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# Insights into Stress-Induced Death Processes during Aging in the Marine Bloom-Forming Dinoflagellate *Karenia brevis*

**Yida Gao [1,2,*] and Deana L. Erdner [1]**

[1] Marine Science Institute, The University of Texas at Austin, Port Aransas, TX 78373, USA
[2] Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, St. Petersburg, FL 33701, USA
* Correspondence: yida.gao@utexas.edu

**Abstract:** Harmful algal blooms (HABs) globally affect marine ecosystems and human health. Significant attention has been paid to understanding the initiation processes of HABs, while much less is known of the mechanisms causing cell demise and bloom decline. Recent evidence reveals that programmed cell death (PCD) can be a possible pathway for HAB termination. However, it is still not clear how PCD expression varies with the growth of marine phytoplankton. In order to characterize how susceptibility to stress-induced cell death changes with the growth of marine harmful algae, we examined the prevalence of PCD markers during 48 h after the addition of $H_2O_2$ in dinoflagellate *Karenia brevis* cultures at the early-log, mid-log and stationary phase. The results show that acute susceptibility to cell death (before time 0.5 h) increased as cultures aged, reflecting a chronological decrease in stress acclimation abilities. However, the youngest cultures showed strong PCD expression and the fastest overall rate of cell loss within the first 24 h, hypothesized to result from the cell-density-dependent $H_2O_2$ detoxifying process. This research highlights the existence of PCD in HAB species and that aging marine phytoplankton are more susceptible to exogenous stress, which agrees with previous observations of significant PCD during bloom decline.

**Keywords:** harmful algal bloom; stress; programmed cell death; growth stages



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## 1. Introduction

Harmful algal blooms (HABs) have profound impacts on coastal areas around the world. Given their detrimental effects on marine ecosystems and human health, significant attention has been given to understanding the initiation processes of HABs. For toxic dinoflagellate *Karenia brevis*, a number of theories have been proposed for bloom generation, including the convergent bloom hypothesis [1], Saharan dust hypothesis [2] and migratory behavior hypothesis [3]. In contrast, much less is known of the mechanisms and processes leading to bloom decline and termination [4,5]. Consumption by zooplankton grazers and sinking have been long assumed to be the major cell loss pathways for marine phytoplankton [6]. However, increasing evidence shows that programmed cell death (PCD), an autocatalytic self-destruction process, may also contribute to the demise of natural phytoplankton blooms [7–9]. Previous research suggests that PCD in *Alexandrium catenella*, a dinoflagellate species prevailing at global temperate waters, could account for up to 14% of cell loss per day during the process of bloom termination [8].

Some studies have observed PCD in marine microalgae induced by external stress from irradiance, temperature, salinity and nutrients [10–14], while none have systematically assessed how stress-induced PCD may vary across growth stages. In cultures of marine phytoplankton, genes responsible for cell defense and stress acclimation are downregulated as cultures age [15]. Therefore, we hypothesized that cell mortality in response to external stress in marine microalgal populations would increase with the chronological age of the culture. All biotic and abiotic stress-induced PCD processes start with a burst of intracellular

reactive oxygen species (ROS; e.g., $H_2O_2$), which serves as signals to trigger caspase-like activity (cysteine-dependent aspartic acid proteases) and subsequent cell death [7,16]. Therefore, exogenous $H_2O_2$ has become a classic stimulus to conveniently and controllably induce downstream cellular cell death responses in a laboratory environment [5,17–20].

The marine dinoflagellate *K. brevis* accounts for frequent HAB events that affect human health and coastal economies in the Gulf of Mexico (GoM) [21]. *K. brevis* cells can produce and accumulate brevetoxins, which would lead to neurotoxic shellfish poisoning (NSP), a type of biological intoxication causing animal death and human sickness [22]. In order to investigate how susceptibility to stress-induced PCD varies with the age of marine microalgal cultures, we examined the prevalence of ROS, caspase-like activity and cell death with/without the addition of the strong oxidant $H_2O_2$ in the early-log phase, mid-log phase and stationary phase cultures of *K. brevis*. The only research on $H_2O_2$-induced PCD in *K. brevis* was conducted by Johnson et al. [5], who measured short-term (10 min) responses of the PCD cascade in log phase cultures after challenge with $H_2O_2$. Given the potential for a lagged cell death reaction to oxidative stress observed in other dinoflagellates [23], we monitored cell death responses for 48 h at different points across culture growth, with the aim of characterizing the PCD cascade at each growth stage.

## 2. Materials and Methods

### 2.1. Culture Conditions

Culture preparation was described in detail in Gao and Erdner [24]. *K. brevis* clone SP3 was inoculated at a starting density of 300 cells/mL in L1 medium [25] on Day 0. The inoculum culture was maintained at salinity 32 under 25 °C. The irradiance environment was set up as 90 μmol $m^{-2}$ $s^{-1}$ on a 12:12 light:dark cycle by white fluorescent lights. After four days of growth, 120 mL aliquots of culture were dispensed into each of the eighteen 200 mL flasks, which were labeled in triplicate as "early-log treatment", "early-log control", "mid-log treatment", "mid-log control", "stationary treatment" and "stationary control". This arrangement reduced repeated samplings of the same flask, as the unarmored structures of *Karenia* are very fragile and sensitive to frequent disturbance/shear force [26–28]. Another flask with 120 mL of culture was inoculated at the same time and maintained under the same conditions, and its cell densities were used as an indicator for the growth stages in other flasks. The growth curve of *K. brevis* cells in the "indicator" flask has been presented in Gao and Erdner [24]. The timing of the early-log, mid-log and stationary stages in the batch cultures were indicated by cell densities of approximately 4000 cells/mL, 9000 cells/mL and 18,000 cells/mL, respectively, from this "indicator" flask [24]. The cell densities relating to each growth stage were derived from previous growth curves of SP3 cultures incubated under the same environmental conditions.

### 2.2. Experimental Setup

Oxidative stress was induced by addition of 180 μM $H_2O_2$ (calculated final concentration) to early-log treatment, mid-log treatment and stationary treatment flasks on day 9, day 14 and day 21, respectively. The level of $H_2O_2$ addition was chosen from a preliminary test (0–260 μM) as a dose that caused death in some cells but did not obliterate them rapidly. The addition of $H_2O_2$ was made at time 0 h, which was set to three hours after the onset of the light phase. Treatments and their corresponding controls were sampled at 0.5 h, 6 h, 24 h, 30 h and 48 h after the $H_2O_2$ addition for measurement of cell densities and percentages of cell staining for cell death, ROS and caspase-like activity. This sampling frequency would allow us to capture a long-term cell death reaction to oxidative stress in *K. brevis*.

### 2.3. Measurement of Stress and Cell Death Markers

Measurement of cell death, ROS and caspase-like activity were described in detail in Gao and Erdner [24]. An epifluorescent microscope (BX41, Olympus, Tokyo, Japan) was used to observe green fluorescence generated by these reagents with a filter that has an excitation wavelength of 450–490 nm (band pass) and emission wavelength of 523 nm (long

pass). Replicate counts of 50–100 cells were determined for each parameter on each culture vessel, using a Sedgewick Rafter chamber.

#### 2.3.1. Cell Death Detection

Cell membrane permeability, which is a hallmark of cell death, was examined using SYTOX-green dye (S7020, Invitrogen, Carlsbad, CA, USA), because it can only access and bind to intracellular ds-DNA when the structure of the cell membrane is compromised. Cells in L1 medium were stained with 0.5 µM SYTOX-green dye and observed after 40 min of dark incubation at room temperature [24]. SYTOX percentages were calculated as: the number of cells with green fluorescence/the number of intact cells.

#### 2.3.2. ROS Production

Carboxy-$H_2$DCFDA (C400, Invitrogen) was used to detect intracellular ROS. This reagent primarily reacts with $H_2O_2$ and has greatly lower sensitivity with other forms of ROS (e.g., superoxide and singlet oxygen). A 100 µM working solution of carboxy-$H_2$DCFDA was prepared by dilution of 10 mM stock solution into phosphate-buffered saline (PBS). Sub-samples of algal cultures were mixed with the stock solution in a 9:1 ratio, making the final concentration of carboxy-$H_2$DCFDA 10 µM. Oxidation of carboxy-$H_2$DCFDA would generate green fluorescence, and it was examined after 20 min of dark incubation at room temperature. Percentages of cells with ROS were calculated as: the number of cells with green fluorescence/the number of intact cells.

#### 2.3.3. Activity of Caspase-like Enzymes

Caspase-like enzyme activity is a distinctive hallmark of PCD. Caspase-like activity was examined by a fluorescent inhibitor of caspases (FLICA) in Image-iT LIVE Green Poly Caspases Detection Kit (I35104, Invitrogen). Two ml aliquots of culture were collected, in duplicate, from each flask and centrifuged at 500× *g* for 5 min. The supernatant was replaced with 1× FLICA working reagent. After 60 min of dark incubation at room temperature, another round of centrifugation (500× *g*, 5 min) was conducted to remove the FLICA solution. Cells were resuspended in wash buffer and examined for caspase-like activity under the epifluorescence microscope. Percentage of cells with caspase-like activity was calculated as: the number of cells with green fluorescence/the number of intact cells.

### *2.4. Cell Density*

The densities of total morphologically intact cells were estimated in duplicate from each culture vessel and counted under the microscope using a Sedgewick Rafter chamber after Lugol's preservation. Live cell densities of each triplicate flask were calculated as: averaged total morphologically intact cells per mL × (1-averaged SYTOX proportion). The percentage of lysed cells within the first 0.5 h was calculated as (1-morphologically intact cells per mL in treatments at time 0.5 h/morphologically intact cells per mL in controls at time 0.5 h) × 100%.

### *2.5. Statistical Analysis*

Two-way repeated measure ANOVA (RM-ANOVA) was used to evaluate differences of PCD markers' prevalence and live cell densities between treatments and controls. The Bonferroni post hoc test was employed to determine significance of the difference in multiple comparisons. The $\alpha$ is equal to 0.05. All statistical analyses were performed using SigmaPlot 14.

## 3. Results

Oxidative stress caused by 180 µM $H_2O_2$ induced extensive cell death responses at all growth stages. In order to compare stress responses right before and after the addition of $H_2O_2$, the data from controls measured at time 0.5 h were used as a proxy for time 0 h in treatments, representing the cellular status right before the induction of oxidative stress.

Within the first 0.5 h after $H_2O_2$ addition, the mid-log and stationary phase cultures exhibited acute cell lysis, losing 26% and 63% of cells, respectively. In contrast, the early-log cultures showed no detectable cell lysis within this same time period. The first measurements of cell death markers occurred at 0.5 h after the addition of $H_2O_2$ and showed drastic increases in stress and cell death markers at all growth stages (Figure 1). During this short period of time (0.5 h), the proportion of cells with ROS increased by 64% in the early-log phase and 40% in mid-log phase (Figure 1A,B). In contrast, ROS prevalence did not change significantly in the stationary phase (80.68 ± 5.73% vs. 87.15 ± 2.59%; $p > 0.05$; Figure 1C). The baseline prevalence of ROS before $H_2O_2$ treatment increased with aging of the cultures (Figure 1A–C). Significant increases in the proportion of cells expressing caspase-like activity were observed in all growth stages after the addition of $H_2O_2$ ($p < 0.05$; Figure 1D–F). Almost 100% of cells displayed caspase-like activity after 0.5 h of treatment in the stationary phase, which was even higher than the ROS prevalence (Figure 1F). At time 0.5 h, the proportions of cells with caspase-like activity and ROS were similar in the early-log phase (~70%) and mid-log phase (~80%) (Figure 1A,B,D,E). The early-log phase and stationary phase cultures shared similar percentages of cells displaying permeable membranes at time 0.5 h (~30%; SYTOX-positive; Figure 1G,I). The mid-log phase cultures exhibited the highest cell death percentages (52.62 ± 7.22%; Figure 1H).

After the initial 30 min acute response phase, *K. brevis* cultures from all growth stages continued to lose cells but at different rates. In the early-log phase (Figure 1J), despite cells experiencing no lysis during the initial acute phase, more than 95% of cells counted at time 0 h were lost by time 24 h, with accompanying prevalence of caspase-like activity and heavy ROS load (Figure 1A,D). In contrast, live cell densities in the mid-log and stationary phases steadily decreased over a 48 h period (Figure 1K,L). This slower culture crash pattern was also reflected in the changes in mortality, as cell death percentages in these two phases were lower than early-log phase and tended to level off after the initial acute shock (Figure 1G–I). Although the prevalence of cells with ROS and caspase-like activity was somewhat variable after time 0.5 h, the expression of these two stress markers was generally significantly higher in treatments vs. controls across all growth stages ($p < 0.05$), except ROS prevalence in stationary phase ($p = 0.16$; Table 1).

While $H_2O_2$-treated cells showed a high prevalence of all three of the stress markers, cells in untreated control cultures also expressed ROS and caspase activity but without cell death (Figure 1, Table 1). ROS was detected in roughly half of the cells at the mid-log phase and stationary phase cells, compared with only 9% of cells in the early-log phase (Table 1). Caspase-like activity was common across growth stages ranging from 24% to 56% of control cells, while the SYTOX percentages in the control cultures remained near zero across all growth stages (Table 1).

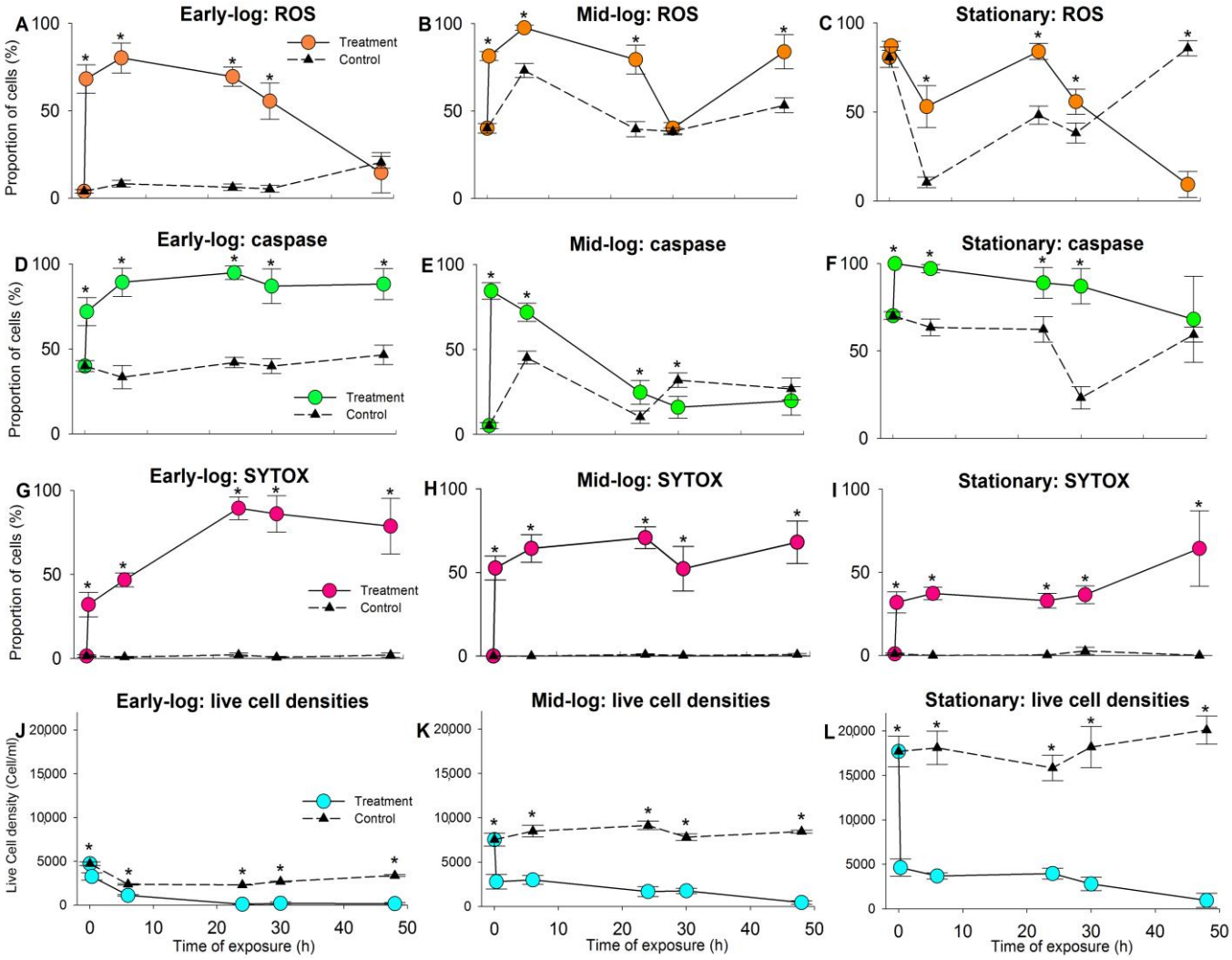


**Figure 1.** Cellular stress responses in *K. brevis* cells exposed to 180 µM $H_2O_2$ (circles) and their corresponding control cultures (triangles) at early-log phase (**A**,**D**,**G**,**J**), mid-log phase (**B**,**E**,**H**,**K**) and stationary phase (**C**,**F**,**I**,**L**). (**A**–**C**) Proportion of cells showing reactive oxygen species (ROS). (**D**–**F**) Proportion of cells showing caspase-like activity. (**G**–**I**) Proportion of cells stained with SYTOX. (**J**–**L**) Live cell density. Data points show average values obtained from triplicate cultures, and error bars show standard deviation of the replicates ($n$ = 6). Asterisks indicate a significant difference between treatments and controls ($p < 0.05$).

**Table 1.** Average percentages of cellular parameters in treatments and controls from all sampling time points during the 48 h experiment at early-log, mid-log and stationary phase. Error bars show standard deviation of the replicates ($n$ = 30). $p$-value between treatments and controls in each growth phase from RM-ANOVA analysis are also listed.

| | ROS (%) | | | Caspase-like Activity (%) | | | SYTOX-Positive (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Treatment** | **Control** | $p$ | **Treatment** | **Control** | $p$ | **Treatment** | **Control** | $p$ |
| Early log | 57.64 ± 14.35 | 8.87 ± 3.64 | <0.001 | 86.24 ± 8.58 | 40.34 ± 4.99 | <0.001 | 66.60 ± 14.94 | 1.42 ± 0.89 | <0.001 |
| Mid log | 76.54 ± 11.30 | 48.93 ± 7.55 | <0.001 | 43.38 ± 15.86 | 23.89 ± 8.40 | 0.003 | 61.59 ± 10.10 | 0.39 ± 0.41 | <0.001 |
| Stationary | 57.86 ±15.78 | 52.67 ± 14.89 | 0.16 | 88.26 ± 13.00 | 55.72 ± 9.78 | 0.002 | 40.84 ± 11.92 | 0.75 ± 1.05 | <0.001 |

## 4. Discussion

This study investigated how PCD responses to $H_2O_2$ stress vary as the cultures grow and age. In response to oxidative stress, the classic PCD cascade was observed in *K. brevis*. Acute susceptibility (within 0.5 h) to oxidative stress increased as cultures aged. In contrast, the overall time to complete culture crash was shortest in the youngest stage (early-log phase) and was accompanied by induction of PCD markers.

PCD could be induced by oxidative stress in *K. brevis* at all growth stages, as prevalence of cell death, ROS and caspase-like activity in treatments were significantly higher than controls after $H_2O_2$ addition. Various stressors can cause a burst of ROS production in microalgae to initiate the PCD cascade [9,10,29]. For example, $CO_2$ limitation during bloom development of the dinoflagellate *Peridinium gatunense* is thought to induce the transfer of photosynthetic electrons from $CO_2$ fixation to oxygen, causing extensive ROS accumulation and PCD induction [9,30]. The addition of catalase, which specifically detoxifies $H_2O_2$, inhibits cell death under $CO_2$ depletion, implicating the role of $H_2O_2$ as an active ROS to trigger PCD in phytoplankton [9,30]. Therefore, the application of external oxidative stress can reproduce cell death responses to general environmental stress [31]. PCD triggered by exogenous $H_2O_2$ has been documented in cyanobacteria, green alga and dinoflagellates (*K. brevis*) [5,17–20]. In the green alga *Micrasterias* addition of 5 mM $H_2O_2$ caused up to 20% mortality in 6 h with the appearance of caspase-like activity, chromatin condensation and ultrastructural changes in mitochondria, indicating the role of $H_2O_2$ in inducing PCD [18]. For *K. brevis,* Johnson et al. [5] observed a cell death pathway that exhibited heavy ROS load, caspase-like activity, DNA fragmentation and specific morphotypes 10 min after addition of $H_2O_2$ to mid-log phase cultures. Caspase-like proteases have been suggested as PCD executioners in phytoplankton under environmental stress, including $CO_2$ limitation, heat shock, UV irradiation and viral infection [9,12,14,28,32]. For example, Zuppini et al. [29] found that caspase inhibitors blocked PCD in green alga *Chlorella saccharophila* triggered by heat shock, demonstrating the involvement of caspase-like enzymes in this cell death process.

In addition to the PCD pathway, other cell death modes may occur in response to oxidative stress. Necrosis, a caspase-independent death pathway caused by acute and irreversible cellular injury from physical or mechanical stress [19,33], may contribute to some of the immediate lysis (before time 0.5 h) of *K. brevis* in the mid-log and stationary phases. Jauzein and Erdner [23] reported that in *Alexandrium tamarense* PCD was not activated in response to 400 μM $H_2O_2$, but that the observed mortality may result from the added oxidant directly causing cell damage.

Short-term stress responses were generally similar at different growth stages in our experiments: sharp increases in ROS (except for stationary phase), caspase-like activity and cell death prevalence at time 0.5 h following exposure. ROS prevalence did not change significantly in the stationary phase at time 0.5 h, as background levels were already high in the controls. While the baseline cell death prevalence was low at all growth stages, the baseline level of ROS and the extent of rapid cell lysis (before time 0.5 h) increased with the aging of cultures. Stronger vulnerability to $H_2O_2$ in old cultures has been observed in freshwater dinoflagellate *P. gatunense*, where self-excreted thiol protease may sensitize cells to oxidative stress and act as a mechanism of cell death synchronization for aged dinoflagellate populations in lakes [34]. The observed increasing acute cell mortality may also be related to biochemical changes in physiology as cultures age. Genes responsible for cell defense and stress acclimation including DNA repair, heat shock proteins and pentatricopeptide repeat proteins are significantly downregulated during the stationary phase in *K. brevis*, which may translate to increased vulnerability to external stress [15,35]. A comparison of gene expression before/after the induction of acute oxidative stress at different growth stages may help further explain the change in susceptibility as cultures age, and the connection between reduced defense response and oxidative-stress-induced mortality.

Although acute susceptibility to oxidative stress (before time 0.5 h) increased with the ages of cultures in *K. brevis*, the youngest cultures (early-log phase) took the shortest time to totally break down. After the initial phase (0.5 h), the cultures in the early-log phase

showed continued increases in the prevalence of caspase activity and SYTOX labelling, indicating a fast and intense PCD activation that caused an almost complete loss of cells during the first 24 h. In the mid-log phase, the percentages of cells with caspase-like activity and SYTOX rapidly increased from nearly 0% to approximately 90% and 50% at time 0.5 h, respectively, (Figure 1E,H), suggesting that 40% of cells at time 0.5 h may be caspase-positive but still alive. They may exemplify a slower PCD process, which is supported by a more gradual increase in STYOX percentages and a slower rate of cell loss, which continued over the next 48 h (Figure 1H,K). Caspase-like enzyme in dead cells could become inactive over time, while intracellular double-strand DNA would be more stable and retain SYTOX staining, which may explain the uncoupling of caspase and SYTOX prevalence in the mid-log phase (Figure 1E,H). Similar mild cell death was observed in stationary phase after 0.5 h, and the constantly high caspase prevalence may be partially explained by the strong baseline level of caspase-like activity observed in controls serving some housekeeping functions [4,7,24,36].

The observed delayed culture crash and slower PCD progression at the latter two growth phases may be related to cell-density-dependent $H_2O_2$ detoxifying processes. Previous research has suggested that the rate of removal of extracellular $H_2O_2$ increases with cell densities due to stronger ROS scavenging in denser cell populations [37,38]. Therefore, after the acute initial phase, the surviving cells from the cultures with higher starting cell densities may experience much lower external $H_2O_2$ concentrations, which would lead to a slower cell death progression [19,39]. In addition, just as kelps can secrete iodide under oxidative stress to detoxify external oxidants [40], *K. brevis* may also have some mechanisms to produce antioxidants in response to elevated external $H_2O_2$ concentrations. With more live cells producing antioxidants in a similar culture volume during the acute initial phase, the concentrations of oxidant in the culture of mid-log and stationary phase would be lower, therefore effectively delaying the breakdown of cultures.

An analysis of the variations and functions of ROS and caspase-like activity from the untreated control cultures alone was previously described in Gao and Erdner [24]. In addition to their functions in the PCD cascade, ROS and caspase-like activity also serve some non-death roles as expressed in the controls (Figure 1A–F) [24]. Strengthened ROS production has been reported during active growth of phytoplankton, which may be due to enhanced chloroplastic or mitochondrial activity [41–43]. Previous research has suggested some housekeeping roles of caspase-like activity in marine micro-organisms, such as proteostasis, cellular division and regulation of chronic aging in cultures [4,23,36,44]. In our research, after addition of $H_2O_2$, the prevalence of ROS, caspase-like activity and dead cells generally increased compared with the controls in all growth stages, indicating the expression of PCD and the cell death roles of ROS and caspase-like activity triggered by external oxidative stress.

## 5. Conclusions

The application of the model oxidative stressor $H_2O_2$ to *K. brevis* revealed general downstream cell death responses. Our results show that acute vulnerability to cell death (within 0.5 h) under oxidative stress increased as cultures of marine phytoplankton aged, which may reflect a chronological decrease in stress acclimation abilities. However, the youngest cultures showed the highest overall rate of cell loss within the first 24 h, hypothesized to result from lower overall activity of cell-density-dependent $H_2O_2$ detoxifying processes. This research highlights that PCD exists in HAB species and that aging marine phytoplankton populations are more susceptible to acute exogenous stress, which may facilitate bloom decline and termination.

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