Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO_2 limitation and oxidative stress

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The phytoplankton assemblage in Lake Kinneret is dominated in spring by a bloom of the dinoflagellate *Peridinium gatunense*, which terminates sharply in summer [1]. The pH in *Peridinium* patches rises during the bloom to values higher than pH9 [2] and results in CO_2 limitation. Here we show that depletion of dissolved CO_2 ($CO_{2(dis)}$) stimulated formation of reactive oxygen species (ROS) and induced cell death in both natural and cultured *Peridinium* populations. In contrast, addition of CO_2 prevented ROS formation. Catalase inhibited cell death in culture, implicating hydrogen peroxide (H_2O_2) as the active ROS. Cell death was also blocked by a cysteine protease inhibitor, E-64, a treatment which stimulated cyst formation. Intracellular ROS accumulation induced protoplast shrinkage and DNA fragmentation prior to cell death. We propose that CO_2 limitation resulted in the generation of ROS to a level that induced programmed cell death, which resembles apoptosis in animal and plant cells. Our results also indicate that cysteine protease(s) are involved in processes that determine whether a cell is destined to die or to form a cyst.

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Results and discussion

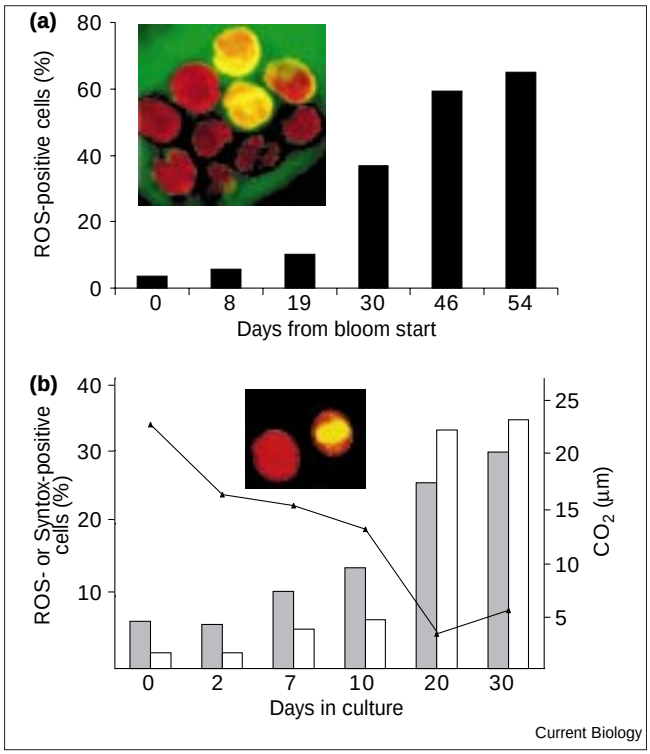
The pH rises that occur during *Peridinium* bloom drastically lower the availability of $CO_{2(dis)}$. This is manifested in the acclimation responses to CO_2 limitation, which include a rise in carbonic anhydrase activity, the ability to accumulate inorganic carbon (Ci), and the apparent photosynthetic affinity for external Ci [3]. The availability of other nutrients such as orthophosphate and nitrogen does not change significantly during the bloom [2]. Increased activity of the antioxidant enzymes towards the end of *Peridinium* bloom [4] suggest a response to oxidative stress. We hypothesized that the CO_2 limitation that develops during the bloom diverts photosynthetic electrons from CO_2 fixation to oxygen, resulting in the production of ROS to a level sufficient to trigger programmed cell death (PCD).

Oxidative stress in *Peridinium* was assessed from the total ROS in a sample (data not shown), and the percentage of ROS-containing cells (Figure 1a). Excellent correlation was observed between these parameters. Individual cells stained with dihydrorhodamine 123 showed variations in the intensity and spectrum of the emitted fluorescence, from red (chlorophyll autofluorescence) in unstressed cells to a yellow or even green color in ROS-containing cells (Figure 1a, inset). The percentage of ROS-positive *Peridinium* cells increased significantly during growth in the lake (Figure 1a) and in batch cultures (Figure 1b). The concentration of H_2O_2 in the cultures also rose with time from undetectable levels at two weeks to 11.5 µM and 102 µM at four and eight weeks, respectively. This increase was accompanied by a rising antioxidative activity, as assessed from the amount of H_2O_2 that remained in the medium 1 hour after the addition of 1 mM H_2O_2. Whereas in the two-week-old culture 60% of added H_2O_2 was found in the medium after 1 hour, only 8% remained in the eight-week-old culture. These data correlate with the antioxidant enzyme activity in the lake during the *Peridinium* bloom [4].

The concomitant rise in antioxidant activities and decline in $CO_{2(dis)}$ concentration suggested a possible link between CO_2 limitation and ROS production. To test this hypothesis, two-week-old cultures grown in the standard medium containing 2 mM Ci were transferred to medium containing either 0.5, 2 or 8 mM Ci, at a constant pH of 7.9. The fraction of ROS-containing cells rose more than fivefold within three hours in 0.5 mM Ci and remained high for at least six days, whereas 8 mM Ci reduced the number of ROS-positive cells to a level below that observed in the standard medium (Figure 2). These data support the suggestion that ROS production in *P. gatunense* depends on the $CO_{2(dis)}$ concentration, even under a light intensity lower than that required to reach maximal photosynthetic rate [3].

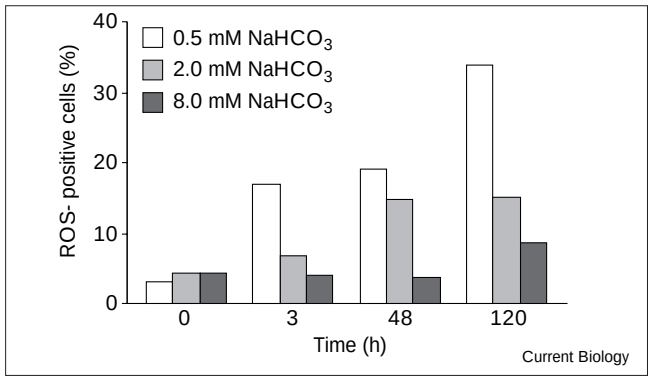
Cellular responses to ROS are largely determined by the ROS concentration and may range from up-regulation of

Figure 1



Cell death and accumulation of ROS in *P. gatunense*. (a) The percentage of ROS-positive cells during the winter–spring bloom in Lake Kinneret. Cells were collected from a depth of 3 m, between 8:00 am and 10:00 am during the 1998 season, and analyzed by epifluorescent microscopy of representative fields (450–600 cells per data point). The inset shows a representative image of *P. gatunense* stained with dihydrorhodamine 123. The red color is due to the autofluorescence of the chlorophyll, whereas yellow or green indicates ROS-containing cells. Video monitoring showed that cells emitting green fluorescence were swimming, albeit slower than the unstressed ones. As the ratio of orange and green colored cells was fairly constant their values were combined. The absolute values varied between experiments, depending on light intensity and the starting inoculum, but similar relative values were obtained in three independent experiments. (b) The percentage of ROS-positive (grey bars) and dead (white bars) cells in batch cultures. Cell death was assayed with Sytox (the inset shows a stained nucleus in the right-hand cell). The graphs show typical progressions in the numbers of ROS-positive and Sytox-positive cells during growth. The exact timing of change in these parameters varied between experiments and depended on light intensity. The line in (b) shows the $CO_{2(dis)}$ concentration in the growth medium.

Figure 2



Influence of Ci concentration on the abundance of ROS-positive *P. gatunense* cells. Cells grown in batch culture in 2 mM Ci were resuspended in a medium containing 0.5, 2, or 8 mM Ci, in 20 mM Hepes–NaOH pH 7.9 and were illuminated with a sub-saturating light intensity (60 µmol quanta m^{-2} s^{-1}). Cells were stained with dihydrorhodamine 123 and the percentage of ROS-positive cells counted under the microscope 3, 48 and 120 h after resuspension.

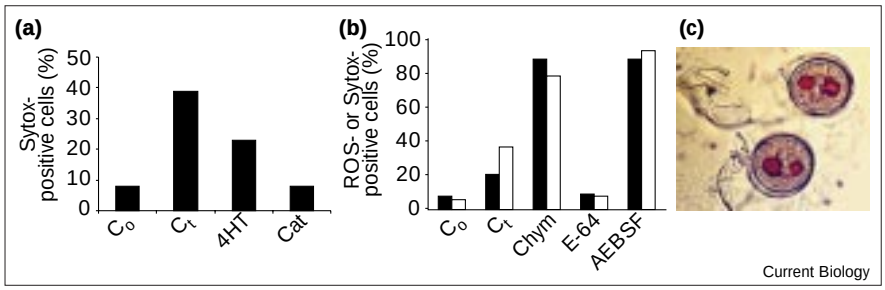
antioxidants to cell death by necrosis. Intermediate ROS concentrations can induce PCD or apoptosis [5,6], a major form of cell death in animals and plants that operates during development and stress responses [6,7]. Activation of cell death by environmental stress was recently described in *Dunaliella* and *Thalassiosira* cultures [8].

To investigate the possible role of oxidative stress in the PCD of *P. gatunense*, we examined whether ROS accumulation during aging coincides with cell autolysis. Cell death was assessed by Sytox, a membrane-impermeable DNA-binding dye [9]. The percentage of Sytox-positive cells rose sharply 20 days after culture initiation, that is, several days after the initial rise in the number of ROS-positive cells (Figure 1b). Sytox-positive cells were unable to swim and were hardly detected in the lake, probably because they sank.

The dependence of cell death on H_2O_2 was examined by the addition of catalase (in dialysis bags) before the rise in the number of ROS-positive cells (Figure 1b). In untreated cultures, the number of Sytox-positive cells increased markedly after six days, but in the presence of catalase it remained stable. The number of Sytox-positive cells was also reduced by a synthetic ROS scavenger, 4-hydroxy-tempo (4HT; Figure 3a). Because catalase specifically detoxifies H_2O_2, these results implicate the latter as the ROS essential for *P. gatunense* cell death.

The suppression of ROS-triggered cell death by the addition of specific metabolic inhibitors, not directly involved in ROS scavenging, would indicate that an intracellular process is initiated by the oxidative stress. The kinase inhibitors K252A and GO6873 had no effect on mortality (data not shown). Addition of the protease inhibitors Pefablock (AEBSF) or chymostatin promoted both H_2O_2 formation and cell death (Figure 3b), probably due to interference with metabolism. In contrast, application of E-64, an inhibitor of cysteine proteases, a family of proteases implicated in animal and plant PCD [7,10], completely suppressed the increase in the number of ROS-and Sytox-positive cells (Figure 3b). E-64 also effectively inhibited cell death following treatment with H_2O_2 (data not shown). Interestingly, E-64

Figure 3



The effect of H_2O_2 scavengers and protease inhibitors on the abundance of ROS-positive and Sytox-positive *Peridinium* cells. (**a**) The H_2O_2 scavenging agents catalase (Cat, 10 mg/ml, added in a dialysis bag with 12,000 MW cutoff) or 4-hydroxy-Tempo (4HT, 0.25 mM) and (**b**) protease inhibitors E-64 (10 μM), chymostatin (60 μM) and AEBSF (0.5 mM) were added to a 14-day-old *Peridinium* culture maintained at near saturating light intensity. (a,b) ROS production and cell death were assayed after 6 days as described in Figure 1. C_0, untreated control at time 0; C_t, control culture after 6 days. (**c**) Typical cysts formed 10 days after the addition of 10 μM E-64 to *P. gatunense* that were harvested from a depth of 1–3 m and resuspended in lake water. The extent of cystation in untreated cells was at least fivefold lower.

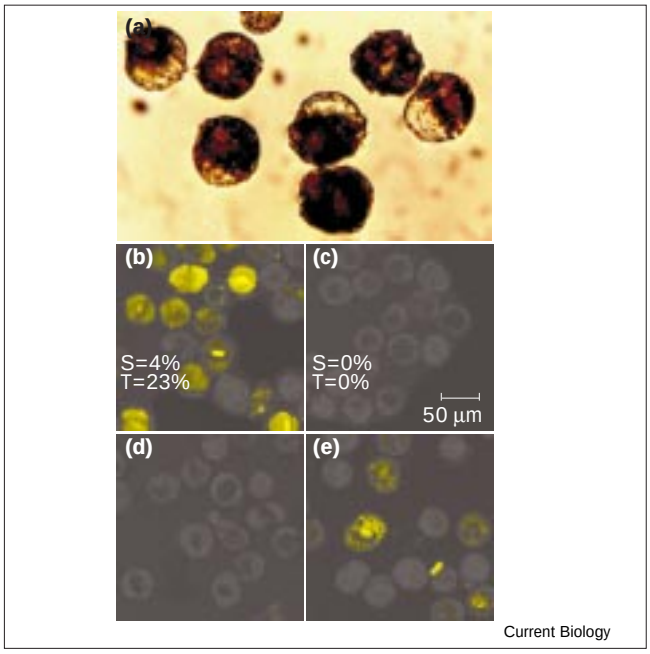
substantially stimulated cyst formation in cells collected from the lake (Figure 3c). The cellular parameters that determine the fate of *Peridinium* cells, that is, cyst formation or death, are poorly understood. E-64 might have intervened in a PCD pathway and induced many of the cells to form cysts.

The experiments with catalase and E-64 indicated that *Peridinium* cell death is a metabolic process mediated by H_2O_2 and specific protease(s). To confirm that the ROS-induced death of *P. gatunense* cells can be defined as PCD, we examined the induction of cytoplasmic shrinkage and DNA fragmentation, two of the most common hallmarks of PCD in plant and animal cells [6,11]. A distinct shrinkage of the protoplast was observed by video microscopy of swimming, ROS-containing cells, and in natural *Peridinium* populations collected from different depths in Lake Kinneret at the end of the bloom (Figure 4a). Protoplast shrinkage preceded the loss of membrane integrity, as assessed by Sytox staining.

To examine whether CO_2 limitation induced DNA fragmentation, swimming cells were collected from an exponentially growing culture and incubated in fresh medium supplemented with either 0.2 or 2 mM Ci. After 16 hours, 4% of the cells in 0.2 mM Ci but none of the cells in 2 mM Ci were Sytox-positive. At this time, 24% of the cells in 0.2 mM Ci but almost none of the cells treated with 2 mM Ci were labeled by the terminal-transferase-mediated dUTP nick-end labeling (TUNEL) method (Figure 4b,c). Staining was localized in the nuclei, as visualised using confocal microscopy. These data clearly indicate that DNA nicking induced by CO_2 limitation preceded the permeabilization of the plasmalemma. Nuclear DNA fragmentation constitutes a point of no return and is considered the strongest evidence for autolysis. But we could not detect nucleosomal laddering of DNA, possibly because dinoflagellates do not have the typical nucleosomal arrangement observed in higher eukaryotes [12], and which is consistent with the absence of ladders in yeast apoptosis [13].

The growth of unicellular algal populations has received considerable attention but little is known about the mechanisms that regulate their mortality. Abiotic factors, such as depletion of essential nutrients, biotic factors and increased grazing, or pathogens were suggested as

Figure 4



Hallmarks of the induction of apoptosis in *P. gatunense*. (**a**) Morphological changes during *P. gatunense* cell death. ROS-containing cells were followed by a video attached to the fluorescent microscope. Progressive shrinkage of the protoplast was observed in cells that were still swimming. (**b–e**) TUNEL analysis of nuclear DNA fragmentation after CO_2 limitation. Swimming cells were resuspended in fresh medium with final Ci concentrations of (b) 0.2 mM or (c) 2 mM (control). S and T, the percentage of Sytox-positive and TUNEL-positive cells, respectively, 16 h after resuspension. (**d**) A sample as in (a) except that the terminal transferase had been omitted was used as a negative control. (**e**) A sample as in (c) except that cells were pretreated with DNase I before TUNEL labeling was used as a positive control.

possible causes of population decline [14–16]. Berges and Falkowski [8] reported that nutrient constraints or darkness induced a cell-death process in batch cultures of *Thalassiosira* and *Dunaliella*, respectively, which also coincided with induction of a protease [8]. Our results show that CO_2 limitation developed during the growth period (Figure 1) and caused oxidative stress (Figure 2), which initiated a coordinated sequence of morphological and biochemical events terminating in *Peridinium* cell death. Inhibition of ROS accumulation blocked cell death (Figure 3a), indicating that H_2O_2 was essential for its induction. The cell-death process could be blocked by a cysteine protease inhibitor, but not by other protease inhibitors (Figure 3b). Moreover, *P. gatunense* also exhibited other hallmarks of apoptosis, such as protoplast shrinkage (Figure 4a) and nuclear DNA fragmentation (Figure 4b). All these phenomena were detected in living cells, indicating initiation of an autolytic process. The concomittant inhibition of cell death and stimulation of cyst formation by E-64 suggests a link between these processes, possibly through cysteine proteases.

Our results show a remarkable parallel between the PCD of *P. gatunense* and other organisms and highlight the central role of ROS in the PCD of diverse organisms [5,6,13]. The evolutionary role of PCD in unicellular organisms that results in the coordinated death of a large population is not understood, although it has been suggested that in unicellular organisms apoptosis could enforce selection to various stresses [17]. It is reasonable to assume that by allowing only the best adapted individuals to establish cysts, while eliminating less healthy members of the community, PCD confers a selective advantage to a population during subsequent seasons.

Materials and methods

Cell growth and harvesting

P. gatunense cells were collected from a depth of 3 m in station H at Lake Kinneret as described in [3]. Batch cultures were grown as described in [3].

Analysis of ROS in the cells and antioxidant activity

Cells were stained with dihydrorhodamine123 [18] in the dark for 15 min and collected on polycarbonate nucleopore filters (Osmonics). Total ROS were measured with 20 μm 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes) [19]. Measurements were from three independent experiments (n = 4 in each data set).

Determination of cell death

Cell death was assessed by incubation with 25 nM Sytox (Molecular Probes) for 15 min in the dark and counted using the fluorescent microscope.

TUNEL labeling

Cells were fixed in 50% acetone or methanol for 20 min, washed in graded ethanol (100%, 80%, 50%, 20%), and 3 times with PBS, and permeabilized with 0.1% Triton X-100. Cells were labeled according to the manufacturer's instructions (Promega), and analyzed with a BioRad confocal microscope with laser beam excitation of 488 nm and emission of 525 nm.

Chemicals and other treatments

Trans-(epoxysuccinyl)-L-leucylamino-4-guanidinobutane (E-64), AEBSF and chymostatin were from Boehringer Manheim. K252A and GO6873 were from Calbiochem. All other chemicals and catalase were from Sigma. Inhibitors were added into the culture medium in a single dose.

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