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# INHIBITION OF CASPASE-LIKE ACTIVITIES PREVENTS THE APPEARANCE OF REACTIVE OXYGEN SPECIES AND DARK-INDUCED APOPTOSIS IN THE UNICELLULAR CHLOROPHYTE *DUNALIELLA TERTIOLECTA*[1]

*María Segovia*[2]

Department of Ecology, Faculty of Sciences, University of Málaga, Bulevar Louis Pasteur s/n, 29071-Málaga, Spain

*and John A. Berges*

Department of Biological Sciences, University of Wisconsin-Milwaukee, P.O. Box 413, Milwaukee, Wisconsin 53201, USA

**When the chlorophyte alga *Dunaliella tertiolecta* Butcher is placed in darkness, a form of programmed cell death with many similarities to apoptosis is induced, including the induction of caspase-like proteases. Many uncertainties about the regulation and mediators that participate in the process remain. To examine the relationship between caspase-like activities and different apoptotic events (i.e., phosphatidylserine [PS] translocation), increases in membrane permeability and numbers of dead cells revealed by SYTOX-green staining, and the generation of reactive oxygen species (ROS), we used the broad-range caspase inhibitor Boc-D-FMK to block the activity of the whole class of caspase-like proteins simultaneously. In the presence of the inhibitor, ROS were not produced, and cells did not die. Loss of membrane asymmetry, indicated by external labeling of PS by annexin V, was apparent at midstages of light deprivation, although it did not conform to the typical pattern for PS exposure observed in metazoans or vascular plants, which occurs at early stages of the apoptotic event. Thus, we have evidence for a link between ROS and cell death involving caspase-like enzymes in an alga. The fact that caspase-like inhibitors prevent not only cell death, but also ROS and loss of cell membrane integrity and asymmetry, suggests that caspase-like proteases might have regulatory roles early in cell death, in addition to dismantling functions.**

*Key index words:* **apoptosis inhibition; caspase-like activities; cell death; cell viability; *Dunaliella tertiolecta*; phosphatidylserine; phytoplankton; reactive oxygen species; unicellular chlorophyte**

*Abbreviations:* **PCD, programmed cell death; PS, phosphatidylserine; ROS, reactive oxygen species**

Apoptosis is a ubiquitous physiological process, essential for the proper growth and development of metazoans (Kerr et al. 1972). It is typically defined as a type of programmed cell death (PCD) that is dependent on the activity of specific proteases called caspases (cysteinyl aspartate-specific proteases (Thornberry 1999) and is accompanied by highly conserved morphological changes, including chromatin condensation and margination and ordered DNA cleavage, while the cytoplasm and organelles remain unaffected (Cohen 1997). Recent studies have demonstrated that unicellular organisms, including chlorophytes (Berges and Falkowski 1998, Segovia et al. 2003, Segovia and Berges 2005), dinoflagellates (Vardi et al. 1999, Franklin and Berges 2004, Segovia 2007), diatoms (Casotti et al. 2005, Bidle and Bender 2008), coccolithophorids (Bidle et al. 2007), yeast (Frohlich and Madeo 2000, Burhans et al. 2003), and bacteria (Cairns 2002, Rice and Bayles 2003), including cyanobacteria (Ning et al. 2002a,b, Berman-Frank et al. 2004), undergo PCD.

The factors that cause cell death in unicells, its roles, and the details of the apoptotic process remain unclear (Franklin et al. 2006, Deponte 2008), but model species, such as *D. tertiolecta*, are proving useful in examining common apoptotic features. When placed in darkness, this unicellular chlorophyte undergoes a form of cell death reminiscent of apoptosis in metazoans. Many morphological criteria of apoptotic cell death are met, including an increase chromatin margination, degradation of the nucleus, and DNA fragmentation (Segovia et al. 2003). Assays using caspase-specific fluorogenic substrates demonstrate that activities increase with time in darkness, in parallel with the morphological changes. Furthermore, these activities are inhibited by caspase-specific inhibitors, and antibodies raised against mammalian caspases cross-reacted with proteins in the alga. The pattern of expression of these immunologically reactive proteins was correlated with the enzymatic activity measured, with the onset of cell death (Segovia et al.

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[2]Author for correspondence: e-mail segovia@uma.es.

2003) and loss of cells into fragments of the submicrometer size (Berges and Falkowski 1998).

Caspases belong to a larger clan of cysteine peptidases, unified by a common histidine–cysteine dyad in the active site, and a common 3-D structure including a ''caspase-hemoglobinase fold'' (Chen et al. 1998). Uren et al. (2000) proposed that protists, yeasts, and higher plants contain variant forms of caspases, designated metacaspases (Bidle and Falkowski 2004); these have been identified in genome sequences of prokaryotic and eukaryotic phytoplankton, including cyanobacteria, the chlorophyte *Chlamydomonas reinhardtii*, the marine diatoms *Phaeodactylum tricornutum* (C. J. Choi and J. A. Berges, unpublished data) and *Thalassiosira pseudonana* (Bidle and Bender 2008), and the marine haptophyte *Emiliania huxleyi* (Bidle et al. 2007). However, in the majority of cases, measurements in unicellular species have been confined to activity assays using artificial substrates designed to be specific for mammalian caspases. Metacaspase activities are completely different from those shown by caspase like-activities (Vercammen et al. 2004). Thus, because we cannot associate the activities measured in *D. tertiolecta* with metacaspases, we describe them as ''caspase-like'' enzymes in this article.

Caspases-like proteins have been measured in several other unicellular species. Cysteine proteases must be central to the PCD mechanism of cultured and natural populations of the dinoflagellate *Peridinium gatunense*, because treatment with the cysteine protease inhibitor E-64 suppresses autolysis and, instead, leads to cyst formation in response to cell death under inorganic carbon limitation as well as the ROS that result from carbon limitation (Vardi et al. 1999). The freshwater cyanobacterium *Anabaena* spp. undergoes PCD with an increase in nonspecific protease activity after exposure to univalent-cation salts (Ning et al. 2002a,b). In the filamentous cyanobacterium *Trichodesmium* sp., PCD is responsible for the mortality of >45% of the biomass in ageing cultures. The increase in immunoreactivity to human caspase-3 polyclonal antisera as well as the increase in caspase-like activity, which correlated with mortality rate, was inhibited by a broad-spectrum caspase inhibitor (Berman-Frank et al. 2004).

Many uncertainties about the apoptotic process in unicells remain. To study the role of caspase-like enzymes in cell death events in different organisms, inhibitors might provide a useful tool. The relationship between the induction and/or activation of proteolytic activities and the cell death event has been examined using inhibitors of cytoplasmic protein synthesis (cycloheximide) and organellar protein synthesis (chloramphenicol) (Segovia and Berges 2005). These inhibitors did not prevent cell death from occurring when cultures were placed in the dark. No effect was observed either for nonspecific protease activities (caseinolysis) or for specific caspase-like activities using the fluorgenic substrates for caspases 1, 3, 8, and 9, suggesting that the cell death program was not dependent on protein synthesis but rather on posttranslational modification of preexisting constitutive proteins. For this reason, the use of specific inhibitors of caspase-like activity seems a more useful approach. Synthetic peptide inhibitors compete for the active site of the enzyme with their physiological substrates. Such peptides can be specific (blocking one or several caspases) or general (blocking anything related). We have used the broad-spectrum Boc-D-FMK caspase inhibitor in our experiments since broad-spectrum caspase inhibitors are the only useful tools for blocking the activity of the entire class of caspase-like enzymes simultaneously (Van der Hoorn and Jones 2004). More importantly, these inhibitors help discriminate between caspase-dependent or caspase-independent cell death.

A possible complication of using caspase inhibitors is that they may not block cell death due to certain caspase-independent pathways (Huettenbrenner et al. 2003, Punj and Chakrabarty 2003). For this reason, specific events of apoptosis, such as plasma membrane changes and changes in ROS, must also be examined. Plasma membrane changes include ''blebbing,'' which is the result of the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer (Leist and Nicotera 1997), that is, a loss of normal plasma membrane asymmetry. PS translocation is one of the most important early signaling events in metazoan PCD. ROS serve as signal transducers of cell death: first, they are the signal during the early phases of cell death, and, second, they are produced because of alterations in mitochondrial permeability, leading to the final destruction of the cell at later stages. The appearance of ROS is known to orchestrate the activation of PCD in animals (Cohen 1997, Leist and Nicotera 1997) and in vascular plants (Pennell and Lamb 1997).

Here, we report that inhibition of caspase-like activities using specific caspase inhibitors prevented dark-induced apoptosis completely, and critical markers of the cell death process, such as ROS production, loss of membrane permeability, and PS labeling, were not seen when inhibitors were used. This provides evidence that *D. tertiolecta* undergoes a caspase-like-dependent PCD, and it suggests a regulatory role of these enzymes during the PCD process.

## MATERIALS AND METHODS

*Culture conditions.* *D. tertiolecta* (CCAP strain 19/6) was grown in 1 L semicontinuous batch cultures in artificial seawater medium (Goldman and McCarthy 1978) enriched with f/2 nutrients (Guillard and Ryther 1962) at 16°C under continuous white light at 200 μmol quanta $\cdot$ $m^{-2} \cdot s^{-1}$, while maintaining gentle stirring and bubbling with filtered air. When cultures reached mid-log-phase, they were placed in complete darkness for 7 d, while maintaining gentle stirring and bubbling with filtered air.

*Inhibition of cell death.* Two separate sets of three independent cultures were placed in darkness. The first set received additions of 50 μM (final concentration) of the irreversible broad-range caspase inhibitor Boc-D-FMK (Calbiochem, San Diego, CA, USA, #218759) according to Segovia et al. (2003), while the second set received no addition and served as a control. Temperature, stirring, and bubbling were maintained as in the light. Cultures were sampled daily at the same time.

*Chl* a *fluorescence and cell counts.* The optimal quantum yield for PSII fluorescence ($F_v/F_m$) was measured with a portable PAM-2000 fluorometer (Waltz, Efeltrich, Germany) as described by Schreiber et al. (1986). The initial fluorescence emitted when all the reaction centers are open ($F_0$) and the maximal fluorescence corresponding to all the reaction centers closed ($F_m$) were determined in 15 min dark-adapted samples after a saturating light pulse closing all the PSII reaction centers. PSII quantum efficiencies, $F_v/F_m$, are defined as $(F_m - F_0)/F_m$. High $F_v/F_m$ values indicate that cells are in good condition, whereas a decrease of $F_v/F_m$ may indicate stress (Foyer et al. 1994). Samples were measured in triplicate. Cells were preserved in Lugol's iodine and counted (in triplicate) under the microscope using a haemocytometer.

*Cell viability.* Viability of cells grown in presence and absence of the caspase inhibitor was studied using fluorescein diacetate (FDA; Invitrogen, Carlsbad, CA, USA, #F1303). FDA is a nonpolar, nonfluorescent stain, which diffuses freely into cells. Inside the cell, the FDA molecule is cleaved (hydrolyzed) by nonspecific esterases to yield the fluorescent product fluorescein and two acetates. Accumulations of fluorescein are the result of intracellular esterase activity and thus indicate metabolic activity and therefore cell viability. For this purpose, 1 mL of culture sample ($1 \times 10^6$ cells) was gently centrifuged (Heraeus Labofuge 400, Thermo Scientific, MA, USA) at 500*g*. Pelleted cells were resuspended in 1 mL of 10 mM PBS buffer at pH 7 containing FDA at 30 μM final concentration. Samples were incubated at 20°C in darkness for 60 min under gentle shaking. Green fluorescence of cells was observed using a Leitz Laborlux D epifluorescence microscope (Leitz, Wetzlar, Germany) with an excitation wavelength of 451 nm and emission of 510 nm. The percentage of stained cells was calculated according to Franklin and Berges (2004). Samples were also analyzed by using a DAKO cytomation flow cytometer (MoFlo; Beckman Coulter, Fullerton, CA, USA). Counts were triggered using forward scatter (FSC) signals. Negative controls were performed according to the manufacturer's instructions: (i) fluorescent probe in PBS without sample, (ii) sample in PBS without fluorescent probe, and (iii) heat shocking cells at 70°C for 1 h (which eliminated FDA cleavage completely).

*Cell death.* We also used the mortal stain SYTOX-green (Invitrogen), which is a high-affinity nucleic acid stain that only penetrates cells with compromised plasma membranes. SYTOX-green staining of cells thus indicates a dead cell, at least until the cell loses integrity and lyses (Veldhuis et al. 1997, 2001). As above, 1 mL of culture sample was centrifuged at 500*g*. Pelleted cells were resuspended in 1 mL of 10 mM PBS buffer at pH 7 containing SYTOX-green at 20 μM final concentration. Samples were incubated at 20°C in darkness for 30 min while shaking. Green fluorescence of cells was observed under the epifluorescence microscope with an excitation wavelength of 450–490 nm and emission of 523 nm. The percentages of stained cells and the negative controls were determined as indicated above. However, in this case, cells that had been heat killed at 70°C for 1 h served as a positive control. The negative control was carried out by substituting distilled water for the fluorescent probe. Cells were also checked using flow cytometry as indicated above.

*ROS.* Reactive oxygen species were assayed with carboxy-$H_2$DCFDA [5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; Invitrogen] a cell-permeable indicator of ROS that is nonfluorescent until oxidation by ROS occurs within the cell. $H_2$DCFDA is the most commonly used reagent for detecting intracellular ROS species, and despite its lack of specificity, it has been proved to be very useful for assessing the overall oxidative stress. One milliliter of culture sample ($1 \times 10^6$ cells) was centrifuged at 500*g*. The pellet was resuspended in 1 mL of 10 mM PBS buffer at pH 7 containing 5 μM $H_2$DCFDA final concentration and incubated for 30 min at 20°C in darkness, washing it thoroughly afterward. Green fluorescence of cells was observed under the epifluorescence microscope with an excitation wavelength of 490 nm and emission of 525 nm and by flow cytometry. The percentages of stained cells and negative controls were calculated as above.

*Loss of membrane asymmetry.* Loss of membrane asymmetry was assayed with annexin-V-FITC (Invitrogen). Annexin V is a calcium-dependent phospholipid-binding protein conjugated to the green-fluorescing fluorescein isothiocyanate (FITC), which shows high affinity for PS exposed on the outer leaflet of the cytoplasmic membrane of apoptotic cells. One milliliter of culture sample ($1 \times 10^6$ cells) was centrifuged at 500*g*. The pellet was resuspended in 400 μL of assay buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$) and 20 μL of annexin V-FITC. Samples were gently vortexed and incubated for 15 min at room temperature in darkness. Green fluorescence of cells was observed under the epifluorescence microscope with an excitation wavelength of 494 nm and emission of 518 nm. The percentages of stained cells and negative controls were determined as above. A positive control for annexin V and propidium iodide (PI) labeling was carried out by osmotically shocking cells. The negative control was carried out by substituting the fluorescent probe by distilled water. Samples were also analyzed by flow cytometry as indicated before.

*Statistics.* Differences in $F_v/F_m$, and cell counts with and without the inhibitor were tested using two-way analyses of variance (ANOVAs). Where significant differences were detected, post hoc multiple comparisons were made using the Tukey's tests ($P < 0.05$). To check for the source of variation in the groups, we carried out one-way ANOVAs followed by Tukey's tests ($P < 0.05$). To quantify the relationship between the variables, we performed the Pearson product moment correlations (considering $P < 0.05$ as significant). The statistical analyses were carried out using the SigmaSTAT 3.1 statistical package (SPSS Inc., Chicago, IL, USA).

## RESULTS

*D. tertiolecta* growing cultures when deprived of light underwent massive cell death between the third and seventh days, very similar, but somewhat faster than that described by Berges and Falkowski (1998). However, in presence of the irreversible broad-range caspase-inhibitor Boc-D-FMK, the cell death event was eliminated. In control cultures (without inhibitor), $F_v/F_m$ remained high during the first 2 d and suddenly dropped 4-fold after 3 d in darkness as previously described (Berges and Falkowski 1998, Segovia et al. 2003, Segovia and Berges 2005). When the caspase inhibitor was added to the cultures at 0 d, $F_v/F_m$ did not decrease and stayed constant during the whole light-deprivation period (Fig. 1A). Cell numbers paralleled the decrease in photochemical efficiency in the control, decreasing almost immediately after the onset of darkness. In presence of the inhibitor, cell numbers did not decline, showing the same initial value as when cells were in light (Fig. 1B).

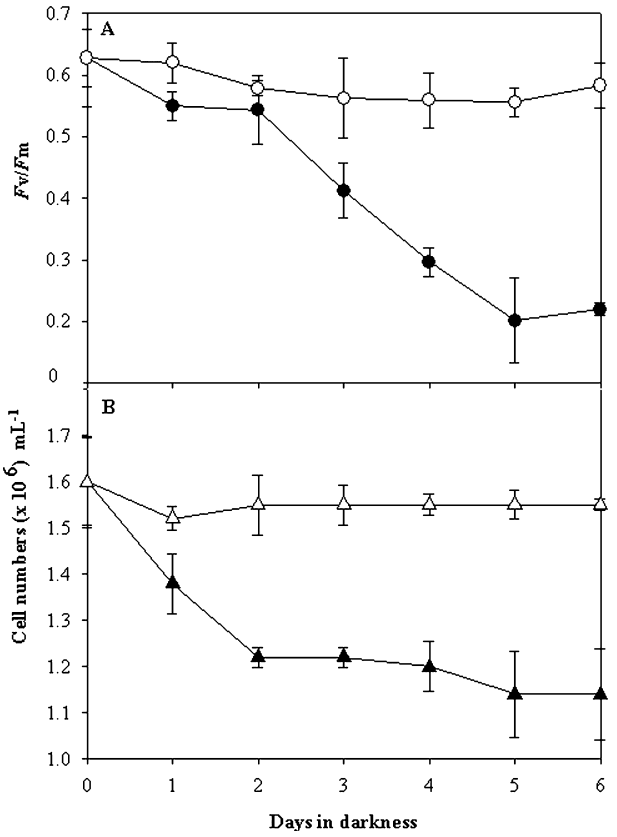


FIG. 1. Variation of PSII optimum quantum yield ($F_v/F_m$) (A) and cell numbers (B) in cultures of *Dunaliella tertiolecta* deprived of light. Cells were placed in darkness immediately after the day 0 measurement (culture in light). Cultures in the absence of Boc-D-FMK (closed symbols), cultures containing 50 μM of Boc-D-FMK (open symbols). Symbols are means of measurements in three independent cultures. Error bars indicate standard deviations. There were statistically significant differences ($P < 0.05$) within treatments.

Cell survival and viability were checked in the cultures in presence and absence of the caspase inhibitor with the fluorescent probes SYTOX-green (Fig. 2) and FDA (Fig. 3), respectively. When the plasma membrane is compromised, cells incubated with the nucleic acid stain will fluoresce green, whereas living cells appear red due to the autofluorescence of chl. Initially (days 1 to 3), under light deprivation, only a small nonsignificant percentage of cells (<5%) showed green fluorescence (Fig. 2E). Cells appeared to be alive as demonstrated by chl red fluorescence (Fig. 2A) and FDA labeling (see below). After the onset of apoptosis, labeling increased and red fluorescence faded from 4 to 7 d, (Fig. 2B) in ~90% of the cells (Fig. 2, E and D).These data were confirmed by flow cytometry. In the presence of Boc-D-FMK, cells never underwent apoptosis and showed red fluorescence and never green fluorescence throughout the whole of the light-deprivation period (Fig. 2C). Negative controls were analyzed using cells that had been in darkness for 4 d by substituting bidistilled and deionized water for the fluorescent probe, and fading of chl red fluorescence could be seen in dead cells (data not shown). Positive controls, consisting of heat-shocked cells, showed green fluorescence, indicative of SYTOX-green binding to DNA, therefore indicative of loss of membrane integrity (data not shown).

FDA is an indicator of esterase activity in the cells; live cells should have active esterases and, hence, fluoresce green, while dead ones lacking esterase activity will appear reddish/yellow under blue excitation. When cells were probed with FDA, the results confirmed the data obtained with SYTOX. During the first 3 d in darkness (Fig. 3A), green labeling indicated that 97% of the cells were alive and had esterase activity. However, from 4 to 7 d in darkness, green fluorescence turned into yellow, indicating a decrease in esterase activity coincidental with the onset of cell death (Fig. 3B), and only about 5% of the cells displayed green fluorescence. When the caspase inhibitor was present, cells showed esterase activity throughout the dark-deprivation period (Fig. 3C). Negative controls were as described above for SYTOX. Also, heat-shocked cells were used as negative controls, and we observed fading of red autofluorescence.

Production of ROS often occurs when the cell has suffered an injury or some sort of stress. When the concentration of ROS is high, cell death is induced by means of activation of the caspase cascade (Cohen 1997). Ninety-five percent of *D. tertiolecta* cells (Fig. 4, B, E, and D) produced ROS after 4 d in darkness as shown by the green fluorescence of algae, in contrast to red autofluorescence observed from 0 to 3 d in darkness (Fig. 4A), and they were not detected before the 4th day. When cells were grown in the presence of the caspase inhibitor, ROS production was inhibited during the 7 d in darkness (Fig. 4C). Negative controls were as described for FDA; that is, no green labeling was observed when the fluorescent probe was substituted with MilliQ. When cells were heat shocked, red fluorescence faded (data not shown).

Under light deprivation, the percentage of cells showing PS translocation was very low on days 0 and 1, but green fluorescence, indicative of annexin-V binding, became apparent after 2 d in darkness; ~4% of the cells were annexin positive, during days 2 and 3 (Fig. 5, C and D, respectively). From the fourth day onward, ~80% of the cells were annexin-positive (Fig. 5, E, F, and G, corresponding to 4, 5, and 6 d in darkness, respectively) due either to translocation of PS to the outer membrane or to entry of the stain to cell and binding to PS on the inner membrane as membrane integrity was lost. In the presence of Boc-D-FMK, cells were healthy and alive as shown by red chl fluorescence (picture not shown) during the entire light-deprivation period. Negative controls were analyzed as above, and cells did not stain. Positive controls consisted of osmotic-shocked cells and revealed green fluorescence, thus

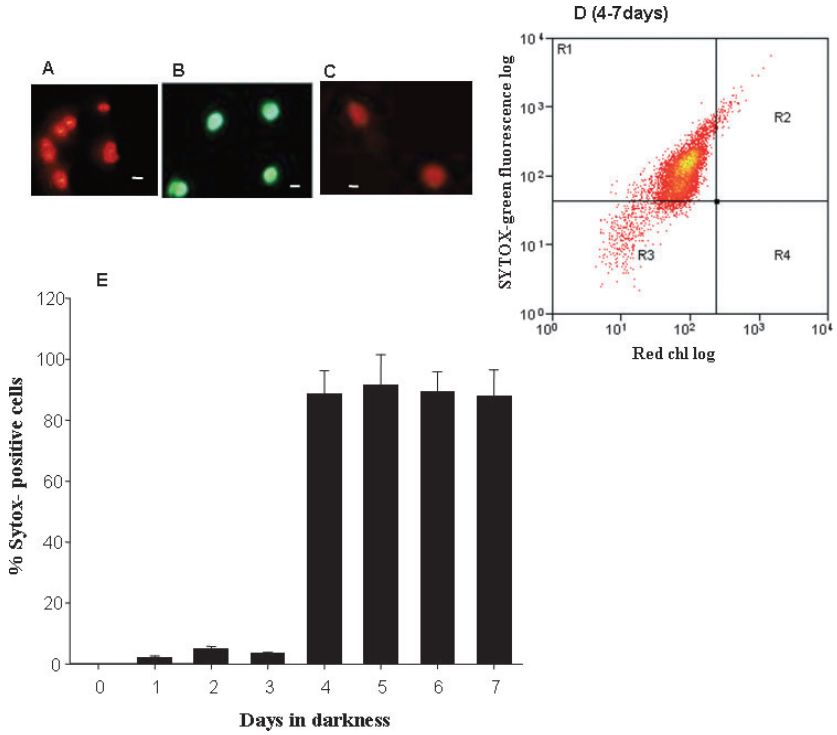


FIG. 2. Changes in staining in cultures of *Dunaliella tertiolecta* deprived of light. Cells staining with SYTOX were judged to be dead on the basis of compromised cell membranes. Micrographs of SYTOX staining of cells under epifluorescence microscopy: (A) cells with intact membranes before the onset of apoptosis (day 0); (B) dead cells with compromised plasma membranes measured after 4 d in darkness; (C) living cells in the presence of the irreversible caspase inhibitor Boc-D-FMK after 4 d in darkness. Scale bars, 10 μm. (D) Cultures of light-deprived cells measured using flow cytometry. The *y*-axes in the flow cytometer charts refer to SYTOX-stained cells, and the *x*-axes refer to red chl autofluorescence. R3 corresponds to the signal given by the control or cells during the first days of darkness. Fluorescence falling within R1 indicates positive labeling. (E) Percentage of stained cells. There were no statistically significant differences ($P > 0.05$) between days 0 to 3 and 4 to 7. There were statistically significant differences ($P < 0.05$) between the two groups on days (0–3 and 4–7).

indicating PS exposure due to loss of membrane integrity (data not shown).

In all cases, the shape of the cells with Boc-D-FMK was identical to the shape of the actively growing cells in light, before stressing them with darkness. However, during light deprivation, without the inhibitor, cell size decreased ~10%, and shape changed with cells becoming thinner (a typical feature of apoptotic cells).

## DISCUSSION

The possible roles of PCD in unicellular organisms, such as bacteria (Lewis 2000), including cyanobacteria (Berman-Frank et al. 2004); yeast (Madeo et al. 1999); trypanosomatids (Ameisen 1998); and unicellular algae (Vardi et al. 1999, Segovia et al. 2003), have received much attention recently. The presence of the basic mechanisms of apoptosis in these organisms indicates that cell death pathways evolved in prokaryotes and unicellular eukaryotes (Bidle and Falkowski 2004, Franklin et al. 2006). The occurrence of apoptosis in unicells is confusing because, unlike multicellular organisms, it results in complete loss of the organism and would appear to be maladaptive. However, there are theories suggesting that PCD in unicellular organisms could allow for the constant selection of the fittest cells (see Welburn et al. 1997, Madeo et al. 1999). Regardless of its origins or evolutionary meaning, the existence of PCD is a major mechanism in phytoplankton that has important implications for aquatic ecosystems (Bidle and Falkowski 2004, Franklin et al. 2006, Segovia 2007).

Several sequenced cyanobacterial genomes (e.g., *Trichodesmium* and *Anabaena*) as well as model phytoplanktonic species, such as *E. huxleyi*, *C. reinhardtii*, and *T. pseudonana*, contain metacaspases orthologues and other putative PCD-related proteins (Bidle and Falkowski 2004, Montsant et al. 2007). Proteases in general are kept quiescent in the cell, prepared to commit the cell to a particular fate. However, it has been speculated that caspase-like enzymes might have housekeeping functions in this species when a cell death stimulus is not present (Segovia et al. 2003) and seem to be constitutive (Segovia and Berges 2005). The question that arises is how important are the housekeeping functions of caspase-like proteases, given that cells can survive with them inhibited, according to our results. Comparable results have been reported for metacaspase activities in *P. tricornutum*, which showed apparently

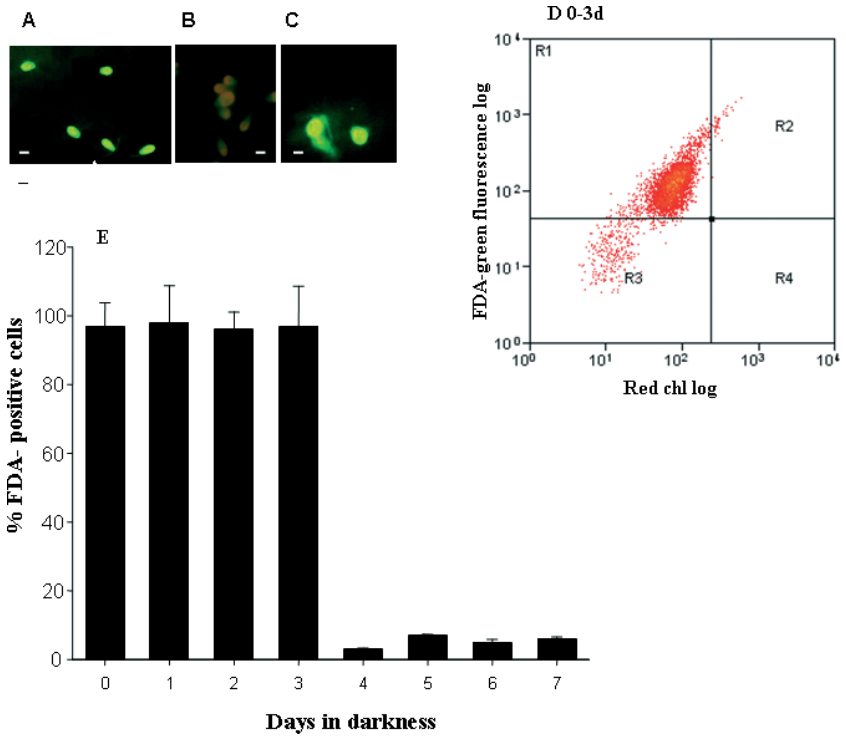


FIG. 3. Cell viability in *Dunaliella tertiolecta* under light deprivation. The percentage of living cells was demonstrated by esterase activity shown by FDA fluorescent staining. (A) Cells showing esterase activity before cell death detected by epifluorescence microscopy and flow cytometry, respectively (day 1). The *y*-axes in the flow cytometer charts refer to FDA-stained cells, and the *x*-axes refer to red chl autofluorescence. R3 corresponds to the signal given by the control or cells during the first days of darkness. Fluorescence falling within R1 indicates positive labeling; (B) fading of green fluorescence of dead cells (day 4) in darkness; (C) live cells in darkness in presence of Boc-D-FMK (day 4); (D) percentage of stained cells. Scale bars (A–C), 10 μm. There were no statistically significant differences ($P > 0.05$) between days 0 to 3 and 4 to 7. There were statistically significant differences ($P < 0.05$) between the two groups of days (0–3 and 4–7). FDA, fluorescein diacetate.

high levels of these enzymes under normal growth conditions (Bidle and Bender 2008).

The data we show here would correspond to the mechanism underlying the PCD events in *D. tertiolecta*. Cell viability fluorescent stain, indicating compromised plasma membranes and esterase activity, showed that from 0 to 3 d in darkness, *D. tertiolecta* cells were alive but probably preparing themselves to die as the percentage of stained cells was not significant statistically. However, at this stage, cells had not yet reached the ''point of no return.'' On the fourth day, intense staining and fading of FDA fluorescence revealed from days 4 to 7 in darkness indicated that cells were dead. This finding suggests that the onset of apoptosis was blocked by the caspase-like inhibitor, indicating that caspase-like activities are responsible for cell death in *D. tertiolecta*, as previously demonstrated by Segovia et al. (2003). An increase of membrane permeability is considered as the ''point of no return'' in the progression of the cell death process in several organisms (Ellis et al. 1991). We suggest that there was an initial, rapid loss of cells and then a phase where cells stained as dead, but cell numbers remained relatively stable (i.e., lysis rate was slower), explaining why the cell counts and the cell staining show different patterns. Though unexpected, there is no inherent ''conflict'' between cell counts and the cell-staining pattern as clearly demonstrated by Veldhuis et al. (2001) in several phytoplanktonic samples.

Exactly at the same timing that esterase activity decreased and compromised plasma membranes emerged, cells started to produce ROS after 3 d in darkness. ROS are common mediators of stress-induced PCD in animals (Cohen 1997, Leist and Nicotera 1997) and a variety of responses that involve activation of a plant-encoded pathway for PCD in vascular plants (Pennell and Lamb 1997, Lam et al. 2001, Chichkova et al. 2004, Van Breusegem and Dat 2006). ROS are also known to mediate PCD in unicellular organisms, such as kinetoplastids (Ridgley et al. 1999, Sen et al. 2004) and yeast (Madeo et al. 1999), and it seems that they are a necessary requirement for viral-induced cell death or with non-virus mediated death processes in *E. huxleyi* (Evans et al. 2006). ROS appearance was concurrent with all the apoptotic events already studied in this species. In plants (note that *D. tertiolecta* belongs to Viridiplantae), chloroplasts and peroxisomes are the main sites for ROS production (Foyer and Noctor 2003, Asada 2006), and some of the ROS can diffuse between the different compartments (Henzler and Steudle

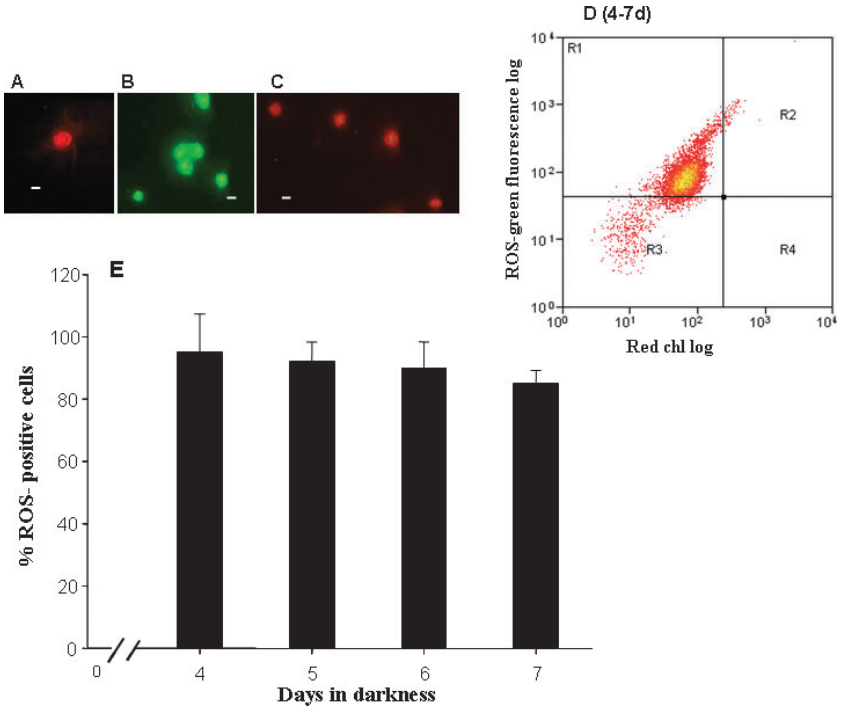


FIG. 4. Accumulation of reactive oxygen species (ROS) in *Dunaliella tertiolecta* under light deprivation. ROS-positive cells were exposed by the fluorescent probe $H_2$DCFDA. (A) Live cells after 1 d in darkness; (B, D) production of ROS after 4 d in darkness detected by epifluorescence microscopy and flow cytometry, respectively. The *y*-axes in the flow cytometer charts refer to ROS-stained cells, and the *x*-axes refer to red chl autofluorescence. R3 corresponds to the signal given by the control or cells during the first days of darkness. Fluorescence falling within R1 indicates positive labeling; (C) healthy and live cells in the presence of Boc-D-FMK (5 d in darkness); (E) percentage of stained cells. From days 0–3 in darkness, no labeling was apparent. Scale bars (A–C), 10 μm. There were no statistically significant differences ($P > 0.05$) between days 4 to 7. There were statistically significant differences ($P < 0.05$) between the two groups of days (0–3 and 4–7).

2000). In *D. tertiolecta*, it seems that until day 4 the chloroplast was active, and probably the balance between ROS production and ROS scavenging was coupled. After the fourth day onward, in parallel with the loss of chloroplastic structure and morphology (see Segovia et al. 2003, Segovia and Berges 2005), the capacity for coping with the excess of ROS disappeared, probably as a consequence of failure in energy dissipation due to the degradation of the xanthophyll cycle in this species (Casper-Lindley and Bjorkman 1998, Masojídek et al. 2004, Chidambara Murthy et al. 2005). It is known that the detection of rapid changes in ROS concentrations that result from metabolic disturbances or external factors is used by cells to activate stress-related responses and to readjust homeostasis as well as to function as signaling agents regulating many biological processes in vascular plants (Gadjev et al. 2008). To answer why there is a jump from zero to all in some of the variables analyzed, we must note that this behavior responds to a catastrophic kind of event, considering that the *D. tertiolecta* population has suffered the divergence from one state to another due to a stress factor. The transition between the states of equilibrium is produced in an abrupt manner, with no intermediates. Such behavior is widespread in biology, and there are several mathematical-ecological theories that model these events, especially regarding population behavior (Gleick 1988, Kingsland 1995). The catastrophic culture decline was already described by Berges and Falkowski 1998, but at that time, it was not known that an apoptotic phenomenon was responsible. This, together with the results using the inhibitor, in which there was no ROS accumulation, clearly suggests that there is a direct link between ROS apparition in this species and the cell death phenomenon observed. Classically, it has been assumed that ROS production precedes cell death. However, we cannot unequivocally identify ROS as the cause driving the entrance of the cells into the death cascade (although literature supports such an idea) or if ROS accumulation is in fact the consequence of the death cascade. Our data suggest that this might not always be the case, or that the two processes occur so closely in time that we cannot resolve the sequence.

Accordingly, the ''point of no return'' was probably reached between days 3 and 4 under light deprivation. Within this narrow band of time (24 h), cells exceeded the thin boundary between life and death and were poised and committed to die during the following hours and days. Interestingly, clear loss of membrane asymmetry was revealed after 4 d in

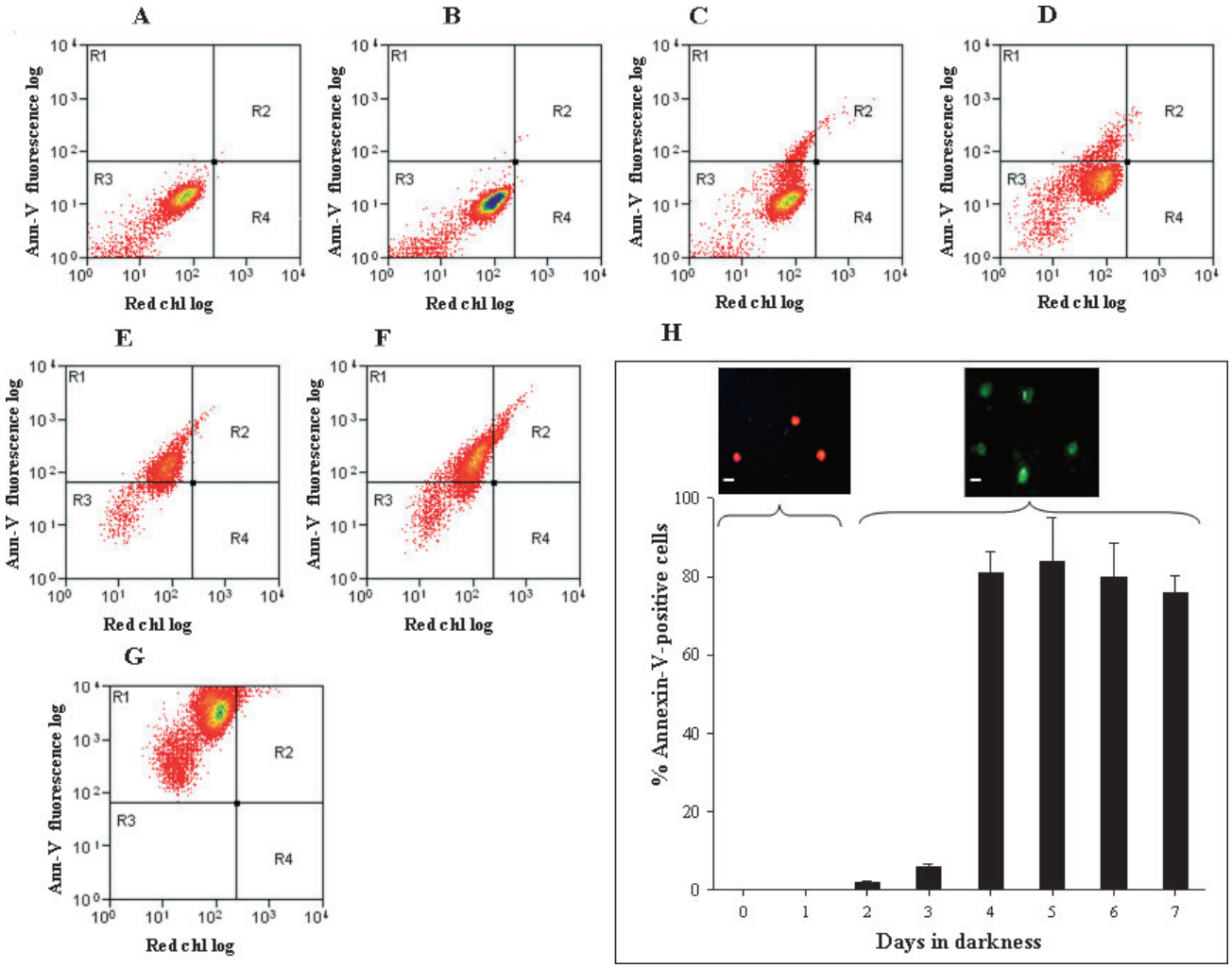


FIG. 5. Loss of membrane asymmetry in *Dunaliella tertiolecta* under light deprivation. The percentage of cells showing phosphatidylserine (PS) translocation was visible by epifluorescence microscopy and flow cytometry due to annexin-V binding to PS. The *y*-axes in the flow cytometer charts refer to annexin-V-stained cells, and the *x*-axes refer to red chl autofluorescence. (A, B) Untranslocated PS on days 0 and 1 in darkness, respectively, showing no binding of annexin-V; (C, D) little annexin-V binding during days 2 and 3, respectively; (E, F, G) annexin-V binding to membranes parallels the highest percentage of annexin-V positive cells on days 4, 5, and 6, respectively, in darkness; (H) percentage of cells showing annexin-V labeling. Live cells in presence of Boc-D-FMK (4 d in darkness) showed no labeling at all. Scale bars, 10 μm. There were no statistically significant differences ($P > 0.05$) between days 0 to 3 and 4 to 7. There were statistically significant differences ($P < 0.05$) between the two groups of days (0–3 and 4–7).

darkness onward, parallel in time with ROS production, loss of membrane permeability, and cell viability.

PS is located in the inner leaflet of the plasma membrane in plants, and its asymmetry is a common feature in all normal cells of living creatures (Takeda and Kasamo 2001). After the onset of apoptosis, PS translocation from the inner to the outer leaflet of the plasma membrane takes place (Hale et al. 1996). By using combination of comet assay and cell electrophoresis, Ning et al. (2002a,b) demonstrated that annexin-V preferentially binds to negatively charged exposed phospholipids (e.g., PS) in plant cells. Annexin-V binding has also been reported to happen prior to the detection of DNA strand breaks but almost at the same time as chromatin condensation in *Nicotiana plumbaginifolia*. Annexin binding was present during the whole process of apoptosis during cell senescence (O'Brien et al. 1997, 1998), and it has also been reported during apoptotic cell death of the parasites *Trichomonas vaginalis* (Chose et al. 2002), *Plasmodium falciparum* (Deponte and Becker 2004), and recently in the iron-starved diatom *T. pseudonana* (Bidle and Bender 2008). In *D. tertiolecta*, the timing for significant PS translocation (4 d in darkness) was a little later (1 d in darkness) than the timing for chromatin condensation and margination at the nuclear envelope (see Segovia et al. 2003, Fig. 2). However, the differences between DNA breakage and PS translocation-chromatin condensation in *D. tertiolecta* could indicate that the sequence of events occurring during the different stages of apoptosis may differ

among cell types and species, suggesting multiple signaling pathways (Martin et al. 1994). This possibility was also noticed by O'Brien et al. (1998) who reported that early stages of the apoptotic pathway in plant cells could be reversible. The results obtained suggest that PS exposure in *D. tertiolecta* does not conform to the pattern observed either in metazoans or in vascular plants. Why membrane permeability loss occurs almost simultaneously to translocation of PS in *D. tertiolecta* is unclear; however, PS inversions serve different purposes in multicellular organisms. In metazoans, PS exposure is known to be a signal that triggers the response of neighboring cells. Thus, annexin-V binding may not be a good apoptotic marker in unicells.

The use of the broad-spectrum inhibitors (Boc-D-FMK) helps discriminate between caspase-dependent and caspase-independent cell death, because broad-spectrum caspase inhibitors are the only useful tools for blocking the activity of the entire class of caspases simultaneously (Van der Hoorn and Jones 2004). When the inhibitors do not block cell death, caspase-independent cell death is often invoked (Huettenbrenner et al. 2003, Punj and Chakrabarty 2003). The phenomenon observed in *D. tertiolecta* is certainly caspase-like dependent. By inhibiting caspase-like activities, neither $F_v/F_m$ nor cell numbers decreased but stayed constant during the whole light-deprivation period. Cell viability shown by esterase activity was undisturbed, and plasma membranes were not compromised. The appearance of ROS and loss of plasma membrane asymmetry were prevented; therefore, PCD in *D. tertiolecta* was eliminated, and all the cells were alive. In the presence of the inhibitor, cells seemed to bypass the ''point of no return,'' indicating once more that caspase-like enzymes are responsible for the execution of the cell in *D. tertiolecta*. Therefore, we add compelling evidence that caspase-like activities are directly linked to the regulation of the PCD. ROS are often viewed as a signal that activates cell death pathways involving caspase-like enzymes, but our data suggest that the enzymes are involved much earlier. Similar results were observed in blooms formed by the freshwater dinoflagellate *P. gatunense*, which undergo catastrophic decline when the pH rises and the availability of dissolved $CO_2$ is drastically reduced. Nevertheless, PCD was prevented when cells were treated with E-64, an inhibitor of cysteine proteases. The inhibitor completely suppressed ROS and SYTOX-positive cells and inhibited cell death following treatment with $H_2O_2$, as it occurs in *D. tertiolecta* in the presence of the inhibitor. Research focused on the inhibition of the PCD process has been mainly carried out using *Arabidopsis thaliana*. Elbaz et al. (2002) showed that plant cells undergo PCD by constitutively expressing the proteins required to run the death program upon induction with the fungal elicitor EIX or by staurosporine in the presence of cycloheximide. The permeable peptide caspase inhibitors zVAD-FMK and zBocD-FMK blocked PCD induced by EIX or staurosporine. Equally, PCD induced by $H_2O_2$ was reduced by addition of the caspase-3 inhibitor Z-YVAD (Tiwari et al. 2002).

It is difficult to decipher which events happen upstream or downstream during PCD in this unicellular chlorophyte. Inhibition of caspase-like enzymes might suppress other signals that commit the cell to suicide, suggesting a central and key role of these enzymes in the process. One possibility is that the critical regulators of PCD in this organism are the caspase-like enzymes themselves or activate other proteins related with cell death processes. They might act by controlling redox signals coming either from the chloroplasts, peroxisomes, or the mitochondria, or they could be linked with signal transduction mechanisms. For instance, during *D. tertiolecta*'s cell death process under light deprivation, we have observed a 4-fold decrease in protein kinase activities (PKs) measured in crude extracts by using a synthetic PKA/PKC pseudosubstrate and a monoclonal antibody, which recognizes the phosphorylated form of that peptide (C. García, M. T. Mata, and M. Segovia, unpublished data). PK activities and caspase-like activities showed a significant negative correlation ($r = 0.99$). PKs are involved in signal transduction pathways activated by extracellular and intracellular stimuli to control cell fate through activating or inhibiting apoptotic pathways (Martin et al. 2005). These results are preliminary and must be taken with caution, but they argue in favor of a regulatory role of caspase-like activities. They might be part of the signaling cascades at some stage, as indicated by protease inhibitor studies, and according to the role that has been suggested for the metacaspases and CDR1 (Van der Hoorn and Jones 2004). In mammalian cells, ROS appears to be upstream of cytochrome c release and caspase activation. However, it can also appear at later stages depending on the organism (Burhans et al. 2003), as seems to be the case for *D. tertiolecta*. The fact that in this species ROS accumulation does not clearly precede the cell death machinery activation suggests that the process might be reversible at the first stages, but once the concentration of signals (namely, ROS accumulation) crosses a certain threshold, the cell is committed to die. For instance, specific caspase inhibitors and E-64 blocked cytochrome c release and partially prevented the permeability transition and ATP depletion in *A. thaliana* (events supposedly occurring upstream of caspase activation) (Tiwari et al. 2002). Caspase-like activities may well have a defensive role against viral infection in phytoplankton given that, as mentioned before, ROS production seems compulsory for viral-mediated cell death processes in *E. huxleyi* (Evans et al. 2006).

We now have one more piece of evidence that the PCD phenomenon is dependent on caspase-like

enzymes, and also, and most importantly, that ROS production seems to follow an on/off switch in this process. Therefore, we propose that caspase-like enzymes might have a regulator role apart from being the executors of the cell in this process. In support of this idea, another species from the genus *Dunaliella* (*D. viridis*) exposed to various environmental stresses showed different cell death morphotypes other than apoptotic, under the stresses mentioned above, depending on the kind and intensity of the stimulus, and it concurred with the activation of the caspase-like activity DEVDase (Jiménez et al. 2009).

It has been traditionally thought that PCD mechanisms in unicellular organisms share characteristics with metazoans, as it indeed happens in a wide number of species. However, the use of inhibitors of caspase-like enzymes in *D. tertiolecta* suggests that some of the concepts associated with metazoan PCD do not operate in the same manner in these organisms, and many pieces of the puzzle are still missing. Finding these pieces will be greatly facilitated by a clearer idea of timescales and sequences of events, some of which are provided in the present work. Therefore, we must consider again that the pathways through which PCD proceeds in unicells present clear divergences from those of metazoans and vascular plants.

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