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DISCOVERY OF A RESTING STAGE IN THE HARMFUL, BROWN-TIDE-CAUSING PELAGOPHYTE, *AUREOUMBRA LAGUNENSIS*: A MECHANISM POTENTIALLY FACILITATING RECURRENT BLOOMS AND GEOGRAPHIC EXPANSION[1]

Yoonja Kang

School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, New York 11794-5000, USA

Ying-Zhong Tang

Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Science, Qingdao 266071, China

Gordon T. Taylor, and Christopher J. Gobler[2]

School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, New York 11794-5000, USA

To date, the life stages of pelagophytes have been poorly described. This study describes the ability of *Aureoumbra lagunensis* to enter a resting stage in response to environmental stressors, including high temperature, nutrient depletion, and darkness, as well as their ability to revert from resting cells back to vegetative cells after exposure to optimal light, temperature, and nutrient conditions. Resting cells became round in shape and larger in size, filled with red accumulation bodies, had smaller and fewer plastids, more vacuolar space, contained lower concentrations of chl *a* and RNA, displayed reduced photosynthetic efficiency, and lower respiration rates relative to vegetative cells. Analysis of vegetative and resting cells using Raman microspectrometry indicated resting cells were enriched in sterols within red accumulation bodies and were depleted in pigments relative to vegetative cells. Upon reverting to vegetative cells, cells increased their chl *a* content, photosynthetic efficiency, respiration rate, and growth rate and lost accumulation bodies as they became smaller. The time required for resting cells to resume vegetative growth was proportional to both the duration and temperature of dark storage, possibly due to higher metabolic demands on stored energy (sterols) reserves during longer period of storage and/or storage at higher temperature (20°C vs. 10°C). Resting cells kept in the dark at 10°C for 7 months readily reverted back to vegetative cells when transferred to optimal conditions. Thus, the ability of *Aureoumbra* to form a resting stage likely enables them to form annual blooms within subtropical ecosystems, resist temperature extremes, and may facilitate geographic expansion via anthropogenic transport.

***Key index words: Aureoumbra*; brown tide; cysts; harmful algal blooms; metabolism; resting stage**

For billions of years, phytoplankton have evolved survival strategies to adapt to changes in environmental conditions, such as the formation of resting cysts or spores (Anderson 1975, Anderson et al. 1985, Matsuoka and Fukuyo 2000, Bravo and Figueroa 2014), the alternation of nutrition modes (Jochem 1999, Wilken et al. 2013), and the reduction in cellular metabolism (Dehning and Tilzer 1989, Popels and Hutchins 2002). Inactive or resting stages are common in the life history of many phytoplankton taxa and may provide tolerance to unfavorable conditions (Bravo and Figueroa 2014). The characteristics of resting stages differ among classes of phytoplankton with akinetes prevalent among cyanobacteria (Kaplan-Levy et al. 2010), statospores in chrysophytes (Sandgren 1983), resting spores in diatoms (Hargraves 1983), and resting cysts in dinoflagellates (Dale 1983), euglenophytes (Olli 1996), and raphidophytes (Kim et al. 2015). Environmental cues that trigger the formation of resting stages include suboptimal temperature (Anderson 1980, Anderson et al. 1985), nutrient stress (Sandgren 1981, Figueroa et al. 2005), sustained darkness (Anderson 1975, Anderson et al. 1987, Itakura et al. 1996), and the presence of allelopathic competitors (Rengefors et al. 1998, Fistarol et al. 2003, 2004). Resting stages refer to all types of cells that reduce metabolic rate, cease cell division, but remain viable (von Dassow and Montresor 2011), and are often characterized by distinct morphological and compositional changes in cells such as thickened membranes and the formation of starch granules (Chapman et al. 1982), lipid droplets (Anderson 1975), and/or red accumulation bodies (Wall and

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[2]Author for correspondence: e-mail christopher.gobler@stonybrook.edu.
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Dale 1969, Matsuoka and Fukuyo 2000). Resting cells that undergo changes in morphology and physiology leading to a resting stage do not undergo major changes in cell surface or enclosing cell structures which distinguishes them from resting cysts or spores (Sicko-Goad et al. 1989).

Picoeukaryotes are known for their broad diversity and importance in marine food webs and biogeochemical cycles (Moon-van der Staay et al. 2001, Cuvelier et al. 2010, Massana 2011). Pelagophytes are important picoeukaryotes in marine ecosystems (Simon et al. 1994, Andersen et al. 1996, John et al. 2007). *Pelagomonas* sp. appears to be one of the most abundant picoeukaryotes in the North Atlantic and Pacific subtropical oceans and its relative abundance increases with depth through the photic zone (Simon et al. 1994). Recently, the metagenomic analyses of Global Ocean Survey data reveal that *Pelagomonas* populations are ubiquitously distributed across world oceans (Worden et al. 2012).

Pelagophytes also have ecological importance within coastal ecosystems. Two species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, are notorious for their ability to create ecosystem disruptive algal blooms (Sunda et al. 2006, Gobler and Sunda 2012). For the past 25 years, harmful brown tides caused by *A. lagunensis* DeYoe et Stockwell have occurred within subtropical, coastal ecosystems of the Northwest Atlantic Ocean (Buskey et al. 2001, Gobler et al. 2013, Koch et al. 2014, Phlips et al. 2014), causing declines in zooplankton populations (Buskey et al. 2001), loss of seagrass beds (Montagna et al. 1993), and the death of fish and juvenile bivalves (Gobler et al. 2013). Brown tides caused by *Aureoumbra* had been confined to coastal Texas, USA, for more than 20 years (Buskey et al. 2001), but recently these blooms have emerged in the Indian River Lagoon and Mosquito Lagoon, Florida, USA (Gobler et al. 2013) and in Guantanamo Bay, Cuba (Koch et al. 2014). While *Aureoumbra* has been historically detected at low levels in the Gulf of Mexico and Florida Bay (Villareal et al. 2002), the emergence of these brown tides in new habitats may be related to long water residence time, high salinity, low nitrate, high DON, and low grazing rates on *Aureoumbra* (Gobler et al. 2013, Kang et al. 2015). Similarly, brown tides caused by the temperate pelagophyte, *Aureococcus*, have expanded to South Africa and China after being confined to the east coast of the United States for more than a decade (Probyn et al. 2010; Gobler and Sunda 2012, Zhang et al. 2012).

Although environmental selection (Baas Becking 1934) could account for the expansion of brown tides into new regions (Koch et al. 2014, Kang et al. 2015), little is known regarding the physiological strategies *Aureoumbra* employs to survive suboptimal conditions for extended periods of time. Previous studies have shown that the temperate brown tide alga, *A. anophagefferens*, is able to endure 30 d of darkness (Popels and Hutchins 2002, Popels et al. 2007), a characteristic some have suggested may facilitate its anthropogenic transport in ship's ballast water (Doblin et al. 2004). However, cyst-like or resting cells have never been documented for *Aureococcus* or any other pelagophyte. Following an serendipitous discovery of a resting stage cell in cultures of the related pelagophyte, *Aureoumbra*, this study was initiated to characterize the ability of *Aureoumbra* to enter a resting stage in response to environmental stressors, including high temperature, nutrient depletion, and darkness as well as their ability to revert back to vegetative cells. The biochemical composition, fine structure, and growth characteristics of resting cells in *Aureoumbra* were comprehensively characterized and contrasted with vegetative cells in exponential and stationary growth phase. These findings have relevance for understanding the ecological mechanism by which brown tides caused by *Aureoumbra* annually recur in a given ecosystem and expand geographically.

MATERIALS AND METHODS

Culture information. Four strains of *A. lagunensis* were used to investigate the intraspecific variation in the response of this species to environmental stressors. A strain originating from the Laguna Madre, Texas, USA (strain CCMP1510; hereafter TX) was obtained from the National Center for Marine Algae and Microbiota (NCMA, formally CCMP), Maine, USA. This TX strain was isolated more than 20 years ago (18 June 1992). A Cuban strain (CB) was isolated by Y.Z. Tang from Guantanamo Bay, Cuba (18 May 2013; Koch et al. 2014) and two Florida strains (FL2 and FL5) were isolated by Y.Z. Tang from the Indian River Lagoon, Florida, USA (5 June 2012). The identity of CB and FL strains was confirmed via sequencing of the 18S rRNA gene (Gobler et al. 2013, Koch et al. 2014). Stock cultures were maintained in modified GSe medium (Doblin et al. 1999), made with autoclaved and then filtered North Atlantic seawater (0.22 μm) with a final salinity of 32.5, and amended with 100 μM NH_4^+ and 6 μM PO_4^- as *Aureoumbra* grows poorly on nitrate (Muhlstein and Villareal 2007). All cultures were maintained with a final concentration of 1% antibiotic solution (Primary stock was a mixture of 10,000 I.U. penicillin and 10,000 $\mu g \cdot mL^{-1}$ streptomycin; Mediatech Inc., Herndon, VA, USA) to minimize bacterial proliferation. Cultures were grown at 21°C with a 12:12 h light:dark cycle, illuminated by a bank of fluorescent lights, covered by one layer of neutral density screening which reduced light levels to 80 μmol photons $\cdot m^{-2} \cdot s^{-1}$.

Resting stage inducement and recovery experiments. To assess the response of *Aureoumbra* to high temperature, triplicate 450 mL cultures of four strains were incubated at 35°C with the initial cell density of 4×10^5 cells $\cdot mL^{-1}$. These experimental cultures were grown in modified GSe medium, with 50 μM NH_4^+, 3 μM PO_4^-, and a final concentration of 1% antibiotic solution. To assess nutrient stress effects, triplicate 450 mL cultures of strain FL2 were grown under different nutrient conditions; N-depletion (No NH_4^+ and 3 μM PO_4^-), P-depletion (50 μM NH_4^+ and No PO_4^-), or without both N and P. Cultures were incubated at 21°C. The density and characteristics of cells were microscopically monitored daily, with the densities of normal vegetative and resting cells (see Results) being differentiated, and a battery of other measurements were made as described below.

To assess the ability of resting cells to revert back to vegetative cells, twenty-four 40 mL cultures of *Aureoumbra* strain FL2 (4×10^5 cells $\cdot$ mL^{-1}) were amended with GSe medium and 50 μM NH_4^+, 3 μM PO_4^-, and a final concentration of 1% antibiotic solution and wrapped with aluminum foil to eliminate light. Cultures were incubated at 10°C and 21°C and 1% antibiotics were added in darkness every 10 d to discourage bacterial degradation of resting cells (Tang and Gobler 2012). Every 5 d for 40 d, three replicates were removed from each temperature to begin the recovery phase of the experiment when fresh medium with 50 μM NH_4^+, 3 μM PO_4^-, and a final concentration of 1% antibiotic solution were added to the triplicate cultures. Cultures were incubated at 21°C and monitored for vegetative and resting cell densities and characteristics every 2–3 d. All experiments were terminated when vegetative cell growth reached the end of the exponential phase. A final experiment was performed to examine the ability of *Aureoumbra* to survive prolonged darkness in which cultures were stored in the dark at 10°C, a temperature representing the thermal minimum in the subtropical environments in which *Aureoumbra* blooms, for 7 months as described above. Once a month, a subset of cultures was transferred to 21°C in the light and cell densities were monitored at 0, 15, and 30 d.

Measurements. Vegetative and resting cells were enumerated on a hemocytometer using a light microscope (Motic® B1 Series) across all experiments. Cell types were distinguished based on obvious differences in size and morphology (see Results). Morphological changes in cells were documented using an inverted light microscope (Nikon Eclipse TS100, Tokyo, Japan) equipped with a Nikon DigiSight Color Digital Camera System (DSVi1). The percentage of resting cells (%) was calculated by comparing the number of resting cells with the total number of cells counted in samples. Maximum quantum efficiency of photosystem II (F_v/F_m) was estimated from in vivo (F_v) and DCMU (3,4-dichlorophenyl-1, 1-dimethylurea)-enhanced in vivo fluorescence (F_m) of each replicate sample measured on a Turner Designs TD-700 fluorometer across all experiments (Parkhill et al. 2001). Samples were dark adapted prior to readings and DCMU was added at a final concentration of 10 μM. Fluorescence readings were corrected using sterile GSe medium as blank. Chl *a* measurements were made on cells collected on a Whatman GF/F glass fiber filter that was frozen, extracted in 90% acetone, and quantified on a Trilogy® Fluorometer (Turner Designs, Sunnyvale, California, USA), and Chl *a* was normalized to cell density (ng $\cdot$ cell^{-1}).

Raman microspectrometer. Differences in the macromolecular composition of vegetative and resting cells were assessed with a Renishaw inVia Confocal Raman Microspectrometer that provides molecular fingerprints based on inelastic scattering of laser light. This instrument enables mapping of intracellular distributions and relative concentrations of biomolecules, such as nucleic acids, proteins, carbohydrates, pigments, and lipids within individual cells (Huang et al. 2010). Vegetative and resting cells of *Aureoumbra* strain FL2 captured on 1 μm polycarbonate membranes were freeze transferred to ultraclean mirror-finished $1 \times 3''$ stainless steel microscope slides. Membrane surfaces were moistened with atomized sterile MilliQ water and placed sample-side down on the clean steel slide surface and both were placed on a −80°C frozen aluminum block. Immediately after freezing, the membrane was peeled away leaving cells frozen to the slide surface. Slides were air dried at room temperature in darkness to minimize loss of pigments. Whole cell or subcellular Raman spectra were acquired through 50× (2.6 μm beam diameter) or 100× (1.3 μm beam diameter) objective lenses, respectively, using a 514 nm Ar ion laser excitation at 1%–50% power, which delivered between 0.01 and 2 mW of energy to the sample. Spectra were processed using Renishaw's® Wire4.1™ software by first subtracting baselines using software's standard best fit polynomial algorithm, and then normalizing intensities to a maximum of 1 because cell-to-cell Raman scattering intensities varied widely. For statistical purposes, relative intensities of sterol and carotenoid peaks from 20 vegetative and 20 resting cells were compared. Previously reported Raman spectra of sterols and carotenoids were used to interpret spectral features (De Gelder et al. 2007, Movasaghi et al. 2007).

Ultrastructural observation with transmission electron microscope. To contrast *Aureoumbra* morphology, 20 mL vegetative cells and resting cells of *Aureoumbra* strain FL2 were collected in a 50 mL conical centrifuge tube and mixed with an equal amount of 6% transmission electron microscope (TEM) grade glutaraldehyde that was buffered with 0.2 M cacodylate (pH = 7.8). Samples were stored at 4°C for 48 h to let cells gently settle to the bottom and overlying supernatant was removed, leaving a minimal glutaraldehyde fixative. Samples were washed in the cacodylate buffer and fixed in 2% osmium tetroxide buffered with cacodylate. Samples were embedded in agar and dissected into small pieces, followed by dehydration in a graded series of acetone (30%, 50%, 70%, 95%, and 100%) for 15 min each and embedding in Epon resin. Ultrathin sections of 80 nm were cut with a Leica EM UC7 ultramicrotome and placed on formvar-coated slot copper grids. Sections were then counterstained with uranyl acetate and lead citrate and viewed with a FEI Tecnai12 BioTwinG^2 TEM at the Microscopy Imaging Center of Stony Brook University, Stony Brook, NY, USA. The digital images were acquired with an AMT XR-60 CCD Digital Camera system mounted on the TEM.

RNA and DNA content. The cellular RNA and DNA content of resting and vegetative cells were quantified. RNA:DNA ratios have been used in many organisms to provide information on the growth condition, with elevated ratios being indicative of more rapid growth (Dortch et al. 1983, Malzahn et al. 2003, Gobler and Talmage 2013). Three 20 mL samples of resting and vegetative cells of *Aureoumbra* strain FL2 were filtered onto 1 μm polycarbonate filters, heated at 50°C for 30 min, and added to 1 mL of cetyltrimethyl-ammonium bromide (CTAB). Samples were then stored at −80°C. For extraction of nucleic acids, frozen samples were immediately placed in a 65°C water bath for 10 min. Nucleic acids of samples were extracted using a modified CTAB technique (Dempster et al. 1999). DNase and RNase digestion of samples were performed using Qubit® dsDNA BR Assay kit (Invitrogen) and Qubit® RNA BR Assay kit (Invitrogen, Carlsbad, CA, USA), respectively, according to manufacturer's protocol. RNA and DNA concentrations in the extracted samples were quantified using Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) compared to a standard curve of nucleic acids.

Respiration rates. Respiration rates of resting and vegetative cells were quantified. Three 20 mL cultures with 3.5×10^5 cells $\cdot$ mL^{-1} were added to 20 mL glass scintillation vials with oxygen sensor spots (OXSP5) inside. All samples were treated with 1% antibiotics and stored at 21°C after wrapped with aluminum foil to produce a dark environment. Oxygen concentrations were measured using a FireSting Oxygen Sensor (PyroScience, Aachen, Germany) for 4 h at the initial and 30 d after the dark storage, according to manufacturer's protocol. Respiration rates were determined from the slope of regression of oxygen concentration (mg $\cdot$ L^{-1}, *y*-axis) against time (sec, *x*-axis). Respiration rates (μM $\cdot$ cell^{-1} $\cdot$ h^{-1}) of vegetative and resting cells were determined.

Statistics. Paired *t*-tests were performed to assess differences in biovolume (μm^3), Chl *a* (ng $\cdot$ cell^{-1}), respiration rates (μM $\cdot$ cell^{-1} $\cdot$ h^{-1}), DNA and RNA contents (pg $\cdot$ cell^{-1}), RNA:DNA ratios, F_v/F_m, and the Raman

TABLE 1. Physiological characteristics of vegetative and resting cells. Paired *t*-tests were performed on all parameters.

Parameters	Vegetative cells	Resting cells	Sample size	T	df	Sig
Biovolume (μm^3)	19 ± 0.32	44 ± 2.9	9	69	16	**
Chl *a* ($ng \cdot cell^{-1}$)	0.29 ± 0.11	0.07 ± 0.03	3	−3.3	4	*
Respiration rate ($\mu M \cdot h^{-1} \cdot cell^{-1}$)	0.05 ± 0.01	0.02 ± 0.01	3	4.4	4	**
DNA ($pg \cdot cell^{-1}$)	0.02 ± 0.01	0.02 ± 0.002	3	ND	ND	ND
RNA ($pg \cdot cell^{-1}$)	0.19 ± 0.04	0.15 ± 0.02	3	4.1	4	*
RNA:DNA	9.7 ± 0.76	6.6 ± 0.37	3	4.4	4	*
F_v/F_m						
High temperature	0.61 ± 0.06	0.11 ± 0.05	3	−9.5	4	**
Nutrient limitation	0.62 ± 0.03	0.13 ± 0.03	9	−44	16	**
Dark storage 10°C	0.65 ± 0.02	0.15 ± 0.03	3	−18	4	**
608: 1518 Raman intensity ratio	0.27 ± 0.01	7.5 ± 0.03	20	99	36	**

*$P < 0.05$, **$P < 0.001$.
ND indicates that the difference between vegetative and resting cells was not statistically significant.

intensity of sterol and β-carotene diagnostic peaks between vegetative and resting cells (Table 1).

RESULTS

Morphological characteristics of resting stage. Compared to vegetative cells that were irregularly shaped and small (<5 μm in size and 19 ± 0.32 μm^3 in biovolume; Fig. 1A), resting cells formed via exposure of vegetative cells to 35°C, nutrient deprivation, or darkness were characterized by an approximate doubling of cell diameter and biovolume (~10 μm in diameter and 44 ± 2.9 μm^3 in biovolume; Fig. 1B; paired *t*-test: $T_{16} = 69$, $P < 0.001$; Table 1). All resting cells contained large (>1 μm) and conspicuous red droplet-like organelles (Fig. 1B). During recovery periods, the resting cells reverted to vegetative cell division, and there was no evidence of an archeopyle (i.e., a distinctive empty cyst typically described for dinoflagellates) or equivalent (e.g., statospore for chrysophytes) in cultures (Fig. 1B). In vegetative cells, plastids were greenish, large, and numerous and contained distinct thylakoids, with plastoglobuli fully formed, the cytoplasm enriched with small vesicles, and the Golgi apparatus prominent (Fig. 1C). In resting cells, the granular cytoplasm became denser, the plastids became pale and less numerous and densely aggregated, vacuole space expanded, and droplet-like organelles formed (Fig. 1D). Single-cell Raman spectra of vegetative cells exhibited distinct β-carotene peaks at 1,008, 1,155, and 1,518 cm^{-1} indicating pigment-rich cells (Fig. 2). In contrast, β-carotene peaks in single-cell Raman spectra of resting cells with red droplets were highly suppressed, although a strong peak appeared at 608 cm^{-1}, which is consistent with sterol enrichment. When vegetative cells transformed to resting cells, the 608:1518 Raman intensity ratio, an indication of the relative prevalence of sterols versus pigments increased 27-fold from 0.27 ± 0.01 to 7.5 ± 0.03 (paired *t*-test: $T_{36} = 99$, $P < 0.001$; Table 1). Attempts to exclusively resolve red droplets by Raman microspectrometry were unsuccessful because of their size. Thus, analyzed spectra represented average cell content.

Resting stage cells of *Aureoumbra* were characterized by lowered RNA:DNA ratios relative to actively growing cells. When vegetative cells transformed to resting cells, RNA content ($pg \cdot cell^{-1}$) declined significantly from 0.19 ± 0.04 $pg \cdot cell^{-1}$ to 0.15 ± 0.02 $pg \cdot cell^{-1}$ (paired *t*-test: $T_4 = 4.1$, $P < 0.05$; Table 1), whereas the DNA content ($pg \cdot cell^{-1}$) was fairly stable being 0.02 ± 0.01 $ng \cdot cell^{-1}$ and 0.02 ± 0.002 $pg \cdot cell^{-1}$ in vegetative and resting cells, respectively (Fig. 3A; Table 1). Accordingly, RNA:DNA ratios were significantly reduced in resting cells from 9.7 ± 0.76 to 6.6 ± 0.37 (paired *t*-test: $P < 0.05$, $T_4 = 4.4$; Fig. 3B; Table 1). Finally, respiration rates decreased by 3-fold when vegetative cells transformed into resting cells from 0.05 ± 0.01 $\mu M \cdot h^{-1} \cdot cell^{-1}$ to 0.2 ± 0.01 $\mu M \cdot h^{-1} \cdot cell^{-1}$ (paired *t*-test: $T_4 = 4.4$, $P < 0.001$; Fig. 3C; Table 1).

Responses to high temperature. All four strains of *Aureoumbra* were incubated at 35°C to assess the formation of resting cells. Upon exposure to 35°C, cell densities of the two FL strains (FL2, FL5) initially increased for 3 d and then declined, the Cuban strain (CB) cell densities remained steady before declining, whereas the TX strain declined immediately after exposure to 35°C (Fig. 4A). Vegetative cells of all strains entered the resting stage by 10th day of incubation (Fig. 4B). During this transformation, Chl *a* ($ng \cdot cell^{-1}$) declined slightly (Fig. 4C), whereas F_v/F_m declined in parallel with the reduction in cell density (Fig. 4D). Upon the subsequent transfer of the newly formed resting cells to 21°C, most strains of *Aureoumbra* entered the exponential growth phase after 12 d of incubation, although the Texas strain grew more slowly (Fig. 4A). Upon exposure to 21°C, F_v/F_m increased gradually from 0.11 ± 0.05 to 0.61 ± 0.06 (paired *t*-test: $T_4 = -9.5$, $P < 0.001$; Table 1), whereas vegetative cell densities increased ~5 d later (Fig. 4, A and D). There was a significant difference in cellular Chl *a* per cell content between vegetative (0.29 ± 0.11 $ng \cdot cell^{-1}$) and resting cells (0.07 ± 0.03 $ng \cdot cell^{-1}$; paired *t*-test:

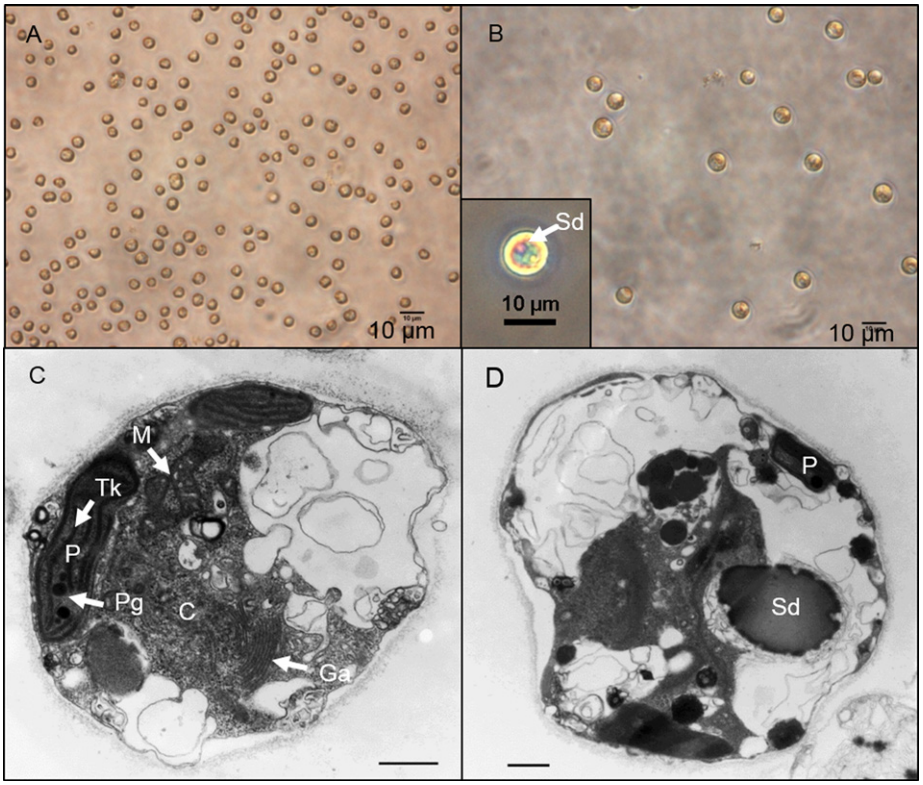

FIG. 1. Light microscope and TEM images of *Aureoumbra lagunensis*. (A) Light microscope image of vegetative cells at 21°C, (B) light microscope image of resting cells formed at 35°C and an inserted figure of an enlarged image of *Aureoumbra* resting cell, (C) TEM image of vegetative cell, (D) TEM image of resting cell. C, cytoplasm; Sd, sterol-enriched droplet; Ga, Golgi apparatus; M, mitochondria; P, plastid; Pg, plastoglobuli; Tk, thylakoids. The size of scale bar of A and B represents 10 μm and that of C and D represents 500 nm. [Color figure can be viewed at wileyonlinelibrary.com].

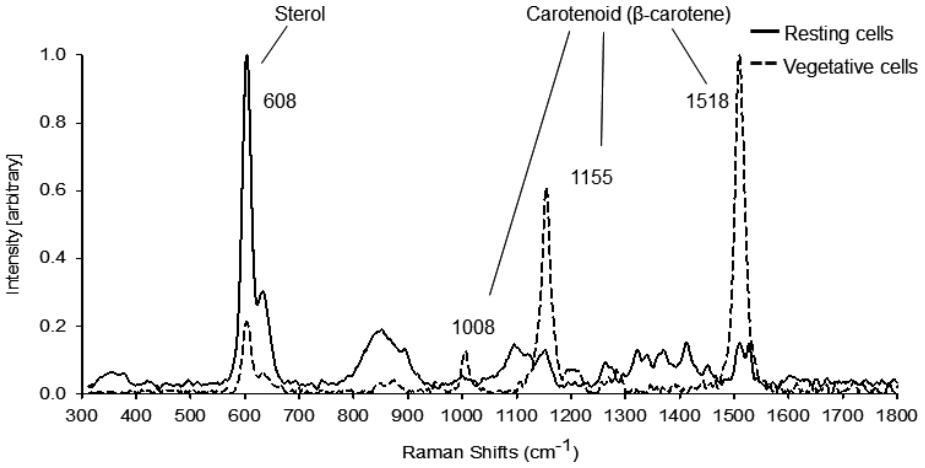

FIG. 2. Examples of single-cell Raman spectra from resting cells and vegetative cells. Raman spectra generated from 20 vegetative cells and 20 resting cells were averaged to obtain the quantitative values that we compared statistically. Dashed and solid lines indicate Raman spectra of vegetative cells and resting cells, respectively. [Color figure can be viewed at wileyonlinelibrary.com].

$T_4 = -3.3$, $P < 0.05$; Table 1). Cellular Chl *a* content increased as cells reverted to vegetative cells with significantly higher Chl *a* per cell in FL strains compared to other strains, although Chl *a* per cell values decreased after cells resumed cellular division (Fig. 4C).

Resting cell production in nutrient limitation. Strain FL2 incubated in the absence of N, P, or both nutrients converted to resting cells, although the rate of transformation was dependent upon the type of nutrient limitation with the onset of changes in the P-deprived cultures being slower than N-deprived or

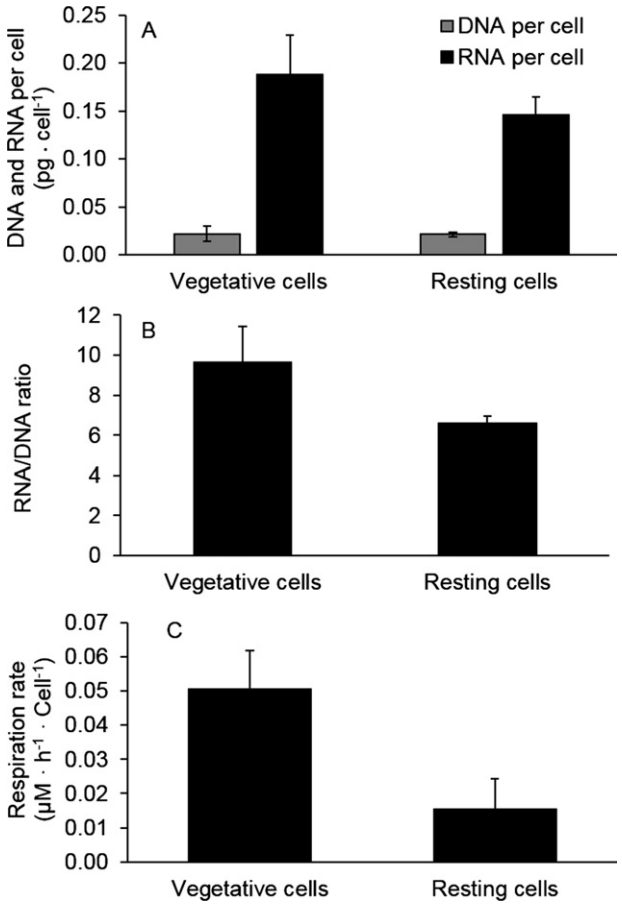

FIG. 3. Physiological characteristics of resting cells including RNA and DNA content per cell, RNA/DNA ratios, and oxygen consumption rate. (A) RNA and DNA content per cell (pg · cell^{-1}), (B) RNA/DNA ratios, and (C) oxygen consumption rate (μM · h^{-1} · cell^{-1}).

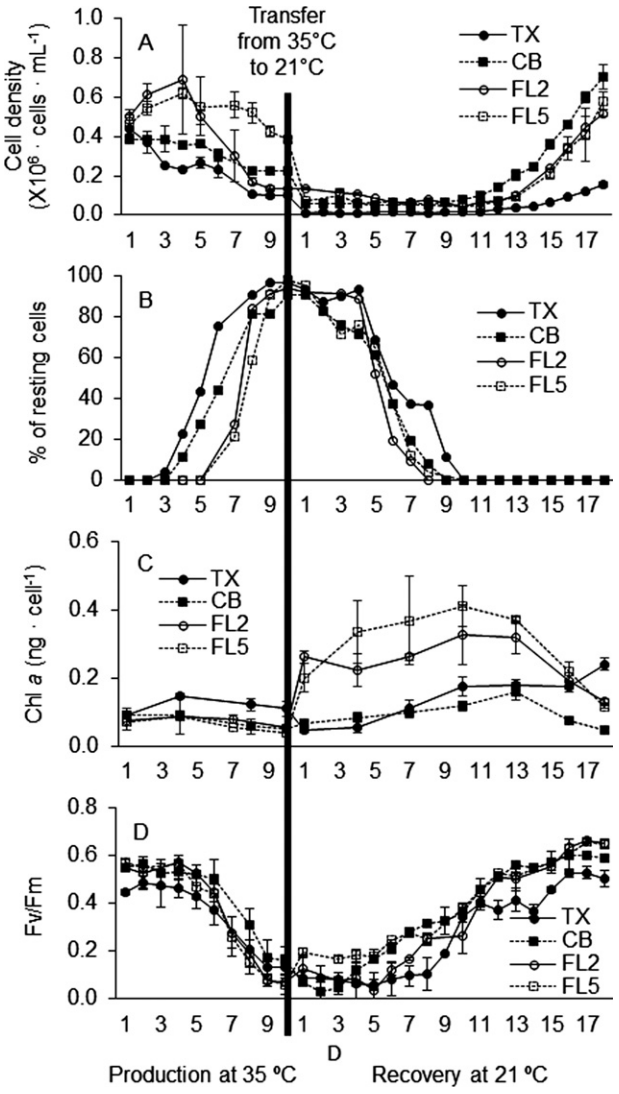

FIG. 4. Responses *Aureoumbra* to high temperature during resting cell production experiments and favorable condition (21°C) during recovery experiments. (A) Cell density, (B) percent of resting cells, (C) Chl *a* per cell (ng · cell^{-1}), and (D) F_v/F_m.

both nutrient-deprived cultures. When cells were grown without N or without both N and P, the density of vegetative *Aureoumbra* cells doubled from 5×10^5 cells · mL^{-1} to 1×10^6 cells · mL^{-1} over ~10 d, and then slowly declined after 35 d of incubation to 3×10^5 cells · mL^{-1} by day 85, and remained uniformly low through 100 d of incubation (Fig. 5A). In P-deprived cultures, vegetative cell densities doubled twice over 5 d to 2×10^6 cells · mL^{-1} and slowly increased to 3×10^6 cells · mL^{-1} through day 40, and then steadily declined to 3×10^5 cells · mL^{-1} through day 90 (Fig. 5A). As vegetative cell density declined, resting cells were produced. From ~60 to ~70 d, the treatments without N went from <30% to >90% resting cells, whereas cells in the P-deprived cultures transformed to resting cells more slowly compared to N-deprived or both nutrient-deprived cultures needing 30 d (day 60 to 90) to go from <30% to >90% resting cells (Fig. 5B). While F_v/F_m of N-deprived and nutrient-deprived cultures dropped sharply over 20 d of incubation, F_v/F_m of P-deprived cultures remained fairly high over 10 d and then decreased sharply. F_v/F_m of all conditions slowly decreased through day 90 and remained low through 100 d of incubation (Fig. 5C). Across all conditions, F_v/F_m of vegetative cells (0.62 ± 0.03) was significantly higher than that of resting cells (0.13 ± 0.03; paired *t*-test: $T_{16} = -44$, $P < 0.001$; Table 1).

Resumption of cell division from the prolonged darkness. The ability of *Aureoumbra* resting cells to resume growth was assessed while cultures were stored in the dark at 10°C and 21°C and then transferred to 21°C at 80 μmol photons · m^{-2} · s^{-1}. For *Aureoumbra* transferred to darkness at both temperatures, resting cells were formed after 15 d of incubation periods (Fig. 6, A and D). After cells were transferred from the dark at 10°C to the light at 21°C, the time needed for resting cells to revert to

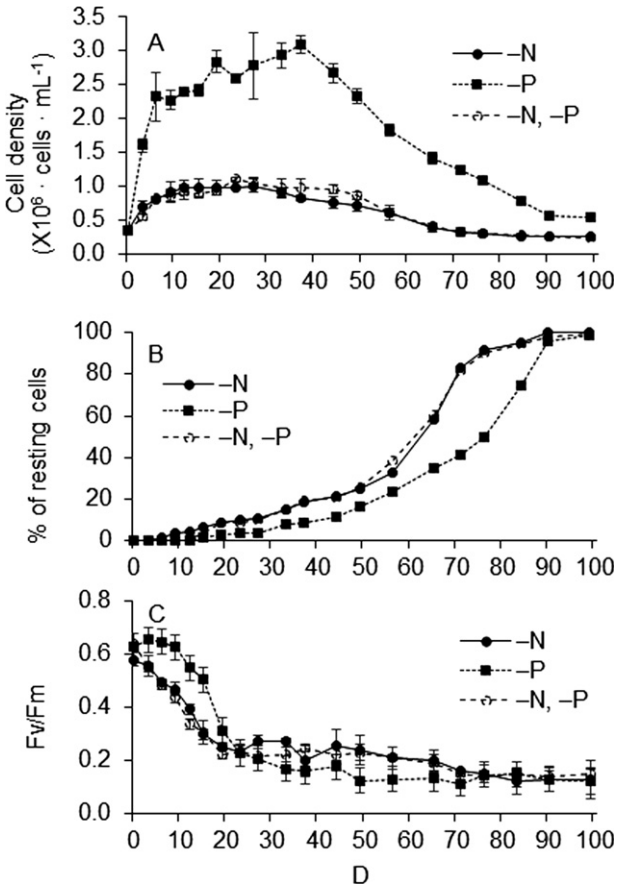

FIG. 5. Responses of *Aureoumbra* to nutrient limitation. (A) Cell density, (B) percent of resting cells, and (C) F_v/F_m.

vegetative cells was proportional to the amount of time the resting cells were stored in the dark (Fig. 6A). Resting cells stored for 15 d completely reverted to vegetative cells after 4 d while resting cells stored for 40 d needed 15 d to completely revert to vegetative cells (Fig. 6A) with cell densities increasing as resting cells declined (Fig. 6B). These changes were also reflected in F_v/F_m values which became maximal when resting cells (0.15 ± 0.03) reverted to vegetative cells (0.65 ± 0.02; paired *t*-test: $T_4 = -18$, $P < 0.001$; Table 1), but prior to exponential growth (Fig. 6C).

Compared to 10°C storage, at 21°C there was a longer lag time for resting cells to transform to vegetative cells during the recovery experiments (Fig. 6, D and E). Resting cells stored for 15 d completely reverted to vegetative cells after 7 d and resting cells stored for 40 d completely reverted to vegetative cells after a 31 d recovery period (Fig. 6D), while the regrowth of *Aureoumbra* vegetative cells initiated as resting cells declined (Fig. 6E). The response of F_v/F_m to the favorable growth conditions was, again, faster than cell density responses (Fig. 6F). Resting cells stored in the dark at 21°C also required at least 5 d more to reach the maximal F_v/F_m after the complete conversion of resting cells into vegetative cells (Fig. 6F).

Aureoumbra resting cells were able to revert to vegetative cells after storage in complete darkness at 10°C for 7 months (Fig. 7). While resting cell densities after the dark storage declined from 5.0×10^5 to 2.6×10^4 cells · mL^{-1} after 7 months (Fig. 7), vegetative cell densities increased to ~1.0×10^5 cells · mL^{-1} 15 d after resting cells were transferred to fresh medium under light and increased to ≥~2.0×10^6 cells · mL^{-1} at day 30 of the incubation (Fig. 7).

DISCUSSION

This study documents the ability of *Aureoumbra* to form resting cells under unfavorable conditions including high temperature, darkness, and nutrient limitation as well as the ability of these resting cells to revert to vegetative cells under optimal light and temperature conditions and following long-term (7 months), dark storage. Compared to vegetative cells, resting cells are larger, rounded, have reduced levels of photosynthetic pigments, RNA, and respiration rates, and are enriched in sterols compared to vegetative cells. This represents the first report of the existence of resting stage or any other life-cycle stage for pelagophytes. Collectively, these findings provide novel insights regarding physiology, life history, and ecology of this harmful alga.

Physiological and biochemical characteristics of resting cells. *Aureoumbra* resting cells are a resting stage that differs from resting cysts or spores that undergo more substantial change in morphology of the cell surface and/or surface structures including thickened walls or modified enclosing inorganic or organic thecae (Sicko-Goad et al. 1989). *Aureoumbra* resting cells also differed from vegetative, stationary phase cells that retained the primary characteristics of vegetative cells with regard to size and shape. In the nutrient limitation experiment (Fig. 5), *Aureoumbra* transitioned from exponential phase cells after 1 week to enter stationary phase for ~1 month, and then transitioned to the characteristic resting cells thereafter. In contrast, when exposed to high temperatures or darkness, cell transitioned rapidly (~1 week) from vegetative cells to resting cells. Finally, the ability of resting cells to persist in darkness for 7 months and then resume vegetative growth is a characteristic not common for stationary phase cells.

The ultrastructure of *Aureoumbra* resting cells was characterized by aggregated plastids, and conspicuous, red, sterol-enriched droplets. Consistent with our results, previous studies have reported that resting cells in other classes of phytoplankton have similarly discernible characteristics including aggregation of thylakoid, the reduction but not elimination of photosynthetic apparatus (i.e., smaller plastid), and the appearance of lipid globules (Bibby

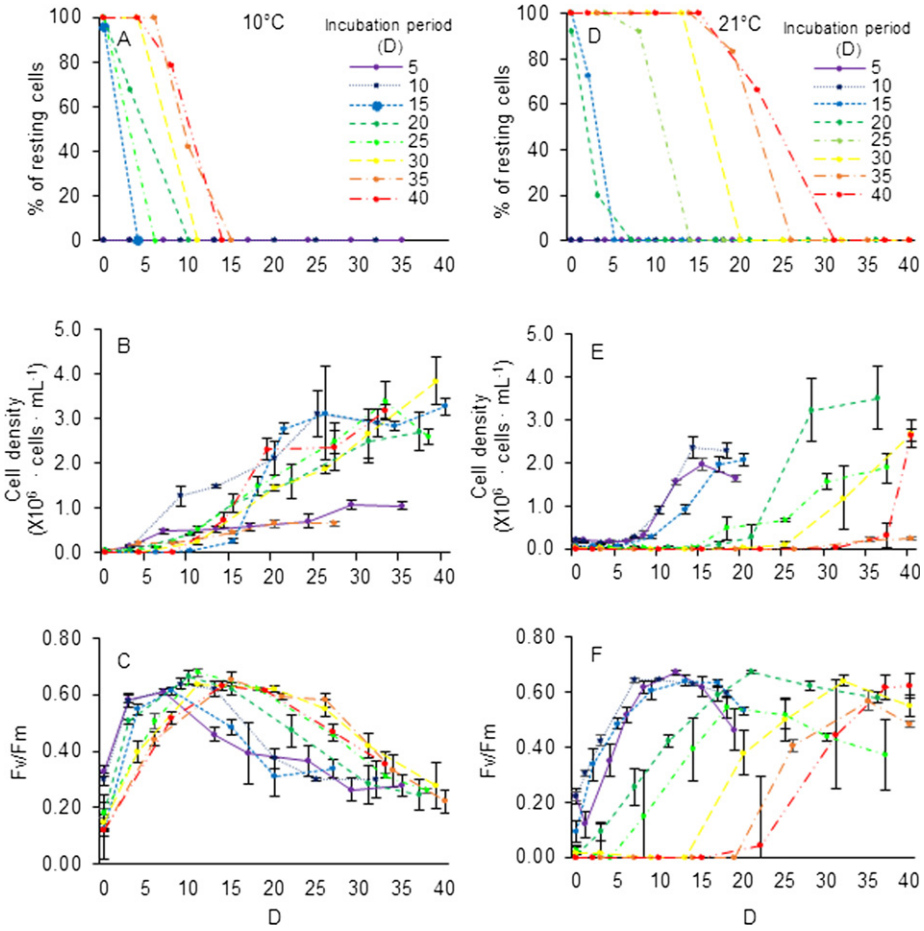

FIG. 6. Responses of *Aureoumbra* cells upon exposure to light at 21°C after stored in the dark at 10°C (A) percent of resting cells, (B) cell density, and (C) F_v/F_m, and responses of *Aureoumbra* cells upon exposure to light at 21°C after stored in the dark at 21°C (D) percent of resting cells, (E) cell density, and (F) F_v/F_m. [Color figure can be viewed at wileyonlinelibrary.com].

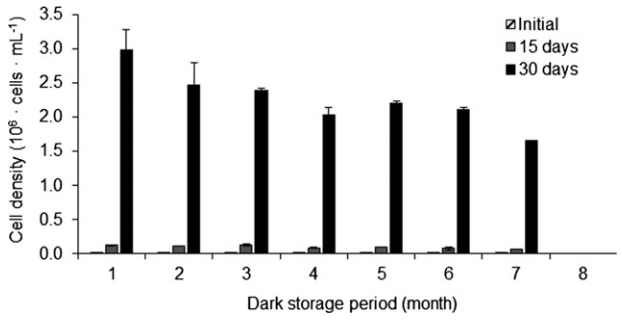

FIG. 7. *Aureoumbra* cell density after cultures were stored in the dark at 10°C for 8 months. Cell densities were evaluated at the start of the 7-month experiment as well as 15 and 30 d after dark-stored cultures were transferred to 21°C with light.

and Dodge 1972, Anderson 1975, 1976, Chapman et al. 1982). Organelles which were reduced in size or were not discernable in resting cells (e.g., thylakoid, plastids, and plastoglobuli) are involved in the metabolic activity associated with photosynthesis, which ceases in resting cells and increases when the favorable condition returns (Anderson 1975, 1976). In our study, ~25% reduction in RNA:DNA ratios and a 3-fold decrease in respiration rates compared to actively growing cells are evidence of lowered metabolism in *Aureoumbra* upon entering a resting stage.

As a unique biomarker, sterol composition has been utilized to characterize the coastal pelagophytes, *A. anophagefferens* (Giner and Boyer 1998) and *A. lagunensis* (Giner et al. 2001), and sterol fatty acid esters can be used for energy storage and reserves (Giner et al. 2009). Raman microspectrometry revealed a prominent peak attributable to sterols in resting cells that was less pronounced in vegetative cells. Spectrometric detection of sterols is consistent with a previous study demonstrating that actively growing *Aureoumbra* cells contain 10 different sterols (Giner et al. 2001). Red, sterol-enriched droplets were visually observed in *Aureoumbra* resting cells via light microscopy and the sterol peak identified by Raman microspectrometry increased 5-fold in resting cells compared to vegetative cells. Hence, it is likely that the red bodies developed by

Aureoumbra resting cells are a type of sterol reserve used as energy storage for cellular metabolism during prolonged resting periods (Anderson 1975). Such bodies biochemically differ from the red accumulation bodies described for dinoflagellate resting cysts that have been hypothesized to be enriched with pigments (Bibby and Dodge 1972, Loeblich and Loeblich 1984, Bravo and Figueroa 2014). Furthermore, sterols are essential components of the membranes of microorganisms to control membrane fluidity and permeability (Bloch 1992, Volkman 2003). Given that resting to vegetative cell transition involves going from large cells with less surface to volume (cell membrane) to small cells with more, sterols may be utilized for membrane production when vegetative growth resumes.

Phytoplankton may increase lipid content in response to stress at the expense of protein and polysaccharides that are considered as short energy storage products (Smith and Morris 1980). Given these findings, we hypothesize that *Aureoumbra* vegetative cells may accumulate photosynthetic products during a light period and produce sterol-enriched droplets from the photosynthetic products under stressful conditions such as darkness, high temperatures, or prolonged nutrient stress as an adaptive mechanism to prepare for persistence as a resting cell. This process seems to take several days as shown during our high temperature incubations. Resting cells likely utilize sterol reserves slowly during the low metabolic, resting periods. Upon transformation of resting cells back to vegetative cells, *Aureoumbra* cells may rely on the remaining sterol reserves in the red droplets to reactivate its photosynthetic apparatus. While we were able to revert resting stage cells back to vegetative cells, even after 7 months of dark storage, it seems likely that an overly long resting stage may completely deplete the sterol bodies and may prevent reversion to vegetative growth and ultimately result in cell death.

Reversion of resting cells to vegetative cells. When phytoplankton encounter suboptimal light or nutrient conditions, photosynthetic capacity can decrease due to the reduction in electron transfer between photosystems I and II (Kulandaivelu and Senger 1976). *Aureoumbra* resting cells exposed to high temperature, darkness, or nutrient stress exhibited multiple signs of reduced or ceased photosynthetic activity, including smaller and fewer plastids, reduced Chl *a* content per cell, and a lowered photosynthetic efficiency. With regard to Chl *a* content and F_v/F_m, these changes preceded the transformation of vegetative cells to resting cells and vice versa. Anderson (1976) reported a lag phase in diatom resting cells during which cells produce substrate required to resume photosynthesis or repair the photosynthetic apparatus allowing energy absorption and transduction. Similarly, it seemed *Aureoumbra* required such a lag phase to re-engage their intracellular photosynthetic apparatus including Chl *a* content before the resumption of vegetative growth. Interestingly, the lag phase was proportional to the time of incubation in the dark and temperature, likely due to utilization of stored energy (sterol reserves) over the extended incubation period and presumably higher metabolic rates depleting more sterols at higher temperature.

Thermal tolerances. Growth rates of phytoplankton typically increase with temperature to an optimal above which growth dramatically declines (Eppley 1972). Four strains of *Aureoumbra* investigated in this study grow at a maximal rate within a range 20°C–30°C (unpublished data) but quickly transform into resting cells at 35°C. These findings suggest that *Aureoumbra* is more resistant to heat stress than other tropical species that perish at such high temperatures (Boyd et al. 2013, Fu et al. 2014) and imply that *Aureoumbra* populations possess a means to survive extreme heat waves in coastal ecosystems that may become more common in the future (Hoegh-Guldberg and Bruno 2010, Thomas et al. 2012). Long-term records show that temperatures of 33°C have been recorded in the Indian River Lagoon, FL (St. Johns River Water Management District 2000–2015), a level that may be high enough to induce resting cell formation and may be more common in the future.

Intraspecific variability can be important for supporting the persistence of phytoplankton blooms (Burkholder and Glibert 2009, Lakeman et al. 2009, Tillmann et al. 2009, Hattenrath-Lehmann and Gobler 2011) and this study provides an evidence in intraspecific variability among four strains of *Aureoumbra* from TX, FL, and Cuba. Specifically, the TX strain was the slowest among the four strains studied here to revert from resting cells to vegetative cells. This TX strain has been cultivated since 1992 while other three strains were isolated more than 20 years after this (i.e., FL2 and FL5 strains in 2012 and CB strain in 2013), suggesting that the TX strain which has not encountered extreme temperatures in the past two decades, may be less capable of quickly responding to such conditions. Consistent with our results, Lakeman et al. (2009) demonstrated that genetic drift during the serial transfers of batch cultures can result in mutation occurring in algal populations with fitness (e.g., growth rate) declining relative to when cultures are first isolated.

Resistance to nutrient limitation. Nitrogen is an essential element for algal growth and its limitation can restrict photosynthetic ability due to a loss of Chl *a* and a decrease in the efficiency of photosystem I and II (Kolber et al. 1988, Geider et al. 1993, Berges et al. 1996). While a sharp decline in F_v/F_m in the N-deprived or both N- and P-deprived treatments was indicative of nutrient limitation, *Aureoumbra* cells remained in a stationary phase for multiple weeks indicating *Aureoumbra* was well adapted to persisting at low nitrogen concentrations, likely because

of its small size, high surface area-to-volume ratio, and thin surface diffusive boundary layer (Sunda et al. 2006, Sunda and Hardison 2007). The gradual decrease in cell density and conversion into resting cells imply that beyond a set tolerance threshold, *Aureoumbra* vegetative cells alter their metabolism to revert to resting stages after 60 d without N.

Unlike N limitation, P limitation does not directly affect the photosynthetic proteins such as the carboxylating protein RUBISCO and the light harvesting fucoxanthin-chl protein (Geider et al. 1993). In P-deprived cultures, cell density increased by 6-fold over 40 d and cells were converted to resting cells more slowly than other conditions. This observation is consistent with the ability of *Aureoumbra* blooms to thrive in P-limited environments with high N:P ratio (Villareal et al. 1998, Liu et al. 2001) and the more vital role of N in the synthesis of photosynthesis apparatus (Falkowski et al. 1989, Geider et al. 1993, 1998). Prolonged P starvation (weeks to months), however, did eventually cause a reduction in photosynthetic efficiency and the conversion of vegetative cells into the resting stages.

Extended dark exposure. The time required for resting cells to revert back to vegetative cells was proportional to the amount of time that cells were stored in the dark at 10°C and 21°C. This finding is similar to studies of the chlorophyte, *Scenedesmus acuminatus* (Dehning and Tilzer 1989), and another coastal pelagophyte, *A. anophagefferens* (Popels and Hutchins 2002, Popels et al. 2007), and is likely related to the time needed to up-regulate synthesis of the photosynthetic apparatus (Dehning and Tilzer 1989, Popels and Hutchins 2002) as well as the relative amount of energy available from energy reserves (Dawes and Senior 1973). The significantly longer lag phase for cells stored at 21°C compared to 10°C may be caused by the larger decrease in photosynthetic efficiency at 21°C and faster use of storage products (i.e., sterol reserves) leaving fewer available for metabolism to reactivate photosynthetic apparatus from energy stores upon reintroduction to light conditions (Dehning and Tilzer 1989).

Even after 7 months of dark storage at 10°C, resting cells were able to revert to vegetative cells showing the potential ability of *Aureoumbra* to "overwinter" during cooler periods in subtropical environments such as TX and FL, USA. Resting cells required at least 15 d of light incubation to return to active growth, showing the potential contribution of *Aureoumbra* resting cells to the reinitiation of blooms 15 d after the subtropical winter when waters warm and growth conditions become favorable. In addition, the high density ($>2.0 \times 10^6$ cells $\cdot$ mL^{-1}) of *Aureoumbra* vegetative cells attained even after the 7 months of dark storage indicates that extended persistence as resting cells does not discourage the subsequent formation of intense blooms upon exposure to optimal growth conditions.

The ability of *Aureoumbra* to form resting cells as a means of tolerating environmental stressors (e.g., high temperature, nutrient stress, and long-term darkness) suggests that this alga may have competitive advantage in ecosystems with extended periods of high temperatures and/or low nutrient conditions and/or lower light. Specifically, within the northern Indian River Lagoon and Mosquito Lagoon, blooms of *Aureoumbra* have been sporadic, emerging during the summer of 2012, dissipating during November only to re-emerge and bloom again 6 months later in May 2013 (Koch et al. 2014, Kang et al. 2015), a pattern consistent with the formation of resting cells, perhaps within darkened sediments, and the conversion of these resting cells into vegetative cells as demonstrated within experiments. Hence, the ability of *Aureoumbra* to form resting cells may serve as a means to form annual blooms within subtropical ecosystems.

Potential role of resting stages in the geographical expansion of brown tides. Given that *Aureoumbra* is able to revert from resting stages to vegetative cells after long-term storage in darkness and that operation of commercial and cruise ships in the Caribbean region has increased during the past two decades (Dwyer and Forsyth 1998, Wood 2000), the potential for the anthropogenic transport of *Aureoumbra* to new regions via ships' ballast water is possible. Phytoplankton in residual ballast water must persist in complete darkness, sometimes under elevated temperatures (Hallegraeff 1998). Prior studies have shown that the temperate brown tide alga, *Aureococcus*, was detected in ballast tanks of commercial ships despite the large disparity between the salinity in ballast tanks (~2) and the salinity required for active growth (>22; Doblin et al. 2004) and that *Aureococcus* can survive 30 d of darkness (Popels and Hutchins 2002, Popels et al. 2007). Consistent with those reports, ability of *Aureoumbra* resting cells to resume vegetative growth after 7 months in the dark suggests *Aureoumbra* cells in ballast tanks could be viable even after extended ocean voyages.

The ability of *Aureoumbra* to form resting cells may also be important for the persistence and potential expansion of *Aureoumbra* populations in subtropical and tropical regions under climate change. Ocean temperatures have increased by an average of 1°C during the past 100 years (Hoegh-Guldberg and Bruno 2010). Temperature changes can have a direct impact on the growth of phytoplankton (Boyd et al. 2013) and may be more important for coastal algal species relative to oceanic species (Hallegraeff 2010). Rising seawater temperatures that increase the growth rates of HABs has been shown to facilitate a temporal and spatial expansion of HABs (Moore et al. 2008, Fu et al. 2012). *Aureoumbra* has traditionally had a limited, subtropical range (Gobler and Sunda 2012), but blooms of this alga have recently expanded to tropical regions of the North Atlantic (e.g., the

Guantanamo Bay, Cuba; Koch et al. 2014), demonstrating its ability to thrive within warmer climates. While some have hypothesized that warming seawater may cause a decline in the phytoplankton diversity and a poleward shift in species (Thomas et al. 2012), the ability of *Aureoumbra* to grow rapidly at temperatures up to 30°C and enter the resting stages at high temperature (~35°C) could allow this alga to geographically expand both northward and southward into tropical systems. Moreover, the minor variation in the production of resting stages among four strains at 35°C further supports the hypothesis that this trait is fixed in this species and may allow it to resist periods of extreme high temperatures that are common in coastal regions of tropics and will likely be more common in the future. Collectively, the ability of *Aureoumbra* to form resting cells under unfavorable conditions, the increased frequency of commercial and cruise ship operation in the Caribbean region, and concurrently rising temperature due to climate change may be facilitating a geographical expansion of this harmful brown tide alga that could continue into the future.

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