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CONDITIONAL SENESCENCE IN *CHLAMYDOMONAS REINHARDTII* (CHLOROPHYCEAE)[1]

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The mechanisms of microalgal senescence may play an important role in nutrient recycling and enhanced survival. However, the aging physiology of microalgae is an understudied phenomenon. To investigate the patterns of conditional senescence in *Chlamydomonas reinhardtii* P. A. Dangeard, we used a cell wall-less strain, transformed with a reporter gene to infer changes in photosynthetic gene expression. We examined plastid ultrastructure, photosynthetic function, and photoprotective mechanisms during aging in batch cultures. LHCII transcription levels decreased before the population entered stationary phase, and the characteristic transcriptional light-shift response was lost. A decline in photosynthetic proteins with a concomitant increase in the photoprotective protein, LHCSR, was observed over time. However, nonphotochemical quenching remained stable during growth and stationary phase, and then declined as alternative quenching mechanisms were up-regulated. Photosynthetic efficiency declined, while Fv/Fm remained stable until the death phases. As the culture progressed through stationary phase, disorganization of the chloroplast was observed along with an increase in cytoplasmic oil bodies. We also observed a partial recovery of function and proteins during the final death phase, and attribute this to the release of nutrients into the medium from cell lysis and/or active secretion while cells were senescing. Allowing open gas exchange resulted in high levels of sustained starch production and maintained maximum cell density, prolonging the stationary phase.

***Key index words:* *Chlamydomonas*; LHCSR; light harvesting complexes of PS II; nonphotochemical quenching; oil bodies; photoacclimation; senescence**

***Abbreviations:* Φ_{NO}, energy loss via non-regulated mechanisms; Φ_{NPQ}, energy loss via energy-dependent regulated mechanisms; Φ_{II}, quantum efficiency of PSII; LHC, light-harvesting complex; LL, low light; ML, medium light; NPQ, nonphotochemical quenching; ROS, reactive oxygen species**

Microbial organisms are key components of most environments. These populations are generally considered "immortal" and any losses in nature are attributed to grazing, viral lysis or sedimentation. However, heterogeneity within the environment leads to nutrient patchiness and, as unicellular populations increase, nutrients become depleted leading to cell death. This phenomenon is analogous to cultures grown in closed systems (batch culture) under laboratory conditions. All batch culture growth is characterized as having a logarithmic growth phase where nutrients are sufficient to support high rates of growth. Then, as cell number increases and nutrients become deplete, there is a transition to a stationary phase where division ceases and cell numbers are maintained, which is generally referred to as conditional senescence (Fredriksson and Nyström 2006). It is under this phase that cells undergo metabolic and structural changes that can lead to death and the loss of cell numbers (Fogg and Thake 1987, Wanner and Egli 1990).

Like most microbes, microalgae grown in batch culture undergo predictable changes in growth rate as nutrient levels decline over time. From exponential phase, where most biochemical experiments are focused, the culture begins a deceleration of growth, reaches stationary phase and then, depending on conditions, enters a death phase (Fogg and Thake 1987, Wanner and Egli 1990, Levert and Xia 2001). There have been several studies looking at the phenomena of senescence in a variety of microalgae, and typically stationary phase is accompanied by a loss in chlorophyll (Ebata and Fujita 1971, Messer and Ben-Shaul 1972) along with a decrease in photosynthesis and respiration (Fogg 1959). There are obvious changes in the structure of the thylakoid membranes in stationary phase, with thylakoid membranes becoming more compact (McLean 1968, Messer and Ben-Shaul 1972, Oliveira and Bisalputra 1977). The capabilities of photosynthesis also change with the age and nutrient status, including the activity of both PSI and PSII (Ebata and Fujita 1971, Vavilin et al. 1999) and the ratio of PSII to PSI (Naus and Melis 1991). One of the most striking observations in microalgae is the accumulation of lipid bodies during stationary phase (Milner 1948, Collyer and Fogg 1954, McLean 1968, Oliveira and Bisalputra 1977). Cytosolic lipid bodies increase in algae as a function of nutrient deprivation and a shift in the carbon/nitrogen ratio, characteristics of stationary growth (Fogg and Thake 1987).

The process of leaf senescence in plants is more thoroughly studied, though similarities in the changes during batch culture growth of microalgae and leaf

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senescence have been noted (McLean 1968). However, being multicellular, leaf senescence is distinct and characterized by the coordinated disassembly of macromolecules and the recovery of nutrients during cell death. These nutrients are then remobilized to other portions of the growing plant (Noodén et al. 1997, Matile 2001, Buchanan-Wollaston et al. 2003). The chloroplast is the primary target for degradation during senescence (Guo and Gan 2005) where it is remodelled into the gerontoplast (Thomas et al. 2003), while the mitochondria, nucleus, and the inner plastid envelope remain functional until the later stages of senescence (Biswal and Biswal 1988, Smart 1994, Noodén et al. 1997). Senescence in plants is also characterized by a loss in chlorophyll and an overall decrease in photosynthetic function (Jenkins et al. 1981, Keskitalo et al. 2005, Zimmermann and Zentgraf 2005, Ananieva et al. 2008). Ultrastructure analysis shows that during leaf senescence the thylakoid membrane becomes unappressed and disorganized and plastoglobuli (lipid bodies contained within the chloroplast) increase in both number and size (Hurkman 1979, Ghosh et al. 2001, Kutík et al. 2001, Prakash et al. 2001). These lipid bodies contain triacylglycerol (Kaup et al. 2002) and α-tocopherol (Rise et al. 1989). Although oil bodies in microalgae are located within the cytoplasm, they bear a striking resemblance in content and structure with senescence-induced plastoglobuli associated with thylakoid turnover in senescing leaves (Matile et al. 1999, Kutík et al. 2001). These cytosolic lipid bodies may function as a sink for electrons generated in the photosystems and may also have a more direct photoprotective role, absorbing excess light and functioning as a "sunscreen" for the chloroplast (Hu et al. 2008).

Photoautotrophs need to optimize photosynthesis for growth and development. When light capture exceeds the ability of the photosynthetic organism to fix carbon, which is likely to happen as nutrients become depleted in aging batch cultures, excitation energy can pass to oxygen, generating reactive oxygen species (ROS) (Asada 2000). This leads to the oxidation of macromolecules and eventual bleaching of the organism. Lipid peroxidation and increases in membrane permeability leading to decreased photosynthetic capacity are classic symptoms of both general oxidative stress (Asada 2000) and plant senescence (Matile 2001). Inducible mechanisms that decrease the capacity to absorb light or dissipate it in a safe and controlled fashion, rather than allowing this energy to reach oxygen, are called photoacclimation mechanisms (Falkowski and Laroche 1991, Anderson et al.1995, Niyogi 2000). It is not clear which photoacclimation mechanisms are functioning in aging microalgae. In plants, there is typically, a decrease in photosynthetic electron flow (Φ_{PSII}) in senescing leaves (Jenkins and Woolhouse 1981) before any observable chlorophyll loss. This is primarily due to the proteolysis of RubisCO (Dertinger et al. 2003) that decreases CO_2 fixation (Lepedŭs et al. 2005) and is associated with losses of PSI and PSII reaction centers (Yordanov et al. 2008). The reaction centers still present are functioning (Jenkins and Woolhouse 1981, Yordanov et al. 2008), so no observable change in quantum yield (Fv/Fm) is detected. Of the proteins associated with photosynthesis, it appears that the light harvesting complexes of PS II (LHCII) are the most stable (Humbeck and Krupinska 2003, Tang et al. 2005). Since LHCII are responsible for light harvesting this seems counterintuitive, however, LHCII stability may be necessary for the continued functioning of the photoprotective mechanism known as NPQ.

There is considerable evidence that oxidative stress plays a role in plant senescence (Keskitalo et al. 2005, Ananieva et al. 2008). This fits well with Harman's (1981) oxidative stress hypothesis in which the accrual of macromolecular damage by ROS is purported to be the principle causal factor promoting aging. Certainly, oxidative stress and antioxidant capacity have been correlated to life span in many organisms (Sohal 2002). The key principle behind the oxidative stress hypothesis of aging is that an imbalance between ROS generation, antioxidant protection, and repair of oxidative damage leads to aging and senescence (Beckman and Ames 1998). Since maintaining this balance is the primary goal of photoacclimation, there is a conceptual connection between photoprotection and aging in photoautotrophs. *Chlamydomonas* is already a model system for examining the structural and functional aspects of photosynthesis and is, therefore, a prime candidate for senescence studies. In this study, we used a transgenic cell wall-less strain of *Chlamydomonas reinhardtii*, containing a reporter gene for LHCII transcriptional regulation (Durnford et al. 2003, Humby et al. 2009) to examine the general process of senescence in microalgae with the goal of examining the photoprotective mechanisms during photoheterotrophic batch culture aging.

MATERIALS AND METHODS

Strains and culturing. A cell wall-less laboratory strain (27.69) containing the ARS:LHC-reporter gene system was used for these experiments (Durnford et al. 2003, Humby et al. 2009). For batch culture aging analysis, two separate experiments, under identical conditions, were conducted using three replicates each. Cultures were inoculated from exponentially growing starter cultures and tracked over time. For all physiological experiments, cultures were started from TAP medium plates (Harris 1989) and grown mixotrophically to mid-exponential phase (4×10^6 cells $\cdot$ mL^{-1}) in 75 mL of TAP in a 250 mL Erlenmeyer flask. These cells were used to inoculate 500 mL of TAP media in 1 L Erlenmeyer flasks at a starting concentration of 3×10^5 cells $\cdot$ mL^{-1}. Flasks closed with a foam stopper fitted with a glass tube and plastic tubing to facilitate sampling of culture with a 50 mL syringe without removing the lid were used. The lid apparatus was covered with two layers of aluminum foil and gently agitated on orbital shakers (20 rpm) in a Conviron growth chamber at 24°C, under continuous white light (cool white). The LL environment was maintained at ~80 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. At

various time points during the experiment, subsamples of the culture were removed using an attached syringe.

To determine the extent that air exchange affects batch culture growth, one set of cultures were plugged with a foam stopper and covered in aluminum foil (no gas exchange) and in the others, a Pasteur pipet was inserted into the lid and sterile, humidified air was blown into the headspace of the flasks (but not bubbled in the culture, as it causes too much foaming).

For microscopy, cultures were started in 75 mL TAP in LL conditions until mid-exponential (ca. 2–5 $\times$ 10^6 cells $\cdot$ mL^{-1}), cultures were then concentrated to a final value of 1×10^8 cells $\cdot$ mL^{-1} and a series of 10 μL drops were placed on TAP Agar (1.3%) plates, parafilmed, and grown in LL conditions.

Cell viability, counts, estimates of size, and chlorophyll analysis. For viability estimates, cells were exposed to Trypan Blue (final concentration 0.4% in phosphate buffered saline) for 15 min (Tolnai 1975), placed on a slide and the total number of cells and nonviable cells were counted. Trypan Blue is a vital stain that penetrates dead cells, rendering them blue, but which living cells can exclude. During these trials with the cell wall-less strain, all cells at all time points were living. This was attributed to rupture of dead cells due to agitation. Therefore, cell number could be used as a proxy for cell survival. To obtain cell numbers, cells were fixed in 1.25% (w/v) glutaraldehyde and counted using a hemocytometer and light microscope. Chlorophyll extraction was done in 80% (v/v) acetone, pigment concentrations were determined as previously described (Porra et al. 1989). Cell size was measured by capturing live cell images using a Leica DC-500 (GmbH, Heidelberg, Germany) and area of cells were estimated using Image J software.

Pulse amplitude modulated fluorometric analysis. Low light-acclimated cultures were dark adapted for 10 min on a shaker plus an additional 5 min dark adaptation following transfer to the reaction vessel at 24°C. Fm was determined by a saturating flash (2000 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}) for 0.6 ms, and then challenged with an actinic light of 500 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} (ML) to determine Fs. Fluorescence was measured using a Walz PAM 101 chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany). Fluorescent parameters were calculated according to the equations described by Genty et al. (1989), Schreiber et al. (1986), and Roháček (2002), unless otherwise noted. The paramaters calculated include: Maximum quantum efficiency of PSII (Fv/Fm = (Fm − Fo)/Fm); Quantum efficiency of photochemical energy under illumination (Φ_{II} = (Fm′ − Fs)/Fm′; Oxborough and Baker 1997); (NPQ = (Fm − Fm′)/Fm′); quantum yield of non-regulated quenching (Φ_{NO} = Fs/Fm); and quantum yield of NPQ (Φ_{NPQ} = (Fs/Fm′) − (Fs/Fm); Kramer et al. 2004, Klughammer and Schreiber 2008).

Protein analyses. A quantity of 20 mL of culture was removed from the liquid culture at the appropriate time, centrifuged at 3,000*g* for 3 min. The supernatant was promptly removed and the pellet frozen in liquid nitrogen and stored at −80°C until processed. Total protein was extracted by addition of equal volume of lysis solution (2% SDS, 0.2M Na_2CO_3, 0.5 mM PMSF). Cells were resuspended and samples sonicated on ice for two 5 s bursts. This solution was centrifuged for 5 min at 12,000*g* at room temperature and the supernatant was collected. Protein quantification was done using Bovine Serum Albumin standards and the BCA Protein Assay kit (Pierce, Rockford, IL, USA). 2.5 μg of each sample was fractionated on a 10% acrylamide SDS-PAGE gel (Sambrook and Russell 2001) and proteins electrotransferred onto ultrapure nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Western blots done as previously described (Durnford et al. 2003). Antisera included the following: Rabbit anti-PsaA (core subunit of PSI) from Agrisera (AS06 172); rabbit anti-CPII from *Chlamydomonas* (courtesy of Dr. K. Hoober); chicken anti-PsbA (D1 subunit of PSII; Agrisera AS05 084); chicken anti-RubisCO (courtesy of Dr. D. Campbell); rabbit anti Cyt f (Agrisera AS06 119) and rabbit anti-LHCSR (courtesy of Dr. M. Guertin). The primary antibody was detected by incubating the membrane for 1 h in horse radish peroxidase conjugated goat anti-rabbit or rabbit anti-chicken secondary IgG (H1L) antibody (Invitrogen, Carlsbad, CA, USA) and visualized with the ECL Western Blotting Detection Kit (Amersham, Fairfield, CT, USA) and detected using the Chemidoc system (BioRad, Hercules, CA, USA).

Arylsulfatase reporter gene assays. Cultures were grown in LL and shifted to ML following the protocol as described in Humby et al. (2009). The arylsulfatase reporter assay (Ohresser et al. 1997) was modified to accommodate the use of 96 well plates as described in Durnford et al. (2003).

Transmission electron microscopy (TEM) and histology. Cells were grown on TAP agar plates as described above then carefully scraped off and resuspended in 1 mL of liquid TAP medium. Cells were allowed to recover for 15 min then fixed with 1.25% glutaraldehyde for 1 h with gentle agitation. Cells were then rinsed in 0.1 M sodium cacodylate buffer two times for 30 min each then postfixed with 1% osmium tetroxide overnight, rinsed twice again and stored in 0.1 M sodium cacodylate buffered solution. Cells were then encased in 2% agarose and cut into 3 mm pieces. These preparations then underwent a dehydration series of increasing acetone: 30%, 50%, 70%, 80%, 90%, 100%, with each treatment lasting 20 min with an additional 20 min step at 100% acetone. Cells were then infiltrated with resin using a 2:1 ratio of acetone: fresh Epon-Araldite resin for 3 h, then 1:2 ratio overnight, followed by a 100% fresh resin infiltration for 2 h. Cells were then embedded in fresh Epon-Araldite resin in Beem capsules and polymerized at 50°C for 24 h then 60°C for 48 h. Polymerized blocks were trimmed and sectioned with a diamond knife on a Leica Ultramicrotome (GmbH). One μm thickness sections were placed on a warm microscope slide and stained with a premixed epoxy tissue stain (EMS # 14950, Hatfield, PA, USA) composed of toluidine blue and basic fuchsin (Spurlock et al. 1966) for 2 min and rinsed with water and visualized with a Leica DC-500 (GmbH). For TEM imaging 70 nm sections were collected onto copper grids (formvar-coated or formvar- and carbon-coated), examined in a JEOL 2011 STEM at 200 kv, and images were captured with a Gatan 4k $\times$ 4k digital camera.

Starch assay. Starch extraction procedure was based on the procedure described by Siaut et al. (2011). Briefly, 2×10^6 cells were centrifuged (13,000*g*, 10 min) and resuspended in 1 mL 100% methanol. The samples were then mixed vigorously, centrifuged again, and the pellet left to dry before being stored at −20°C. Pellets were resuspended in 500 μL of dH_2O and autoclaved for 30 min at 120°C to solubilize the starch. Total starch content was analyzed using an enzymatic starch assay kit (SA20; Sigma-Aldrich, St Louis, MO, USA).

Statistical analyses. One-way or two-way ANOVAs were performed where appropriate using DataDesk 6 (Ithaca, NY, USA). Significance was defined as 95% confidence with an $\alpha = 0.05$. When significance was detected, post hoc analysis was conducted using the Bonferroni adjustment. This test divides the error rate among the tests performed and is highly conservative in risking a Type 1 error.

RESULTS

Characterization of aging cultures of Chlamydomonas. To determine the pattern of senescence in *Chlamydomonas*, cells were grown in batch culture. Six

phases of the growth curve were identified according to Nyström (2004), shown as a gray scale in Figure 1 (note: phase 1 – lag phase is not discussed in this study). Up to 3 d post inoculation, the cells grew exponentially (phase 2). Deceleration of growth began after 3 d and approached zero growth at approximately 4 d (phase 3). These two phases combined described a logistic growth curve. Stationary phase (phase 4), defined by zero growth, has a cell density of ~1.3 × 10^7 cells · mL^{-1} under experimental conditions. This cell density was maintained for ~3–4 d, after which cell death commenced. The end of stationary phase is delineated by an increase in the rate of cell death and a reduction in cell numbers between days 8 and 10, defined as the accelerated death phase (phase 5). This is followed by a steady decrease in cell numbers during the logarithmic death phase (phase 6), though eventually the rate of cell loss slowed down. Overall, the culture declined by ~72% to a density of ~5 × 10^6 cells · mL^{-1} over the course of the experiment (Fig. 1A). We also examined a wildtype strain (CC125) with a cell wall, and while the cell number did not decline with age, the cell viability, as assayed using Trypan Blue, followed a similar trend as the cell wall-less strain used in this study (Fig. 1A). Other than an initial high level during exponential phase, chl per cell was steady at ~2.3 pg. The apparent increase in chl per cell late in the death phase (phase 6; Fig. 1B) was not significant due to the greater variability in cell counts as a result of cellular debris. To confirm this observation, we also followed chl content in wildtype cells and chlorophyll levels were constant, with some variability, and did not show an increase in older cells. Chl *a*/*b* ratio remained essentially constant at ~2.3, though with a small increase in phase 6 (Fig. 1C).

Changes in cell size, or estimated spherical diameter (ESD), were measured from cells grown on TAP plates in exponential (phase 2), early stationary (phase 4), and accelerated death phase (phase 5). The size frequency distribution followed a normal curve in each phase with a shift to larger size categories in older cells (Fig. 1D). Mean ESD shifted from 6.38 ± 0.15 μm, to 6.64 ± 0.15 μm, and 6.87 ± 0.17 μm, respectively,

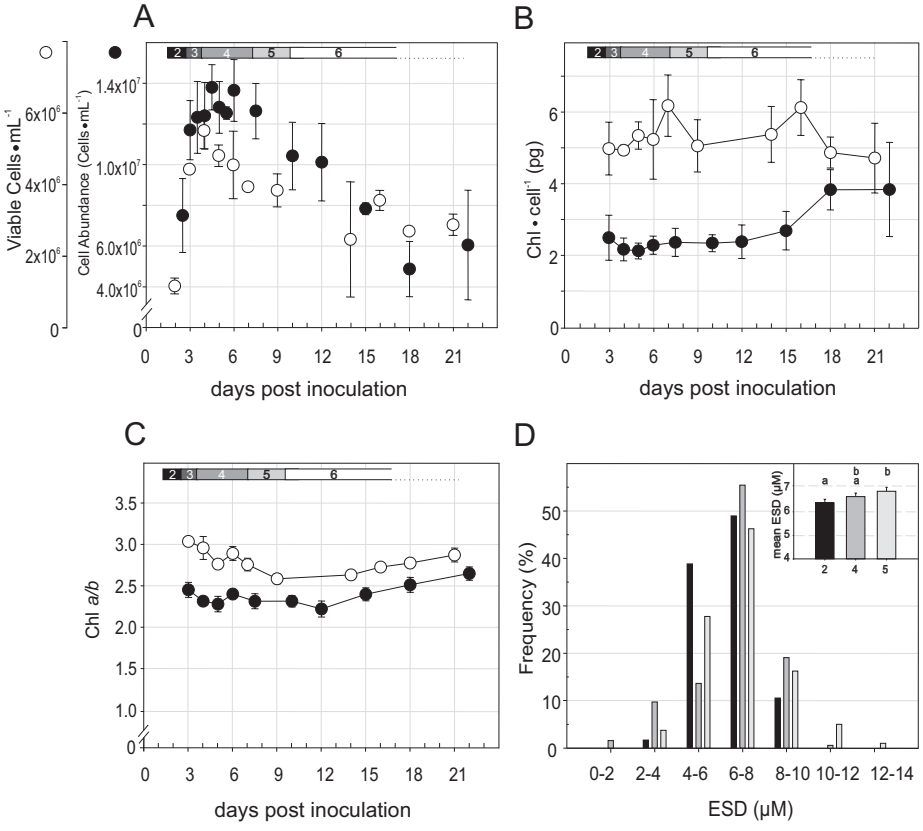

FIG. 1. Growth and chlorophyll (chl) data over time. Cultures were grown in TAP under LL. Filled circles are the cell wall-less strain used in this study while the open circles represent the cell-walled wildtype strain (CC125). (A) Growth curves. Five phases are described and indicated by gray scale bar: 2 – exponential, 3 – deceleration of growth, 4 – stationary phase, 5 – accelerated death phase, and 6 – logarithmic death phase. Open circles are cell viability (walled strain) as measured with Trypan Blue, rather than cell abundance (wall-less strain). (B) Chlorophyll per cell (pg). (C) Chl*a*/*b* ratio. (D) Frequency distribution and mean cell size. Cells were grown on TAP-agar plates and estimated spherical diameter (ESD) was calculated when cells were in exponential (black bar), early stationary (dark gray), and late stationary/early death phase (light gray). Inset: mean ESD, bar color corresponds to frequency histogram, numbers represent the phases as described in A. Bars labelled with a different letter are significantly different. Error bars = SD, $n = 3$.

indicating a significantly different volume ratio change of 1.25 between phase 2 and phase 5 cells (One-way ANOVA, $F_{2,1148} = 6.90$, $P = 0.0011$; Fig. 1D inset). It should be noted that no conversion was made to account for stain or mounting effects so the calculated values are likely lower than the actual cell size; however, the size distribution and statistical differences are still valid.

Age-related changes in Chlamydomonas *ultrastructure.* There were clear structural differences between exponentially growing cells and cells in death phase (phase 6; Fig. 2). One of the most diagnostic features of aged photoautotrophic organisms was the production of lipid bodies in the cytoplasm (McLean 1968) and this feature is easily visualized in *Chlamydomonas* using histological stains (Fig. 2, A and B). The lipid drops were observed as orange globules within the cytoplasm and although present in the exponentially growing cells (Fig. 2A) were larger and more numerous in death phase cells (Fig. 2B). These droplets can also be visualized in TEM images as osmiophilic amorphous bodies within the cytoplasm (Fig. 2, C and D) and we confirmed these lipid bodies contained both polar and neutral lipids using fluorescent observation with Nile Red (data not shown).

TEM images showed the structural changes in more detail (Fig. 2, E–H). In exponentially growing cells the thylakoids were tightly compacted and showed clear granal stacking connected by stromal lamellae (Fig. 2, E and G), whereas in death phase cells they were more disorganized, appear fragmented, and few stromal lamellae were observed. The membranes appeared tightly appressed and tended to curve around structures such as starch grains in a serpentine fashion (Fig. 2, F and H). Other cell features, such as the nucleus and mitochondrion, appeared intact (Fig. 2, G and H). The prominent nucleolus found in the majority of younger cells was typically absent in cells from older cultures, though not entirely, as judged from a casual observation of various TEM images.

Changes in photosynthetic proteins. Western blots were conducted to determine the levels of photosynthetic proteins LHCII, D1, PsaA, Cyt f, RubisCO, and an LHC-like protein (LHCSR) as the culture aged (Fig. 3). All photosynthetic proteins began to decrease as cells entered stationary phase (phase 4) and reached their lowest levels in the mid-death phase (Phases 5–6). Interestingly, there was a partial recovery of PSI, PSII, and LHCII late in the death phase. LHCSR levels, which are low during active growth, began to increase in stationary (Phase 4), reaching maximum levels in the death phase (Phase 6). It is also of interest that Cyt f, a component of the Cyt b_6f complex, had a continuous decline without recovery in the death phase. RubisCO also declined in the death phase and had no recovery compared to the photosystem complexes (Fig. 3).

*Transcriptional light harvesting complex gene (*Lhcbm4*) regulation in aging cultures.* Light-harvesting complex

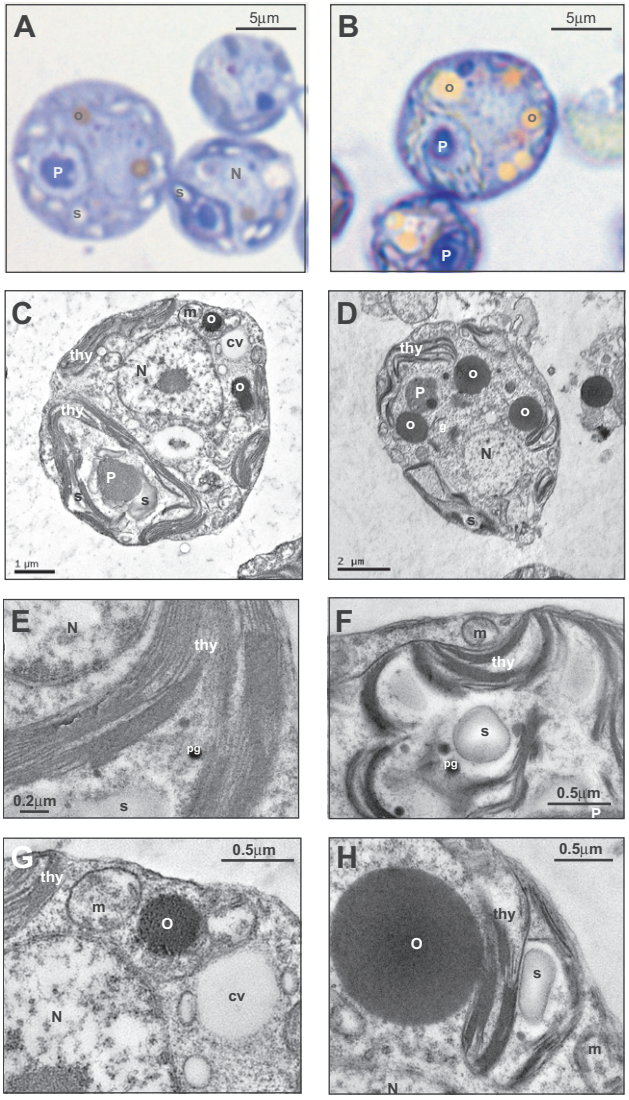

FIG. 2. Ultrastructure. Panels on left represent exponentially growing cultures (phase 2) and panels on right represent cultures in death phase (phase 6). (A and B) Polychrome stained cultures. Epoxy embedded cells were double stained with toluidine blue and basic fuschin distinctly coloring membranes, cytoplasm, and lipid bodies. (C and D) TEM images. (E–H) TEM images of cells showing changes in thylakoid structure (E and F) and size of lipid bodies (G and H). Note intact mitochondria and nuclear membrane in older cultures. P = pyrenoid, N = nucleus, s = starch, cv = contractile vacuole, m = mitochondrion, thy = thylakoid membrane, pg = plastoglobuli, O = osmophilic (lipid) body.

transcript levels were inferred using an LHC-reporter gene that has the *Lhcbm4* promoter fused to the *Chlamydomonas* arylsulfatase gene (Fig. 4). While interpretation of inferred transcription using a reporter requires there to be no changes in the stability of the transcript, our previous studies have found it to be robust measure of transcriptional activity (Durnford et al. 2003, Humby et al. 2009). Constitutive transcript levels were high in LL-acclimated cultures during mid-exponential phase (2.75 d – phase 2), but

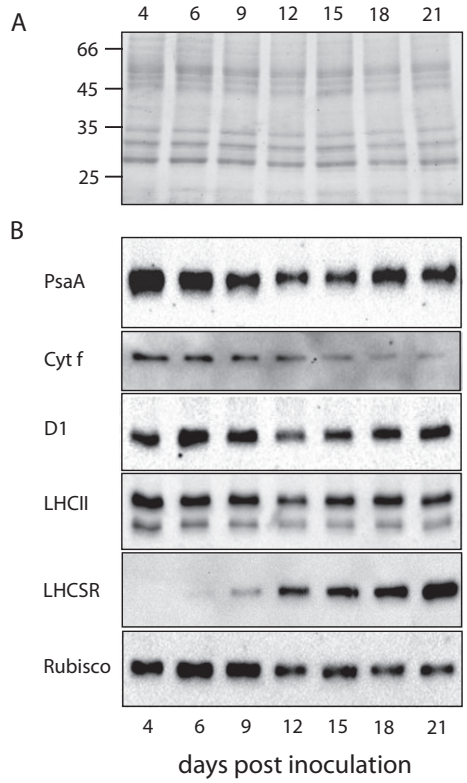

FIG. 3. Changes in photosynthetic proteins over time. (A) Coomassie Brilliant Blue-stained acrylamide gel used as a loading control. (B) Western blots using a variety of antibodies as described in methods. A quantity of 2.5 μg protein was loaded per lane. *X*-axis is age of culture in days post inoculation.

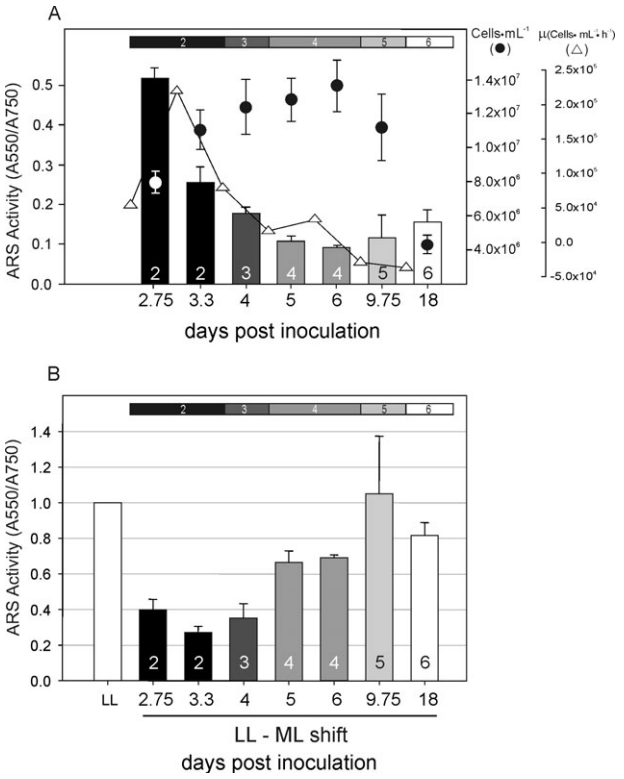

FIG. 4. Transcriptional activity inferred from ARS expression. (Gray scale pattern represents phases as described in Figure 1A, phase numbers are superimposed on histogram bars) (A) Absolute LL levels of ARS expression (standarized to cell number). Closed circles represent density of cultures (cells · mL^{-1}) at time of experiment. Open triangles represent growth rate between time points. Note rate scale goes below zero. (B) Relative ARS expression from 70 μmol photons · m^{-2} · s^{-1} (LL) acclimated challenged with a 2 h ML (500 μmol photons · m^{-2} · s^{-1}) shift (values normalized to LL). Error bars = SD, $n = 3$.

dropped significantly in late exponential phase (3.3 d – phase 2) – even when growth rate was at its highest (Fig. 4A; One-way ANOVA, $F_{6,14} = 68.17$, $P \leq 0.0001$). It reached its lowest levels in late stationary (6 d – phase 4; Fig. 4A). As constitutive transcription levels dropped over time, the high-light responsiveness of inferred *Lhcbm4* transcription also declined (Fig. 4B). During the growth phases (phase 2 and 3), *Lhcbm4* transcription dropped 60%–70% upon a 2 h exposure to a higher light intensity (ML – 500 μmol photons · m^{-2} · s^{-1}; Fig. 4B). In stationary phase (phase 4), inferred *Lhcbm4* transcription was less responsive to an increase in light intensity, decreasing only 40% compared to the LL control. During the accelerated death phases (phase 5 and 6), the transcriptional response to light stress was significantly less responsive than both growth phases, showing a light-dependent decline of 20% or less (Fig. 4B; one-way ANOVA, $F_{6,14} = 13.056$, $P \leq 0.0001$).

Shifts in photosynthesis and photoprotection during batch culture. There were significant differences detected over time in all fluorescent parameters measured during conditional senescence. Maximum quantum yield of PSII (Fv/Fm) remained constant through growth and stationary phases, but decreased significantly at the onset of phase 5 (accelerated death), dropping from 0.74 ± 0.004 to 0.63 ± 0.032 (Fig. 5A; one-way ANOVA, $F_{5,12} = 11.498$, $P = 0.0003$). During phase 6, Fv/Fm recovered to 0.68 ± 0.04 and was not significantly different than any time point. While stationary (phase 4) showed no difference to earlier phases in Fv/Fm, it was significantly lower in photosynthetic efficiency (Φ_{II}; one-way ANOVA, $F_{5,12} = 25.14$, $P < 0.05$). The lowest point again was the death phase 5 and there was a significant recovery in phase 6 (one-way ANOVA, $F_{5,12} = 25.14$, $P = .0019$; Fig. 5A).

The quantum yield of the non-regulated pathway (Φ_{NO}) increased at the onset of stationary phase (phase 4), and reached the highest level at the end of phase 5, which was significantly different than all other time points (Fig. 5B; one-way ANOVA, $F_{5,12} = 18.06$, $P < 0.05$). NPQ decreased significantly in phase 6 (Fig. 5B; one-way ANOVA, $F_{5,12} = 18.06$, $P = 0.035$) coincident with the increase in Φ_{II}. The quantum yield of NPQ (Φ_{NPQ}) remained at or below 0.17 throughout the entire growth curve, although the final time points (death phase 6) were significantly lower than those levels during stationary (Fig. 5B; one-way ANOVA, $F_{5,12} = 8.67$, $P < 0.015$).

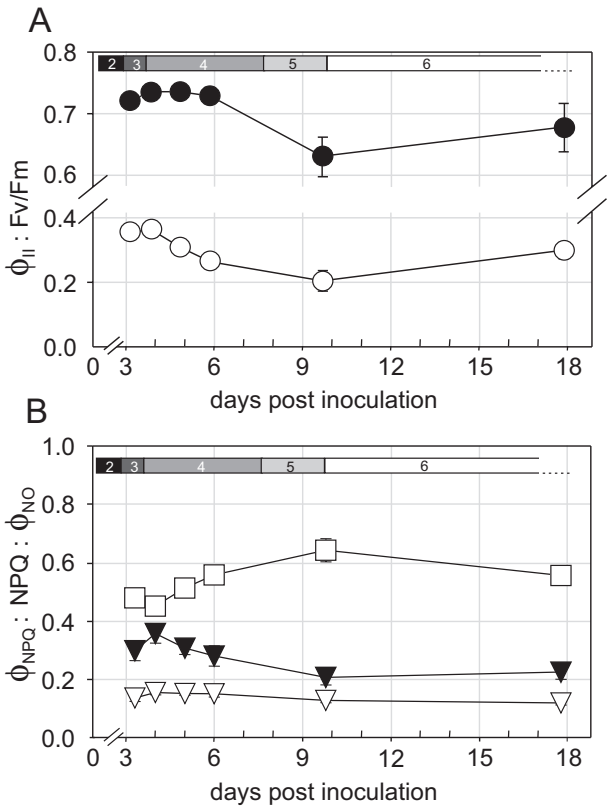

FIG. 5. Fluorescence parameters over time. Gray scale bars represent growth phases as described in Figure 1A. (A) Fv/Fm (closed circles) and quantum yield of PSII (Φ_{II} – open circles) (B) Principal quenching parameters. NPQ (closed triangles), yield of NPQ (Φ_{NPQ} – open triangles), and yield from other energy losses (Φ_{NO} – open squares). Error bars = SD, $n = 3$.

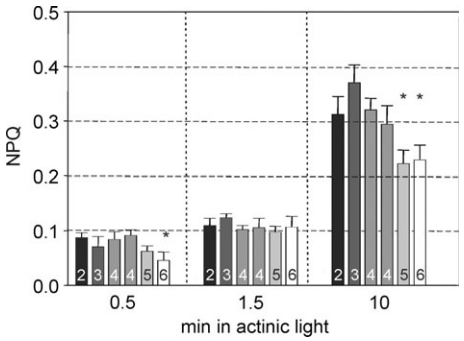

FIG. 6. NPQ induction kinetics over time. Gray scale follows growth curve phases as described in Figure 1A (phase numbers are superimposed on histogram bars). Cultures were grown in LL, dark adapted for 15 min, and challenged with ML for 10 min. NPQ levels after 0.5, 1.5 (fast induction) and 10 min (slow induction) for each time point. *Denotes statistically different values, one-way ANOVA, $P < 0.05$. Error bars = SD, $n = 3$.

With the strong induction of LHCSR in senescent cultures, a protein associated with photoprotection, we tested for differences in NPQ over a 10 min exposure to ML (Fig. 6). NPQ induction is marked by a fast initial pH-gradient-dependent induction and a subsequent slow rise in NPQ as zeaxanthin is being accumulated (Niyogi et al. 1997). There were no significant differences in the initial induction of NPQ on cultures except the accelerated death phase (Fig. 6; 0.5 and 1.5 min; ANOVA, $F_{5,12} = 5.07$, $P = 0.01$). Over the following 10 min time course, NPQ levels rose in all cultures, however, both death phases showed significantly lower NPQ induction (Fig. 6; 10 min; one-way ANOVA, $F_{5,12} = 11.45$, $P = 0.0003$).

In additional experiments, we noticed that the culturing condition was very important in the duration of stationary phase. Figure 7 shows one such experiment where the same strain that was inoculated at 3×10^5 cells $\cdot$ mL^{-1} as before, but one was plugged with a foam stopper and covered in aluminum foil as with all previous experiments, while the other had humidified air blown into the headspace of the culture. The difference was dramatic with the low gas-exchange culture starting to die off soon after reaching stationary phase, while the culture with air exchange had no decline in cell numbers over the 14 d experiment (Fig. 7A). Physiologically, the cultures were quite different in terms of starch accumulation. The cultures with free gas exchange started to accumulate starch during stationary, with a plateau 9 d post inoculation. The cultures without gas exchange had little starch accumulation until after day 9 where it rose significantly (Fig. 7B; one-way ANOVA $F_{4,15} = 8.45$, $P = 0.0009$). This time period corresponds to the final death phase (phase 6; Figs. 1A and 7A).

DISCUSSION

Conditional senescence includes changes in photosynthetic proteins. Chlamydomonas grown mixotrophically in batch culture grows exponentially and rapidly reaches stationary, where the cultures shift from a growth to a non-dividing stage. The chloroplast undergoes significant changes as thylakoid membranes become disorganized and the lamellae are compacted and more parallel, similar to senescence in another unicellular green alga, *Spongiochloris typica* (McLean 1968). In this study, we observe a significant (albeit small) increase in cell size between growth and death phases, which may reflect the residual cell expansion in the absence of cell division in stationary and/or the accumulation of oil bodies, the latter being a well-described phenomenon in several alga groups. Oil accumulation is related to a variety of abiotic stresses, especially nitrogen deficiency (Collyer and Fogg 1954, McLean 1968, Aaronson et al. 1983, Fogg and Thake 1987, Liu and Lin 2001, Liang et al. 2006, Siaut et al. 2011). Although the source of cytosolic oil bodies is still not known, there is evidence that plastoglobuli can be exuded into the cytoplasm (Oliveira and Bisalputra 1977, Guiamét et al. 1999) and it now appears that plastoglobuli and cytosolic lipid bodies are at the very least similar in nature if not origin (Kaup et al. 2002).

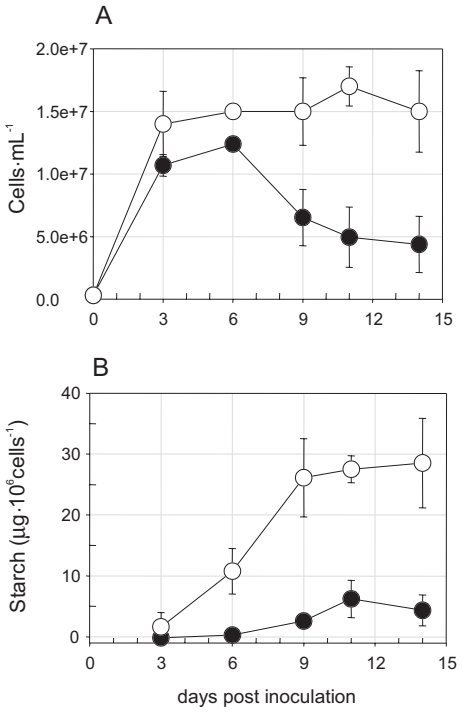

FIG. 7. Growth and starch production differences in an open versus closed system. Air exchange (open circles) and limited air exchange (closed circles). (A) Growth curve. (B) Starch content per 1×10^6 cells. Error bars = SD, $n = 4$.

As the culture enters stationary phase, the concentration of LHCII, D1, PsaA, Cyt f, and RubisCO in cells declined relative to total protein levels, implying significant changes in the photosynthetic complexes and thylakoid membranes in general. The declines of photosynthetic proteins are a classic response to nutrient deprivation in *Chlamydomonas* (Plumley and Schmidt 1989, Peltier and Schmidt 1991, Wykoff et al. 1998) that reflects a decrease in photosynthetic rate (Wykoff et al. 1998). The partial recovery of PSI, PSII and LHCII beginning at day 15 is intriguing; especially since the abundance of Cyt f and RubisCO did not recover. This suggests that while the cells were taking advantage of an increase in nutrient availability to rebuild, presumably from nutrients released from dead individuals, there were still limitations in photosynthetic electron transport. This is similar to the situation in plants where the Cyt b_6f complex and plastocyanin concentration in aging leaves closely mirrors the carbon assimilation potential (Schöttler et al. 2004) while PSI and II are more stable.

Surprisingly, the amount of measured chlorophyll per cell did not change significantly while in stationary or early death phases. While it is conceivable that other chlorophyll-binding proteins scavenged this chlorophyll as the photosystems turned over, it is also possible that some were stored in oil bodies (Guiamét et al. 1999). The accumulation of chlorophyll in cytoplasmic oil bodies has been found for senescing soybean (Guiamét et al. 1999) but this possibility requires further investigation. The initial decline in the photosynthetic complexes after day 9 may have proceeded via an autophagy mechanism that was induced as a result of nutrient deprivation. Pérez-Pérez et al. (2012) observed the induction of an autophagy marker, ATG8, as cells transitioned to the stationary phase, indicating that this is an active process stimulated by cell stress.

While LHCII protein levels declined during stationary phase (though partially rebounding later), we observed inferred *Lhcbm4* constitutive transcript levels dropping significantly even though the cultures were still growing. In bacteria, declines in transcription and cessation of protein synthesis are commonly observed in senescing batch cultures (Nyström 2004) and transcriptional regulation of photosynthetic proteins is also common in senescing leaves (Jiang et al. 1993, Humbeck and Krupinska 2003, Andersson et al. 2004). An association of transcription levels with growth rate has been made with a variety of genes in yeast (Brauer et al. 2008). Presumably there are feedback signals related to growth rate of the culture after it passes the asymptote of the logarithmic growth curve. Thus, steady state regulation of LHCII transcripts is potentially tied to the rate of cell division more than light levels per se. In *Chlamydomonas*, short-term light stress responses result in transient changes in transcript expression (Teramoto et al. 2002, Durnford et al. 2003, Elrad and Grossman 2004, Humby et al. 2009), and we observed the predicted transcriptional response to a high-light shift during the growth phases in our experiments; however, as the population aged, transcriptional changes became increasingly resistant to light shifts. This suggests that the mechanism used to signal light shifts or the ability to respond to such shifts is compromised during senescence. Alternatively, the cells may not be experiencing the same light stress due to changes in plastid ultrastructure, and induction of photoprotection mechanisms, effectively reducing the activation of the signal that leads to transcriptional repression.

The changes in the light responsiveness imply that there may be an enhanced capability to resist light stress, which would make sense given the limited ability to replace protein components in stationary phase. The classic long-term regulatory mechanisms, which inherently rely on cell division rather than active degradation, would likely have a limited role. This suggests alternative long-term approaches to reducing oxidative stress in the face of light stress, which may include alternative energy sinks, enhanced detoxification, or reduction of protein photosynthetic complexes by degradation. Certainly plastid and cytosolic lipid body formation has been suggested to function in just such a role (Tevini and Steinmuller 1985, Kaup et al. 2002), that being a storage compartment for degradation products that is presumably tied to reducing light stress.

Photoprotection during conditional senescence. In response to stress, one would predict the induction of short-term, reversible photoacclimatory mechanisms, such as NPQ (Horton et al. 1992, Anderson et al. 1995, Niyogi 2000, Horton et al. 2005). As cultures transitioned from active growth into stationary, quantum yield (Fv/Fm) remained stable while photosynthetic efficiency (Φ_{II}) declined, indicating that while the reaction centers were functioning, there was less energy directed through the photochemical reactions and more being dissipated by other mechanisms. As *Chlamydomonas* aged further (phase 5 and 6), Fv/Fm and Φ_{PSII} both declined, indicating the accumulation of damaged PSII due to light stress. In plants, there is a similar decline in photosynthetic efficiency (Φ_{II}) while quantum efficiency remains stable until it reaches later stages of senescence (Dai et al. 2004, Wingler et al. 2004). In diatoms, a rapid decrease in Φ_{II} as the culture approached stationary phase was observed but Fv/Fm was also decreased (Liang et al. 2006). Our cultures followed the same pattern as seen in higher plants. While Φ_{II} declined at the onset of stationary, Fv/Fm remained stable during stationary and only declined during the death phases. This suggests that alternative mechanisms are being utilized to redirect energy from reaction centers during stationary and only in the death phases do we see the accumulation of damaged reaction centers or photoinhibition.

Photons reaching the reaction center are shunted into three possible routes: photochemistry (Φ_{II}); energy-dependent quenching – principally NPQ (Φ_{NPQ}); or non-regulated, constitutive pathways that include inactive reaction centers (Φ_{NO}; Kramer et al. 2004, Klughammer and Schreiber 2008). If photoprotective mechanisms are intact, then a compensatory increase in Φ_{NPQ} with the decline in Φ_{II} is expected (Kramer et al. 2004) and there is often an increase in NPQ observed in maturing leaves that correlates with the decline in Φ_{II} (Dai et al. 2004, Wingler et al. 2004). NPQ then declines in late senescence; the absolute level of decline varies depending on the plant species (Dai et al. 2004, Wingler et al. 2004). Our study shows the same trend, with NPQ level remaining relatively constant from exponential growth to early stationary (while Φ_{II} decreases) and then a decline in NPQ during the death phases. In *Chlamydomonas*, various stresses induce LHCSR (Naumann et al. 2007, Peers et al. 2009), a protein directly associated with NPQ activation, qE in particular (Peers et al. 2009, Bonente et al. 2011). Surprisingly, our data show that LHCSR increases from the onset of stationary phase, with maximal induction in the death phase (phase 6), the point where we observed declines in NPQ. Although we did not distinguish between the different components of NPQ in this study, there was no coincident induction of NPQ capability with LHCSR up-regulation, indicating a role for this stress-response protein in aging cultures independent of NPQ. Perhaps the senescence-associated changes in the thylakoid membrane prevent generation of a sufficient pH gradient to allow for significant NPQ (qE) induction. Induction of LHCSR despite such conditions may offer a rapid photoacclimation mechanism should the nutrient status improve suddenly.

What is interesting is that quantum yield of NPQ (Φ_{NPQ}) remains essentially unchanged through the growth curve and the compensatory increase is through the alternative, non-regulated pathways (Φ_{NO}). Generally, as Φ_{NO} represents basal, light-independent quenching mechanisms, little change is expected under steady state conditions but increases in Φ_{NO} reflect decreased capacity for photoprotection (Klughammer and Schreiber 2008) as does the steady low value of Φ_{NPQ}. As senescence continues and the thylakoid membrane organization is changed, the regulated dissipation of energy via NPQ (qE specifically) may not be possible and becomes less relevant. Certainly both death phases showed significantly lower initiation of the rapid stage of NPQ and a reduced level after 10 min induction. Under conditions of limited growth, and limited gas exchange, *Chlamydomonas* uses alternative mechanisms to attempt to bleed excess photons from the functioning reaction centers. A possible photoprotective mechanism is the temporary inactivation of reaction centers. It has been shown that a change in the carbon/nitrogen ratio during late stationary results in PSII inactivation (Vavilin et al. 1999), and these inactive reaction centers are proposed to have a photoprotective role to compensate for the loss of NPQ (Dai et al. 2004).

Is senescence a unicellular adaptation to limited nutrients?. The long-term goal of our work is to decipher the adaptive strategies that, presumably, maximize viability of a culture under defined conditions. In our culture set up, shortly after stationary phase, cells begin a rapid decline in numbers and over half have lysed by 18 d post inoculation. This represents a putative source of carbon, nitrogen, phosphate, and other nutrients, which have presumably become limiting as cells entered stationary phase (Fogg and Thake 1987). Evidence for the uptake of nutrients by the survivors is present in the partial recovery of photosynthetic complexes and function. In this study, a cell wall-less strain was used, and cells lysed easily. In a cell-walled strain, the cell viability pattern was the same, however, the cells did not lyse during the experimental procedure, thus the fate of cell contents of less frangible cells is uncertain in natural populations and needs further investigation. While it is obvious that the lysing of cells may provide nutrients that survivors can utilize, whether or not aging *Chlamydomonas* cells are programmed to make this process more beneficial for survivors, perhaps by actively secreting nutrients, is not certain. Durand et al. (2011) found that the supernatants from *Chlamydomonas* cells, triggered to undergo programmed cell death (PCD) by

a heat treatment, were more effective at promoting growth of untreated cells than supernatants from non-PCD (sonicated) cells. This suggests that how cells die may indeed affect the survivors of the population. In our system, we have no evidence for a "PCD" but the possibility that, under these senescent conditions, the cells enter a physiological state that could benefit the surviving members of a population when the cells do die remains.

Such questions are receiving attention lately, especially with regard to the ecological significance of cell death in microplankton populations (Franklin and Berges 2004, Franklin et al. 2006). In these examples, cell death of phytoplankton has been hypothesized to play a role in the late summer blooms of eutrophic lakes as nutrients during cell lysis are released into the upper epilimnion (Sigee et al. 2007). Beneficial release of nutrients into the media has been observed both in the lab and in the field. Marine algae mobilize and exude photosynthate products as polysaccharides and lipids during nutrient deplete conditions (Myklestad 1995, Chin et al. 2004) and cell death in yeast populations has been proposed to function in providing nutrients to young isogenic relatives (Fröhlich and Madeo 2000).

In our study we maintained a closed system, such that gas exchange with the external environment was limited. If cultures had free exchange of air, the duration of stationary phase was dramatically changed. In fact, under such conditions, cultures maintained a high cell density for at least a month without any significant cell decline (data not shown). This suggests that, although the onset of the stationary phase is most likely a response to nutrient availability, the shortening of this phase is a response to an abiotic stress. In this case, we predict that CO_2 limitation blocks photosynthesis in the closed cultures, as is indirectly evidenced by the lack of significant starch accumulation in these cells compared to those with free air exchange. The delayed, smaller increase of starch biosynthesis in the cultures without gas exchange is likely due to the capture of CO_2 released from respiration that was likely stimulated from nutrient assimilation from the dying culture during this time frame, and correlated with the recovery of some of the photosynthetic complexes. Just after stationary, however, there would have been a photooxidative stress that leads to rapid cell deterioration and the precipitation of the death phase. This observation fits well with the oxidative stress hypothesis of aging (Harman 1981).

There is considerable interest in the commercial use of algae as a feedstock for biofuels. For maximal oil production, current approaches require cultures to be grown into stationary phase or starved for nitrogen (Myklestad 1995, Liu and Lin 2001, Chin et al. 2004, Liang et al. 2006, Sigee et al. 2007, Hu et al. 2008). Thus, understanding the physiological and biochemical responses to entering stationary phase will be required for effective mass culturing of microalgae.

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