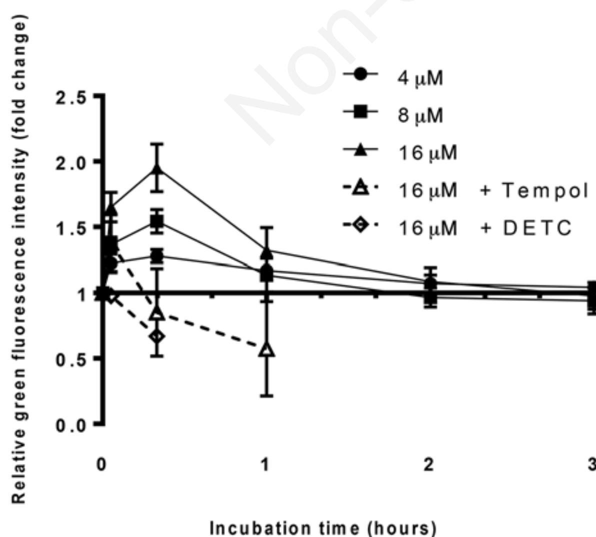

Fig. 3. ROS production in *S. tropicum* exposed to different concentrations of OCTA. Data are values of DHR123-derived green fluorescence normalized to the control values. Data are means ±SD from three biological replicates.

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