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Physiological stress and cell death in marine phytoplankton: Induction of proteases in response to nitrogen or light limitation

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Abstract

The physiological processes of natural phytoplankton mortality due to environmental stress (vs. that caused by sedimentation and predation) are poorly understood. Cell survival was examined in batch cultures of the diatom *Thalassiosira weissflogii* and the chlorophyte *Dunaliella tertiolecta* during deprivation of fixed nitrogen or light. Despite severe impairment of photosynthetic efficiencies, both species remained viable during 2 weeks of N starvation. Under N stress, a specific protease was induced in the diatom, overall activity of proteases doubled, and there was gradual, selective loss of certain proteins, especially ribulose 1,5-bisphosphate carboxylase/oxygenase. Light-deprived diatoms were virtually unaffected, but the chlorophyte underwent catastrophic cell death after about 6 d of darkness. Cell death coincided with a large increase in protease activity and the induction of a specific protease. Although we cannot completely rule out roles for viruses or bacteria in the losses of cells, the consistent timing, the unique response to stress, and the coincident expression of a specific protease strongly suggest that the process is a form of autocatalyzed cell death, such as apoptosis. While of uncertain adaptive significance, phytoplankton cell death may have implications for species succession and cycling of organic matter in aquatic ecosystems.

Despite the considerable contribution of marine phytoplankton to global primary production (*see* Falkowski 1994), many aspects of the ecology and physiology of these highly diverse organisms are poorly understood. For example, mortality of phytoplankton populations is often considered to be caused only by external factors such as sedimentation and herbivore grazing (e.g. Walsh 1983), but only rarely have the physiological processes involved in natural, internally driven cell mortality been addressed. Indeed, there is a temptation to view photoautotrophic, unicellular eukaryotic algae as being essentially immortal. There are, however, reports of mass cell lysis in natural aquatic ecosystems (Brussaard et al. 1995; Van Bleijswijk et al. 1994). Such lysis could be due to viral pathogens (Bratbak et al. 1993; Suttle 1992), but an alternative possibility, that mortality is caused by factors internal to the cells, has not been adequately investigated.

Very little is known about the processes of cell death in unicellular organisms. By analogy with processes in multicellular organisms, cell death might be necrotic (swelling and immediate lysis, as in a response to injury) or apoptotic (shrinkage and fragmentation, as in some forms of programmed cell death) (Raff 1992; Trump 1966). Only very recently have cell death programs have been identified in prokaryotes (Naito et al. 1995) and unicellular eukaryotes (Welburn et al. 1997), and we are aware of only a single example in a freshwater alga (Kirk 1994).

Because phytoplankton cell lysis events apparently occur after blooms, when growth conditions become suboptimal (Brussaard et al. 1995; Van Bleijswijk et al. 1994), we hypothesized that environmentally stressful conditions might trigger cell death. We examined the responses of two taxonomically divergent phytoplankters—the diatom *Thalassiosira weissflogii* and the chlorophyte *Dunaliella tertiolecta*—to severe, but ecologically relevant, physiological stresses. The conditions selected were nitrogen starvation (in the presence of saturating irradiance) and light deprivation (in the presence of sufficient nutrients). The former stress limits the ability of the cell to use photosynthetically fixed carbon for protein synthesis (Falkowski et al. 1989; Berges et al. 1996; Turpin 1991), but does not prevent the formation and utilization of photosynthetic storage products. The latter condition does not directly prevent protein synthesis, but eliminates the ability of the cell to acquire reduced organic carbon to meet metabolic demands for energy or for new cell synthesis.

To monitor cell responses, we measured changes in variable fluorescence emissions (F_v/F_m), which provides an index of photosynthetic capability (*see* Falkowski and Kolber 1995); changes in SDS-PAGE protein profiles; and changes in levels of cell-associated proteolytic enzymes. Proteases may be involved in reprocessing of endogenous protein during nutrient deprivation, as well as in stress responses (Vierstra 1993; Harrington et al. 1994).

Materials and methods

Cultures of the diatom *Thalassiosira weissflogii* (Gru.) Fryxell et Hasle (clone T-VIC) and the chlorophyte *Dunaliella tertiolecta* Butcher (clone DUN) were grown in 3-liter semicontinuous batch cultures at 18°C, as previously described (Berges and Falkowski 1996). For N deprivation

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experiments, duplicate cultures were transferred into medium containing 75 μM nitrate and allowed to exhaust the nutrient in continuous saturating (but not supraoptimal) irradiance (200 μmol quanta m^{-2} s^{-1}). In light-deprivation experiments, media contained 880 μM nitrate. Duplicate cultures were grown to one-half the cell concentration at which growth ceased to be logarithmic, and were placed in continuous darkness, achieved by wrapping flasks in alternating layers of aluminum foil and black cloth. All cultures were stirred at low speed and gently bubbled with filtered air throughout the experiments.

Cell counts were monitored using a model TAII Coulter Counter. Chlorophyll and carotenoid pigments were measured spectrophotometrically in 90% aqueous acetone extracts (Dubinsky et al. 1986; Jeffrey and Humphrey 1975). Photochemical efficiencies in the PSII reaction centers, indicated by quantum yields for fluorescence (F_v/F_m), were measured on dark-adapted cells using a Turner Designs model 10 fluorometer, before and after addition of 10 μM DCMU (*see* Geider et al. 1993). Carbon and N content and C:N ratio were determined on filtered samples with a Perkin Elmer CHN analyzer, and nitrate concentrations were determined as in Falkowski et al. (1989).

For protease measurements, cells were harvested by centrifugation at 3,000 × *g* and protease activities determined fluorimetrically as previously described (Berges and Falkowski 1996). Both caseinolytic activity (measuring the hydrolysis of fluorescein-isothiocyanate [FITC]-labeled casein) and leucine aminopeptidase (LAP) activity (measuring hydrolysis of leucine β-naphthylamide) were measured (Berges and Falkowski 1996). Units of activity (U) were defined as micromoles of fluorescent product (TCA-soluble FITC, or free β-naphthylamide) produced per minute.

Samples for SDS-PAGE were collected by centrifuging 100-ml volumes of culture, and were prepared, loaded on an equal protein basis, separated by electrophoresis, and silver stained as previously described (Berges and Falkowski 1996). Zymograms of proteases were prepared as described in Berges and Falkowski (1996). Samples collected by centrifugation were homogenized by a combination of freeze-thawing and sonication in buffer without SDS or reducing agents. Samples were not boiled and were spun briefly at 7,500 × *g* just prior to loading to remove cell fragments. After electrophoresis, protease activities were detected by diffusing casein into the gels, followed by Coomassie staining (Sarath et al. 1989).

Bacterial cell counts and cell dimensions were measured with an epifluorescence microscope on filtered samples stained with DAPI (Porter and Feig 1980). While axenic cultures of both species could be maintained during logarithmic growth phases, bacteria were detected in cultures under light and nutrient stress; however, keeping cultures axenic by the use of antibiotics was ruled out due to potentially serious effects on algal metabolism (e.g. Cotrell and Suttle 1993). We estimated the potential contribution of bacterial protein to measurements by two methods. First, from knowledge of bacterial numbers and volumes, we applied a C:vol conversion (0.15 pg C μm^{-1}; Norland 1993) to calculate the fraction of C contained in bacteria, relative to the total C in algal cultures determined by CHN analysis. Assuming a constant ratio between C and protein (probably a worst-case scenario), these data were used to estimate the proportion of bacterial protein represented in the samples. Additionally, we determined the number of bacteria harvested with the algae by performing DAPI counts of the culture before and (in the supernatant) after centrifugal harvesting.

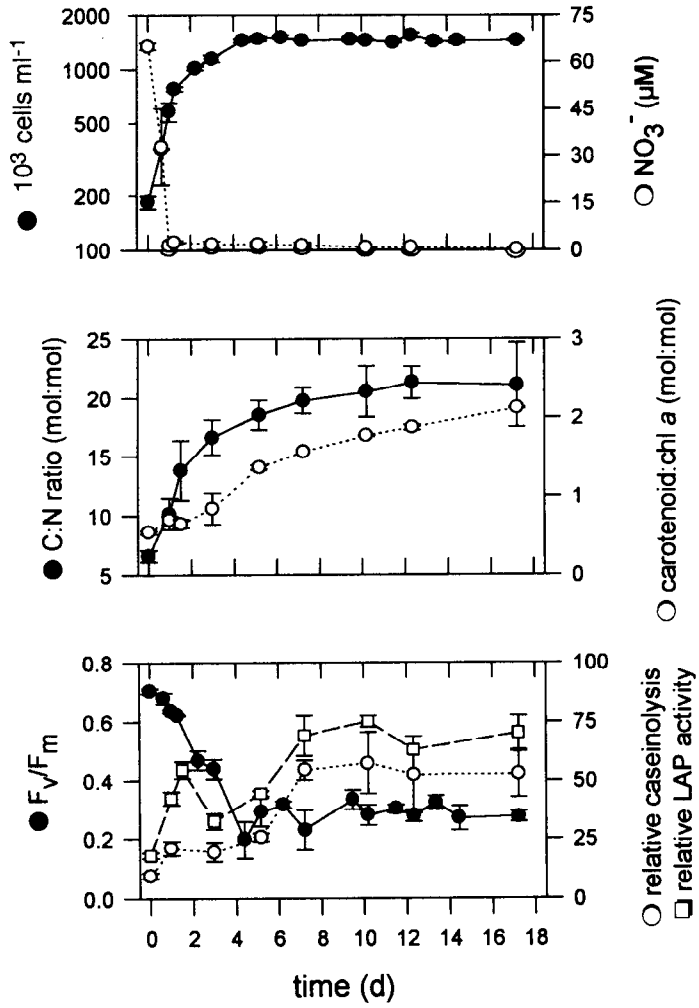

Fig. 1. Responses of cultures of *Dunaliella tertiolecta* to nitrogen starvation. For relative protease activities, 100 corresponds to 4×10^{-5} U (mg protein)$^{-1}$ caseinolytic activity and 3×10^{-4} U (mg protein)$^{-1}$ leucine aminopeptidase (LAP) activity. Symbols represent means of measurements in two replicate cultures with standard errors.

Results

In both species, N starvation led to gradual increases in cellular C reserves without a concomitant increase in organic N (reflected in increased C:N ratios), loss of Chl *a* (demonstrated by increased carotenoid:Chl *a* ratios), and declines in photochemical efficiency (Figs. 1, 2). The increases in carotenoid:Chl *a* ratios in both species were almost entirely due to loss of Chl *a*; carotenoid concentrations did not change appreciably under N starvation. Judging from F_v/F_m values, N starvation effects were more severe in the diatom

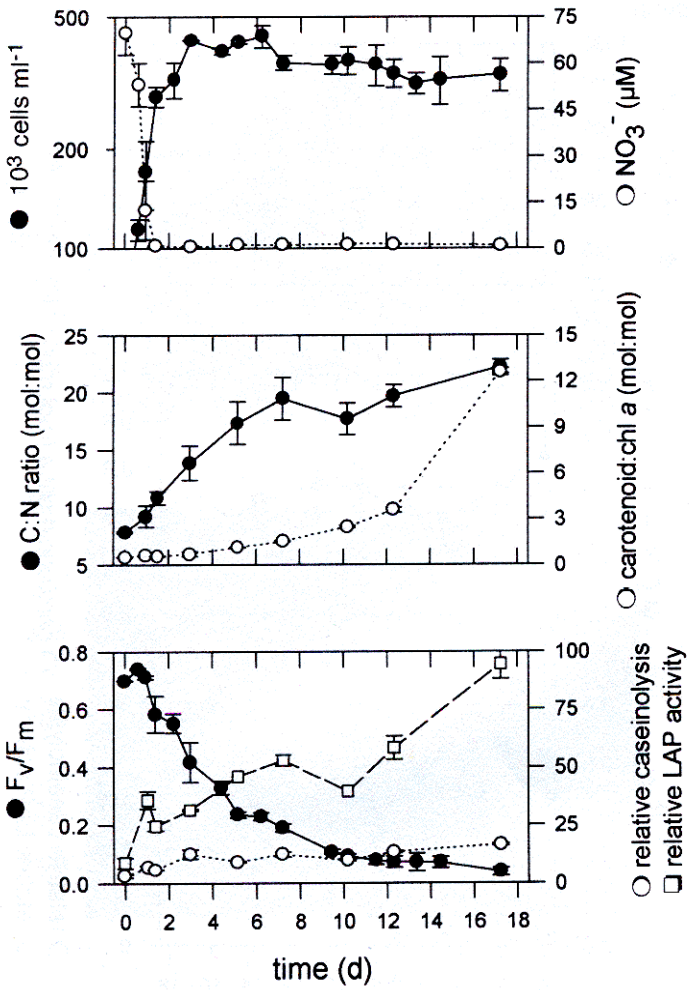

Fig. 2. Responses of cultures of *Thalassiosira weissflogii* to nitrogen starvation (compare with Fig. 1).

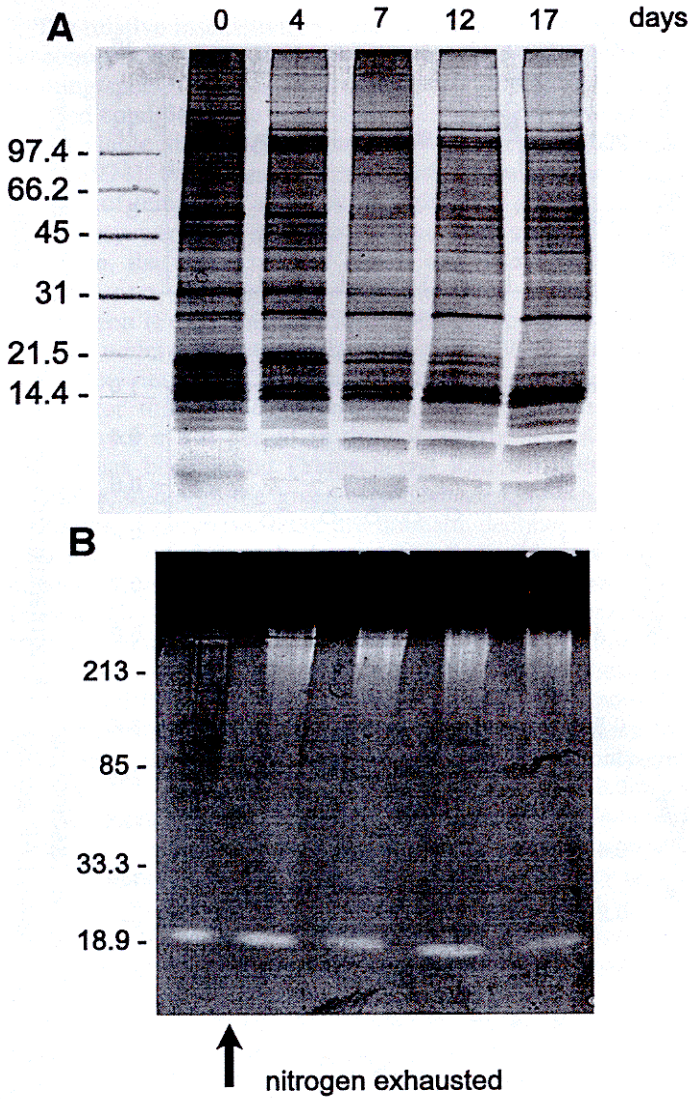

Fig. 3. Protein composition of *Thalassiosira weissflogii* during nitrogen starvation. A. Silver-stained 4–20% gradient SDS-polyacrylamide gel of samples prepared from cultures shown in Fig. 2. B. Casein zymograms of protease activities detected after separation of proteins using 10% native polyacrylamide gels under nondenaturing conditions. Time in days corresponds to that in Fig. 2. Labels to the left indicate location of molecular weight standards in kDa.

than in the chlorophyte. F_o and F_m each decreased with increasing N starvation; the decline in F_v/F_m was thus caused by more rapid decrease in F_m. Silver-stained SDS-PAGE patterns in both species revealed a rapid loss of the photosynthetic carboxylation enzyme Rubisco (at ~55 kDa), as well as the light-harvesting pigment protein complexes (at ~20 kDa) (data shown only for *T. weissflogii,* Fig. 3A).

Nitrogen starvation also led to an increase in protease activity in both species (Figs. 1, 2). There were some differences between the species. LAP activities increased 12-fold for the diatom, but only 4-fold for the chlorophyte, whereas only in the chlorophyte were appreciable changes in caseinolytic activity seen. For *D. tertiolecta,* casein zymograms revealed a single constitutive protease. Because gels were necessarily run under nondenaturing conditions, the relative molecular mass of this protease cannot be specified; however, it ran reproducibly just below a denatured protein standard of 20 kDa. For convenience, we refer to proteases by their "apparent" molecular relevant mass in the results and following discussion. No changes in the relative activity of this protease were seen during starvation (data not shown; cf. Berges and Falkowski 1996). For *T. weissflogii,* a similar, constitutive protease of similar size was observed. As in the chlorophyte, this protease did not change in activity as cells became increasingly N starved, but there were substantial increases in a protease with an apparent molecular mass >200 kDa (Fig. 3B). Despite the considerable declines in photosynthetic energy conversion efficiency, cell numbers remained constant for a period greater than 2 weeks in both species.

T. weissflogii was hardly affected by almost 2 weeks of darkness—cell numbers, pigment ratios, and photosynthetic capacity remained very stable (Fig. 4). After a rapid, initial decline, owing mainly to a loss of cell carbon, C:N ratios also remained essentially unchanged. No changes were observed in protease activities (Fig. 4) or protein patterns in silver-stained gels (data not shown).

In contrast, light deprivation induced catastrophic declines

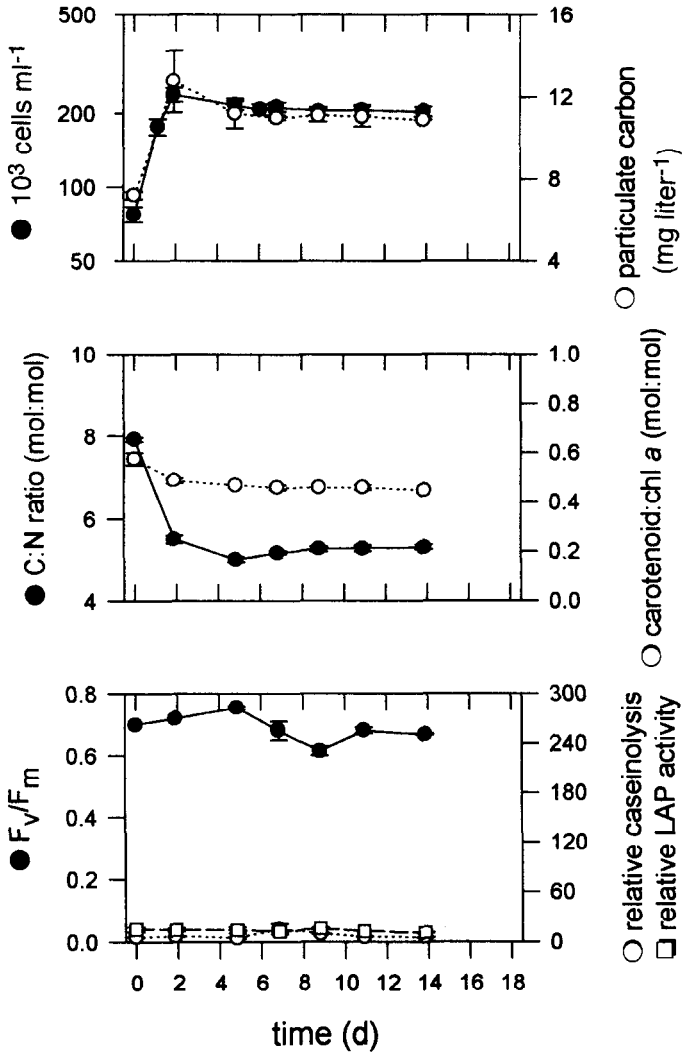

Fig. 4. Responses of cultures of *Thalassiosira weissflogii* to a transition from saturating irradiance (200 μmol quanta m^{-2} s^{-1}) at 18°C to darkness after the day 1 sampling.

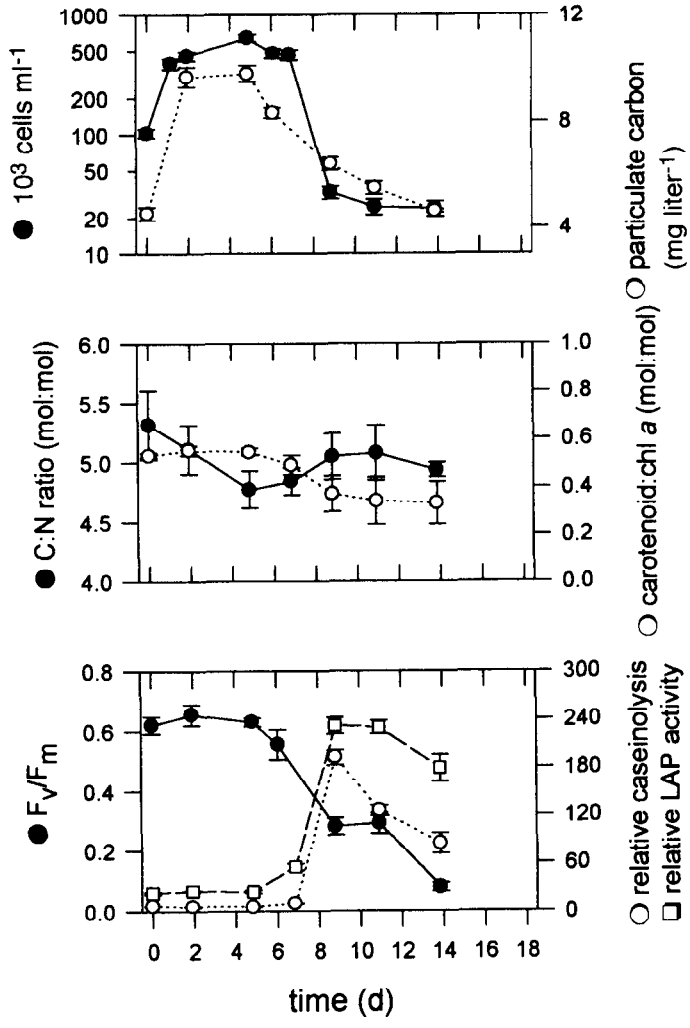

Fig. 5. Responses of cultures of *Dunaliella tertiolecta* to a transition from saturating irradiance (200 μmol quanta m^{-2} s^{-1}) at 18°C to darkness after the day 1 sampling.

in cell numbers in cultures of *D. tertiolecta* (Fig. 5). Declines were very sharp (unlike the gradual declines seen in N-starved cells), beginning on days 6–7 after light deprivation. Within 48 h, the formerly dense green cultures became virtually transparent. These declines were reproducible and the timing was consistent, irrespective of initial culture density. Decreases in cell numbers coincided with >60% reduction in particulate C in the cultures, indicating that a substantial portion of the products of cell lysis were less than the 1-μm (nominal) pore size of the Gelman A/E filters used to collect samples.

Culture declines coincided with a rapid loss of photosynthetic efficiency. In this case, F_m alone decreased over the first 5 d, after which both F_o and F_m declined to virtually zero. Furthermore, there was an increase in protease activities more than a factor of three greater than levels measured during N deprivation (Fig. 5). Silver-stained SDS-PAGE revealed marked qualitative changes in the electrophoretic profile of cell proteins (Fig. 6A), which accompanied increases in protease activities. We observed that during the lytic process, the constitutive protease (<20 kDa) apparent molecular weight disappeared and a new protease with an apparent molecular weight of 60 kDa was induced (Fig. 6B). A 60-kDa protease was also induced in cells shocked by a temperature shift from 18°C to >30°C (data not shown).

Upon reexposure to light, light-deprived *Dunaliella* cultures regrew to previous culture densities over the course of 3 weeks. The lag phase was unusually long, but revealed that viable cells remained even after 2 weeks of total darkness.

Discussion

In both species, N starvation led to characteristic symptoms of N limitation (Falkowski et al. 1989; Geider et al. 1993), but the vast majority of cells survived the stress.

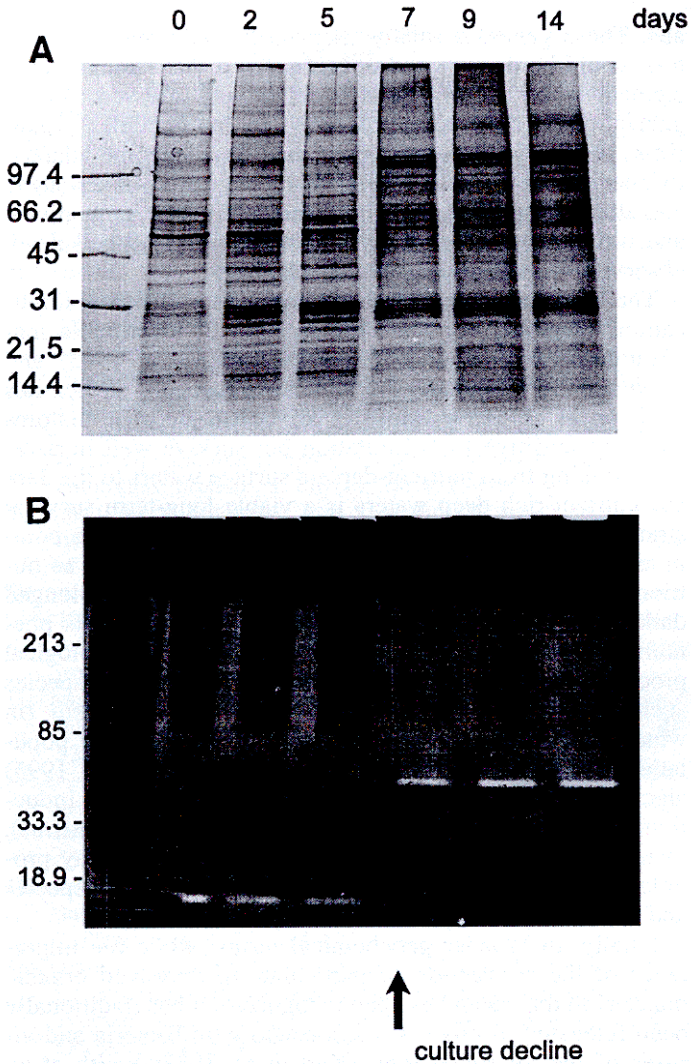

Fig. 6. Protein composition of *Dunaliella tertiolecta* following a transition from saturating irradiance (200 μmol quanta m^{-2} s^{-1}) to darkness after the day 1 sampling. A. Silver-stained 4–20% gradient SDS-polyacrylamide gel of samples prepared from cultures shown in Fig. 4. B. Casein zymograms of protease activities detected after separation of proteins using 10% native polyacrylamide gels under nondenaturing conditions. Time in days corresponds to that in Fig. 4. Labels to the left indicate location of molecular weight standards in kDa.

There was a gradual increase in protease activities in both species, and evidence that there were differences between the species in the proteases responding to N starvation. In bacteria, increased proteolytic activity is often found under conditions of environmental stress (Matin et al. 1989; Reeve et al. 1984; Teichert et al. 1989; Vierstra 1993). In fact, proteases can be critical to cell survival—nutrient-limited fungal and bacterial mutants that lack specific proteases die far more quickly than do wild types (Reeve et al. 1984; Teichert et al. 1989). The proteases detected are found in nutrient-sufficient cells and are probably involved in normal protein turnover. Increases in protease activity may be related to recovery of N from nonessential proteins, and use of such N for synthesis of new proteins (*see* Vierstra 1993).

The relative insensitivity of the diatom to light deprivation is concordant with previous observations that nonspore-forming species such as *T. weissflogii* survive several weeks in good condition. The congeneric *Thalassiosira gravida* remains viable after 90 d of darkness (Smayda and Mitchell-Innes 1974). Peters and Thomas (1996*a*) demonstrated that a range of temperate and Antarctic diatoms are able to survive many weeks of darkness in vegetative states, and, furthermore, that *Thalassiosira rotula* and *Thalassiosira antarctica* can also survive simultaneous light and N deprivation (Peters and Thomas 1996*b*). In contrast, *D. tertiolecta* undergoes catastrophic cell death. While such dramatic declines in cell numbers have not been previously reported for this species, precipitous loss of photosynthetic ability over a 5-d period in the congeneric *Dunaliella euchlora* has been found (Yentsch and Reichert 1963). Furthermore, although the green alga *Scenedesmus* can survive 3 months of darkness, there are dramatic declines in protein over the first 4–5 d, and a large proportion of the population undergoes lysis in the short term (Dehning and Tizler 1989). Interestingly, even very short exposures to subcompensation irradiance (such as brief exposure to light during sampling) have been shown to prolong dark survival (Hellebust and Terborgh 1967). Our results were consistent only under the most rigorous control of light conditions, and this may explain why the phenomenon has not been commonly observed.

The mechanism of culture decline is intriguing. Whereas viral lysis could cause such a phenomenon, this is unlikely for two reasons: (1) the timing of the decline is very reproducible and insensitive to the cell density of the *D. tertiolecta* culture (unlike the situation for previously characterized lytic viruses, e.g. Bratbak et al. 1993), and (2) declines in nutrient-stressed cultures inoculated from the same stock cultures (which would have contained viruses as well) were never observed. Furthermore, a 0.22-μm filtrate from lysed *Dunaliella* cultures has no effect on logarithmically growing cultures of this species (data not shown). Bacteria were in the cultures, and cases of bacterially mediated algal cell lysis are known (Cole 1982). However, there is little information about this phenomenon and the arguments against viruses being responsible would also apply for bacteria.

The possibility that the changes in cell proteins or specific proteases were due to bacteria is also unlikely. Maximum bacterial numbers of 8×10^5 ml^{-1} were seen in N-starved *T. weissflogii* cultures, but in *D. tertiolecta* cultures they were always $<1 \times 10^5$ ml^{-1}. Based on their low biomass and small size (average volumes of 1–2 μm^3), we calculated that bacterial protein could not contribute >0.4% of total protein in the worst case. More than 90% of the bacteria remained in the supernatant after centrifugation. The resulting levels of bacterial protein would have been undetectable in silver-stained gels. Unless one accepts the presence of bacterial enzymes with extraordinarily high specific activities (i.e. 10^5-fold that of algal protease), it seems unlikely that zymograms would show bacterial proteases either. Although we cannot exclude bacterial influence, a conservative argument would suggest that the proteases measured are algal rather than bacterial in origin.

An alternative hypothesis is that the mortality was induced

by the algae themselves. The evidence for this rests on the specificity of the triggering factor (darkness), the reproducibility of the declines, the strong correlation between physiological indicators and the declines, and the specific induction of a protease at the point of decline. While we cannot yet show cause and effect, we suggest that the evidence is compelling.

The phenomenon we have described has some similarities to apoptotic cell death in multicellular organisms. In higher plants, apoptosis can be induced by stresses such as pathogen exposure or the production of superoxides (Greenberg et al. 1994; Greenberg 1997; Jabs et al. 1996), and specifically induced proteases seem to play key roles in animal tissues (e.g. Enari et al. 1995). The definition of apoptosis is controversial—some authors use it as a synonym for programmed cell death, whereas others choose to use it as a descriptive term for the cell state following death (Trump 1996). The phenomenon is usually characterized by chromatin condensation, degradation of DNA resulting in a "laddering" on electrophoretic gels, and membrane "blebbing" (Ryerson and Heath 1996). It is not clear what the morphologies characteristic of tissues might look like in a unicellular organism. By using a dUTP nick-labeling reaction (Gavrieli et al. 1992), we failed to detect evidence of the DNA fragmentation characteristic of apoptosis in permeablized *D. tertiolecta* cells fixed on glass slides. However, there are also plant cell death phenomena that have not yet been identified as apoptotic, including plant hypersensitive responses (Greenberg et al. 1994) and death and autolysis of higher plant tracheary elements (Ye and Varner 1996). The latter process also involves expression of specific proteases with molecular masses in the ranges we have observed. The rapidity of the cell death phenomenon in *D. tertiolecta* suggests that either most cells responded to the same stress simultaneously, or that the initiation of the lytic process had a positive feedback on viable cells (i.e. a type of "quorum sensing"; *see* Raff 1992). That the inoculation of a healthy culture with cell-free filtrate did not stimulate lysis suggests that the latter hypothesis is not responsible for the cell death process.

If declines in *D. tertiolecta* cultures represent activation of a cell death program, then there are potential evolutionary implications. While the origins of cell autolysis are obscure, recent reports indicate that bacteria and protozoa undergo programmed cell death (Naito et al. 1995; Yarmolinsky 1995; Welburn et al. 1997), and there is at least one report of such an occurrence in a colonial alga (Kirk 1994). Presence of cell death in bacteria and algae suggests that this phenomenon arose early in evolutionary history. Although the occurrence of bacterial and protozoan cell death has fostered the idea that it is adaptive or "altruistic" in the sense that surviving cells benefit from the organic compounds released by dying ones (Yarmolinsky 1995; Welburn et al. 1997), autolysis in an obligate autotroph such as *Dunaliella* (which cannot benefit from organic molecules; *see* Wheeler et al. 1974) would require a very different interpretation.

We hypothesize that genes encoding stress-induced proteases might have been incorporated into bacterial and eukaryotic genomes from relict viral infections, comparable to the endogenous retroviral protease genes found in metazoans. These genes, normally silenced by host repressor factors, could be derepressed or reorganized via a transposable element when the organism is selectively stressed. As organizational complexity proceeded to multicellular organisms, such relict protease genes may have become adapted to phenomena such as the death of vegetative macroalgae and alternations of gametophyte and sporophyte generations, and ultimately the developmentally programmed cell death observed in metazoa and higher plants.

The results of our study have important ecological implications. Diatoms dominate the fraction of identifiable material in many sediment trap records (Billett et al. 1983), and rapid sinking rates result in viable cells reaching the benthos (Cahoon et al. 1994; Smith et al. 1996). Because diatoms are very sensitive to N limitation but survive well in darkness, sinking from nutrient-deplete surface waters to the dark but nutrient-rich deep waters is a viable long-term survival strategy (Smetacek 1985). In contrast, *D. tertiolecta* commonly occurs in tidepools where it could be subject to nutrient deprivation, but would rarely experience prolonged darkness. The ecological significance of the cell lysis phenomenon in *Dunaliella* is uncertain, but the physiological process may be common to other groups of algae. If species such as *Emiliania huxleyi* and *Phaeocystis pauchetii* (in which lytic losses can account for up to 75% of the population; *see* Van Bleijswijk et al. 1994; Brussaard et al. 1995) also show lysis events with a characteristic protease induction (perhaps subject to different triggering mechanisms), then the phenomenon described for *D. tertiolecta* may provide an understanding of the dynamics of individual species and the ecology of species succession.

Finally, in broader geochemical terms, while the importance of the conversion of particulate to dissolved organic material in the ocean has been recognized, it has traditionally been relegated to processes associated with bacteria and viruses of the microbial loop (Karl et al. 1988; Smith et al. 1992). The results presented here suggest that autocatalytic cell death could accomplish this conversion as well.

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