# Stony Brook University

**An investigation of pelagophyte ecology**

A Dissertation Presented

by

**Yoonja Kang**

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

**Doctor of Philosophy**

in

**Marine and Atmospheric Science**

Stony Brook University

**December 2016**

**Stony Brook University**

The Graduate School

**Yoonja Kang**

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

**Dr. Christopher J. Gobler – Dissertation Advisor**
**Professor, School of Marine and Atmospheric Sciences**

**Dr. Gordon T. Tayor - Chairperson of Defense**
**Professor, School of Marine and Atmospheric Sciences**

**Dr. Jackie L. Collier – Committee Member**
**Associate Professor, School of Marine and Atmospheric Sciences**

**Dr. Stephen B. Baines – Committee Member**
**Associate Professor, Department of Ecology and Evolution**

**Dr. Raphael M. Kudela – Committee Member**
**Professor, Ocean Sciences**
**University of California, Santa Cruz**

This dissertation is accepted by the Graduate School

Charles Taber

Dean of the Graduate School

Abstract of the Dissertation

**An investigation of pelagophyte ecology**

by

**Yoonja Kang**

**Doctor of Philosophy**

in

**Marine and Atmospheric Science**

Stony Brook University

**2016**

Pelagophytes are important picoplankton in the open ocean and in coastal waters where *Aureococcus anophagefferens* and *Aureoumbra lagunensis* form ecosystem-disruptive brown tides. Despite decades of research, factors facilitating brown tides are not completely understood. This dissertation is a comparative study of pelagophytes assessing processes facilitating brown tides and transcriptional similarities among open ocean and coastal pelagophytes. During my dissertation, top-down (e.g. grazing) and bottom-up (e.g. nutrients) processes shaping phytoplankton communities were examined during brown tides in the Indian River Lagoon, Florida, USA. While moderate levels of ammonium promoted *Aureoumbra* growth over picoprokaryotes, grazing pressure on *Aureoumbra* was near absent permitting this alga to outgrow co-existing phytoplankton. My research also discovered that *Aureoumbra* forms resting cells in response to environmental stressors such as high temperature, nutrient limitation, and darkness. Resting cells were characterized by larger biovolumes compared to vegetative cells, development of red accumulation bodies, and enriched sterol content. In addition, resting cells could revert to vegetative cells after seven months of dark storage, an observation

consistent with field observations showing re-occurrence of brown tides in Indian River Lagoon six months after an initial bloom. This dissertation also discovered the strong allelopathic effects of *Aureococcus* and *Aureoumbra* on co-occurring algae. Allelopathic effects were broad, causing significant dose-dependent inhibition in the growth of multiple phytoplankton species at brown tide densities of $>2.5 \times 10^5$ cells $mL^{-1}$ suggesting allelopathy may be important for the intensification and persistence of blooms. Sorting flow cytometry was combined with species-specific immune-detection of *Aureococcus* to contrast the nutritional ecology of the brown tide alga with other co-occurring picoplankton during blooms and provided a comparison of nitrogen uptake on a per-cell basis that affirmed the importance of urea in promoting *Aureococcus* blooms. Lastly, comparative transcriptomics of four pelagophytes demonstrated that these algae commonly express functional genes associated with cleavage of organic nitrogen under low N conditions, the utilization of intracellularly recycled phosphate under low P conditions, and the use of flavodoxins over ferredoxins under low light conditions. Collectively, this research provides a new understanding of the mechanisms facilitating brown tides and transcriptional similarities among pelagophytes.

## Dedication Page

I dedicate this dissertation to the memory of my late father.

Table of Contents

## List of Figures

### Chapter 2



### Chapter 3

**Chapter 6**

## List of Tables

### Chapter 2



### Chapter 3



### Chapter 4



### Chapter 5

**Chapter 6**

## Acknowledgments

Last five years were the most interesting and rewarding time of my life. Through graduate school, I have been supported by many amazing people. First of all, I wish to thank my advisor Dr. Christopher Gobler. To me, sometimes he was like an anchor that holds me to stay on a right path and sometimes he was like a lighthouse that helps me figure out where I need to step forward. Without his guidance and encouragement, I would not been able to complete this journey. It was very pleasant to work with such a talented scientist and teacher who I hope to emulate his integrity and passion for science, teaching, and students. I also appreciate my committee, Dr. Gordon Taylor, Dr. Jackie Collier, Dr. Stephen Baines, and Dr. Raphael Kudela for their guidance during my Ph.D and thoughtful comments on my dissertation. Special thanks to Dr. Taylor and Dr. Kudela for their help to analyze my samples. I also thank to Theresa and Jen for being such good friends and fantastic people who have supported me and shared a lot of laughs for the last five years. I also thank to other Gobler lab people for their help and awesomeness. I have been blessed with amazing friends in New York and Miami for the last eight years. I wish to thank all who made my time in both places fun and memorable. Lastly, but not least, I would like to thank my family for their tireless support and love through my life. I especially thank to my mother who supported me to pursue what I wish to do.

# Chapter 1

## Dissertation Introduction

## Introduction

For decades, oceanographers have recognized the central role that picoplankton play in global ocean ecology and biogeochemical cycles (Li et al., 1983; Platt et al., 1983; 1994; Richardson and Jackson, 2007). For much of that time, the bulk of research on ocean picoplankton has focused on picocyanobacteria, particularly within the genera of *Synechococcus* and *Prochlorococcus* (Waterbury et al., 1986; Chisholm et al., 1992; Partensky et al., 1999; Rocap et al., 2002). In contrast, picoeukaryotes have been less studied despite their broad diversity and importance in food webs and biogeochemical cycles (Moon-van der Staay et al., 2001; Cuvelier et al., 2010; Massana, 2011). Within their great diversity, there is accumulating evidence that among the picoeukaryotes, pelagophytes may be some of the most important members. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans demonstrated that pelagophytes are one of the two most abundant groups of microeukaryotes at these locations (along with Prymnesiophytes; Andersen et al., 1996). Field studies in the open waters of the Gulf of Mexico suggest pelagophyte productivity is largely responsible for the large, annual $CO_2$ drawdown occurring in this region based on the co-occurrence of elevated RuBisCO gene transcript levels from this group and reduced seawater $pCO_2$ levels (John et al., 2007). Flow cytometric and electron microscopic analyses revealed that *Pelagomonas* sp. appears to be the most abundant picoeukaryote in the North Atlantic and Pacific subtropical oceans and its relative abundance increases with depth (Simon et al., 1994). Dimier et al. (2009) showed that the maximum growth rate of this low light-adapted *Pelagomonas calceolata* is 0.35 $d^{-1}$ at low light intensity (10 – 50 $\mu$mol photons $m^{-2}$ $s^{-1}$). Quantitative PCR (qPCR) amplification of the rbcL gene of pelagophytes showed the peak and fluctuation of pelagophyte abundance in and around the deep chlorophyll maximum (DCM; Li et al., 2013) that may result

from a variation in the annual light cycle (Letelier et al., 2004) and the sporadic eddy events with intrusion of nutrient-rich water into the euphotic zone (Sakamoto et al., 2002). Recent research on natural field samples of picoeukaryotes through the cell sorting using a flow cytometer and the whole-genome sequencing documented the ubiquitous distribution and abundance of *Pelagomonas calceolata* in the World Ocean (Worden et al., 2012). However, despite the one of the most abundant picoeukaryotes in the subtropical open ocean and the cosmopolitan distribution (Andersen et al., 1993; Simon et al., 1994), the ecological characteristics of pelagophytes and *Pelagomonas* are poorly understood.

The importance of pelagophytes in several coastal ecosystems has also become clear in recent decades. Specifically, two species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, have become notorious for their ability to create ecosystem disruptive algal blooms (Sunda et al 2006). These two algae are phylogenically the most closely related pelagophytes and share a highly similar ecological niche (Gobler and Sunda 2012). Also known as brown tides these blooms can cause harm or mortality within multiple classes of marine organisms including zooplankton, shellfish, and seagrass as a consequence of disruption of feeding or filtering and drastic light attenuation (Onuf, 1996; Buskey et al., 1997; Harke et al., 2011; Gobler et al., 2013a). *Aureoumbra* blooms had been localized in and around Laguna Madre, Texas, from 1990 – 2012 but have recently expanded to the East Coast of Florida (Gobler et al., 2013a) and to Cuba (Koch et al., 2014). Similarly brown tide blooms caused by *Aureococcus* that had been detected across the Mid-Atlantic US (RI to VA) for decades and have also emerged in the coastal waters of China (Zhang et al., 2012) and South Africa (Probyn et al., 2001).

To date, several mechanisms have been proposed to account for the occurrence of brown tides including the ability to utilize DON at low DIN levels (Berg et al., 2002; Mulholland et al.,

2002; Muhlstein and Villareal, 2007), the ability to resist zooplankton grazing (Buskey and Hyatt, 1995; Gobler et al., 2002b), and the maximal growth at low light levels (Milligan and Cosper, 1997; Pustizzi et al., 2004). However, other mechanisms that have been shown to be important for facilitating harmful algal blooms (HABs) such as allelopathic inhibition in the growth of competing algae, the ability to form resting cells in response to unfavorable growth conditions, or nutritional competition with co-occurring algae have not been studied for brown tides (Granéli and Turner, 2006).

*Nutritional ecology*

Compared to open ocean pelagophytes, the nutritional ecology of brown tide algae, *Aureococcus* and *Aureoumbra* has been well studied. Brown tide algae can utilize DON in low dissolved inorganic nitrogen (DIN) environments (Berg et al., 1997; Muhlstein and Villareal, 2007) putatively using a suite of transporters for DON compounds (e.g. urea) that are metabolically unavailable for other phytoplankton (Gobler et al., 2011). Upregulating DON-related transcripts responsive to nitrogen limitation may also permit *Aureococcus* to outcompete co-existing phytoplankton and bloom (Wurch et al., 2011b; Frischkorn et al., 2014). Even if nitrate is available in brown tide habitats, the DIN may not support bloom populations, especially for *Aureoumbra* as they have been reported to be unable to grow on nitrate (Deyoe and Suttle, 1994).

Regarding phosphorus (P), prior studies have suggested that *Aureoumbra* is well adapted to P-limited environments (Liu et al., 2001). Liu et al. (2001) specifically found that this species undergoes a cellular transition from N-limitation to P-limitation at extremely high N:P critical ratio (~130), a characteristic potentially linked to a high alkaline phosphatase activity (Sun et al., 2012). Low dissolved inorganic nutrient levels are not likely to favor the growth of most co-occurring

phytoplankton, but brown tide algae can proliferate because of their ability to exploit organic forms of nitrogen and phosphorus (Gobler et al., 2011). While the molecular underpinnings that facilitate dominance under low nutrient conditions have been well-studied in *Aureococcus* (Wurch et al., 2011b; Wurch et al., 2013; Frischkorn et al., 2014), very little is known regarding the physiological pathways that may facilitate similar success in other pelagophytes including *Aureoumbra* and *Pelagomonas*.

Comparisons of traits among species can reveal the extent to which their niches overlap and to which they compete in nature (Hutchinson, 1961; Smayda, 1997). There are multiple lines of evidence suggesting that pico-cyanobacteria are prime competitor with brown tide species. Likewise, there is also the potential for strong competition between *Pelagomonas*, which is highly abundant in many regions (Simon et al., 1994; Andersen et al., 1996; Worden et al., 2012) and picocyanobacteria, which are also known to dominate the DCM zone (Campbell and Vaulot, 1993; Casey et al., 2007). In New York, *Aureococcus* blooms are often succeeded by *Synechococcus* sp. (Gobler et al., 2004a; Koch et al., 2013), while Texas brown tides caused by *Aureoumbra* blooms co-occur with phycocyanin (PC) and phycoerythrin (PE)-containing cyanobacteria (Buskey et al., 2001; DeYoe et al., 2007). Collectively, these observations suggest multiple types of *Synechococcus* may compete with brown tide algae for resources during blooms. However, similarity in cell size has made physically separating these populations in coastal waters, and thus differentiating their nutrient ecology, a challenge. For example, prior tracer studies examining assimilation of carbon, N, and vitamins by HABs have utilized filter size fraction and comparisons of bloom and non-bloom waters (Mulholland et al., 2002; Mulholland et al., 2004; Koch et al., 2013) making identification of the precise phytoplankton populations responsible for nutrient uptake somewhat ambiguous. Therefore, methods that can assign the definitive uptake rates to

different co-occurring phytoplankton populations during bloom and non-bloom periods would more clearly define the nutritional niche of distinct plankton populations during brown tides.

*Flow cytometry*

Flow cytometry is a technique with multiple advantages over conventional microscopy techniques when identifying and quantifying picoplankton (Jacquet et al., 1998; Shi et al., 2011; Balzano et al., 2012). As individual cells pass through 488 nm blue argon-ion laser and 635 red diode laser, size is estimated based on scattering of the laser beam and pigmentation is estimated based on fluorescence (Olson et al., 1989). Cells are detected based on size (i.e. light scattering) and fluorescence. Modern flow cytometers used for enumerating phytoplankton (e.g. FACSCalibur flow cytometer, Becton Dickinson, San Jose, CA) can have four channels for detecting fluorescence: one for green light that is deal for indicating the presence of fluorescein isothiocyanate (FITC, FL1, 500-560 nm), one for orange light that is used to indicate the presence of phycoerythrin (FL2, 540-630 nm), and two others for dark red (FL3, 660-700 nm) and red light (FL4, 645-677 nm) (Olson et al., 1989). Use of these different channels can permit the differentiation of multiple sizes and types of small eukaryotic and prokaryotic phytoplankton. Fluoroscent dyes can also be detected via flow cytometers and recently, Koch et al. (2014) demonstrated that flow cytometric detection of *Aureoumbra* with a fluorescent antibody was more accurate and faster than conventional microscopy-based identification. Similarly, I have routinely used an immunofluorescent tag to quantify *Aureococcus* via flow cytometry (Stauffer et al., 2008). Single-cell sorting using a flow cytometer has been shown to be a useful tool for conventional research on microbiology (Collier, 2000; Brehm-Stecher and Johnson, 2004) and has recently become useful for assessing nutrient uptake and assimilation among picoplankton (Talarmin et al., 2011; Duhamel et al., 2012). Furthermore, analyses of phytoplankton after incubation with

$H^{14}CO_3^-$ have shown no statistically significant difference between unsorted and sorted cells (Rivkin et al., 1986). Hence, sorting flow cytometry may be a useful tool to detect nutrient assimilation among plankton groups of interest during brown tides.

*Grazing*

Zooplankton grazing is an important factor in both controlling phytoplankton biomass and transferring primary production to upper trophic levels of marine food webs (Stoecker and Capuzzo, 1990; Landry et al., 1997; Behrenfeld, 2010). Selective grazing by zooplankton has the potential to alter phytoplankton community composition as well as carbon transfer to the higher trophic levels (Strom, 2008). The disruption of zooplankton grazing has been implicated as a causal mechanism for harmful algal blooms (Smayda, 2008) including brown tides (Sunda et al., 2006). Several studies have reported selective avoidance of *Aureococcus* by zooplankton during blooms (Gobler et al., 2002b; Caron et al., 2004) while Deonarine et al. (2006) found that intense *Aureococcus* blooms deter zooplankton grazing, but that zooplankton grazing on the brown tide alga recovers during the demise of blooms. Dense blooms of *Aureococcus* (> 1 x $10^6$ cells $mL^{-1}$) can also lead to a reduction in egg production of copepods and growth rates of ciliates (Bricelj and Lonsdale, 1997). Brown tide algae exude extracellular polymeric substances (EPS) that may clog feeding apparatus and alter the swimming behavior of ciliates (Liu and Buskey, 2000a). Specifically, a particulate form of EPS (e.g. transparent exopolymer particles; TEP) seems to be an indicator of stress in *Aureoumbra* as it produces more TEP than dissolved carbohydrate under hypersalinity (Liu and Buskey, 2000b) and low N:P ratio (Liu and Buskey, 2003). In contrast to Texas brown tides and the Northeast brown tides, the grazing susceptibility of the recently emerged brown tides of Florida and Cuba are unknown. Furthermore, it is hypothesized that successful

escape of *Aureoumbra* from zooplankton grazing may facilitate the proliferation of brown tides in new regions such as Florida.

*The ability of Aureoumbra lagunensis to form resting cells*

The formation of resting stage cells is one survival strategy for phytoplankton to adapt to environmentally unfavorable conditions (Anderson, 1975; Anderson et al., 1985; Matsuoka and Fukuyo, 2000b; Bravo and Figueroa, 2014). Resting stages refer to cells that reduce metabolic rate, cease cell division, but remain viable (von Dassow and Montresor, 2010) and are often characterized by distinct morphological and compositional changes 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 Dale, 1969; Matsuoka and Fukuyo, 2000b). Resting cells that undergo changes in morphology and physiology leading to a resting stage typically 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).

There are many groups of phytoplankton known to produce resting stages including cyanobacteria (Kaplan-Levy et al., 2010), chrysophytes (Sandgren, 1983), diatoms (Hargraves, 1983), dinoflagellates (Dale, 1983), euglenophytes (Olli, 1996), and raphidophytes (Kim et al., 2015). Specific cues that induce the formation of resting stages include sub-optimal 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; Fistarol et al., 2004a). To date, non-vegetative stage or resting cell production has not been documented among pelagophytes. Interestingly, *Aureococcus* cultures have shown the ability to survive and regrow after 30 days of

dark storage (Popels and Hutchins, 2002), an attribute that some have hypothesized could facilitate ballast water transport of this species (Doblin et al., 2004). However, no study has ever documented any morphological changes that might be indicative of resting cell formation in *Aureococcus*. Given that resting stages have been associated with the recurrence of annual HABs and geographical expansion of algal populations (Matsuoka and Fukuyo, 2000a; Garces et al., 2001; Tang and Gobler, 2012), the ability of brown tide algae to form resting cells might explain their ability to form continuous blooms in NY and TX (Gobler and Sunda, 2012) or their ability to expand across ocean basins (Zhang et al., 2012; Koch et al., 2014) perhaps via ballast water transport.

*Allelopathic effects on the growth of competing algae*

Allelopathy is an interspecies interaction whereby one plant can have detrimental effects on another via allelochemicals (Rice, 2013). This process has been cited as a key factor facilitating the occurrence of some HABs (Granéli and Turner, 2006). Allelopathic chemicals associated with secondary metabolites (e.g. hemolytic compounds or toxins) can cause reductions in photosynthetic efficiency (Figueredo et al., 2007; Prince et al., 2008) and metabolism (Zheng et al., 2016), lysis of target algae (Igarashi et al., 1996; Rengefors and Legrand, 2001; Fistarol et al., 2003), target cell immobilization (Tillmann et al., 2007; Tillmann et al., 2009), and/or the formation of temporary cysts (Fistarol et al., 2004a).

Allelopathic interactions between harmful microalgae and macroalgae have been considered not only as a mechanism for competing co-occurring phytoplankton for niches (Prince et al., 2008; Tang and Gobler, 2010; Hattenrath-Lehmann and Gobler, 2011b), but also for mitigating some types of harmful algal blooms (Tang and Gobler, 2011; Tang et al., 2014). In an

ecosystem setting, densities of brown tides caused by *Aureococcus* or *Aureoumbra* are often inversely correlated with densities of other picoplankton such as cyanobacteria and picoeukaryotes and especially, cyanobacterial densities often increase rapidly as brown tides diminish. Past field observations (Sieracki et al., 2004; Gobler et al., 2011, Gobler et al., 2013) coupled with preliminary lab experiments suggest that brown tide algae may indeed affect other phytoplankton via allelopathy.

*Transcriptomics*

In the past decade, dozens of phytoplankton species have had their complete genome sequenced. However, a genome, being comprised of the total DNA sequence within an organism, only reflects the potential physiological capabilities of that organism. For that potential to be realized, genes must be transcribed into corresponding messenger RNAs that are subsequently translated into proteins. With development of high throughput sequencing techniques (i.e., next generation sequencing; NGS) and recent advances in bioinformatic approaches (Soneson and Delorenzi, 2013), the transcriptomes of eukaryotes and prokaryotes have been widely used to assess gene - environment interactions via the quantification of each transcript in organism. Global transcriptome sequencing has recently been applied to some HABs and has been useful for identifying novel and important aspects of HAB physiological ecology (Erdner and Anderson, 2006; Moustafa et al., 2010; Wurch et al., 2011b). Wurch et al. (2011b) found that *Aureococcus* up-regulates a variety of ammonium transporters and hydrolases in response to nitrogen limitation. Intraspecific homogeneity of gene expression in response to low nutrient levels indicates the importance of species-specific nutritional strategy for the population growth (Frischkorn et al., 2014) for example, the exploitation of DON by *Aureococcus* under low N conditions (Gobler et al., 2011; Wurch et al., 2011b). By generating and comparing transcriptomes of pelagophytes

under conditions that are commonly found in an ecosystem setting (low levels of nutrients and light), the biochemical pathways that facilitate their dominance in coastal and open ocean ecosystems can be identified.

**Objectives**

Despite decades of research, ecophysiological characteristics of open ocean pelagophytes are poorly understood. Although more is known regarding the ecology of brown tide algae, factors facilitating their range of expansion are still unknown. This dissertation is focused on comparative studies of pelagophytes to assess processes facilitating brown tides and transcriptional similarities among open ocean and coastal bloom-forming pelagophytes.

1. Assess the role of nutrients and zooplankton grazing in the expansion of brown tide blooms caused by *Aureoumbra lagunensis* in the Indian River Lagoon and Mosquito Lagoon, FL, USA.
2. Examine the ability of *Aureoumbra lagunensis* to produce resting cells in response to environmental stressors including high temperature, nutrient depletion, and darkness.
3. Assess allelopathic effects of brown tide pelagophytes on competing microalgae
4. Utilize sorting flow cytometry to study nitrogen uptake by *Aureococcus anophagefferens* and groups of picoplankton during brown tide in NY, USA.
5. Describe and quantify differential gene expression of pelagophytes under light and nutrient limitation.

## References


Andersen, R.A., Bidigare, R.R., Keller, M.D., Latasa, M., 1996. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans. Deep-Sea Res PT II 43 (2–3), 517-537.

Andersen, R.A., Saunders, G.W., Paskind, M.P., Sexton, J.P., 1993. Ultrastructure and 18S ribosomal RNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov. and the description of a new algal class, the Pelagophyceae classis nov. J. Phycol. 29 (5), 701-715.

Anderson, D.M., 1980. Effects of temperature conditioning on development and germination of *Gonyaulax tamarensis* (Dinophyceae). J. Phycol. 16 (2), 166-172.

Anderson, D.M., Coats, D.W., Tyler, M.A., 1985. Encystment of the dinoflagellate *Gyrodinium uncatenum*: temperature and nutrient effects. J. Phycol. 21 (2), 200-206.

Anderson, D.M., Taylor, C.D., Armbrust, E.V., 1987. The effects of darkness and anaerobiosis on dinoflagellate cyst germination. Limnol. Oceanogr 32 (2), 340-335.

Anderson, O.R., 1975. The ultrastructure and cytochemistry of resting cell formation in *Amphora coffaeformis* (Bacillariophyceae). J. Phycol. 11 (3), 272-281.

Balzano, S., Marie, D., Gourvil, P., Vaulot, D., 2012. Composition of the summer photosynthetic pico and nanoplankton communities in the Beaufort Sea assessed by T-RFLP and sequences of the 18S rRNA gene from flow cytometry sorted samples. The ISME journal 6 (8), 1480-1498.

Behrenfeld, M.J., 2010. Abandoning Sverdrup's Critical Depth Hypothesis on phytoplankton blooms. Ecology 91 (4), 977-989.

Berg, G.M., Glibert, P.M., Lomas, M.W., Burford, M.A., 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. Mar. Biol. 129 (2), 377-387.

Berg, G.M., Repeta, D.J., Laroche, J., 2002. Dissolved organic nitrogen hydrolysis rates in axenic cultures of Aureococcus anophagefferens (Pelagophyceae): Comparison with heterotrophic bacteria. Appl. Environ. Microbiol. 68 (1), 401-404.

Bravo, I., Figueroa, R.I., 2014. Towards an ecological understanding of dinoflagellate cyst functions. Microorganisms 2 (1), 11-32.

Brehm-Stecher, B.F., Johnson, E.A., 2004. Single-cell microbiology: tools, technologies, and applications. Microbiol. Mol. Biol. Rev. 68 (3), 538-559.

Bricelj, V.M., Lonsdale, D.J., 1997. *Aureococcus anophagefferens*: Causes and ecological consequences of brown tides in US mid-Atlantic coastal waters. Limnol. Oceanogr. 42 (5), 1023-1038.

Buskey, E.J., Hyatt, C.J., 1995. Effects of the Texas (USA) brown tide alga on planktonic grazers. Mar. Ecol. Prog. Ser. 126 (1-3), 285-292.

Buskey, E.J., Liu, H.B., Collumb, C., Bersano, J.G.F., 2001. The decline and recovery of a persistent Texas brown tide algal bloom in the Laguna Madre (Texas, USA). Estuaries 24 (3), 337-346.

Buskey, E.J., Montagna, P.A., Amos, A.F., Whitledge, T.E., 1997. Disruption of grazer populations as a contributing factor to the initiation of the Texas brown tide algal bloom. Limnol. Oceanogr. 42 (5), 1215-1222.

Campbell, L., Vaulot, D., 1993. Photosynthetic picoplankton community structure in the subtropical North Pacific Ocean near Hawaii (station ALOHA). Deep Sea Research Part I: Oceanographic Research Papers 40 (10), 2043-2060.

Caron, D.A., Gobler, C.J., Lonsdale, D.J., Cerrato, R.M., Schaffner, R.A., Rose, J.M., Buck, N.J., Taylor, G., Boissonneault, K.R., Mehran, R., 2004. Microbial herbivory on the brown tide alga, *Aureococcus anophagefferens*: results from natural ecosystems, mesocosms and laboratory experiments. Harmful Algae 3 (4), 439-457.

Casey, J.R., Lomas, M.W., Mandecki, J., Walker, D.E., 2007. *Prochlorococcus* contributes to new production in the Sargasso Sea deep chlorophyll maximum. Geophys. Res. Lett. 34 (10).

Chapman, D., Dodge, J., Heaney, S., 1982. Cyst formation in the freshwater dinoflagelalte *Ceratium hirundinella* (Dinophyceae). J. Phycol. 18 (1), 121-129.

Chisholm, S.W., Frankel, S.L., Goericke, R., Olson, R.J., Palenik, B., Waterbury, J.B., West-Johnsrud, L., Zettler, E.R., 1992. *Prochlorococcus marinus* nov. gen. nov. sp.: an oxyphototrophic marine prokaryote containing divinyl chlorophyll *a* and *b*. Arch. Microbiol. 157 (3), 297-300.

Collier, J.L., 2000. Flow cytometry and the single cell in phycology. J. Phycol. 36 (4), 628-644.

Cosper, E.M., Dennison, W.C., Milligan, A.J., Carpenter, E.J., Lee, C., Holzapfel, J., Milanese, L., 1989. An examination of the environmental factors important to initiating and sustaining 'brown tide' blooms., In: Cosper, E.M., Briceli, V., Carpenter, E.J. (Eds.), Novel phytoplankton blooms: causes and impacts of recurrent brown tides and other unusual blooms. Springer Verlag, Berlin, pp. 317-340.

Cuvelier, M.L., Allen, A.E., Monier, A., McCrow, J.P., Messie, M., Tringe, S.G., Woyke, T., Welsh, R.M., Ishoey, T., Lee, J.H., Binder, B.J., DuPont, C.L., Latasa, M., Guigand, C., Buck, K.R., Hilton, J., Thiagarajan, M., Caler, E., Read, B., Lasken, R.S., Chavez, F.P., Worden, A.Z., 2010. Targeted metagenomics and ecology of globally important uncultured eukaryotic phytoplankton. Proc. Natl. Acad. Sci. 107 (33), 14679-14684.

Dale, B., 1983. Dinoflagellate resting cysts: "benthic plankton", In: Fryxell, G.A. (Ed.), Survival strategies of the algae. Cambridge University Press, Cambridge, p. 144.

Deonarine, S.N., Gobler, C.J., Lonsdale, D.J., Caron, D.A., 2006. Role of zooplankton in the onset and demise of harmful brown tide blooms (*Aureococcus anophagefferens*) in US mid-Atlantic estuaries. Aquat. Microb. Ecol. 44 (2), 181-195.

DeYoe, H.R., Buskey, E.J., Jochem, F.J., 2007. Physiological responses of *Aureoumbra lagunensis* and *Synechococcus* sp to nitrogen addition in a mesocosm experiment. Harmful Algae 6 (1), 48-55.

Deyoe, H.R., Suttle, C.A., 1994. The inability of the Texas brown tide alga to use nitrate and the role of nitrogen in the initiation of a persistent bloom of this organism. J. Phycol. 30 (5), 800-806.

Dimier, C., Brunet, C., Geider, R., Raven, J., 2009. Growth and photoregulation dynamics of the picoeukaryote *Pelagomonas calceolata* in fluctuating light. Limnol. Oceanogr. 54 (3), 823-836.

Duhamel, S., Björkman, K.M., Karl, D.M., 2012. Light dependence of phosphorus uptake by microorganisms in the subtropical North and South Pacific Ocean. Aquat. Microb. Ecol. 67, 225-238.

Erdner, D.L., Anderson, D.M., 2006. Global transcriptional profiling of the toxic dinoflagellate *Alexandrium fundyense* using massively parallel signature sequencing. BMC Genomics 7 (1), 88.

Figueredo, C.C., Giani, A., Bird, D.F., 2007. Does allelopathy contribute to *Cylindrospermopsis raciborskii* (Cyanobacteria) bloom occurrence and geographic expansion? J. Phycol. 43 (2), 256-265.

Figueroa, R.I., Bravo, I., Garcés, E., 2005. Effects of nutritional factors and different parental crosses on the encystment and excystment of *Alexandrium catenella* (Dinophyceae) in culture. Phycologia 44 (6), 658-670.

Fistarol, G.O., Legrand, C., Granéli, E., 2003. Allelopathic effect of *Prymnesium parvum* on a natural plankton community. Mar. Ecol. Prog. Ser. 255, 115-125.

Fistarol, G.O., Legrand, C., Rengefors, K., Graneli, E., 2004. Temporary cyst formation in phytoplankton: a response to allelopathic competitors? Environ. Microbiol. 6 (8), 791-798.

Frischkorn, K.R., Harke, M.J., Gobler, C.J., Dyhrman, S.T., 2014. De novo assembly of *Aureococcus anophagefferens* transcriptomes reveals diverse responses to the low nutrient and low light conditions present during blooms. Name: Frontiers in Microbiology 5, 375.

Garces, E., Zingone, A., Reguera, B., 2001. Life histories of microalgal species causing harmful blooms. Calvi, Majorca, Spain: European Commission Directorate General Science, Research and Development.

Gobler, C.J., Berry, D.L., Dyhrman, S.T., Wilhelm, S.W., Salamov, A., Lobanov, A.V., Zhang, Y., Collier, J.L., Wurch, L.L., Kustka, A.B., Dill, B.D., Shah, M., VerBerkmoes, N.C.,

Kuo, A., Terry, A., Pangilinan, J., Lindquist, E.A., Lucas, S., Paulsen, I.T., Hattenrath-Lehmann, T.K., Talmage, S.C., Walker, E.A., Koch, F., Burson, A.M., Marcoval, M.A., Tang, Y.Z., LeCleir, G.R., Coyne, K.J., Berg, G.M., Bertrand, E.M., Saito, M.A., Gladyshev, V.N., Grigoriev, I.V., 2011. Niche of harmful alga *Aureococcus anophagefferens* revealed through ecogenomics. Proc. Natl. Acad. Sci. 108 (11), 4352-4357.

Gobler, C.J., Boneillo, G.E., Debenham, C.J., Caron, D.A., 2004. Nutrient limitation, organic matter cycling, and plankton dynamics during an *Aureococcus anophagefferens* bloom. Aquat. Microb. Ecol. 35 (1), 31-43.

Gobler, C.J., Koch, F., Kang, Y., Berry, D.L., Tang, Y.Z., Lasi, M., Walters, L., Hall, L., Miller, J.D., 2013. Expansion of harmful brown tides caused by the pelagophyte, *Aureoumbra lagunensis* DeYoe et Stockwell, to the US east coast. Harmful Algae 27 (0), 29-41.

Gobler, C.J., Renaghan, M.J., Buck, N.J., 2002. Impacts of nutrients and grazing mortality on the abundance of *Aureococcus anophagefferens* during a New York brown tide bloom. Limnol. Oceanogr. 47 (1), 129-141.

Gobler, C.J., Sunda, W.G., 2012. Ecosystem disruptive algal blooms of the brown tide species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*. Harmful Algae 14, 36-45.

Granéli, E., Turner, J.T., 2006. Ecology of harmful algae. Springer.

Hargraves, P., 1983. Diatom resting spores: significance and strategies, In: Fryxell, G.A. (Ed.), Survival strategies of the algae. Cmbridge University Press, Cambridge, p. 144.

Harke, M.J., Gobler, C.J., Shumway, S.E., 2011. Suspension feeding by the Atlantic slipper limpet (*Crepidula fornicata*) and the northern quahog (*Mercenaria mercenaria*) in the presence of cultured and wild populations of the harmful brown tide alga, *Aureococcus anophagefferens*. Harmful Algae 10 (5), 503-511.

Hattenrath-Lehmann, T.K., Gobler, C.J., 2011. Allelopathic inhibition of competing phytoplankton by North American strains of the toxic dinoflagellate, *Alexandrium fundyense*: Evidence from field experiments, laboratory experiments, and bloom events. Harmful Algae 11 (0), 106-116.

Hutchinson, G.E., 1961. The paradox of the plankton. The American Naturalist 95 (882), 137-145.

Igarashi, T., Satake, M., Yasumoto, T., 1996. Prymnesin-2: a potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. J. Am. Chem. Soc. 118 (2), 479-480.

Itakura, S., Nagasaki, K., Yamaguchi, M., Imai, I., 1996. Cyst formation in the red tide flagellate *Heterosigma akashiwo* (Raphidophyceae). J. Plankton Res. 18 (10), 1975-1979.

Jacquet, S., Lennon, J.-F., Marie, D., Vaulot, D., 1998. Picoplankton population dynamics in coastal waters of the northwestern Mediterranean Sea. Limnol. Oceanogr. 43 (8), 1916-1931.

John, D.E., Wang, Z.A., Liu, X., Byrne, R.H., Corredor, J.E., López, J.M., Cabrera, A., Bronk, D.A., Tabita, F.R., Paul, J.H., 2007. Phytoplankton carbon fixation gene (RuBisCO) transcripts and air-sea $CO_2$ flux in the Mississippi River plume. The ISME journal 1 (6), 517-531.

Kaplan-Levy, R.N., Hadas, O., Summers, M.L., Rücker, J., Sukenik, A., 2010. Akinetes: dormant cells of cyanobacteria, In: Lubzens, E., Cerda, J., Clark, M. (Eds.), Dormancy and resistance in harsh environments. Springer, Berlin Heidelberg, pp. 5-27.

Kim, J.-H., Park, B.S., Wang, P., Kim, J.H., Youn, S.H., Han, M.-S., 2015. Cyst morphology and germination in *Heterosigma akashiwo* (Raphidophyceae). Phycologia 54 (5), 435-439.

Koch, F., Kang, Y., Villareal, T.A., Anderson, D.M., Gobler, C.J., 2014. Novel immunofluorescence flow cytometry technique detects the expansion of harmful brown tides caused by *Aureoumbra lagunensis* to the Caribbean Sea. Appl. Environ. Microbiol. 80 (16), 4947-4957.

Koch, F., Sañudo-Wilhelmy, S.A., Fisher, N.S., Gobler, C.J., 2013. Effect of vitamins B and B 12 on bloom dynamics of the harmful brown tide alga, *Aureococcus anophagefferens* (Pelagophyceae). Limnol. Oceanogr 58 (5), 1761-1774.

Landry, M.R., Barber, R.T., Bidigare, R.R., Chai, F., Coale, K.H., Dam, H.G., Lewis, M.R., Lindley, S.T., McCarthy, J.J., Roman, M.R., Stoecker, D.K., Verity, P.G., White, J.R., 1997. Iron and grazing constraints on primary production in the central equatorial Pacific: An EqPac synthesis. Limnol. Oceanogr. 42 (3), 405-418.

Letelier, R.M., Karl, D.M., Abbott, M.R., Bidigare, R.R., 2004. Light driven seasonal patterns of chlorophyll and nitrate in the lower euphotic zone of the North Pacific Subtropical Gyre. Limnol. Oceanogr 49 (2), 508-519.

Li, B., Karl, D.M., Letelier, R.M., Bidigare, R.R., Church, M.J., 2013. Variability of chromophytic phytoplankton in the North Pacific Subtropical Gyre. Deep-Sea Res. Part II-Top. Stud. Oceanogr. 93, 84-95.

Li, W., Rao, S., Harrison, W., Smith, J., Cullen, J., Irwin, B., Platt, T., 1983. Autotrophic picoplankton in the tropical ocean. Science 219 (4582), 292-295.

Li, W.K.W., Beaumont, A.R., Hunter, J.R., 1994. Primary production of prochlorophytes, cyanobacteria, and eucaryotic ultraphytoplankton: measurements from flow cytometric sorting. Mar. Biol 114, 85-95.

Liu, H.B., Buskey, E.J., 2000a. The exopolymer secretions (EPS) layer surrounding *Aureoumbra lagunensis* cells affects growth, grazing, and behavior of protozoa. Limnol. Oceanogr. 45 (5), 1187-1191.

Liu, H.B., Buskey, E.J., 2000b. Hypersalinity enhances the production of extracellular polymeric substance (EPS) in the Texas brown tide alga, *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 36 (1), 71-77.

Liu, H.B., Buskey, E.J., 2003. Effects of media N: P ratio on extracellular polymeric substances (EPS) production of the brown tide-forming alga, *Aureoumbra lagunensis*. Plankton Biol. Ecol. 50 (2), 55-60.

Liu, H.B., Laws, E.A., Villareal, T.A., Buskey, E.J., 2001. Nutrient-limited growth of *Aureoumbra lagunensis* (Pelagophyceae), with implications for its capability to outgrow other phytoplankton species in phosphate-limited environments. J. Phycol. 37 (4), 500-508.

Massana, R., 2011. Eukaryotic picoplankton in surface oceans. Annu. Rev. Microbiol. 65, 91-110.

Matsuoka, K., Fukuyo, Y., 2000a. Technical guide for modern dinoflagellate cyst study. WESTPAC-HAB, Japan Society for the Promotion of Science, Tokyo, Japan.

Matsuoka, K., Fukuyo, Y., 2000b. Technical guide for modern dinoflagellate cyst study. WESTPAC-HAB, Japan Society for the Promotion of Science, Tokyo, Japan.

Milligan, A.J., Cosper, E.M., 1997. Growth and photosynthesis of the 'brown tide' microalga *Aureococcus anophagefferens* in subsaturating constant and fluctuating irradiance. Mar. Ecol. Prog. Ser. 153, 67-75.

Moon-van der Staay, S.Y., De Wachter, R., Vaulot, D., 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature 409 (6820), 607-610.

Moustafa, A., Evans, A.N., Kulis, D.M., Hackett, J.D., Erdner, D.L., Anderson, D.M., Bhattacharya, D., 2010. Transcriptome profiling of a toxic dinoflagellate reveals a gene-rich protist and a potential impact on gene expression due to bacterial presence. PLoS One 5 (3), e9688.

Muhlstein, H.I., Villareal, T.A., 2007. Organic and inorganic nutrient effects on growth rate-irradiance relationships in the Texas brown-tide alga *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 43 (6), 1223-1226.

Mulholland, M.R., Boneillo, G., Minor, E.C., 2004. A comparison of N and C uptake during brown tide (*Aureococcus anophagefferens*) blooms from two coastal bays on the east coast of the USA. Harmful Algae 3 (4), 361-376.

Mulholland, M.R., Gobler, C.J., Lee, C., 2002. Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. Limnol. Oceanogr. 47 (4), 1094-1108.

Olli, K., 1996. Resting cyst formation of *Eutreptiella gymnastica* (Euglenophyceae) in the northern coatal Baltic Sea. J. Phycol. 32 (4), 535-542.

Olson, R.J., Zettler, E.R., Anderson, O.K., 1989. Discrimination of eukaryotic phytoplankton cell - types from light scatter and autofluorescence properties measured by flow cytometry. Cytometry 10 (5), 636-643.

Onuf, C.P., 1996. Seagrass responses to long-term light reduction by brown tide in upper Laguna Madre, Texas: Distribution and biomass patterns. Mar. Ecol. Prog. Ser. 138 (1-3), 219-231.

Partensky, F., Hess, W., Vaulot, D., 1999. *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiol. Mol. Biol. Rev. 63 (1), 106-127.

Platt, T., Rao, D.S., Irwin, B., 1983. Photosynthesis of picoplankton in the oligotrophic ocean.

Popels, L.C., Hutchins, D.A., 2002. Factors affecting dark survival of the brown tide alga *Aureococcus anophagefferens* (Pelagophyceae). J. Phycol. 38 (4), 738-744.

Prince, E.K., Myers, T.L., Kubanek, J., 2008. Effects of harmful algal blooms on competitors: allelopathic mechanisms of the red tide dinoflagellate Karenia brevis. Limnol. Oceanogr. 53 (2), 531-541.

Probyn, T., Pitcher, G., Pienaar, R., Nuzzi, R., 2001. Brown tides and mariculture in Saldanha Bay, South Africa. Mar. Pollut. Bull. 42 (5), 405-408.

Pustizzi, F., MacIntyre, H., Warner, M.E., Hutchins, D.A., 2004. Interaction of nitrogen source and light intensity on the growth and photosynthesis of the brown tide alga *Aureococcus anophagefferens*. Harmful Algae 3 (4), 343-360.

Rengefors, K., Karlsson, I., Hansson, L.A., 1998. Algal cyst dormancy: a temporal escape from herbivory. P. Roy. Soc. B-Biol. Sci. 265 (1403), 1353-1358.

Rengefors, K., Legrand, C., 2001. Toxicity in *Peridinium aciculiferum*—an adaptive strategy to outcompete other winter phytoplankton? Limnol. Oceanogr. 46 (8), 1990-1997.

Rice, E.L., 2013. Allelopathy. Academic Press.

Richardson, T.L., Jackson, G.A., 2007. Small phytoplankton and carbon export from the surface ocean. Science 315 (5813), 838-840.

Rivkin, R.B., Phinney, D.A., Yentsch, C.M., 1986. Effects of flow cytometric analysis and cell sorting on photosynthetic carbon uptake by phytoplankton in cultures and from natural populations. Appl. Environ. Microbiol. 52 (4), 935-938.

Rocap, G., Distel, D.L., Waterbury, J.B., Chisholm, S.W., 2002. Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. Appl. Environ. Microbiol. 68 (3), 1180-1191.

Sakamoto, C., Karl, D., Jannasch, H., Bidigare, R., Letelier, R., Johnson, K., 2002. Nitrate variability measured in situ on the HALE ALOHA open ocean mooring: Influence of mesoscale eddy activity on phytoplankton community dynamics, AGU Fall Meeting Abstracts, p. 0198.

Sandgren, C.D., 1981. Characteristics of sexual and asexual resting cyst (statospore) formation in *Dinobryon cylindricum* Imhof (Chrysophyta). J. Phycol. 17 (2), 199-210.

Sandgren, C.D., 1983. Survival strategies of chrysophycean flagellates: Reproduction and the formation of resistant resting cysts, In: Fryxell, G.A. (Ed.), Survival Strategies of the Algae. Cambridge University Press, Cambridge, pp. 23-49.

Shi, X.L., Lepère, C., Scanlan, D.J., Vaulot, D., 2011. Plastid 16S rRNA gene diversity among eukaryotic picophytoplankton sorted by flow cytometry from the South Pacific Ocean. PLoS One 6 (4), e18979.

Sicko-Goad, L., Stoermer, E., Kociolek, J., 1989. Diatom resting cell rejuvenation and formation: time course, species records and distribution. J. Plankton Res. 11 (2), 375-389.

Simon, N., Barlow, R.G., Marie, D., Partensky, F., Vaulot, D., 1994. Characterization of oceanic photosynthetic picoeukaryotes by flow cytometry. J. Phycol. 30 (6), 922-935.

Smayda, T.J., 1997. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnol. Oceanogr. 42 (5), 1137-1153.

Smayda, T.J., 2008. Complexity in the eutrophication-harmful algal bloom relationship, with comment on the importance of grazing. Harmful Algae 8 (1), 140-151.

Soneson, C., Delorenzi, M., 2013. A comparison of methods for differential expression analysis of RNA-seq data. BMC Bioinformatics 14 (1), 91.

Stauffer, B.A., Schaffner, R.A., Wazniak, C., Caron, D.A., 2008. Immunofluorescence flow cytometry technique for enumeration of the brown-tide alga, *Aureococcus anophagefferens*. Appl. Environ. Microbiol. 74 (22), 6931-6940.

Stoecker, D.K., Capuzzo, J.M., 1990. Predation on protozoa: its importance to zooplankton. J. Plankton Res. 12 (5), 891-908.

Strom, S.L., 2008. Microbial ecology of ocean biogeochemistry: a community perspective. Science 320 (5879), 1043-1045.

Sun, M.M., Sun, J., Qiu, J.W., Jing, H.M., Liu, H.B., 2012. Characterization of the proteomic profiles of the brown tide alga *Aureoumbra lagunensis* under phosphate- and nitrogen-limiting conditions and of its phosphate limitation-specific protein with alkaline phosphatase activity. Appl. Environ. Microbiol. 78 (6), 2025-2033.

Sunda, W.G., Graneli, E., Gobler, C.J., 2006. Positive feedback and the development and persistence of ecosystem disruptive algal blooms. J. Phycol. 42 (5), 963-974.

Talarmin, A., Van Wambeke, F., Duhamel, S., Catala, P., Moutin, T., Lebaron, P., 2011. Improved methodology to measure taxon-specific phosphate uptake in live and unfiltered samples. Limnol. Oceanogr. Methods 9, 443-453.

Tang, Y.Z., Gobler, C.J., 2010. Allelopathic effects of Cochlodinium polykrikoides isolates and blooms from the estuaries of Long Island, New York, on co-occurring phytoplankton. Mar. Ecol. Prog. Ser. 406, 19-31.

Tang, Y.Z., Gobler, C.J., 2011. The green macroalga, Ulva lactuca, inhibits the growth of seven common harmful algal bloom species via allelopathy. Harmful Algae 10 (5), 480-488.

Tang, Y.Z., Gobler, C.J., 2012. The toxic dinoflagellate *Cochlodinium polykrikoides* (Dinophyceae) produces resting cysts. Harmful Algae 20, 71-80.

Tang, Y.Z., Kang, Y., Berry, D., Gobler, C.J., 2014. The ability of the red macroalga, Porphyra purpurea (Rhodophyceae) to inhibit the proliferation of seven common harmful microalgae. J. Appl. Phycol. 27, 531-544.

Tillmann, U., Alpermann, T.L., da Purificacao, R.C., Krock, B., Cembella, A., 2009. Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. Harmful Algae 8 (5), 759-769.

Tillmann, U., John, U., Cembella, A., 2007. On the allelochemical potency of the marine dinoflagellate *Alexandrium ostenfeldii* against heterotrophic and autotrophic protists. J. Plankton Res. 29 (6), 527-543.

von Dassow, P., Montresor, M., 2010. Unveiling the mysteries of phytoplankton life cycles: patterns and opportunities behind complexity. J. Plankton Res.

Wall, D., Dale, B., 1969. The 'hystrichosphaerid' resting spore of the dinoflagellate *Pyrodinium bahamense*, plate, 1906. J. Phycol. 5 (2), 140-149.

Waterbury, J.B., Watson, S.W., Valois, F.W., Franks, D.G., 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. Can Bull Fish Aquat Sci 214 (71), 120.

Worden, A.Z., Janouskovec, J., McRose, D., Engman, A., Welsh, R.M., Malfatti, S., Tringe, S.G., Keeling, P.J., 2012. Global distribution of a wild alga revealed by targeted metagenomics. Curr. Biol. 22 (17), R675-R677.

Wurch, L.L., Gobler, C.J., Dyhrman, S.T., 2013. Expression of a xanthine and phosphate transporter in cultures and field populations of the harmful alga *Aureococcus anophagefferens*: tracking nutritional deficiency during brown tides. Environ. Microbiol., 2444-2457.

Wurch, L.L., Haley, S.T., Orchard, E.D., Gobler, C.J., Dyhrman, S.T., 2011. Nutrient-regulated transcriptional responses in the brown tide-forming alga *Aureococcus anophagefferens*. Environ. Microbiol. 13 (2), 468-481.

Zhang, Q.C., Qiu, L.M., Yu, R.C., Kong, F.Z., Wang, Y.F., Yan, T., Gobler, C.J., Zhou, M.J., 2012. Emergence of brown tides caused by *Aureococcus anophagefferens* Hargraves et Sieburth in China. Harmful Algae 19, 117-124.

Zheng, J.-W., Li, D.-W., Lu, Y., Chen, J., Liang, J.-J., Zhang, L., Yang, W.-D., Liu, J.-S., Lu, S.-H., Li, H.-Y., 2016. Molecular exploration of algal interaction between the diatom *Phaeodactylum tricornutum* and the dinoflagellate *Alexandrium tamarense*. Algal Research 17, 132-141.

## Chapter 2

# The interactive roles of nutrient loading and zooplankton grazing in facilitating the expansion of harmful algal blooms caused by the pelagophyte, *Aureoumbra lagunensis,* to the Indian River Lagoon, FL, USA

A version of this chapter has been published in:

**Kang, Y.**, F. Koch, C.J. Gobler. 2015. The interactive roles of nutrient loading and zooplankton grazing in facilitating the expansion of harmful algal blooms caused by the pelagophyte, *Aureoumbra lagunensis*, to the Indian River Lagoon, FL, USA. Harmful Algae, 49: 162-173.

**Abstract**

Confined to Texas, USA, for more than 20 years, brown tides caused by *Aureoumbra lagunensis* emerged in the Indian River Lagoon and Mosquito Lagoon, Florida, USA, during 2012 and 2013, affording the opportunity to assess hypotheses regarding the occurrence of these blooms are ecosystem-specific. To examine the extent to which top-down (e.g., grazing) and bottom-up (e.g. nutrients) processes controlled the development of *Aureoumbra* blooms in Florida, nitrogen (N) uptake of bloom and non-bloom water was measured and nutrient amendment and seawater-dilution, zooplankton grazing experiments were performed. During the study, *Aureoumbra* comprised up to 98% of total phytoplankton biomass, achieved cell densities exceeding 2 x $10^6$ cells $mL^{-1}$, and contained isotopically lighter N compared to non-bloom plankton populations, potentially reflecting the use of recycled N. Consistent with this hypothesis, N-isotope experiments revealed that urea and ammonium accounted for > 90% of N uptake within bloom populations whereas nitrate was a primary N source for non-bloom populations. Low levels (10µM) of experimental ammonium enrichment during blooms frequently enhanced the growth of *Aureoumbra* and resulted in the growth rates of *Aureoumbra* exceeding those of phycoerythrin-containing, but not phycocyanin-containing, cyanobacteria. A near absence of grazing pressure on *Aureoumbra* further enabled this species to out-grow other phytoplankton populations. Given this alga is generally known to resist zooplankton grazing under hypersaline conditions, these findings collectively suggest that moderate loading rates of reduced forms of nitrogenous nutrients (e.g ammonium, urea) into other subtropical, hypersaline lagoons could make them susceptible to future brown tides caused by *Aureoumbra*.

## Introduction

Harmful algal blooms (HAB) are pervasive along most coastlines (Hallegraeff, 1993; Anderson et al., 2008). Excessive nutrient loading is commonly cited as a factor contributing to the expansion of HAB (Anderson et al., 2002; Heisler et al., 2008). However, zooplankton grazing plays an important role in constraining phytoplankton abundance in aquatic ecosystems (Burkill et al., 1987; Landry et al., 1997; Calbet and Landry, 2004) and a failure of predator control can facilitate phytoplankton blooms (Irigoien et al., 2005; Modigh and Franzè, 2009). Zooplankton grazing has also been shown to have a primary effect on the outbreak of HAB (Smayda, 2008) and for some HAB such as caused by the pelagophyte, *Aureoumbra lagunensis*, blooms may be promoted via positive feedback between grazing disruption and altered nutrient cycling (Sunda et al., 2006).

For the last 25 years, brown tides caused by *Aureoumbra* have plagued the Laguna Madre, Texas, USA (Buskey et al., 1997; Buskey et al., 2001). These persistent blooms have had a series of adverse ecosystem impacts including reduction of zooplankton populations (e.g. microzooplankton and copepods; Buskey et al., 2001), losses of seagrass (Onuf, 1996), losses of shellfish (Montagna et al., 1993), and slowed growth of polychaete larvae (Ward et al., 2000). During the summer of 2012, a brown tide bloom caused by *Aureoumbra* emerged in the Indian River Lagoon and Mosquito Lagoon, Florida, USA (Gobler et al., 2013a). The bloom returned to this region in the spring of 2013 with densities up to $2 \times 10^6$ cells $mL^{-1}$ (Koch et al., 2014). Previous field and experimental observations of brown tides caused by the alga most closely related to *Aureoumbra*, *Aureococcus anophagefferens*, have demonstrated that high levels of dissolved organic matter can promote dense blooms in coastal ecosystems (Gobler et al., 2004b; Gobler et al., 2011). Although some isolates of *Aureoumbra* are not capable of growing on

nitrate (Deyoe and Suttle, 1994), *Aureoumbra* grows well at high N:P ratios, suggesting that it can adapt to low P environments via a flexible cellular P requirement (Liu et al., 2001) and a high affinity for low levels of ammonium (Sunda and Hardison, 2010). A mesocosm study showed that *Aureoumbra* competes with *Synechococcus* sp. for an open niche and in the presence of the high levels of ammonium (40 µM), *Synechococcus* sp. increased its growth rates while *Aureoumbra* did not (Buskey et al., 2003; DeYoe et al., 2007).

Beyond nutrients, zooplankton grazing may shape phytoplankton community composition during blooms caused by *Aureoumbra*. In the Laguna Madre, Texas, feeding inhibition of grazers reduced the mortality of *Aureoumbra* and was hypothesized to have contributed to the longest continuous HAB on record (7 years; Buskey et al. 1997). The small size of brown tide cells (<5 µm; Buskey and Stockwell, 1993) and excretion of high levels of extracellular polymeric substances (EPS) by *Aureoumbra* (Buskey and Hyatt, 1995; Liu and Buskey, 2000a, 2000b) have been implicated as factors contributing to the inhibition of zooplankton grazing during brown tide blooms. Although grazing on the total phytoplankton community by microzooplankton during Texas brown tides has been measured using chlorophyll *a* (Chl *a*; Buskey and Stockwell, 1993), no studies measuring *in situ* grazing rates on *Aureoumbra* or examining the effects of nutrients and zooplankton grazing on *Aureoumbra* have ever been performed outside of Texas.

The goal of this study was to understand the role of nutrients and zooplankton grazing in the expansion of brown tides caused by *Aureoumbra* to Florida, USA. The spread of *Aureoumbra* to Florida (Gobler et al., 2013a) or Guantanamo Bay, Cuba (Koch et al., 2014) is emblematic of other brown tides (e.g. *Aureococcus anophagefferens* spread to South Africa or China; Probyn et al., 2001; Zhang et al., 2012) and other HAB that continue to expand globally

(Glibert et al., 2005; Kudela and Gobler, 2012). The emergence of *Aureoumbra* blooms in Florida afforded the opportunity to assess whether hypotheses developed regarding the promotion of *Aureoumbra* brown tides are ecosystem-specific. To assess competition between *Aureoumbra* and other small phytoplankton, I sampled locations and times when *Aureoumbra* was and was not the dominant alga. Across all locations, the manner in which nutrients and zooplankton grazing favored the dominance of *Aureoumbra* or competing phytoplankton was assessed.

## Materials and Methods

### *Field samples*

The Indian River Lagoon and Mosquito Lagoon are shallow estuarine systems on the east coast of Florida surrounded by barrier islands that experience weak tidal forcing and water residence times that can exceed one year (Fig. 1; Sheng et al., 1990; Smith, 1993; Phlips et al. 2004, 2010, 2014). During field surveys in September 2012, June 2013, and August 2013, eleven sites were sampled extending 80 km across the northern Indian River Lagoon and southern Mosquito Lagoon (Fig. 1), where a brown tide bloom first emerged in 2012 (Gobler et al., 2013a). Using a small vessel operated by St. Johns River Water Management District, Florida, temperature and salinity were measured at each sampling site using a Hydrolab Minisonde® 5 Multiprobe SE. Lagoon water from bloom and non-bloom sites was collected in triplicate, acid-washed 20 L polyethylene carboys and transported to the Marine Discovery Center (MDC) in New Smyrna Beach, Florida, for experiments with water from some, but not all, sites surveyed.

Triplicate samples were collected at each site and were preserved to a final concentration of 1% formalin and 1% glutaraldehyde to quantify abundance of major phytoplankton populations and *Aureoumbra*, respectively. The abundance of cyanobacteria and photosynthetic picoeukaryotes was determined using a Fluorescence Activated Cell Scan (FACScan; Becton, Dickinson and Company) flow cytometer using fluorescence patterns and particle size derived from side angle light scatter (Collier, 2000). *Aureoumbra* abundances were enumerated using the species-specific antibody on a FACScan flow cytometer (Koch et al., 2014). Abundance of heterotrophic bacteria was quantified after stained with SYBR Green I (Jochem, 2001). Phycocyanin-containing cyanobacteria (PC cyanobacteria) and phycoerythrin-containing cyanobacteria (PE cyanobacteria) were distinguished based on their chlorophyll *a* (Chl *a*) and phycoerythrin content using the program Cyflogic (CyFlo Ltd., Finland). For example, PC cyanobacteria lack phycoerythrin, resulting in a low PE: Chl *a* ratio and creating a distinct population when compared to PE cyanobacteria which contain high levels of PE (high PE : Chl *a* ratio) or eukaryotic algae which lack phycoerythrin and had significantly higher levels of Chl *a* compared to PC cyanobacteria (Berry et al., 2015). Prior studies that have identified PE and PC cyanobacteria in Florida lagoons contiguous with the Indian River Lagoon using these methods concurrently used DNA sequences to affirm their identify as PE and PC cyanobacteria and found these populations belonged to clades III and VIII of accepted *Synechococcus* phylogenies, respectively (Berry et al., 2015). To determine the relative biomass of the major phytoplankton populations quantified flow cytometrically, carbon contents of 900 and 200 fg carbon cell$^{-1}$ were used for *Aureoumbra* (Liu et al., 2001) and cyanobacteria (Kana and Glibert, 1987), respectively. For >2 μm non-brown tide eukaryotes, a carbon to Chl *a* ratio of 60 was applied to the >2 μm

Chl *a* concentrations (analytical method described below; Gobler et al., 2013) and the carbon content of *Aureoumbra* was subtracted.

*Nutrient analysis and $^{15}N$ uptake experiments*

Nutrient samples were collected at each site by filtering 40 mL lagoon water through pre-combusted (2h at 450°C) glass fiber filters (GF/F). Nitrate, nitrite, ammonium, phosphate, silicate, and urea were analyzed in duplicate by standard spectrophotometric methods (Jones, 1984; Parsons et al., 1984; Price and Harrison, 1987). Total dissolved nitrogen (TDN) was analyzed in duplicate by persulfate oxidation (Valderrama, 1981). Dissolved organic nitrogen (DON) concentrations were calculated by subtracting dissolved inorganic nitrogen (DIN; e.g. nitrate, nitrite, and ammonium) from TDN. Samples spiked with SPEX Certi-Prep$^{INC}$ standard reference material at environmentally representative concentrations were quantified and provided full recoveries (mean ± SE) for TDN, nitrate, ammonium, and phosphate.

Uptake rates of nitrogenous compounds by bloom and non-bloom communities in the Indian River Lagoon and Mosquito Lagoon were measured using $^{15}N$-labeled compounds at six survey sites with and without blooms (Table 3). Triplicate, 50 mL, sterile, polycarbonate flasks with screw caps were filled with surface water and $^{15}N$-labeled nitrate, nitrite, ammonium, urea, and glutamic acid were added at concentrations of 0.1, 0.25, 0.2, 0.05 and 0.01 μM, respectively, which were all ≤ 10% of ambient concentrations (Table 2). Flasks were incubated under one layer of neutral density screening which reduced light levels by 33%, mimicking ~0.2 m in the water column during a dense bloom. Incubations were terminated after 30 min by filtering the water from experimental flasks onto pre-combusted GF/F filters. The natural abundance of $^{15}N$ within particulate organic matter was determined by filtering unamended water from

experimental sites onto pre-combusted GF/F filters. Samples collected during the summer of 2012 were analyzed at the U.C. Davis Stable Isotope Facility on a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples collected during the summer of 2013 were analyzed at the U.C. Santa Cruz Stable Isotope Facility on a Finningan Delta Plus XP isotope ratio mass spectrometer (Thermo Fisher Scientific). Uptake rates were determined using equations described by Orcutt et al. (2001) and normalized to particulate organic nitrogen.

*Nutrient amendment experiments*

Nutrient amendment experiments were conducted to assess how specific types of nutrient loading would affect the growth of multiple groups of phytoplankton during brown tide blooms in Florida. Fourteen nutrient amendment experiments were performed with water collected from the Indian River Lagoon and Mosquito Lagoon on 24 and 26 September, 2012, 10 and 12 June, 2013, and 26 and 28 August, 2013 from nine survey sites with multiple experiments using water from multiple sites being established on each date (Table 1, 2). Within 2 h of collection, lagoon water was mixed and transferred to 330 mL Nalgene PETG bottles. Triplicate bottles were enriched with nitrate (10 μM), ammonium (10 μM), phosphate (1 μM), ammonium (10 μM) + phosphate (1 μM), or urea (5 μM = 10 μM N) while another set of triplicate bottles was left unamended as a control treatment. Concentrations of amended nutrients matched spikes in N and P levels historically observed in this system (Gobler et al., 2013a). Experimental bottles were incubated in the Mosquito Lagoon at New Smyrna Beach, Florida, under one layer of neutral density screening. After 24 h, two 4.5 mL samples from each bottle were preserved separately with a final concentration of 1% formalin and 1% glutaraldehyde, respectively, to quantify the abundance of major phytoplankton populations and abundance of *Aureoumbra*, respectively, as described above. Net growth rates (k) of individual phytoplankton populations

were calculated using the formula $k = \ln(B_t/B_0)/t$, where k is the net growth rates in each treatment, $B_t$ is cell density at the end of the experiments, $B_0$ is cell density at the initiation of the experiments, and t is the duration of the experiments in days. Differences in net growth rates between *Aureoumbra* and co-existing cyanobacteria in each treatment were examined to assess how nutrients may alter the relative dominance of *Aureoumbra* within the phytoplankton community.

*Dilution experiments*

Fourteen dilution experiments (Landry et al., 1995) were conducted with water from nine survey sites with and without blooms to quantify the grazing rates by zooplankton on *Aureoumbra* and other major populations including phycocyanin-containing cyanobacteria (PC cyanobacteria), phycoerythrin-containing cyanobacteria (PE cyanobacteria), and heterotrophic bacteria. Filtered seawater was made using Pall pleated filter capsule with 0.2 μm Versapor® Membrane. Unfiltered seawater was diluted with the filtered seawater from each experimental site using 330 mL Nalgene PETG square media bottles whereby the unfiltered seawater represented 100, 70, 40, and 15% of the total volume. Triplicate bottles were established for each dilution level and bottles were amended with 10 μM nitrate and 1 μM phosphate to ensure nutrient-replete conditions (Landry et al., 1995). While *Aureoumbra* grows preferentially on reduced forms of nitrogen (Muhlstein and Villareal, 2007), there was excess ammonium present in water used for all experiments and *Aureoumbra* isolates from this region grow well on mixtures of ammonium and nitrate. Bottles were placed under neutral density screening as described above and incubated in the Mosquito Lagoon. After 24 h of incubation, two 4.5 mL samples were preserved and analyzed as described above to quantify *Aureoumbra* and other major pico-phytoplankton groups. Net growth rates were calculated as described above and

grazing mortality rates (m) were determined from the slope of a linear regression of the calculated net growth rates (y-axis) against the dilution factor (x-axis) following Landry et al (1995). Three-point regressions of dilution curves during this study did not indicate saturation of grazing during experiments (Gallegos, 1989).

*Data analysis*

A site map of the Indian River Lagoon and Mosquito Lagoon was produced using ArcGIS 10.0 (Fig. 1). One-way analyses of variance (Epifanova and Terskikh) with a post-hoc Tukey test were performed to assess statistically significant differences among uptake rates of various N sources and growth rates during nutrient amendment experiments while G-tests were used to compare frequencies of experiments in which grazing rates were measureable on major eukaryotic and prokaryotic populations and in which growth rates of various plankton groups increased in response to nutrient enrichment. For comparative purposes, sites were considered 'bloom' when cell densities were $>5.0 \times 10^5$ cells $mL^{-1}$ or 'non-bloom' when cell densities were $<5.0 \times 10^5$ cells $mL^{-1}$ (Gobler et al., 2013a). T-tests were used to compare environmental parameters between bloom and non-bloom samples. Treatments were deemed significantly different when the probability of outcomes was less than 0.05.

**Results**

*Phytoplankton dynamics*

During September 2012, the *Aureoumbra* bloom was constrained to the southern Mosquito Lagoon where it reached high cell densities ($1.8 \times 10^6$ cells $mL^{-1}$) and comprised >98% of microalgal biomass whereas phycocyanin-containing cyanobacteria dominated cyanobacteria biomass at other sites (Fig. 2; Table 1). During June 2013, *Aureoumbra* bloom densities

exceeded $10^6$ cells $mL^{-1}$ at all locales and *Aureoumbra* comprised the majority of pico-phytoplankton biomass across all sampling sites (34 to 92%; Fig. 2; Table 1). During August 2013, *Aureoumbra* cell densities were lower by an order of magnitude at all stations (~$10^5$ cells $mL^{-1}$; Fig. 2) and PC cyanobacteria had become the dominant picoplankton group comprising from 34 to 85% of algal biomass (Table 1; Fig. 2). While temperatures were fairly consistent during the study (26.8°C at IRLML169 on September 2012 to 30.3°C at Scotts Moor on August 2013), hypersaline conditions (mean = 39.3 g $kg^{-1}$) prevailed during blooms whereas salinity was significantly lower during non-bloom conditions (35.9 g $kg^{-1}$; *t*-test, $p < 0.05$; Table 2).

*Nutrient dynamics, assimilation rates, and $^{15}N$ signature of particulate organic matter*

Mean levels of DIN, DIP, and DOP were relatively low during this study, 2.39 ± 0.351, 0.540 ± 0.150, and 0.939 ± 0.305 $\mu$M, respectively (mean ± SE; Table 2) with the highest DIN levels being 4.02 $\mu$M at IRLML02 during blooms and 5.81 $\mu$M at IRLI10 during non-bloom periods (Table 1; Fig 1). Levels of DON (59.1 ± 10.3 $\mu$M) and silicate (114 ± 14.5 $\mu$M) were higher (Table 2). Levels of DIN, DIP, and DOP did not differ significantly between bloom (2.14 ± 0.510, 0.634 ± 0.276, 1.00 ± 0.216 $\mu$M, respectively) and non-bloom conditions (2.64 ± 0.585, 0.445 ± 0.0937, 0.877 ± 0.272 $\mu$M, respectively) whereas DON and silicate were significantly higher during blooms (73.9 ± 6.61, 150 ± 8.93 $\mu$M, respectively; *t*-test, $p < 0.05$) than those during non-bloom periods (45.4 ± 9.18, 79.2 ± 17.3 $\mu$M, respectively; *t*-test, $p < 0.05$). While the DIN:DIP ratios (4.74 ± 0.703) were lower than the Redfield ratio (16) across all sites, the TDN:TDP (51.0 ± 9.00) and the DON:DOP ratios (124 ± 45.4) were significantly higher, ranging from 18.1 to 141 and from 25.1 to 661, respectively, except for the IRLI10 site (TDN:TDP of 13.5 and DON:DOP of 11.1; Table 2). The DON:DIN ratios (54.3 ± 23.5) were high, particularly during blooms of September 2012 and June 2013 (Table 2). The DIN:silicate

ratios (0.0318 ± 0.00957) were below 0.1 across all sites, except for the IRLI10 site (0.134; Table 2).

Stable N isotope ($\delta^{15}N$) values of particulate organic matter differed between bloom and non-bloom conditions with the $\delta^{15}N$ of bloom communities (mean ± SE; 3.20 ± 0.36‰) being significantly lower than non-bloom communities (5.10 ± 0.71‰; *t*-test, $p < 0.05$; Fig. 3). $^{15}N$-labeled ammonium (mean ± SE; 0.028 ± 0.009 $h^{-1}$; Table 3) and urea (0.056 ± 0.010 $h^{-1}$; Table 3) dominated N-specific uptake rates (89 % of total uptake; Fig. 4a) during dense *Aureoumbra* blooms (Fig. 4a). In experiments where *Aureoumbra* was less abundant, the relative importance of ammonium and urea uptake declined to 18 and 37% of total, respectively (Fig. 4a) while uptake of $^{15}N$-labeled nitrate and glutamic acid increased from 8 to 38% and from 3 to 6%, respectively (Fig. 4a). The uptake of $^{15}N$-labeled nitrite was always <2% of the total (Fig. 4a). The highest uptake percent of $^{15}N$-labeled ammonium (42%) and urea (82%) was detected at sites IRLML02 and IRLI02, respectively, during the *Aureoumbra* bloom in September 2012 (Fig. 4b), while the highest uptake per cent of $^{15}N$-labeled nitrate (51%) and glutamic acid (12%) was detected at the Scotts Moor and NPS sites, respectively during August 2013 (Fig. 4b) when the abundance of *Aureoumbra* was low (Table 3).

*Growth rates and nutrient amendment experiments*

Enrichment of lagoon water with nutrients significantly increased net growth rates of one or more phytoplankton populations in 12 of 14 experiments (Fig. 5). Collectively, net growth rates of *Aureoumbra*, PC cyanobacteria, PE cyanobacteria, and heterotrophic bacteria were significantly enhanced by nutrients in 6, 4, 5, and 7 of 14 of experiments (Table 5; 43, 29, 36, and 50% of experiments, respectively, ANOVA, $p < 0.05$; Fig. 5). While frequency of response

to each individual nutrient did not differ among groups (G-test, $p > 0.05$; Table 5), *Aureoumbra* was enhanced by ammonium more frequently than PC and PE cyanobacteria, PC and PE cyanobacteria were enhanced by nitrate more frequently than *Aureoumbra*, and bacteria were enhanced by phosphate more frequently than *Aureoumbra* (Table 5).

Nutrient amendments resulted in differential changes in the growth rates of *Aureoumbra* and cyanobacteria and thus altered the competitive balance among these autotrophs. During bloom events, enhancement of lagoon water with ammonium, phosphorus, or urea resulted in *Aureoumbra* growth rates outpacing PE cyanobacteria in 71%, 57%, and 57% of experiments, respectively (Fig. 6a). In contrast, *Aureoumbra* growth rates rarely exceeded those of PC cyanobacteria in response to nutrient amendment during bloom or non-bloom experiments (Fig. 6a). During non-bloom periods, nutrient enrichments seldom caused the growth rates of *Aureoumbra* to exceed those of PE cyanobacteria (Fig. 6b). The singular exception was urea additions which led to the growth of *Aureoumbra* exceeding PE cyanobacteria in 57% of experiments and exceeding PC cyanobacteria in 29% of experiments (Fig. 6b).

*Dilution experiments*

Significant grazing mortality rates on at least one major plankton population were observed in all dilution experiments (Table 4). While zooplankton grazing rates on PC and PE cyanobacteria as well as heterotrophic bacteria were quantified in two-third of experiments, only one in 14 experiments yielded detectable grazing on *Aureoumbra* (Fig. 7). Frequencies of grazing rates on microbial populations in the 14 dilution experiments were not significantly different among PC cyanobacteria, PE cyanobacteria and heterotrophic bacteria (57, 64, and 64% of experiments; G-test, $p > 0.05$; Fig. 7a) whereas the frequency of successful grazing on those

three prokaryotic populations were all higher than on *Aureoumbra* (7% of experiments; G-test, $p < 0.001$; Fig. 7a). Grazing rates on prokaryotic populations were high, averaging 0.74 ± 0.13 $d^{-1}$ (mean ± SE) on PC cyanobacteria, 1.13 ± 0.24 $d^{-1}$ on heterotrophic bacteria, and 2.69 ± 0.61 $d^{-1}$ on PE cyanobacteria (Fig. 7b; Table 4). In contrast, in the single case a grazing rate on *Aureoumbra* was measurable was during a non-bloom period and yielded a grazing rate lower than rates for all other populations, 0.24 $d^{-1}$ (Fig. 7b; Table 4). During two experiments performed with bloom water, the growth rates of *Aureoumbra* significantly declined as seawater was progressively diluted (Table 4) suggesting that, unlike the other algae and experiments performed, in these cases, there was more grazing in more dilute seawater. The grazing rates on PC and PE cyanobacteria during blooms and non-bloom periods were not significantly different whereas grazing rates on heterotrophic bacteria population were lower during blooms (0.49 ± 0.10 $d^{-1}$) compared to non-bloom periods (1.31 ± 0.25 $d^{-1}$; Table 4).

## Discussion

In this study, bloom study sites were hypersaline, grazing mortality of *Aureoumbra* by zooplankton was low, ammonium and urea were the primary N sources utilized by plankton during brown tides, and these compounds significantly enhanced the net growth rates of *Aureoumbra* beyond those of competing phytoplankton during blooms. In contrast, competing algae were grazed more rapidly and assimilated and benefited from nitrate enrichment more frequently than *Aureoumbra*. Collectively, therefore, the proliferation of brown tide blooms in the hypersaline Indian River Lagoon and Mosquito Lagoon systems was controlled by nutrient availability, grazing mortality, hypersalinity, and competition with other picoplankton.

### *Nutrients and hypersalinity*

An examination of nutrient concentrations, $\delta^{15}N$ values of particulate organic matter, nutrient enrichment experimental results, and N-uptake rates provides significant insight into the nutritional ecology of *Aureoumbra* blooms in this ecosystem. During the 2012 and 2013 surveys, the Indian River Lagoon and Mosquito Lagoon had consistently low levels of DIN (mean = 2.39 $\mu$M) and DIP (0.540 $\mu$M) and DON levels that were 70% higher ($p < 0.05$) during brown tides. To some extent, this nutrient regime (high DON, low inorganic nutrients) was conducive for the formation of the dense brown tides that occurred during this study. Beyond direct use of compounds within the larger DON pool (Gobler and Sunda, 2012), DON may have been consistently remineralized to more labile compounds such as ammonium and urea (Kirchman et al., 1989) that were subsequently assimilated by *Aureoumbra* during blooms. Still, *Aureoumbra* experienced significantly increased growth rates in response to ammonium and urea loading and was able to outgrow PE cyanobacteria in response to these nutrients during blooms suggesting that moderate loading of these nutrients can intensify brown tides. Smaller phytoplankton are generally more competitive at low nutrient concentrations due to larger surface area-to-volume ratios (Pasciak and Gavis, 1974; Hein et al., 1995; Sunda and Hardison, 2007). The smaller size of *Synechococcus* sp. cells (~ 1 µm; Olson et al., 1990) compared to *Aureoumbra* (2.5 ~ 5 µm; DeYoe et al., 1997) suggests this brown tide alga may be more likely to benefit from moderate inorganic nitrogen loading (10µM) compared to significantly smaller, picocyanobacteria, a hypothesis consistent with the experimental results in this study (Fig 5).

Incubations with $^{15}N$-labeled compounds demonstrated that ammonium and urea comprised the majority of total N assimilated by *Aureoumbra*-dominated plankton populations and thus likely contributed to the proliferation of brown tides in the Indian River Lagoon and Mosquito Lagoon. In contrast to these reduced forms of N, nitrate loading diminished the

relative abundance of *Aureoumbra* within the phytoplankton community. During nutrient amendment experiments nitrate never enhanced the growth of *Aureoumbra* but sometimes led to PC cyanobacteria outgrowing *Aureoumbra.* When the abundance of *Aureoumbra* declined and cyanobacteria became more abundant, uptake of $^{15}$N-nitrate increased nearly five-fold suggesting that the cyanobacteria population may be largely responsible for nitrate uptake within non-bloom populations. This conclusion is consistent with prior studies that reported that an *Aureoumbra* isolate could not grow on nitrate as a sole N source (Deyoe and Suttle, 1994), that this alga grows maximally on ammonium and urea as N sources (Muhlstein and Villareal, 2007), and that the Florida *Aureoumbra* bloom initiated during a period of below average nitrate concentrations (Gobler et al., 2013a).

Traditionally, $\delta^{15}$N values of particulate organic matter have been used to trace sources of N used by primary producers with low values (<3‰) often being associated with fertilizer or atmospheric deposition and higher values (>3‰) being indicative of wastewater N (Lapointe et al., 2004; Kendall and McDonnell, 2012). During this study, $\delta^{15}$N values of particulate organic nitrogen (PON$^{15}$) at non-bloom sites (~ +5‰) were within the range of sewage-enriched N (Lapointe et al., 2004) and macroalgal populations in the Indian River Lagoon (Lapointe et al., 2015) whereas the PON$^{15}$ at brown tide sites was significantly lower ($p$<0.05; ~ +3‰) suggesting that *Aureoumbra* populations were exploiting a different N source. While lighter $\delta^{15}$N values have traditionally been associated with fertilizer- or atmospherically-derived N, the large majority of N in the Indian River Lagoon originates from on-site sewage disposal systems within the watershed (Lapointe and Krupa, 1995; Lapointe et al., 2015), suggesting other processes may influence differences in the $\delta^{15}$N values of N that was ultimately derived from sewage. For example, $\delta^{15}$N-nitrate values from both ocean water and wastewater have been

shown to be heavier than $\delta^{15}$N-ammonium or recycled $\delta^{15}$N (Clark et al., 2008; Hinkle et al., 2008; Fawcett et al., 2011). Therefore, while the possible use of N ultimately derived from fertilizer and/or the atmosphere cannot be excluded, the lighter $\delta^{15}$N values associated with brown tides may reflect its heavier reliance on ammonium and DON as N sources instead of nitrate.

Consistent with the previous study showing that both nitrate and lower salinity discourage brown tides (Gobler et al., 2013a), it would seem that elevated discharge of freshwater (river, run-off, groundwater) would be likely to suppress the formation of *Aureoumbra* blooms. Conversely, below average rainfall likely contributed toward not only low nitrate levels but also hypersalinity during these brown tides (Gobler et al., 2013a). The mean salinity observed during blooms in 2012 and 2013 (39.3 g $kg^{-1}$) was 50% higher than historical mean values from 1996 to 2010 (Gobler et al., 2013a). This observation is consistent with brown tides within the Laguna Madre, TX, where salinity reached ~ 60 PSU, which enhanced the development and persistence of brown tides there (Buskey et al., 1998). For both regions, high evaporation rates that exceeded precipitation caused the hypersaline condition (Buskey et al., 1998; Gobler et al., 2013a). *Aureoumbra* can grow in a wide range of salinity from 10 to 90 PSU and the maximum growth can be reached in a range of salinity from 20 to 60 PSU (Buskey et al., 1998), indicating the salinity during the survey in this study was the most optimal condition for the growth of *Aureoumbra*, but not competitors and predators (Buskey et al., 1997). In addition, the regions of the northern Indian River Lagoon and Mosquito Lagoon where the brown tide was most intense experience low freshwater input compared to the central Indian River Lagoon (Phlips et al., 2010). Consistent with this observation, during the outbreak of brown tides, high densities of *Aureoumbra* occurred throughout the northern Indian River Lagoon and Mosquito

Lagoon but this alga was present only at low levels in the central and southern Indian River Lagoon (Koch et al., 2014). The ability of *Aureoumbra* to achieve the maximum growth rates under low nitrate and hypersaline conditions (Buskey et al., 1998; Liu et al., 2001) that restrict the growth of other small phytoplankton (Sunda and Hardison, 2010) likely contributes to the proliferation of these events.

*Zooplankton grazing mortality of bloom populations*

Microzooplankton grazing can account for the consumption of more than half of phytoplankton production in coastal and estuarine systems (Calbet and Landry, 2004) and preferential grazing by zooplankton can play a key role in altering the composition of phytoplankton communities (Strom, 2008). During this study, zooplankton grazed on PE cyanobacteria and heterotrophic bacteria in more than two-thirds of experiments and grazing rates on these cells were significantly higher than the rates on *Aureoumbra* and PC cyanobacteria (Fig. 7). In contrast, non-significant regressions between the dilution of seawater and the growth rate of *Aureoumbra* suggested that zooplankton avoided grazing on *Aureoumbra* during all but one of fourteen experiments (Goleski et al., 2010) in which the grazing rate was a fraction of rates measured on other groups. A recent study of PC and PE cyanobacteria in Florida Bay, a system physically contiguous with the Indian River Lagoon, demonstrated that zooplankton preferentially graze on PE cyanobacteria and avoid PC cyanobacteria, a characteristic that facilitates blooms of PC cyanobacteria blooms caused by Clade VIII *Synechococcus* (Goleski et al., 2010). When *Aureoumbra* bloomed, however, zooplankton populations grazed on PC cyanobacteria more frequently and at a more rapid rate than on *Aureoumbra*, suggesting this brown tide alga is more noxious to grazers than unpalatable cyanobacteria.

The link between the growth of *Aureoumbra* and reduced grazing pressure by zooplankton has been explored during brown tides in Texas (Buskey and Stockwell, 1993). During those HAB, macrozooplankton and microzooplankton population densities declined and the body size of macrozooplankton were reduced (Buskey and Stockwell, 1993). Furthermore, the gut pigment contents of mesozooplankton were significantly reduced and microzooplankton grazing on the total phytoplankton community declined from 95% to 5% of the community per day (Buskey and Stockwell, 1993). During experiments conducted here, there was no detectable grazing on *Aureoumbra* during 13 out of 14 experiments and the singular experiment in which *Aureoumbra* was grazed, the rate amounted to only 20% of the population per day. *Aureoumbra* produces extracellular polysaccharides (EPS) that can impede the grazing activity of zooplankton (Liu and Buskey, 2000a, 2000b). In addition, *Aureoumbra* can remain physiologically intact after passing the digestive system of zooplankton due to cell protection by EPS (Bersano et al., 2002). The EPS production can be exacerbated by the hypersalinity (Liu and Buskey, 2000b) that was present during this study. Transmission electron microscopy (TEM) images of *Aureoumbra* (Gobler et al., 2013a) isolated from Florida bloom waters in 2012 revealed a thick layer of EPS around cells that would likely contribute to the failure of zooplankton grazing on the Florida brown tide alga. In fact, during two experiments performed during dense blooms, there was a positive relationship between the dilution of seawater and growth rates of *Aureoumbra* (Table 4) providing evidence of a density-dependent inhibition of grazing (Buskey, 2008). In such cases, the benefit of high cell densities and the high EPS-production rates by dense populations were likely important for inhibiting zooplankton grazing and actually outweighed the importance of decreased encounter rates between *Aureoumbra* and zooplankton

created by seawater dilution (Landry et al., 1995). Collectively, this information affirms that lowered grazing mortality promotes and sustains brown tides.

Both nutrients and grazing likely altered the competitive balance between *Aureoumbra* and cyanobacteria during blooms. Given the ability of *Aureoumbra* to outgrow PE cyanobacteria during blooms in response to ammonium and urea additions (Fig. 6a), the loading of these nutrients may support the proliferation of brown tides. Higher grazing pressure on PE cyanobacteria but not *Aureoumbra* and the more rapid growth of *Aureoumbra* populations under specific nutrient conditions both likely contributed to *Aureoumbra* dominating over PE cyanobacteria during blooms. However, the relatively faster growth response of PC cyanobacteria with and without nutrient additions and lower grazing mortality rates of PC cyanobacteria compared to PE cyanobacteria kept abundances of this population elevated during bloom and non-bloom periods. As such, these populations may be considered co-dominant in this system. As noted above, PC cyanobacteria also form HAB in estuaries contiguous with the sites examined in this study (Berry et al., 2015).

*Ecological implications of low zooplankton grazing rates during brown tides*

Complex interactions of top-down and bottom-up processes impact the dynamics of ecosystem disruptive algal blooms including brown tides (Gobler et al., 2002b; Sunda et al., 2006; Sunda and Shertzer, 2012). Prolonged brown tides have numerous negative consequences for ecosystems including the reduction in net energy transfer to higher trophic levels (Sunda et al., 2006; Sunda and Shertzer, 2012). Being nutritionally insufficient to support the growth of zooplankton, the brown tide alga is hypothesized to not support higher trophic levels (Buskey and Hyatt, 1995) and prolonged picoplankton blooms are known to decrease the efficiency of

energy fluxes through food webs (Pomeroy et al., 2007; Fenchel, 2008). During consecutive bloom years in FL, a picoplankton-based food web, reduced zooplankton grazing rates, and a >50% loss of seagrass beds, a critical fish habitat, due to severe shading (Koch et al., 2014) all likely depressed food web productivity within the Indian River and Mosquito Lagoons (Sunda et al., 2006; Sunda and Shertzer, 2012). Consistent with this hypothesis, NOAA scientists investigating a Marine Mammal Unusual Mortality Event (MMUME) in 2013 concluded that dolphins in the Indian River Lagoon and Mosquito Lagoon had perished due to starvation (NOAA, 2013).

*Expansion of brown tides in subtropical lagoons*

Human-driven introduction of non-indigenous species to a new habitat, (e.g. ballast water) has been also hypothesized to be one of principle vectors for HAB outbreaks in exotic ecosystems (Hallegraeff and Bolch, 1991; Smayda, 2007). However, the historical detection of low levels of *Aureoumbra* in the Gulf of Mexico and Florida Bay (Villareal et al., 2002) and the optimal environment for *Aureoumbra* proliferation (e.g. high salinity, low nitrate, and high DON) make Baas-Becking's (1934) hypothesis 'Everything is everywhere, but the environment selects' seem to be the most parsimonious explanation for the emergence of brown tides caused by *Aureoumbra* this region (Koch et al., 2014). As described previously, hypersalinity discourages zooplankton grazing and the growth of algal competitors, is associated with low inorganic nutrient levels, and is generally indicative of slow physical water exchange (Buskey et al., 1997; Gobler et al., 2013a), all conditions likely central to the formation of brown tides in Florida. The large expansion of domiciles with on-site septic systems in the watershed of the northern Indian River Lagoon and Mosquito Lagoon (Lapointe et al., 2015) likely provide the enhanced level of nitrogen loading required to support high biomass HAB such as brown tides

(Heisler et al., 2008) in a manner not plausible before this expansion. This anthropogenic N loading has also increased N:P ratios in this system (Lapointe et al., 2015), another condition known to promote blooms of *Aureoumbra* (Liu et al., 2001). It is notable that the only other known *Aureoumbra* blooms on the planet in Texas and Cuba also occurred under hypersaline conditions suggesting this aspect of *Aureoumbra*'s niche is not ecosystem-specific. The seasonality of the brown tides in Florida (Kaplan-Levy et al.) but not in Texas (year-round) suggests that the physical conditions within Florida are more dynamic with regard to salinity, freshwater flow, and/or tidal exchange. The existence of background levels of *Aureoumbra* along the Gulf of Mexico including the southern and eastern Florida and the recent expansion of this species to the Caribbean region (Koch et al., 2014) indicate the potential for future expansion of brown tides to other hypersaline, moderately eutrophied subtropical lagoons in this region.

## References


Anderson, D., Burkholder, J., Cochlan, W., Glibert, P., Gobler, C., Heil, C., Kudela, R., Parsons, M., Rensel, J., Townsend, D., 2008. Harmful algal blooms and eutrophication: Examining linkages from selected coastal regions of the United States. Harmful Algae 8 (1), 39-53.

Anderson, D., Glibert, P., Burkholder, J., 2002. Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences. Estuaries 25 (4), 704-726.

Baas-Becking, L.G.M., 1934. Geobiologie; of inleiding tot de milieukunde. WP Van Stockum & Zoon NV.

Berry, D.L., Goleski, J.A., Koch, F., Wall, C.C., Peterson, B.J., Anderson, O.R., Gobler, C.J., 2015. Shifts in cyanobacterial strain dominance during the onset of harmful algal blooms in Florida Bay, USA. Microb. Ecol., 1-11.

Bersano, J., Buskey, E., Villareal, T., 2002. Viability of the Texas brown tide alga, *Aureoumbra lagunensis*, in fecal pellets of the copepod *Acartia tonsa*. Plankton Biol. Ecol. 49 (2), 88-92.

Boissonneault-Cellineri, K.R., Mehta, M., Lonsdale, D.J., Caron, D.A., 2001. Microbial food web interactions in two Long Island embayments. Aquat. Microb. Ecol. 26 (2), 139-155.

Burkill, P., Mantoura, R., Llewellyn, C., Owens, N., 1987. Microzooplankton grazing and selectivity of phytoplankton in coastal waters. Mar. Biol. 93 (4), 581-590.

Buskey, E.J., 2008. How does eutrophication affect the role of grazers in harmful algal bloom dynamics? Harmful Algae 8 (1), 152-157.

Buskey, E.J., Deyoe, H., Jochem, F.J., Villareal, T.A., 2003. Effects of mesozooplankton removal and ammonium addition on planktonic trophic structure during a bloom of the Texas 'brown tide': a mesocosm study. J. Plankton Res. 25 (2), 215-228.

Buskey, E.J., Hyatt, C.J., 1995. Effects of the Texas (USA) brown tide alga on planktonic grazers. Mar. Ecol. Prog. Ser. 126 (1-3), 285-292.

Buskey, E.J., Liu, H.B., Collumb, C., Bersano, J.G.F., 2001. The decline and recovery of a persistent Texas brown tide algal bloom in the Laguna Madre (Texas, USA). Estuaries 24 (3), 337-346.

Buskey, E.J., Montagna, P.A., Amos, A.F., Whitledge, T.E., 1997. Disruption of grazer populations as a contributing factor to the initiation of the Texas brown tide algal bloom. Limnol. Oceanogr. 42 (5), 1215-1222.

Buskey, E.J., Stockwell, D.A., 1993. Effects of a persistent “brown tide” on zooplankton populations in the Laguna Madre of South Texas, Toxic phytoplankton blooms in the sea. Proc. 5th Int. Conf. on Toxic Marine Phytoplankton. Elsevier, pp. 659-665.

Buskey, E.J., Wysor, B., Hyatt, C., 1998. The role of hypersalinity in the persistence of the Texas 'brown tide' in the Laguna Madre. J. Plankton Res. 20 (8), 1553-1565.

Calbet, A., Landry, M.R., 2004. Phytoplankton growth, microzooplankton grazing, and carbon cycling in marine systems. Limnol. Oceanogr. 49 (1), 51-57.

Clark, I., Timlin, R., Bourbonnais, A., Jones, K., Lafleur, D., Wickens, K., 2008. Origin and Fate of Industrial Ammonium in Anoxic Ground Water—$^{15}$N Evidence for Anaerobic Oxidation (Anammox). Ground Water Monit. Remediat. 28 (3), 73-82.

DeYoe, H.R., Buskey, E.J., Jochem, F.J., 2007. Physiological responses of *Aureoumbra lagunensis* and *Synechococcus* sp to nitrogen addition in a mesocosm experiment. Harmful Algae 6 (1), 48-55.

DeYoe, H.R., Stockwell, D.A., Bidigare, R.R., Latasa, M., Johnson, P.W., Hargraves, P.E., Suttle, C.A., 1997. Description and characterization of the algal species *Aureoumbra lagunensis* gen. et sp. nov. and referral of *Aureoumbra* and *Aureococcus* to the Pelagophyceae. J. Phycol. 33 (6), 1042-1048.

Deyoe, H.R., Suttle, C.A., 1994. The inability of the Texas brown tide alga to use nitrate and the role of nitrogen in the initiation of a persistent bloom of this organism. J. Phycol. 30 (5), 800-806.

Fawcett, S.E., Lomas, M., Casey, J.R., Ward, B.B., Sigman, D.M., 2011. Assimilation of upwelled nitrate by small eukaryotes in the Sargasso Sea. Nature Geoscience 4 (10), 717-722.

Fenchel, T., 2008. The microbial loop–25 years later. J. Exp. Mar. Biol. Ecol. 366 (1), 99-103.

Gallegos, C.L., 1989. Microzooplankton grazing on phytoplankton in the Rhode River, Maryland - Nonlinear feeding kinetics. Mar. Ecol. Prog. Ser. 57 (1), 23-33.

Glibert, P.M., Anderson, D.M., Gentien, P., Graneli, E., Sellner, K., 2005. The global, complex phenomena of harmful algal blooms. Oceanography 18 (2), 132-141.

Gobler, C.J., Berry, D.L., Dyhrman, S.T., Wilhelm, S.W., Salamov, A., Lobanov, A.V., Zhang, Y., Collier, J.L., Wurch, L.L., Kustka, A.B., Dill, B.D., Shah, M., VerBerkmoes, N.C., Kuo, A., Terry, A., Pangilinan, J., Lindquist, E.A., Lucas, S., Paulsen, I.T., Hattenrath-Lehmann, T.K., Talmage, S.C., Walker, E.A., Koch, F., Burson, A.M., Marcoval, M.A., Tang, Y.Z., LeCleir, G.R., Coyne, K.J., Berg, G.M., Bertrand, E.M., Saito, M.A., Gladyshev, V.N., Grigoriev, I.V., 2011. Niche of harmful alga *Aureococcus anophagefferens* revealed through ecogenomics. Proc. Natl. Acad. Sci. 108 (11), 4352-4357.

Gobler, C.J., Deonarine, S., Leigh-Bell, J., Gastrich, M.D., Anderson, O.R., Wilhelm, S.W., 2004. Ecology of phytoplankton communities dominated by *Aureococcus anophagefferens*: the role of viruses, nutrients, and microzooplankton grazing. Harmful Algae 3 (4), 471-483.

Gobler, C.J., Koch, F., Kang, Y., Berry, D.L., Tang, Y.Z., Lasi, M., Walters, L., Hall, L., Miller, J.D., 2013. Expansion of harmful brown tides caused by the pelagophyte, *Aureoumbra lagunensis* DeYoe et Stockwell, to the US east coast. Harmful Algae 27 (0), 29-41.

Gobler, C.J., Renaghan, M.J., Buck, N.J., 2002. Impacts of nutrients and grazing mortality on the abundance of *Aureococcus anophagefferens* during a New York brown tide bloom. Limnol. Oceanogr. 47 (1), 129-141.

Gobler, C.J., Sunda, W.G., 2012. Ecosystem disruptive algal blooms of the brown tide species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*. Harmful Algae 14, 36-45.

Goleski, J.A., Koch, F., Marcoval, M.A., Wall, C.C., Jochem, F.J., Peterson, B.J., Gobler, C.J., 2010. The role of zooplankton grazing and nutrient loading in the occurrence of harmful cyanobacterial blooms in Florida Bay, USA. Estuaries and Coasts 33 (5), 1202-1215.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32 (2), 79-99.

Hallegraeff, G.M., Bolch, C.J., 1991. Transport of toxic dinoflagellate cysts via ships' ballast water. Mar. Pollut. Bull. 22 (1), 27-30.

Hein, M., Pedersen, M.F., Sand-Jensen, K., 1995. Size-dependent nitrogen uptake in micro-and macroalgae. Mar. Ecol. Prog. Ser. 118 (1), 247-253.

Heisler, J., Glibert, P., Burkholder, J., Anderson, D., Cochlan, W., Dennison, W., Dortch, Q., Gobler, C., Heil, C., Humphries, E., 2008. Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8 (1), 3-13.

Hinkle, S.R., Böhlke, J.K., Fisher, L.H., 2008. Mass balance and isotope effects during nitrogen transport through septic tank systems with packed-bed (sand) filters. Sci. Total Environ. 407 (1), 324-332.

Irigoien, X., Flynn, K.J., Harris, R.P., 2005. Phytoplankton blooms: a 'loophole' in microzooplankton grazing impact? J. Plankton Res. 27 (4), 313-321.

Jochem, F.J., 2001. Morphology and DNA content of bacterioplankton in the northern Gulf of Mexico: analysis by epifluorescence microscopy and flow cytometry. Aquat. Microb. Ecol. 25 (2), 179-194.

Jones, M.N., 1984. Nitrate reduction by shaking with cadmium - alternative to cadmium columns. Water Res. 18 (5), 643-646.

Kana, T.M., Glibert, P.M., 1987. Effects of irradiances up to 2000 μE $m^{-2}$ $s^{-1}$ on marine *Synechococcus* WH7803.1. Growth, pigmentation and cell composition. Deep-Sea Res. 34 (4), 479-495.

Kendall, C., McDonnell, J.J., 2012. Isotope tracers in catchment hydrology. Elsevier.

Kirchman, D.L., Keil, R.G., Wheeler, P.A., 1989. The effect of amino acids on ammonium utilization and regeneration by heterotrophic bacteria in the subarctic Pacific. Deep-Sea Res. 36 (11), 1763-1776.

Koch, F., Kang, Y., Villareal, T., Anderson, D., Gobler, C., 2014. Novel immunofluorescence flow cytometry technique detects the expansion of harmful brown tides caused by *Aureoumbra lagunensis* to the Caribbean Sea. Appl. Environ. Microbiol., AEM. 00888-00814.

Kudela, R.M., Gobler, C.J., 2012. Harmful dinoflagellate blooms caused by *Cochlodinium* sp.: Global expansion and ecological strategies facilitating bloom formation. Harmful Algae 14 (0), 71-86.

Landry, M.R., Barber, R.T., Bidigare, R.R., Chai, F., Coale, K.H., Dam, H.G., Lewis, M.R., Lindley, S.T., McCarthy, J.J., Roman, M.R., Stoecker, D.K., Verity, P.G., White, J.R., 1997. Iron and grazing constraints on primary production in the central equatorial Pacific: An EqPac synthesis. Limnol. Oceanogr. 42 (3), 405-418.

Landry, M.R., Kirshtein, J., Constantinou, J., 1995. A refined dilution technique for measuring the community grazing impact of microzooplankton, with experimental tests in the central equatorial Pacific. Mar. Ecol. Prog. Ser. 120 (1-3), 53-63.

Lapointe, B., Krupa, S., 1995. Jupiter Creek septic tank water quality investigation. Final Report to the Loxahatchee River Environmental Control District. Jupiter, FL.

Lapointe, B.E., Barile, P.J., Matzie, W.R., 2004. Anthropogenic nutrient enrichment of seagrass and coral reef communities in the Lower Florida Keys: discrimination of local versus regional nitrogen sources. J. Exp. Mar. Biol. Ecol. 308 (1), 23-58.

Lapointe, B.E., Herren, L.W., Debortoli, D.D., Vogel, M.A., 2015. Evidence of sewage-driven eutrophication and harmful algal blooms in Florida's Indian River Lagoon. Harmful Algae 43 (0), 82-102.

Liu, H.B., Buskey, E.J., 2000a. The exopolymer secretions (EPS) layer surrounding *Aureoumbra lagunensis* cells affects growth, grazing, and behavior of protozoa. Limnol. Oceanogr. 45 (5), 1187-1191.

Liu, H.B., Buskey, E.J., 2000b. Hypersalinity enhances the production of extracellular polymeric substance (EPS) in the Texas brown tide alga, *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 36 (1), 71-77.

Liu, H.B., Laws, E.A., Villareal, T.A., Buskey, E.J., 2001. Nutrient-limited growth of *Aureoumbra lagunensis* (Pelagophyceae), with implications for its capability to outgrow other phytoplankton species in phosphate-limited environments. J. Phycol. 37 (4), 500-508.

Modigh, M., Franzè, G., 2009. Changes in phytoplankton and microzooplankton populations during grazing experiments at a Mediterranean coastal site. J. Plankton Res. 31 (8), 853-864.

Montagna, P.A., Stockwell, D.A., Kalke, R.D., 1993. Dwarf surfclam *Mulinia lateralis* (Say, 1822) populations and feeding during the Texas brown tide event J. Shellfish Res. 12 (2), 433-442.

Muhlstein, H.I., Villareal, T.A., 2007. Organic and inorganic nutrient effects on growth rate-irradiance relationships in the Texas brown-tide alga *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 43 (6), 1223-1226.

NOAA, 2013. Marine mammal unusual mortality event (MMUME).

Olson, R.J., Chisholm, S.W., Zettler, E.R., Armbrust, E.V., 1990. Pigments, size, and distributions of *Synechococcus* in the North Atlantic and Pacific Oceans. Limnol. Oceanogr. 35 (1), 45-58.

Olson, R.J., Chisholm, S.W., Zettler, E.R., Dusenberry, J.A., 1991. Advances in oceanography through flow cytometry., In: Demers, S. (Ed.), Individual Cell and Particle Analysis in Oceanography. Springer Verlag, pp. 352-402.

Onuf, C.P., 1996. Seagrass responses to long-term light reduction by brown tide in upper Laguna Madre, Texas: Distribution and biomass patterns. Mar. Ecol. Prog. Ser. 138 (1-3), 219-231.

Orcutt, K.M., Lipschultz, F., Gundersen, K., Arimoto, R., Michaels, A.F., Knap, A.H., Gallon, J.R., 2001. A seasonal study of the significance of $N_2$ fixation by *Trichodesmium* spp. at the Bermuda Atlantic Time-series Study (BATS) site. Deep-Sea Research Part Ii-Topical Studies in Oceanography 48 (8-9), 1583-1608.

Parsons, T.R., Maita, Y., Lalli, C.M., 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Oxford.

Pasciak, W.J., Gavis, J., 1974. Transport limitation of nutrient uptake in phytoplankton. Limnol. Oceanogr. 19 (6), 881-888.

Phlips, E., Badylak, S., Christman, M., Lasi, M., 2010. Climatic trends and temporal patterns of phytoplankton composition, abundance, and succession in the Indian River Lagoon, Florida, USA. Estuaries and Coasts 33 (2), 498-512.

Phlips, E.J., Badylak, S., Lasi, M.A., Chamberlain, R., Green, W.C., Hall, L.M., Hart, J.A., Lockwood, J.C., Miller, J.D., Morris, L.J., 2014. From red tides to green and brown tides: bloom dynamics in a restricted subtropical lagoon under shifting climatic conditions. Estuaries and Coasts, 1-19.

Phlips, E.J., Badylak, S., Youn, S., Kelley, K., 2004. The occurrence of potentially toxic dinoflagellates and diatoms in a subtropical lagoon, the Indian River Lagoon, Florida, USA. Harmful Algae 3 (1), 39-49.

Pomeroy, L.R., Williams, P.J.I., Azam, F., Hobbie, J.E., 2007. The Microbial Loop. Oceanography 20 (2), 28-33.

Price, N.M., Harrison, P.J., 1987. Comparison of methods for the analysis of dissolved urea in seawater. Mar. Biol. 94 (2), 307-317.

Probyn, T., Pitcher, G., Pienaar, R., Nuzzi, R., 2001. Brown tides and mariculture in Saldanha Bay, South Africa. Mar. Pollut. Bull. 42 (5), 405-408.

Smayda, T., 2007. Reflections on the ballast water dispersal—harmful algal bloom paradigm. Harmful Algae 6 (4), 601-622.

Smayda, T.J., 2008. Complexity in the eutrophication-harmful algal bloom relationship, with comment on the importance of grazing. Harmful Algae 8 (1), 140-151.

Smith, N.P., 1993. Tidal and nontidal flushing of Florida Indian River Lagoon. Estuaries 16 (4), 739-746.

Strom, S.L., 2008. Microbial ecology of ocean biogeochemistry: a community perspective. Science 320 (5879), 1043-1045.

Sunda, W., Hardison, D., 2010. Evolutionary tradeoffs among nutrient acquisition, cell size, and grazing defense in marine phytoplankton promote ecosystem stability. Mar. Ecol. Prog. Ser. 401, 63-76.

Sunda, W.G., Graneli, E., Gobler, C.J., 2006. Positive feedback and the development and persistence of ecosystem disruptive algal blooms. J. Phycol. 42 (5), 963-974.

Sunda, W.G., Hardison, D.R., 2007. Ammonium uptake and growth limitation in marine phytoplankton. Limnol. Oceanogr. 52 (6), 2496-2506.

Sunda, W.G., Shertzer, K.W., 2012. Modeling ecosystem disruptive algal blooms: positive feedback mechanisms. Mar. Ecol. Prog. Ser. 447, 31-U69.

Valderrama, J.C., 1981. The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar. Chem. 10 (2), 109-122.

Villareal, T.A., Chirichella, T., Buskey, E.J., 2002. Regional distribution of the Texas Brown Tide (*Aureoumbra lagunensis*) in the Gulf of Mexico. Harmful Algae 2004, 374-376.

Ward, L.A., Montagna, P.A., Kalke, R.D., Buskey, E.J., 2000. Sublethal effects of Texas brown tide on *Streblospio benedicti* (Polychaeta) larvae. J. Exp. Mar. Biol. Ecol. 248 (1), 121-129.

Zhang, Q.C., Qiu, L.M., Yu, R.C., Kong, F.Z., Wang, Y.F., Yan, T., Gobler, C.J., Zhou, M.J., 2012. Emergence of brown tides caused by *Aureococcus anophagefferens* Hargraves et Sieburth in China. Harmful Algae 19, 117-124.

**Table 1** Size fractionated Chl *a* concentrations, cell density and relative abundance of the major phytoplankton populations at experimental sites. Abbreviations for table are as follows: *Aureoumbra* = *Aureoumbra lagunensis*, Non-BT euks = non-brown tide eukaryotes, PC cyano = phycocyanin-containing cyanobacteria, and PE cyano = phycoerythrin-containing cyanobacteria.

| | Date | Station | Chl *a* (μg $L^{-1}$) | | | % in > 2 μm | Cell density (cells $ml^{-1}$) | | | | Relative abundance (% of total) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.2 μm | 0.2-2 μm | > 2 μm | | *Aureoumbra* | Non-BT euks | PC cyano | PE cyano | *Aureoumbra* | Non-BT euks | PC cyano | PE cyano |
| Bloom | 9/24/2012 | IRLML02 | 40.5 ± 0.3 | 8.4 ± 1.6 | 32.1 ± 1.6 | 79.3 | 1,250,000 | 0 | 264,000 | 248 | 57 | 41 | 3 | 0 |
| | 9/24/2012 | SBL | 41.1 ± 6.8 | 3.8 ± 6.9 | 37.3 ± 0.2 | 90.7 | 1,820,000 | 0 | 126,000 | 2,930 | 85 | 14 | 1 | 0 |
| | 9/26/2012 | SBL | 31.9 ± 6.3 | 4.6 ± 6.4 | 27.3 ± 0.6 | 85.5 | 1,270,000 | 0 | 597,000 | 2,750 | 65 | 28 | 7 | 0 |
| | 6/10/2013 | IRLI02 | 43.7 ± 9.4 | 10.2 ± 9.4 | 33.5 ± 0.6 | 76.6 | 1,990,000 | 299,000 | 240,000 | 502 | 79 | 10 | 11 | 0 |
| | 6/10/2013 | Scotts Moor | 35.4 ± 2.3 | 3.8 ± 4.2 | 31.6 ± 3.6 | 89.1 | 2,080,000 | 0 | 920,000 | 192 | 90 | 1 | 9 | 0 |
| | 6/12/2013 | SBL | 40.3 ± 3.3 | 20.4 ± 4.8 | 19.9 ± 3.5 | 49.4 | 608,000 | 0 | 2,020,000 | 1,260 | 34 | 40 | 25 | 0 |
| | 6/12/2013 | MDC | 41.3 ± 3.2 | 15.2 ± 3.4 | 26.2 ± 1.2 | 63.3 | 1,880,000 | 0 | 762,000 | 1,550 | 92 | 0 | 8 | 0 |
| Non-bloom | 9/26/2012 | IRLML169 | 9.5 ± 2.3 | 4.7 ± 2.4 | 4.7 ± 0.3 | 49.5 | 43,800 | 314,000 | 2,910,000 | 456,000 | 4 | 26 | 61 | 10 |
| | 6/10/2013 | Moore Creek | 8.6 ± 1.1 | 0.9 ± 1.1 | 7.7 ± 0.1 | 89.2 | 179,000 | 19,800 | 663,000 | 724,000 | 22 | 41 | 18 | 20 |
| | 8/26/2013 | IRLI10 | 8.2 ± 1.3 | 1.1 ± 1.5 | 7.0 ± 0.8 | 86.1 | 7,950 | 24,500 | 172,000 | 197,000 | 1 | 84 | 7 | 8 |
| | 8/26/2013 | Scotts Moor | 25.4 ± 1.2 | 10.7 ± 1.3 | 14.7 ± 0.4 | 57.8 | 143,000 | 325,000 | 3,170,000 | 1,600 | 8 | 50 | 42 | 0 |
| | 8/28/2013 | NPS | 8.8 ± 0.6 | 3.9 ± 0.7 | 4.9 ± 0.3 | 55.4 | 17,400 | 556,000 | 838,000 | 552,000 | 3 | 49 | 29 | 19 |
| | 8/28/2013 | SBL | 11.5 ± 1.3 | 6.0 ± 1.3 | 5.6 ± 0.3 | 48.3 | 30,900 | 265,000 | 2,880,000 | 1220 | 3 | 34 | 63 | 0 |
| | 8/28/2013 | Scotts Moor | 43.8 ± 0.4 | 8.7 ± 1.0 | 35.1 ± 1.0 | 80.1 | 47,000 | 278,000 | 1,570,000 | 454 | 2 | 85 | 13 | 0 |

**Table 2** Levels of inorganic and organic nutrients as well as physical data for the experimental sites in the Indian River Lagoon and Mosquito Lagoon during field surveys conducted in 2012 and 2013. Values depicted are mean ± standard error. BDL = 'below detectable levels' and N/A = 'data not available'. Abbreviation of Si indicates silicates.

| | Date | Station | Nutrients ($\mu$M) | | | | | | | | | | Physical data | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | DIN | DIP | DON | DOP | Si | TDN:TDP | DIN:DIP | DON:DOP | DON:DIN | DIN:Si | T (°C) | S |
| Bloom | 9/24/2012 | IRLML02 | 4.02 ± 0.170 | 0.547 ± 0.131 | 93.2 ± 2.52 | 0.141 ± 0.168 | 128 ± 20.5 | 141 | 7.36 | 661 | 23.2 | 0.0315 | 27.9 | 38.0 |
| | 9/24/2012 | SBL | 1.56 ± 0.150 | 0.211 ± 0.0491 | 75.4 ± 7.94 | 0.772 ± 0.128 | 180 ± 12.5 | 78.2 | 7.40 | 97.7 | 48.3 | 0.00869 | 28.5 | 39.2 |
| | 9/26/2012 | SBL | 1.16 ± 0.121 | 0.300 ± 0.048 | 96.9 ± 1.28 | 1.87 ± 0.243 | 168 ± 18.1 | 45.2 | 3.88 | 51.8 | 83.3 | 0.00695 | 27.4 | 38.5 |
| | 6/10/2013 | IRLI02 | 0.160 ± 0.0289 | 0.528 ± 0.137 | 55.5 ± 7.78 | 0.748 ± 0.198 | 119 ± 8.95 | 43.6 | 0.303 | 74.2 | 347 | 0.00135 | 27.8 | 40.3 |
| | 6/10/2013 | Scotts Moor | 1.88 ± 0.260 | 0.205 ± 0.068 | 58.8 ± 7.70 | 0.867 ± 0.156 | 135 ± 34.1 | 56.6 | 9.18 | 67.7 | 31.3 | 0.00139 | 28.9 | 40.4 |
| | 6/12/2013 | SBL | 2.75 ± 0.278 | 0.387 ± 0.130 | 56.1 ± 2.69 | 1.56 ± 0.357 | 172 ± 23.6 | 30.1 | 7.11 | 35.8 | 20.4 | 0.0160 | N/A | 40.0 |
| | 6/12/2013 | MDC | 3.46 ± 0.194 | 2.26 ± 0.411 | 81.1 ± 12.0 | 1.04 ± 0.330 | 148 ± 9.58 | 25.7 | 1.53 | 78.2 | 23.5 | 0.0234 | N/A | 39.0 |
| Non-bloom | 9/26/2012 | IRLML169 | 0.968 ± 0.0549 | 0.255 ± 0.0692 | 82.5 ± 4.56 | 0.847 ± 0.253 | 128 ± 6.32 | 75.8 | 3.80 | 97.4 | 85.2 | 0.00759 | 26.8 | 37.0 |
| | 6/10/2013 | Moore Creek | 3.11 ± 0.292 | 0.696 ± 0.236 | 59.7 ± 1.19 | 0.850 ± 0.299 | 38.4 ± 6.34 | 40.6 | 4.47 | 70.2 | 19.2 | 0.0811 | 28.0 | 37.7 |
| | 8/26/2013 | IRLI10 | 5.81 ± 0.481 | BDL | 26.5 ± 0.620 | 2.39 ± 0.109 | 43.4 ± 0.343 | 13.5 | - | 11.1 | 4.57 | 0.134 | 28.8 | 32.7 |
| | 8/26/2013 | Scotts Moor | 1.95 ± 0.0464 | 0.535 ± 0.0466 | 32.9 ± 1.92 | 0.264 ± 0.101 | 49.0 ± 2.16 | 46.4 | 3.64 | 133.2 | 18.0 | 0.0398 | 28.5 | 32.8 |
| | 8/28/2013 | NPS | 2.62 ± 0.0218 | 0.463 ± 0.0544 | 20.5 ± 1.84 | 0.817 ± 0.119 | 70.2 ± 3.19 | 18.1 | 5.66 | 25.1 | 7.8 | 0.0374 | 28.7 | 37.0 |
| | 8/28/2013 | SBL | 1.87 ± 0.0436 | 0.694 ± 0.101 | 67.6 ± 0.196 | 0.224 ± 0.136 | 158 ± 6.94 | 75.6 | 2.69 | 301.3 | 36.2 | 0.0119 | 29.2 | 43.8 |
| | 8/28/2013 | Scotts Moor | 2.16 ± 0.0131 | 0.474 ± 0.0855 | 25.7 ± 0.225 | 0.748 ± 0.192 | 68.5 ± 0.369 | 22.8 | 4.55 | 34.4 | 11.9 | 0.0315 | 30.3 | 30.0 |

**Table 3** N-specific uptake rates ($h^{-1}$) of different nitrogen species by plankton communities during *Aureoumbra* bloom and non-bloom conditions. Samples were collected from the Indian River Lagoon and Mosquito Lagoon during surveys in 2012 and 2013.

| | Date | Station | N-specific uptake rate ($h^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|
| | | | $NO_3^-$ | $NO_2^-$ | $NH_4^+$ | Urea | Glutamic acid |
| Bloom | 9/24/2012 | IRLML02 | 0.006 ± 0.001 | 0.001 ± 0.000 | 0.058 ± 0.000 | 0.073 ± 0.022 | 0.002 ± 0.000 |
| | 9/24/2012 | SBL | 0.001 ± 0.000 | 0.000 ± 0.000 | 0.027 ± 0.001 | 0.055 ± 0.006 | 0.002 ± 0.000 |
| | 9/26/2012 | SBL | 0.007 ± 0.001 | 0.001 ± 0.000 | 0.015 ± 0.000 | 0.019 ± 0.002 | 0.002 ± 0.000 |
| | 6/10/2013 | IRLI02 | 0.004 ± 0.000 | 0.000 ± 0.000 | 0.006 ± 0.000 | 0.057 ± 0.001 | 0.002 ± 0.001 |
| | 6/10/2013 | Scotts Moor | 0.013 ± 0.000 | 0.000 ± 0.000 | 0.033 ± 0.001 | 0.078 ± 0.035 | 0.002 ± 0.000 |
| Non-bloom | 9/26/2012 | IRLML169 | 0.013 ± 0.001 | 0.003 ± 0.001 | 0.031 ± 0.004 | 0.055 ± 0.003 | 0.002 ± 0.000 |
| | 8/26/2013 | Scotts Moor | 0.052 ± 0.001 | 0.001 ± 0.000 | 0.014 ± 0.001 | 0.033 ± 0.001 | 0.003 ± 0.000 |
| | 8/28/2013 | NPS | 0.091 ± 0.013 | 0.001 ± 0.000 | 0.018 ± 0.000 | 0.050 ± 0.007 | 0.021 ± 0.007 |

**Table 4** Growth rates of and grazing rates on the major plankton populations during the dilution experiments. The $r^2$ values denote the correlation coefficient of the regressions of dilution fraction versus the net growth rate, m is the grazing rate ($d^{-1}$) and k is the net growth rate ($d^{-1}$) without nutrients. *Aureoumbra* = *Aureoumbra lagunensis*, PC cyanobacteria = phycocyanin-containing cyanobacteria, PE cyanobacteria = phycoerythrin-containing cyanobacteria and Hetero-bacteria = heterotrophic bacteria. A dash indicates values were not significantly different from zero while an asterisk indicates that the regression of dilution and net growth rate yielded a positive slope suggesting the existence of density-dependent grazing.

| | Date | Station | Total phytoplankton | | | *Aureoumbra* | | | PC cyanobacteria | | | PE cyanobacteria | | | Hetero- bacteria | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $r^2$ | m | k | $r^2$ | m | k | $r^2$ | m | k | $r^2$ | m | k | $r^2$ | m | k |
| Bloom | 9/24/2012 | IRLML02 | 0.80 | 0.63 | 0.07 | 0.85 | - | * | - | - | 0.49 | 0.58 | 2.22 | 0.80 | 0.67 | - | * |
| | 9/24/2012 | SBL | 0.90 | 0.75 | 0.10 | - | - | 0.35 | 0.59 | 1.12 | 0.10 | 0.37 | 1.63 | 0.32 | - | - | -0.32 |
| | 9/26/2012 | SBL | 0.42 | 0.29 | -0.03 | - | - | -0.06 | 0.38 | 0.30 | 0.34 | 0.58 | 1.47 | 0.33 | - | - | -0.82 |
| | 6/10/2013 | IRLI02 | 0.81 | 0.63 | -0.12 | - | - | 0.27 | 0.54 | 0.52 | 0.06 | 0.76 | 2.83 | 0.51 | 0.58 | 0.67 | 0.46 |
| | 6/10/2013 | Scotts Moor | 0.90 | 0.78 | 0.21 | 0.46 | - | * | 0.43 | - | * | - | - | 0.31 | - | - | 0.49 |
| | 6/12/2013 | SBL | 0.47 | 0.33 | 0.16 | 0.39 | 0.24 | 0.05 | 0.36 | 0.36 | 0.40 | - | - | 0.11 | 0.79 | 0.86 | 0.55 |
| | 6/12/2013 | MDC | 0.72 | 1.34 | 0.08 | - | - | 0.03 | - | - | 0.21 | 0.84 | 3.28 | -0.68 | 0.36 | 0.31 | 0.38 |
| Non-bloom | 9/26/2012 | IRLML169 | 0.73 | 0.66 | -0.14 | - | - | 0.98 | 0.67 | 0.60 | -0.31 | 0.57 | 0.61 | 0.06 | - | - | -0.84 |
| | 6/10/2013 | Moore Creek | 0.69 | 0.47 | -0.03 | - | - | 0.45 | - | - | 0.41 | - | - | 0.27 | 0.38 | 0.32 | 0.73 |
| | 8/26/2013 | IRLI10 | 0.70 | 0.45 | 0.30 | - | - | 0.49 | 0.78 | 1.12 | 0.16 | 0.53 | 1.21 | 0.24 | 0.81 | 1.75 | 0.41 |
| | 8/26/2013 | Scotts Moor | 0.84 | 1.31 | -0.04 | - | - | -0.07 | - | - | 0.51 | 0.85 | 6.35 | 0.72 | 0.55 | 1.02 | 0.20 |
| | 8/28/2013 | NPS | 0.79 | 1.44 | -0.33 | - | - | 0.13 | 0.55 | 1.24 | 0.90 | - | - | 0.99 | 0.88 | 2.48 | 1.41 |
| | 8/28/2013 | SBL | 0.84 | 1.13 | -0.31 | - | - | -0.15 | 0.54 | 0.67 | 0.01 | 0.89 | 4.61 | 0.03 | 0.51 | 1.58 | -0.84 |
| | 8/28/2013 | Scotts Moor | - | 0.21 | -0.21 | - | - | -0.01 | - | - | 0.61 | - | - | 1.80 | 0.64 | 1.14 | 0.13 |

**Table 5** Significant responses of major phytoplankton populations to treatments during nutrient amendment experiments. One asterisk denotes $p < 0.05$ and two asterisks denote $p < 0.005$. Abbreviations for table are as follows: PC cyanobacteria = phycocyanin-containing cyanobacteria, PE cyanobacteria = phycoerythrin-containing cyanobacteria and Hetero-bacteria = heterotrophic bacteria, N = nitrate, A = ammonium, P = phosphate, A+P = ammonium + phosphate and U = urea.

| | Date | Station | *Aureoumbra lagunensis* | | | | | PC cyanobacteria | | | | | PE cyanobacteria | | | | | Heterotrophic bacteria | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | N | A | P | A+P | U | N | A | P | A+P | U | N | A | P | A+P | U | N | A | P | A+P | U |
| Bloom | 9/24/2012 | IRLML02 | | | | | | | | | | | | | | | | | | | | |
| | 9/24/2012 | SBL | | | | | | | | | | | | | | | | | | ** | ** | ** |
| | 9/26/2012 | SBL | | | | ** | | | | | | | | | | | | | | | | ** |
| | 6/10/2013 | IRLI02 | | * | | | | | * | | | ** | | | | | | | | | | |
| | 6/10/2013 | Scotts Moor | | | | | | | | | | | | | | ** | ** | | | | | |
| | 6/12/2013 | SBL | | | | | | | | | | | | | | | | | | | | |
| | 6/12/2013 | MDC | | * | | * | | | | | | | ** | | * | | ** | | | | | |
| Non-bloom | 9/26/2012 | IRLML169 | | | | | | | | | | | | * | | | | ** | | ** | ** | ** |
| | 6/10/2013 | Moore Creek | | | | | | | | | | | | | | * | | | | | | |
| | 8/26/2013 | IRLI10 | | | | | | | | | | | | | | | | * | | * | ** | * |
| | 8/26/2013 | Scotts Moor | | | | * | * | * | | | | | | | | | | | | | | * |
| | 8/28/2013 | NPS | | | | | | * | | | * | * | | | | | | | | | | |
| | 8/28/2013 | SBL | | * | | | * | | | | * | ** | | | * | ** | * | | | | ** | ** |
| | 8/28/2013 | Scotts Moor | | | | | * | | | | | | | | | | | | | | * | ** |

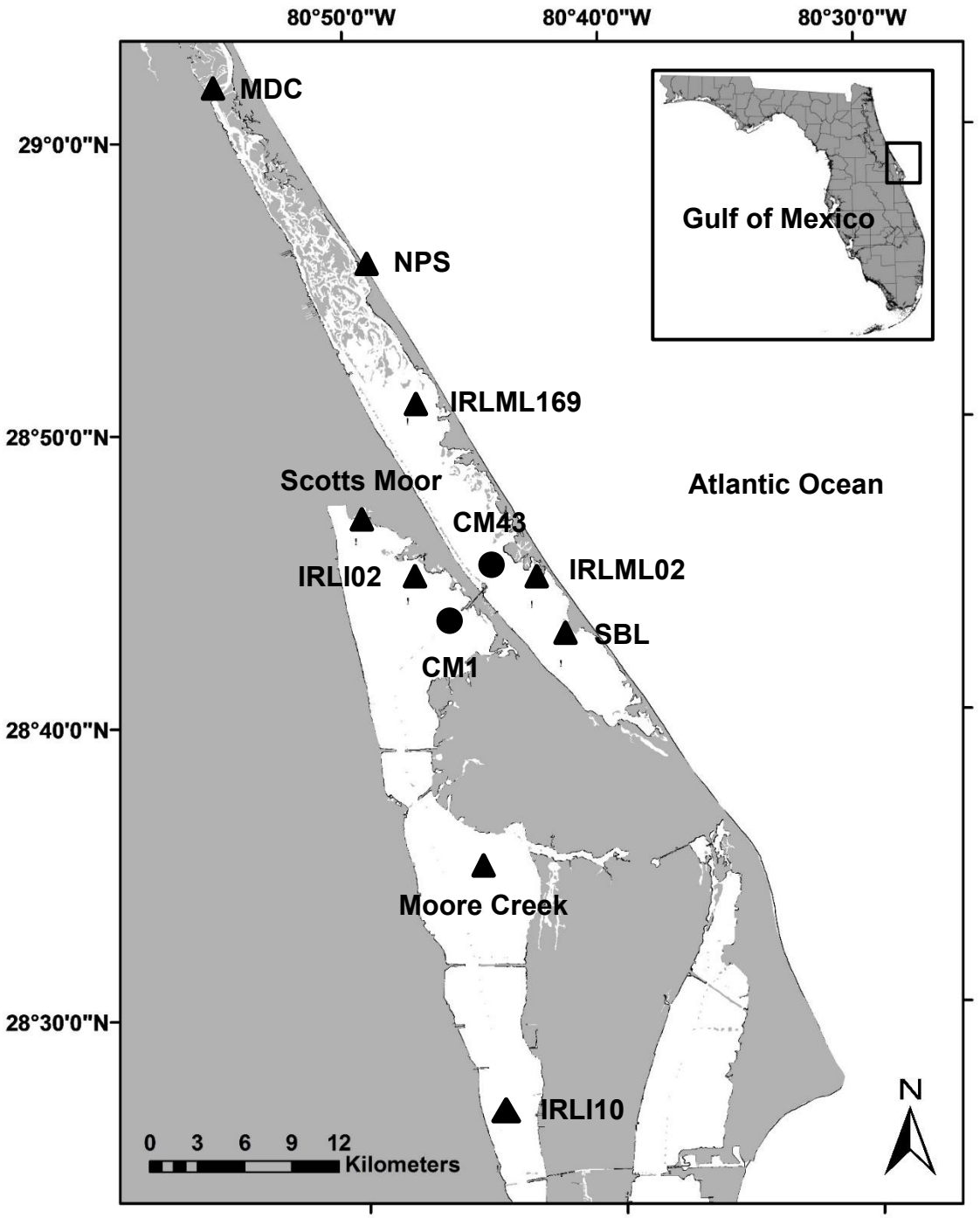



**Figure 1.** Study sites in the Indian River Lagoon and Mosquito Lagoon on the Atlantic coast of Florida, USA. Solid circles and triangles indicate study sites where samples were collected during field surveys in 2012 and 2013. Triangles denote sites, where water was collected for nutrient amendment and grazing experiments.

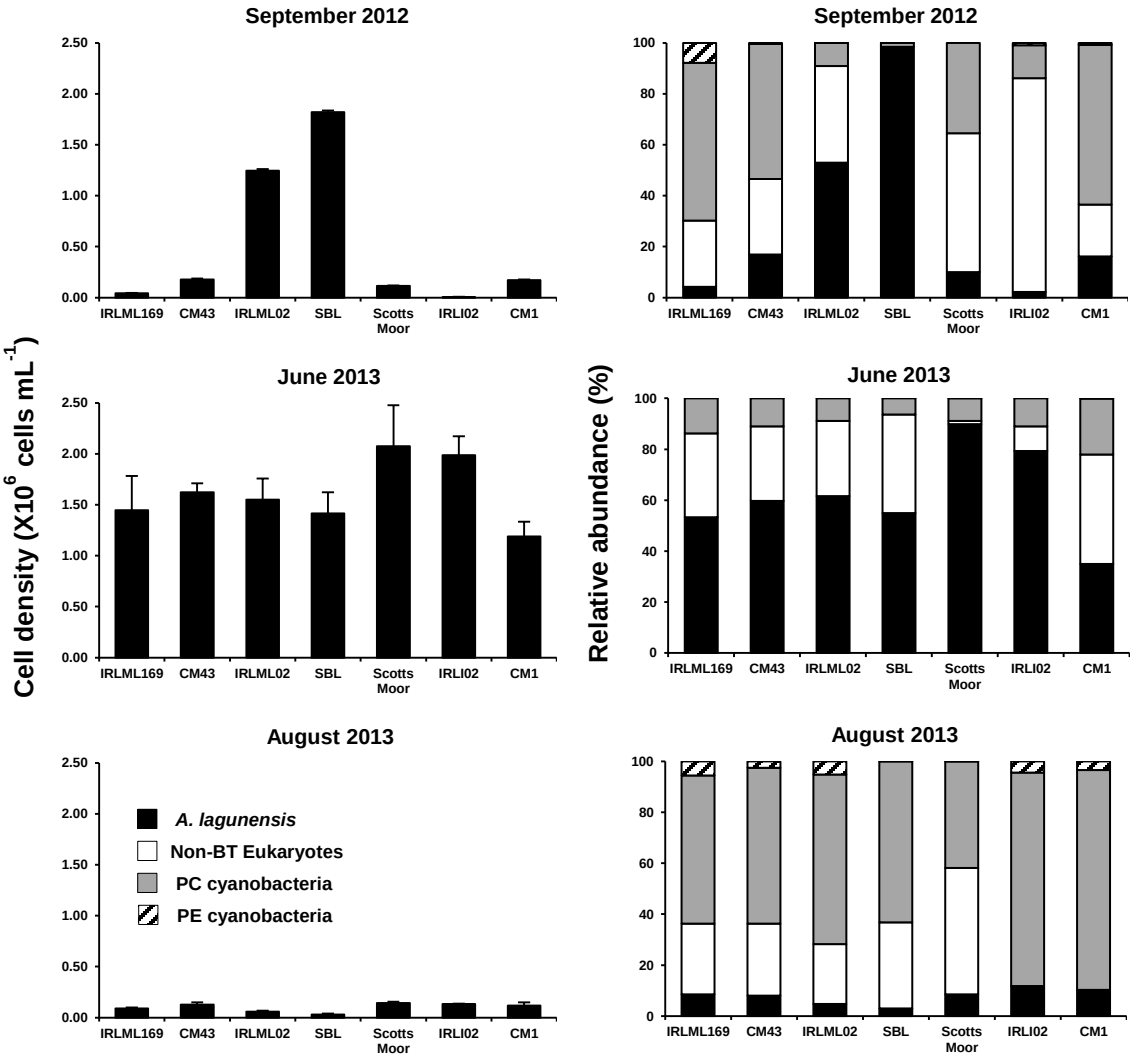



**Figure 2.** Cell density and relative abundance of major phytoplankton populations presented from north to south along the Indian River Lagoon and Mosquito Lagoon. Abbreviations for major phytoplankton populations are as follows: *Aureoumbra* = *Aureoumbra lagunensis*, Non-BT eukaryotes = non-brown tide eukaryotes, PC cyanobacteria = phycocyanin-containing cyanobacteria and PE cyanobacteria = phycoerythrin-containing cyanobacteria.

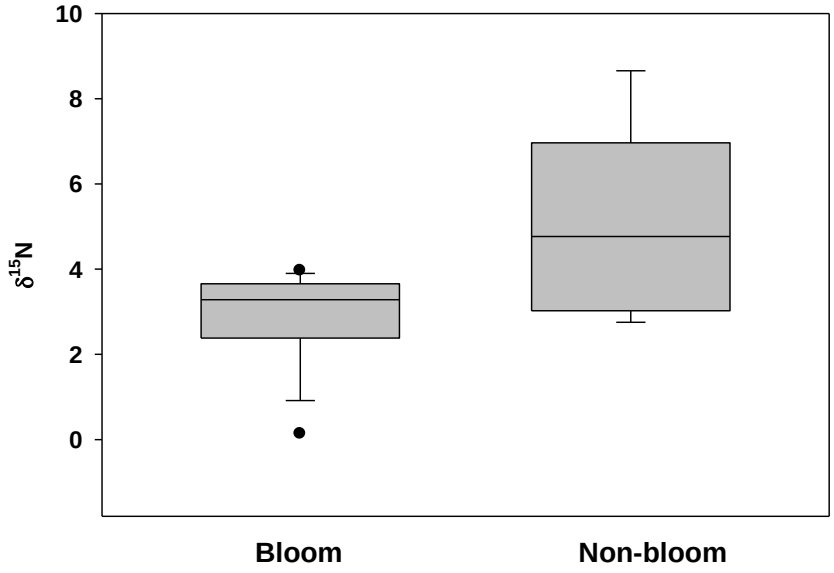



**Figure 3.** Box plot of $\delta^{15}N$ values of phytoplankton populations detected in the Indian River Lagoon and Mosquito Lagoon during bloom and non-bloom conditions of 2012 and 2013.

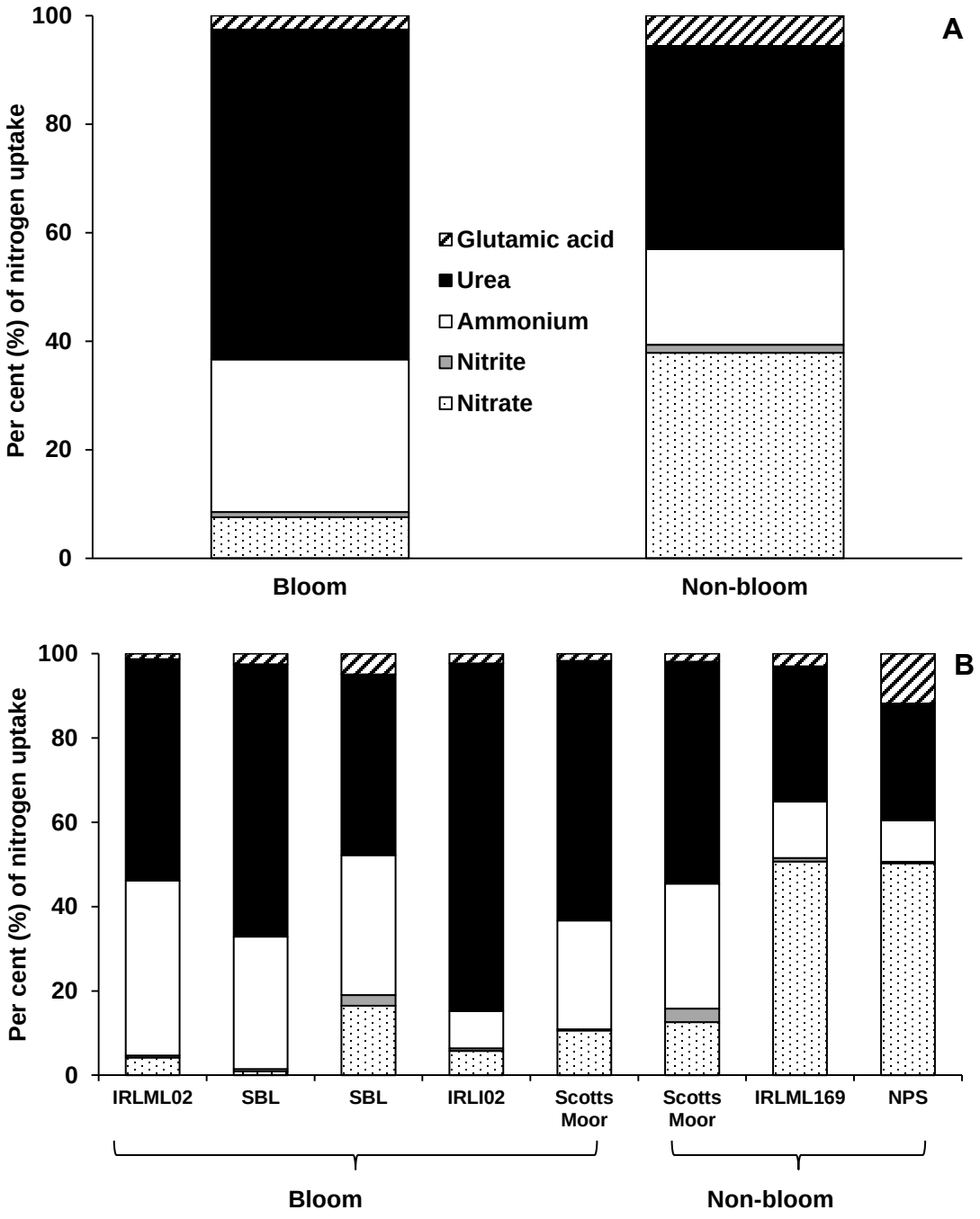



**Figure 4.** Average (n=3) uptake of various nitrogen species shown as per cent (%) of total N uptake within bloom and non-bloom populations (A) and at the experimental sites (B) in the Indian River Lagoon and Mosquito Lagoon during the summers of 2012 and 2013. Please note that bloom and non-bloom sites used for this figure are presented in Table 3.

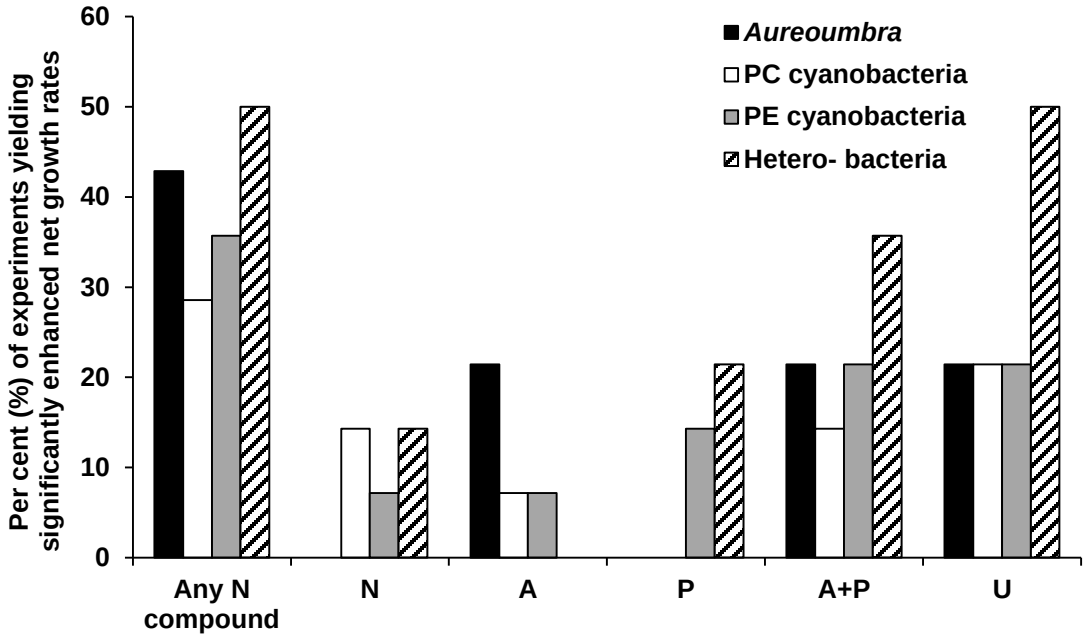



**Figure 5.** Percent (%) of experiments yielding significantly enhanced net growth rates of major plankton populations during nutrient amendment experiments conducted with water collected from Indian River Lagoon and Mosquito Lagoon during 2012 and 2013. Abbreviations of phytoplankton populations are as described in Fig. 2. Hetero-bacteria = heterotrophic bacteria. Stations and dates of data used in this figure are presented in Table 5. Abbreviations for treatments are as follows: N = nitrate, A = ammonium, P = phosphate, A+P = ammonium + phosphate, and U = urea.

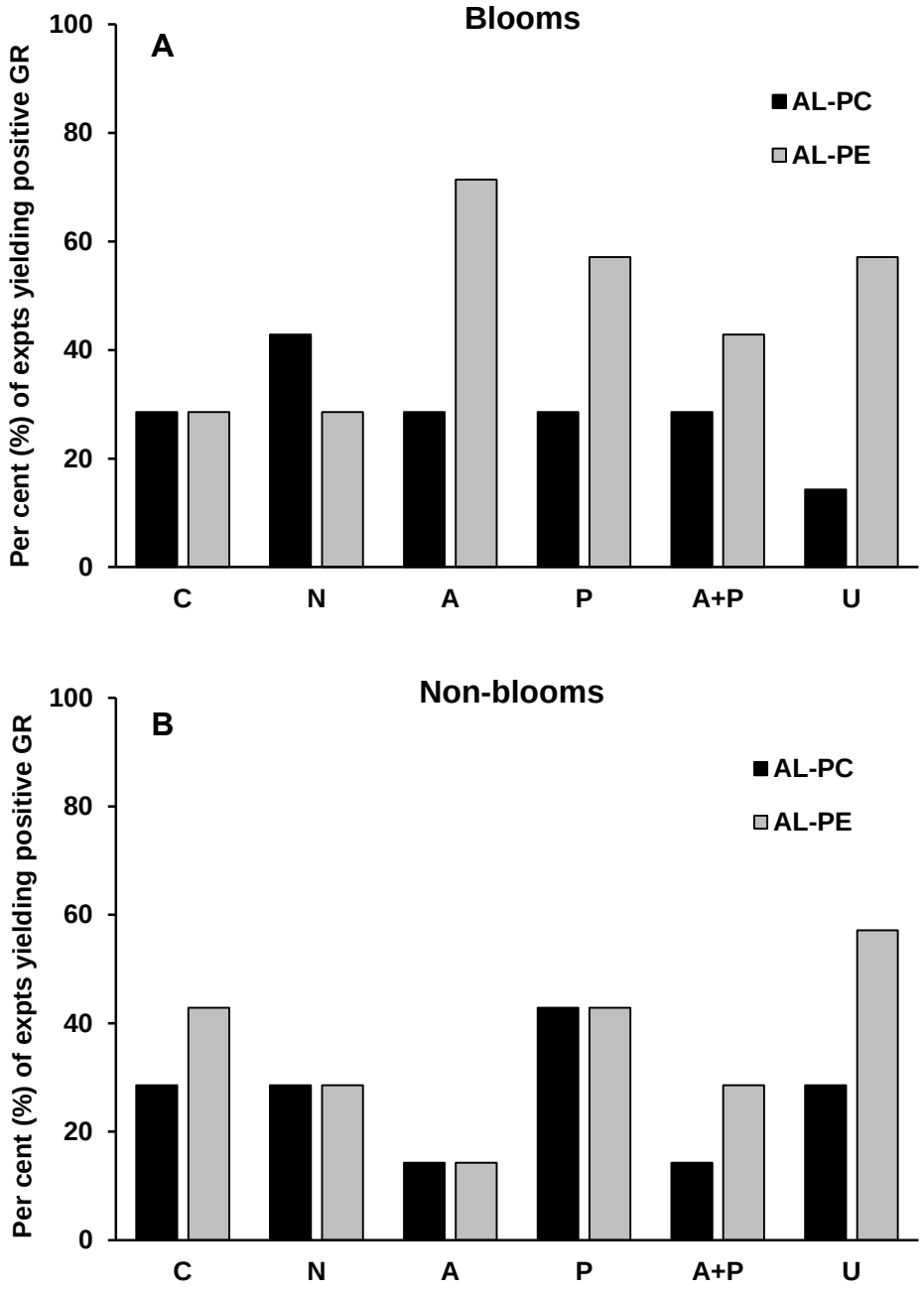



**Figure 6.** Percent (%) of experiments where each treatment yielded a positive difference in growth rates between *Aureoumbra* and competing cyanobacteria populations during nutrient amendment experiments. Difference between *Aureoumbra* and PC cyanobacteria (AL-PC) and the difference between *Aureoumbra* and PE cyanobacteria (AL-PE) during bloom (A) and non-bloom experiments (B). Stations and dates of data used in this figure are presented in Table 5. Abbreviation of phytoplankton populations are as described in Fig. 2. Abbreviations for treatments are as described in Fig. 5.

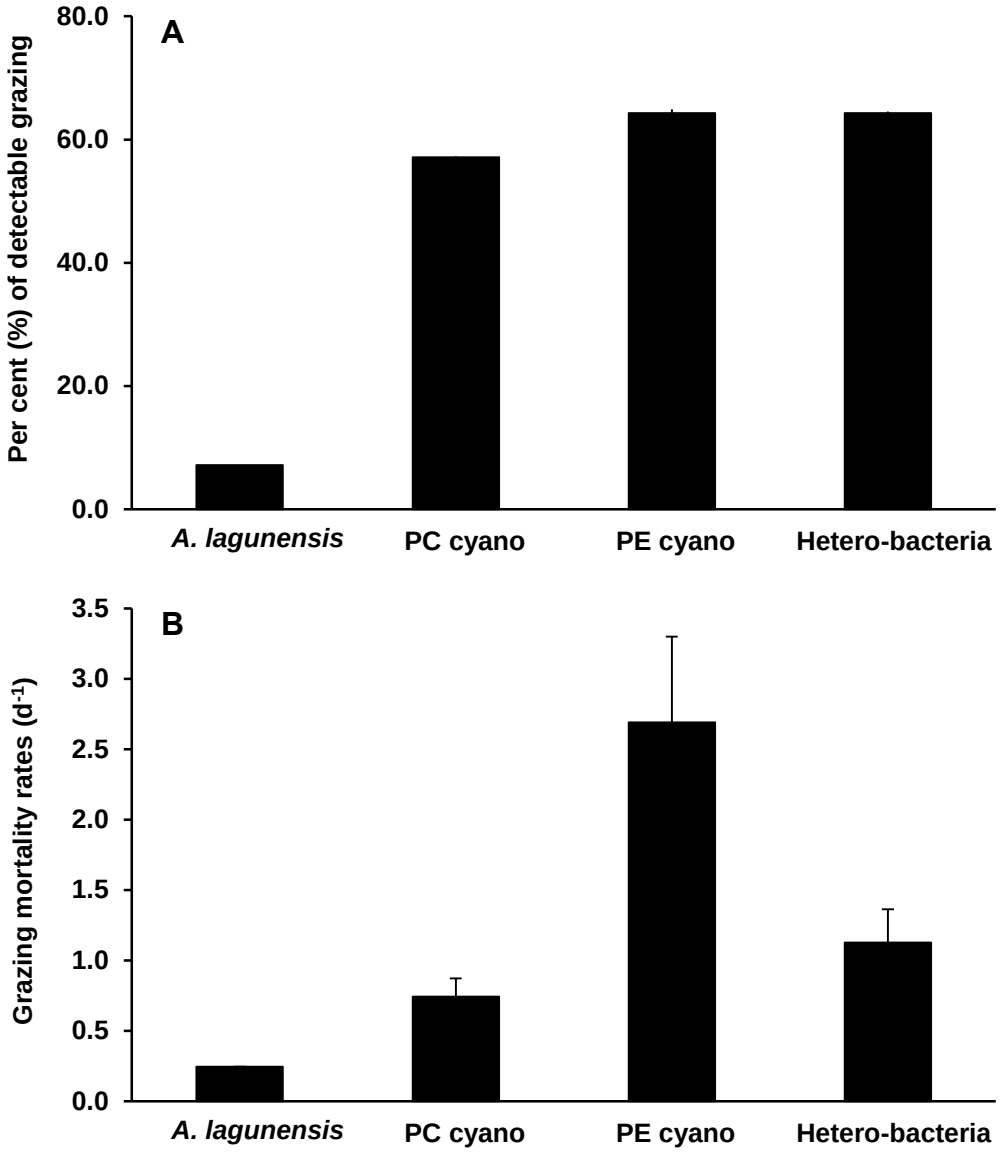



**Figure 7.** Percent (%) of detectable grazing on major phytoplankton populations (A) and average grazing mortality rates for all experiments ($d^{-1}$, mean ± SD; B) using the dilution method. Stations and dates of data used in this figure are presented in Table 4. Abbreviations of phytoplankton populations are as described in Figs. 2 and 5.

# Chapter 3

## Discovery of a resting stage in the harmful, brown tide-causing pelagophyte, *Aureoumbra lagunensis*: A mechanisms potentially facilitating recurrent blooms and geographic expansion

A version of this chapter has been published in:

**Kang**, **Y.**, Y.Z. Tang, G.T. Taylor, C.J. Gobler. 2016. Discovery of a resting stage in the harmful, brown tide-causing pelagophyte, *Aureoumbra lagunensis*: a mechanism facilitating its recent expansion? Journal of Phycology.

**Abstract**

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 were rounder in shape and larger in size than vegetative cells. They also were filled with red accumulation bodies, had smaller and fewer plastids, more vacuolar space, contained lower concentrations of chlorophyll *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 the red accumulation bodies and were depleted in pigments relative to vegetative cells. Upon reverting to vegetative cells, cells increased their chlorophyll *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 v 10°C). Resting cells kept in the dark at 10°C for seven 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 subtropic ecosystems, resist temperature extremes, and may facilitate geographic expansion via anthropogenic transport.

## Introduction

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, 2000b; Bravo and Figueroa, 2014), the alternation of nutrition modes (Jochem, 1999; Wilken et al., 2013), and the reduction of 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 groups 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 sub-optimal 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; Fistarol et al., 2004a). Resting stages refer to all types of cells that reduce metabolic rate, cease cell division, but remain viable (von Dassow and Montresor, 2010) and are often characterized by distinct morphological and compositional changes 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 Dale, 1969; Matsuoka and Fukuyo, 2000b). 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 reveals 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 *Aureoumbra lagunensis* DeYoe et Stockwell have occurred within sub-tropical, coastal ecosystems of the Northwest Atlantic Ocean (Buskey et al., 2001; Gobler et al., 2013b; 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., 2013b). 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., 2013b) 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., 2013b; 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 US for more than a decade (Probyn et al. 1997; 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 sub-optimal conditions for extended periods of time. Previous studies have shown that the temperate brown tide alga, *Aureococcus anophagefferens*, is able to endure 30 days 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 a 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 *Aureoumbra* resting cells 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 *Aureoumbra 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 identities of CB and FL strains were confirmed via sequencing of the 18S rRNA gene (Gobler et al., 2013b; Koch et al., 2014). Stock cultures were maintained in modified GSe medium (Doblin et al., 1999b), 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., 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 quanta $\cdot m^{-2} \cdot s^{-1}$.

*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 $4 \times 10^5$ 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 to back vegetative cells, twenty-four 40 mL cultures of *Aureoumbra* strain FL2 ($4 \times 10^5$ cells $\cdot$ $mL^{-1}$) amended in GSe medium, with 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 days to discourage bacterial degradation of resting cells (Tang and Gobler 2012). Every five days for 40 days, 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 days. 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 sub-tropical environments in which *Aureoumbra* blooms, for seven 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 days.

*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) 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. Chlorophyll *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) 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 membrane were freeze-transferred to ultraclean mirror-finished 1 x 3" stainless steel microscope slides. Membrane surfaces were moistened with atomized sterile MilliQ water and placed

sample side down on 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 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 50x (2.6 µm beam diameter) or 100x (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* (TEM)

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% TEM grade glutaraldehyde which 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$^2$ transmission electron microscope 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 ºC waterbath for 10 min. Nucleic acids 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), respectively, according to manufacturer's protocol. RNA and DNA concentrations in the extracted samples were quantified using Qubit® 2.0 Fluorometer (Invitrogen) 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 x $10^5$ 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, Germany) for 4 h at the initial and after 30 days of 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 ($\mu M \cdot cell^{-1} \cdot h^{-1}$) of vegetative and resting cells were determined.

*Statistics*

Paired *t*-tests were performed to assess differences in biovolume ($\mu m^3$), Chl *a* ($ng \cdot cell^{-1}$), respiration rates ($\mu M \cdot cell^{-1} \cdot h^{-1}$), DNA and RNA contents ($pg \cdot cell^{-1}$), RNA:DNA ratios, $F_v/F_m$, and the Raman 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 are irregularly shaped and small (<5 μm in size and 19 ± 0.32 $\mu m^3$ 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 $\mu m^3$ in biovolume; Fig. 1b; $p<0.001$; paired *t*-test; Table 1). Resting cells all 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 1008, 1155, and 1518 $cm^{-1}$ 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 vs pigments increased 27-fold from 0.27 ± 0.01 to 7.5 ± 0.03 ($p<0.001$; paired *t*-test; Table 1). Attempts to exclusively resolve red droplets by Raman microspectrometry were unsuccessful because of their size. Thus, analyzed spectra represent 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 $pg \cdot cell^{-1}$ ($p<0.05$; paired *t*-test; 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 ($p>0.05$; paried *t*-test; Fig. 3A; Table1). Accordingly, RNA:DNA ratios were significantly reduced in resting cells from 9.7 ± 0.76 to 6.6 ± 0.37 ($p<0.05$; paired *t*-test; Fig. 3B; Table 1). Finally, respiration rates decreased by three-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 $\mu M \cdot h^{-1} \cdot cell^{-1}$ ($p<0.001$; paired *t*-test; 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 three days and then declined, the Cuban strain (CB) cell densities remained steady before declining, while the TX strain declined immediately after exposure to 35°C (Fig. 4A). Vegetative cells of all strains entered the resting stage by $10^{th}$ 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 days 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 ± 0.049 to 0.61 ± 0.064 ($p<0.001$; paired *t*-test; Table 1) whereas vegetative cell densities increased ~ five days later (Fig. 4A, 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 (0.065 ± 0.031 $ng \cdot cell^{-1}$; $p<0.05$; paired *t*-test; 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 both nutrient-deprived. When cells were grown without N or without both N and P, the density of vegetative *Aureoumbra* cells doubled from $5 \times 10^5$ $cells \cdot mL^{-1}$ to $1 \times 10^6$ $cells \cdot mL^{-1}$ over ~10 days, and then slowly declined after 35 days of incubation to $3 \times 10^5$ $cells \cdot mL^{-1}$ by day 85, and remained

uniformly low through 100 days of incubation (Fig. 5A). In P-deprived cultures, vegetative cell densities doubled twice over 5 days to 2 x $10^6$ cells · $mL^{-1}$ and slowly increased to 3 x $10^6$ cells · $mL^{-1}$ through day 40, and then steadily declined to 3 x $10^5$ cells · $mL^{-1}$ through day 90 (Fig. 5A). As vegetative cell density declined, resting cells were produced. From ~60 to ~70 days, 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 days (day 60 to day 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 days of incubation, $F_v/F_m$ of P-deprived cultures remained fairly high over 10 days and then decreased sharply. $F_v/F_m$ of all conditions slowly decreased through day 90 and remained low through 100 days 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 ± 0.02; $p<0.001$; paired *t*-test; Table 1).

*Resumption of cell division from prolonged darkness*

The ability of *Aureoumbra* resting cells to resume growth was assessed while cultures were stored in the dark at 10 and 21°C and then transferred to 21°C with fluences of 80 µmol · $m^{-2}$ · $s^{-1}$. For *Aureoumbra* transferred to darkness at both temperatures, resting cells were formed after 15 days of incubation (Fig. 6A, 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 vegetative cells was proportional to the amount of time the resting cells were stored in the dark (Fig. 6A). Resting cells stored for 15 days completely reverted to vegetative cells after 4 days while resting cells stored for 40 days needed 15 days 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 ± 0.029) reverted to vegetative cells (0.65 ± 0.024; paired *t*-test; 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. 6D, 6E). Resting cells stored for 15 days completely reverted to vegetative cells after seven days and resting cells stored for 40 days completely reverted to vegetative cells after a 31 day 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 five days more to reach the maximal $F_v/F_m$ after the complete conversion of resting cells to vegetative cells (Fig. 6F).

*Aureoumbra* resting cells were able to revert to vegetative cells after storage in complete darkness at 10°C for seven months (Fig. 7). While resting cell densities after the dark storage declined from $5.0 \times 10^5$ to $2.6 \times 10^4$ cells $\cdot$ mL$^{-1}$ after seven months (Fig. 7), vegetative cell densities increased to ~ $1.0 \times 10^5$ cells $\cdot$ mL$^{-1}$ 15 days after resting cells were transferred to fresh medium under light and increased to $\geq$ ~$2.0 \times 10^6$ cells $\cdot$ 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 (seven 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 one week to enter stationary phase for ~one month, and then transitioned to the characteristic resting cells thereafter. In contrast, when exposed to high temperatures or darkness, cell transitioned rapidly (~one week) from vegetative cells to resting cells. Finally, the ability of resting cells to persist in darkness for seven 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 my 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 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 my study, ~25% reduction in RNA:DNA ratios and a three-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, *Aureococcus anophagefferens* (Giner and Boyer, 1998) and *Aureoumbra 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 five-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, I 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 high temperature incubations. Resting cells likely utilize sterol reserves slowly during 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 I was able to revert resting stage cells back to vegetative cells, even after seven months of dark storage, it seems likely that an overly long resting stage may completely deplete the sterol bodies and may ultimately 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 of 20–30°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, 2011a) 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 whereas 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 my 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 to resting cells imply that beyond a set tolerance threshold, *Aureoumbra* vegetative cells alter their metabolism to revert to resting stages after 60 days 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-chlorophyll protein (Geider et al., 1993). In P-deprived cultures, cell density increased by six-fold over 40 days 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; Geider et al., 1998). Prolonged P starvation (weeks to months), however, did eventually cause a reduction in photosynthetic efficiency and the conversion of vegetative cells to 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 and 21°C. This finding is similar to studies of the chlorophyte, *Scenedesmus acuminatus* (Dehning and Tilzer, 1989) and another coastal pelagophyte, *Aureococcus anophagefferens* (Popels and Hutchins, 2002; Popels et al., 2007), and is likely related to the time needed to upregulate 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 re-introduction to light conditions (Dehning and Tilzer, 1989).

Even after seven 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 sub-tropical environments such as TX and FL, USA. Resting cells required at least 15 days of light incubation to return to active growth, showing the potential contribution of *Aureoumbra* resting cells to the re-initiation of blooms 15 days after the sub-tropical winter when waters warm and growth conditions become favorable. In addition, the high density (>2.0 x $10^6$ cells $\cdot$ $mL^{-1}$) of *Aureoumbra* vegetative cells attained even after the seven 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 six 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 to 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 subtropic 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 ppt) and the salinity required for active growth (>22 ppt; Doblin et al., 2004) and that *Aureococcus* can survive 30 days of darkness (Popels and Hutchins, 2002; Popels et al., 2007). Consistent with those reports, ability of *Aureoumbra* resting cells to resume vegetative growth after seven 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 sub-tropical and tropical regions under climate change. Ocean temperatures have increased by an average of 1ºC 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 have 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 tropic regions 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.

## References


Andersen, R. A., Bidigare, R. R., Keller, M. D. & Latasa, M. 1996. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans. *Deep-Sea Res PT II* 43:517-37.

Anderson, D. M. 1980. Effects of temperature conditioning on development and germination of *Gonyaulax tamarensis* (Dinophyceae). *J. Phycol.* 16:166-72.

Anderson, D. M., Coats, D. W. & Tyler, M. A. 1985. Encystment of the dinoflagellate *Gyrodinium uncatenum*: temperature and nutrient effects. *J. Phycol.* 21:200-06.

Anderson, D. M., Taylor, C. D. & Armbrust, E. V. 1987. The effects of darkness and anaerobiosis on dinoflagellate cyst germination. *Limnol. Oceanogr* 32:340-35.

Anderson, O. R. 1975. The ultrastructure and cytochemistry of resting cell formation in *Amphora coffaeformis* (Bacillariophyceae). *J. Phycol.* 11:272-81.

Anderson, O. R. 1976. Respiration and photosynthesis during resting cell formation in Amphora coffeaeformis (Ag.) Kutz. *Limnol. Oceanogr.* 21:452-56.

Baas Becking, L. G. M. 1934. *Geobiologie of inleiding tot de Milieukunde.* Van Stockum W. P. & Zoon N. V., The Hague, The Netherlands,

Berges, J. A., Charlebois, D. O., Mauzerall, D. C. & Falkowski, P. G. 1996. Differential effects of nitrogen limitation on photosynthetic efficiency of photosystems I and II in microalgae. *Plant Physiol.* 110:689-96.

Bibby, B. T. & Dodge, J. D. 1972. The encystment of a freshwater dinoflagellate: a light and electron-microscopical study. *Brit Phycol J.* 7:85-100.

Bloch, K. 1992. Sterol molecule: structure, biosynthesis, and function. *Steroids* 57:378-83.

Boyd, P. W., Rynearson, T. A., Armstrong, E. A., Fu, F., Hayashi, K., Hu, Z., Hutchins, D. A., Kudela, R. M., Litchman, E., Mulholland, M. R., Passow, U., Strzepek, R. F., Whittaker, K. A., Yu, E. & Thomas, M. K. 2013. Marine phytoplankton temperature versus growth responses from polar to tropical waters - outcome of a scientific community - wide study. *Plos One* 8:e63091.

Bravo, I. & Figueroa, R. I. 2014. Towards an ecological understanding of dinoflagellate cyst functions. *Microorganisms* 2:11-32.

Burkholder, J. M. & Glibert, P. M. 2009. The importance of intraspecific variability in harmful algae-Preface to a collection of topical papers. *Harmful Algae* 8:744-45.

Buskey, E. J., Liu, H. B., Collumb, C. & Bersano, J. G. F. 2001. The decline and recovery of a persistent Texas brown tide algal bloom in the Laguna Madre (Texas, USA). *Estuaries* 24:337-46.

Chapman, D., Dodge, J. & Heaney, S. 1982. Cyst formation in the freshwater dinoflagelalte *Ceratium hirundinella* (Dinophyceae). *J. Phycol.* 18:121-29.

Cuvelier, M. L., Allen, A. E., Monier, A., McCrow, J. P., Messie, M., Tringe, S. G., Woyke, T., Welsh, R. M., Ishoey, T., Lee, J. H., Binder, B. J., DuPont, C. L., Latasa, M., Guigand, C., Buck, K. R., Hilton, J., Thiagarajan, M., Caler, E., Read, B., Lasken, R. S., Chavez, F. P. & Worden, A. Z. 2010. Targeted metagenomics and ecology of globally important uncultured eukaryotic phytoplankton. *Proc. Natl. Acad. Sci.* 107:14679-84.

Dale, B. 1983. Dinoflagellate resting cysts: "benthic plankton". *In*: Fryxell, G. A. [Ed.] *Survival strategies of the algae.* Cambridge University Press, Cambridge, pp. 144.

Dawes, E. A. & Senior, P. J. 1973. The role and regulation of energy reserve polymers in micro-organisms. *In*: Rose, A. H. & Tempest, D. W. [Eds.] *Adv. Microb. Physiol.* Academic Press, pp. 135-266.

De Gelder, J., De Gussem, K., Vandenabeele, P. & Moens, L. 2007. Reference database of Raman spectra of biological molecules. *Journal of Raman Spectroscopy* 38:1133-47.

Dehning, I. & Tilzer, M. M. 1989. Survival of *Scenedesmus acuminatus* (Chlorophyceae) in darkness. *J. Phycol.* 25:509-14.

Dempster, E. L., Pryor, K. V., Francis, D., Young, J. E. & Rogers, H. J. 1999. Rapid DNA extraction from ferns for PCR-based analyses. *BioTechniques* 27:66-68.

Doblin, M. A., Blackburn, S. I. & Hallegraeff, G. M. 1999. Growth and biomass stimulation of the toxic dinoflagellate *Gymnodinium catenatum* (Graham) by dissolved organic substances. *J. Exp. Mar. Biol. Ecol.* 236:33-47.

Doblin, M. A., Popels, L. C., Coyne, K. J., Hutchins, D. A., Cary, S. C. & Dobbs, F. C. 2004. Transport of the harmful bloom alga *Aureococcus anophagefferens* by oceangoing ships and coastal boats. *Appl. Environ. Microbiol.* 70:6495-500.

Dortch, Q., Roberts, T. L., Clayton Jr, J. R. & Ahmed, S. I. 1983. RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. *Mar. Ecol. Prog. Ser.* 13:61-71.

Dwyer, L. & Forsyth, P. 1998. Economic significance of cruise tourism. *Ann. Tourism Res.* 25:393-415.

Eppley, R. W. 1972. Temperature and phytoplankton growth in the sea. *Fish. Bull* 70:1063-85.

Falkowski, P. G., Sukenik, A. & Herzig, R. 1989. Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). II Relative abundance of chloroplast proteins. *J. Phycol.* 25:471-78.

Figueroa, R. I., Bravo, I. & Garcés, E. 2005. Effects of nutritional factors and different parental crosses on the encystment and excystment of *Alexandrium catenella* (Dinophyceae) in culture. *Phycologia* 44:658-70.

Fistarol, G. O., Legrand, C. & Granéli, E. 2003. Allelopathic effect of *Prymnesium parvum* on a natural plankton community. *Mar. Ecol. Prog. Ser.* 255:115-25.

Fistarol, G. O., Legrand, C., Rengefors, K. & Graneli, E. 2004. Temporary cyst formation in phytoplankton: a response to allelopathic competitors? *Environ. Microbiol.* 6:791-98.

Fu, F., Yu, E., Garcia, N. S., Gale, J., Luo, Y., Webb, E. A. & Hutchins, D. A. 2014. Differing responses of marine $N_2$ fixers to warming and consequences for future diazotroph community structure. *Aquat. Microb. Ecol.* 72:33-46.

Fu, F. X., Tatters, A. O. & Hutchins, D. A. 2012. Global change and the future of harmful algal blooms in the ocean. *Mar. Ecol. Prog. Ser.* 470:207-33.

Geider, R., Macintyre, Graziano, L. & McKay, R. M. 1998. Responses of the photosynthetic apparatus of *Dunaliella tertiolecta* (Chlorophyceae) to nitrogen and phosphorus limitation. *Eur. J. Phycol.* 33:315-32.

Geider, R. J., Roche, J., Greene, R. M. & Olaizola, M. 1993. Response of the photosynthetic apparatus of *Phaeodactylum tricornutum* (Bacillariophyceae) to nitrate, phosphate, or iron starvation1. *J. Phycol.* 29:755-66.

Giner, J.-L., Li, X. & Boyer, G. L. 2001. Sterol composition of *Aureoumbra lagunensis*, the Texas brown tide alga. *Phytochemistry* 57:787-89.

Giner, J. L. & Boyer, G. L. 1998. Sterols of the brown tide alga *Aureococcus anophagefferens*. *Phytochemistry* 48:475-77.

Giner, J. L., Zhao, H., Boyer, G. L., Satchwell, M. F. & Andersen, R. A. 2009. Sterol chemotaxonomy of marine pelagophyte algae. *Chem. Biodivers.* 6:1111-30.

Gobler, C. & Talmage, S. 2013. Short-and long-term consequences of larval stage exposure to constantly and ephemerally elevated carbon dioxide for marine bivalve populations. *Biogeosciences* 10:2241-53.

Gobler, C. J., Koch, F., Kang, Y., Berry, D. L., Tang, Y. Z., Lasi, M., Walters, L., Hall, L. & Miller, J. D. 2013. Expansion of harmful brown tides caused by the pelagophyte, *Aureoumbra lagunensis* DeYoe et Stockwell, to the US East Coast. *Harmful Algae* 27:29-41.

Gobler, C. J. & Sunda, W. G. 2012. Ecosystem disruptive algal blooms of the brown tide species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*. *Harmful Algae* 14:36-45.

Hallegraeff, G. M. 1998. Transport of toxic dinoflagellates via ships' ballast water: bioeconomic risk assessment and efficacy of possible ballast water management strategies. *Mar. Ecol. Prog. Ser.* 168:10-53.

Hallegraeff, G. M. 2010. Ocean climate change, phytoplankton community responses and harmful algal blooms: a formidable predictive challenge. *J. Phycol.* 46:220-35.

Hargraves, P. 1983. Diatom resting spores: significance and strategies. *In*: Fryxell, G. A. [Ed.] *Survival strategies of the algae.* Cmbridge University Press, Cambridge, pp. 144.

Hattenrath-Lehmann, T. K. & Gobler, C. J. 2011. Allelopathic inhibition of competing phytoplankton by North American strains of the toxic dinoflagellate, *Alexandrium fundyense*: Evidence from field experiments, laboratory experiments, and bloom events. *Harmful Algae* 11:106-16.

Hoegh-Guldberg, O. & Bruno, J. F. 2010. The impact of climate change on the world's marine ecosystems. *Science* 328:1523-28.

Huang, W. E., Li, M., Jarvis, R. M., Goodacre, R. & Banwart, S. A. 2010. Chapter 5 - Shining light on the microbial world: the application on Raman Microspectroscopy. *In*: Allen, I. L., Sima, S. & Geoffrey, M. G. [Eds.] *Adv. Appl. Microbiol.* Academic Press, pp. 153-86.

Itakura, S., Nagasaki, K., Yamaguchi, M. & Imai, I. 1996. Cyst formation in the red tide flagellate *Heterosigma akashiwo* (Raphidophyceae). *J. Plankton Res.* 18:1975-79.

Jochem, F. J. 1999. Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. *Mar. Biol.* 135:721-28.

John, D. E., Wang, Z. A., Liu, X., Byrne, R. H., Corredor, J. E., López, J. M., Cabrera, A., Bronk, D. A., Tabita, F. R. & Paul, J. H. 2007. Phytoplankton carbon fixation gene (RuBisCO) transcripts and air-sea $CO_2$ flux in the Mississippi River plume. *The ISME journal* 1:517-31.

Kang, Y., Koch, F. & Gobler, C. J. 2015. The interactive roles of nutrient loading and zooplankton grazing in facilitating the expansion of harmful algal blooms caused by the pelagophyte, Aureoumbra lagunensis, to the Indian River Lagoon, FL, USA. *Harmful Algae* 49:162-73.

Kaplan-Levy, R. N., Hadas, O., Summers, M. L., Rücker, J. & Sukenik, A. 2010. Akinetes: dormant cells of cyanobacteria. *In*: Lubzens, E., Cerda, J. & Clark, M. [Eds.] *Dormancy and resistance in harsh environments.* Springer, Berlin Heidelberg, pp. 5-27.

Kim, J.-H., Park, B. S., Wang, P., Kim, J. H., Youn, S. H. & Han, M.-S. 2015. Cyst morphology and germination in *Heterosigma akashiwo* (Raphidophyceae). *Phycologia* 54:435-39.

Koch, F., Kang, Y., Villareal, T. A., Anderson, D. M. & Gobler, C. J. 2014. Novel immunofluorescence flow cytometry technique detects the expansion of harmful brown tides caused by *Aureoumbra lagunensis* to the Caribbean Sea. *Appl. Environ. Microbiol.* 80:4947-57.

Kolber, Z., Zehr, J. & Falkowski, P. 1988. Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in photosystem II. *Plant Physiol.* 88:923-29.

Kulandaivelu, G. & Senger, H. 1976. Changes in the reactivity of the photosynthetic apparatus in heterotrophic ageing cultures of *Scenedesmus obliquus*. *Physiol. Plant.* 36:157-64.

Lakeman, M. B., von Dassow, P. & Cattolico, R. A. 2009. The strain concept in phytoplankton ecology. *Harmful Algae* 8:746-58.

Liu, H. B., Laws, E. A., Villareal, T. A. & Buskey, E. J. 2001. Nutrient-limited growth of *Aureoumbra lagunensis* (Pelagophyceae), with implications for its capability to outgrow other phytoplankton species in phosphate-limited environments. *J. Phycol.* 37:500-08.

Loeblich, A. R. I. & Loeblich, L. A. 1984. Dinoflagellate Cysts. *In*: Spector, D. L. [Ed.] *Dinoflagellates.* Academic Press, London, pp. 560.

Malzahn, A., Clemmesen, C. & Rosenthal, H. 2003. Temperature effects on growth and nucleic acids in laboratory-reared larval coregonid fish. *Mar. Ecol. Prog. Ser.* 259:285-93.

Massana, R. 2011. Eukaryotic picoplankton in surface oceans. *Annu. Rev. Microbiol.* 65:91-110.

Matsuoka, K. & Fukuyo, Y. 2000. Technical guide for modern dinoflagellate cyst study. WESTPAC-HAB, Japan Society for the Promotion of Science, Tokyo, Japan.

Montagna, P. A., Stockwell, D. A. & Kalke, R. D. 1993. Dwarf surfclam *Mulinia lateralis* (Say, 1822) populations and feeding during the Texas brown tide event *J. Shellfish Res.* 12:433-42.

Moon-van der Staay, S. Y., De Wachter, R. & Vaulot, D. 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* 409:607-10.

Moore, S. K., Trainer, V. L., Mantua, N. J., Parker, M. S., Laws, E. A., Backer, L. C. & Fleming, L. E. 2008. Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environ. Health* 7:S4.

Movasaghi, Z., Rehman, S. & Rehman, I. U. 2007. Raman spectroscopy of biological tissues. *Applied Spectroscopy Reviews* 42:493-541.

Muhlstein, H. I. & Villareal, T. A. 2007. Organic and inorganic nutrient effects on growth rate-irradiance relationships in the Texas brown-tide alga *Aureoumbra lagunensis* (Pelagophyceae). *J. Phycol.* 43:1223-26.

Olli, K. 1996. Resting cyst formation of *Eutreptiella gymnastica* (Euglenophyceae) in the northern coatal Baltic Sea. *J. Phycol.* 32:535-42.

Parkhill, J. P., Maillet, G. & Cullen, J. J. 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. *J. Phycol.* 37:517-29.

Phlips, E. J., Badylak, S., Lasi, M. A., Chamberlain, R., Green, W. C., Hall, L. M., Hart, J. A., Lockwood, J. C., Miller, J. D. & Morris, L. J. 2014. From red tides to green and brown tides: bloom dynamics in a restricted subtropical lagoon under shifting climatic conditions. *Estuaries and Coasts* 38:886-904.

Popels, L. C. & Hutchins, D. A. 2002. Factors affecting dark survival of the brown tide alga *Aureococcus anophagefferens* (Pelagophyceae). *J. Phycol.* 38:738-44.

Popels, L. C., MacIntyre, H. L., Warner, M. E., Zhang, Y. H. & Hutchins, D. A. 2007. Physiological responses during dark survival and recovery in *Aureococcus anophagefferens* (Pelagophyceae). *J. Phycol.* 43:32-42.

Rengefors, K., Karlsson, I. & Hansson, L. A. 1998. Algal cyst dormancy: a temporal escape from herbivory. *P. Roy. Soc. B-Biol. Sci.* 265:1353-58.

Sandgren, C. D. 1981. Characteristics of sexual and asexual resting cyst (statospore) formation in *Dinobryon cylindricum* Imhof (Chrysophyta). *J. Phycol.* 17:199-210.

Sandgren, C. D. 1983. Survival strategies of chrysophycean flagellates: Reproduction and the formation of resistant resting cysts. *In*: Fryxell, G. A. [Ed.] *Survival Strategies of the Algae.* Cambridge University Press, Cambridge, pp. 23-49.

Sicko-Goad, L., Stoermer, E. & Kociolek, J. 1989. Diatom resting cell rejuvenation and formation: time course, species records and distribution. *J. Plankton Res.* 11:375-89.

Simon, N., Barlow, R. G., Marie, D., Partensky, F. & Vaulot, D. 1994. Characterization of oceanic photosynthetic picoeukaryotes by flow cytometry. *J. Phycol.* 30:922-35.

Smith, A. E. & Morris, I. 1980. Synthesis of lipid during photosynthesis by phytoplankton of the Southern Ocean. *Science* 207:197-99.

Sunda, W. G., Graneli, E. & Gobler, C. J. 2006. Positive feedback and the development and persistence of ecosystem disruptive algal blooms. *J. Phycol.* 42:963-74.

Sunda, W. G. & Hardison, D. R. 2007. Ammonium uptake and growth limitation in marine phytoplankton. *Limnol. Oceanogr.* 52:2496-506.

Thomas, M. K., Kremer, C. T., Klausmeier, C. A. & Litchman, E. 2012. A global pattern of thermal adaptation in marine phytoplankton. *Science* 338:1085-88.

Tillmann, U., Alpermann, T. L., da Purificacao, R. C., Krock, B. & Cembella, A. 2009. Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. *Harmful Algae* 8:759-69.

Villareal, T., Mansfield, A. & Buskey, E. 1998. Growth and chemical composition of the Texas brown tide forming pelagophyte *Aureoumbra lagunensis*. *Harmful algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Vigo, Spain*:359-62.

Villareal, T. A., Chirichella, T. & Buskey, E. J. 2002. Regional distribution of the Texas Brown Tide (*Aureoumbra lagunensis*) in the Gulf of Mexico. *Harmful Algae* 2004:374-76.

Volkman, J. 2003. Sterols in microorganisms. *Appl. Microbiol. Biotechnol.* 60:495-506.

von Dassow, P. & Montresor, M. 2010. Unveiling the mysteries of phytoplankton life cycles: patterns and opportunities behind complexity. *J. Plankton Res.*

Wall, D. & Dale, B. 1969. The 'hystrichosphaerid' resting spore of the dinoflagellate *Pyrodinium bahamense*, plate, 1906. *J. Phycol.* 5:140-49.

Wilken, S., Huisman, J., Naus-Wiezer, S. & Van Donk, E. 2013. Mixotrophic organisms become more heterotrophic with rising temperature. *Ecol. Lett.* 16:225-33.

Wood, R. E. 2000. Caribbean cruise tourism: globalization at sea. *Ann. Tourism Res.* 27:345-70.

Worden, A. Z., Janouskovec, J., McRose, D., Engman, A., Welsh, R. M., Malfatti, S., Tringe, S. G. & Keeling, P. J. 2012. Global distribution of a wild alga revealed by targeted metagenomics. *Curr. Biol.* 22:R675-R77.

**Table 1** Physiological characteristics of vegetative and resting cells. Paired *t*-test was performed on all parameters. One asterisk indicates $p<0.05$ and two asterisks indicate $p<0.001$. ND indicates the difference between vegetative and resting cells was not statistically significant.

| Parameters | Vegetative cells | Resting cells | Sample size | Significance |
|---|---|---|---|---|
| Biovolume ($\mu m^3$) | 19 ± 0.32 | 44 ± 2.9 | 9 | ** |
| Chl. *a* ($ng \cdot cell^{-1}$) | 0.29 ± 0.11 | 0.07 ± 0.03 | 3 | * |
| Respiration rate ($\mu M \cdot h^{-1} \cdot cell^{-1}$) | 0.05 ± 0.01 | 0.02 ± 0.01 | 3 | ** |
| DNA ($pg \cdot cell^{-1}$) | 0.02 ± 0.01 | 0.02 ± 0.002 | 3 | ND |
| RNA ($pg \cdot cell^{-1}$) | 0.19 ± 0.04 | 0.15 ± 0.02 | 3 | * |
| RNA:DNA | 9.7 ± 0.76 | 6.6 ± 0.37 | 3 | * |
| Fv/Fm | | | | |
| High temperature | 0.61 ± 0.06 | 0.11 ± 0.05 | 3 | ** |
| Nutrient limitation | 0.62 ± 0.03 | 0.13 ± 0.03 | 3 | ** |
| Dark storage 10ºC | 0.65 ± 0.02 | 0.15 ± 0.03 | 3 | ** |
| 608:1518 Raman intensity ratio | 0.27 ± 0.01 | 7.5 ± 0.03 | 20 | ** |

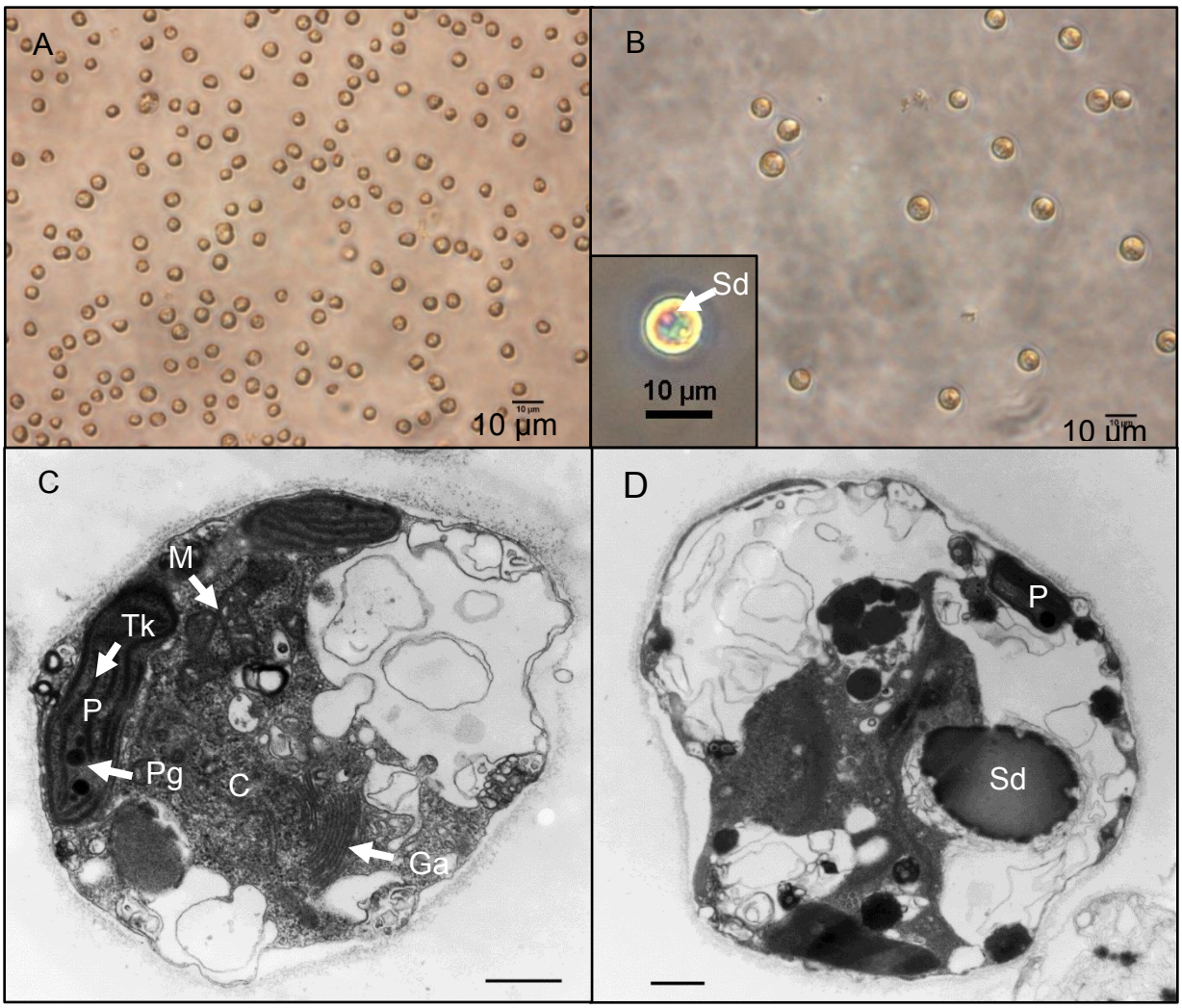



**Figure 1.** Light microscope and TEM images of *A. lagunensis*. (A) light microscope image of vegetative cells at 21ºC, (B) light microscope image of resting cells formed at 35°C and an inserted figure of an enlarged image of *Aureoumbra* resting cell , (C) TEM image of vegetative cell, (D) TEM image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

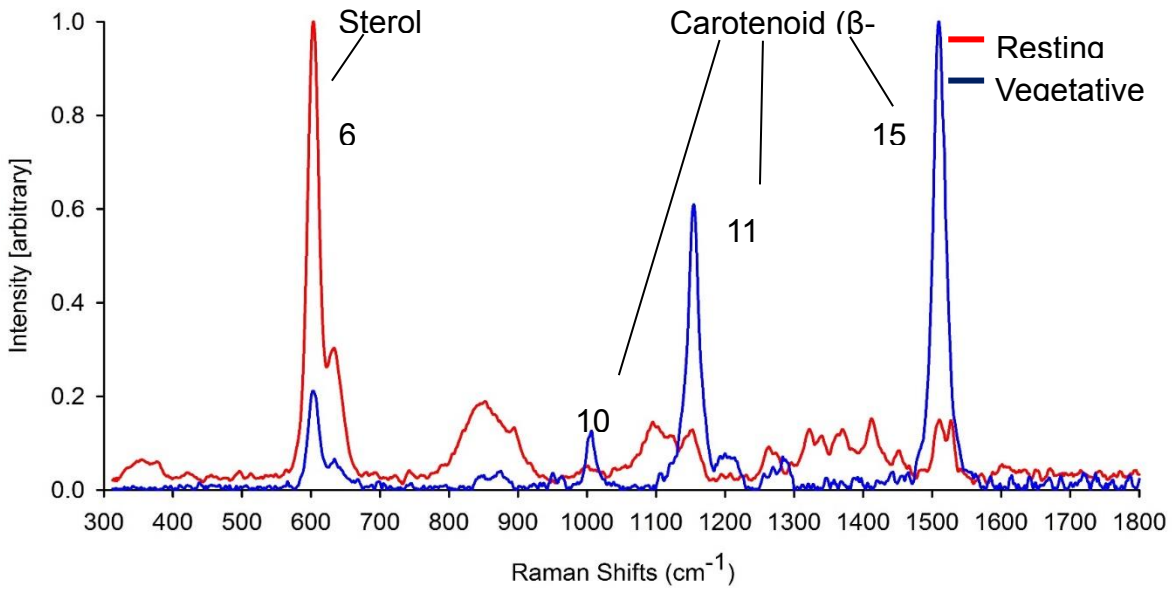



**Figure 2.** Examples of single-cell Raman spectra from resting cells and vegetative cells. Raman spectra generated from 20 vegetative cells and 20 resting cells were averaged to obtain the quantitative values that I compared statistically. Blue and red lines indicate Raman spectra of vegetative cells and resting cells, respectively.

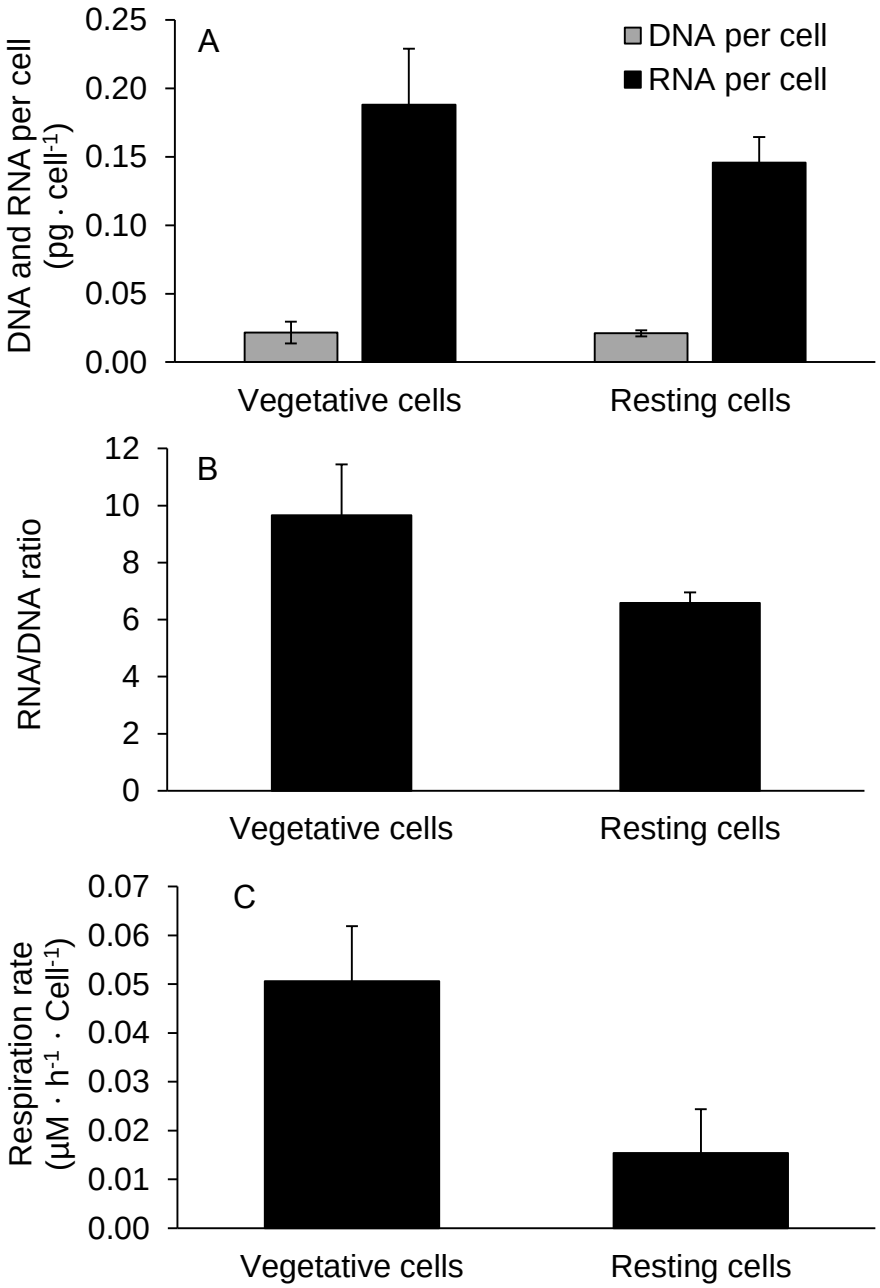



**Figure 3.** Physiological characteristics of resting cells including RNA and DNA content per cell, RNA/DNA ratios, and oxygen consumption rate. (A) RNA and DNA content per cell (pg · cell$^{-1}$), (B) RNA/DNA ratios and (C) oxygen consumption rate (µM · h$^{-1}$ · cell$^{-1}$).

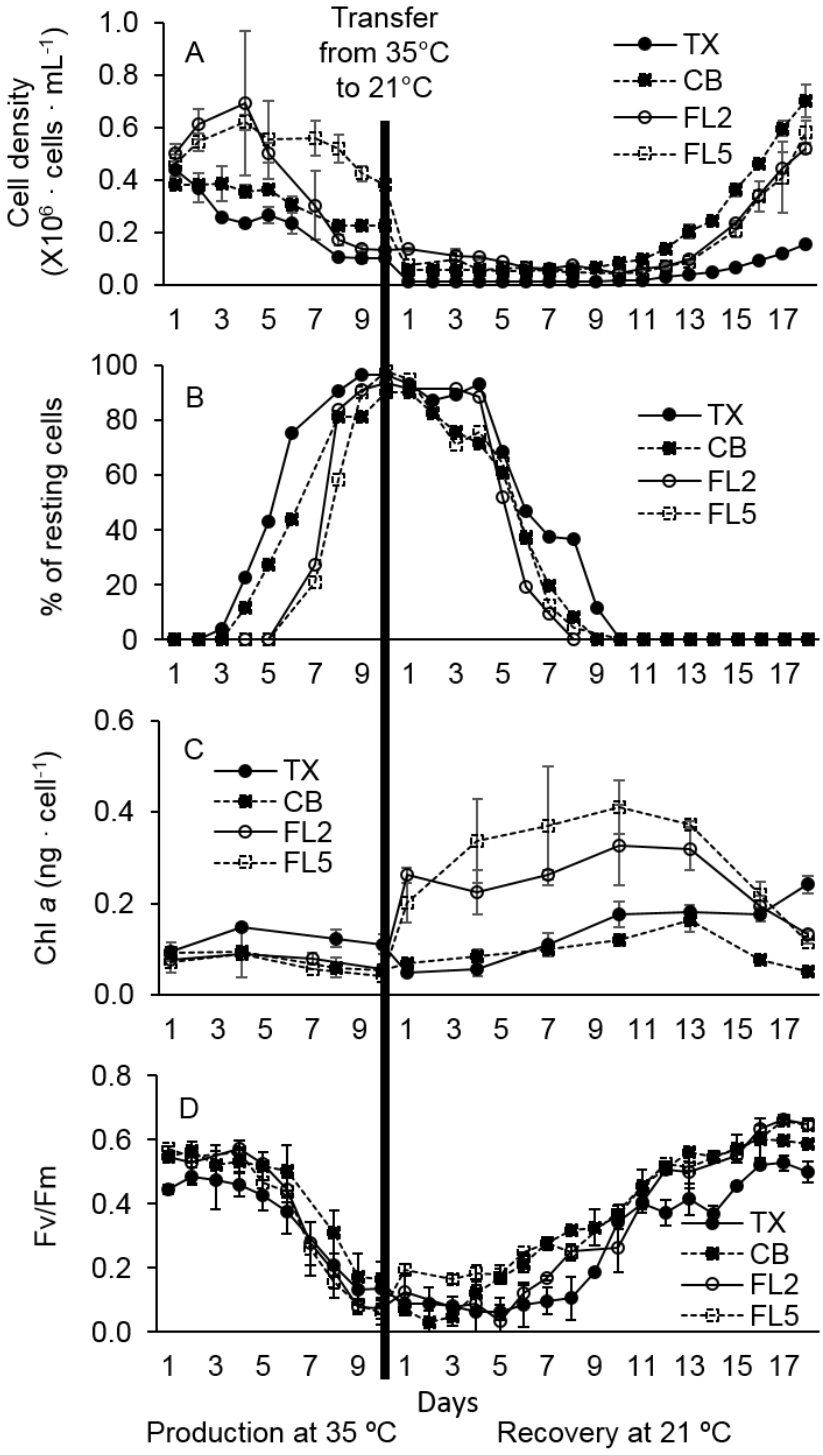



**Figure 4.** Responses of *Aureoumbra* to high temperature during resting cell production experiments and favorable condition (21°C) during recovery experiments. (A) cell density, (B) percent of resting cells, (C) Chl *a* per cell (ng · cell$^{-1}$), (D) $F_v/F_m$.

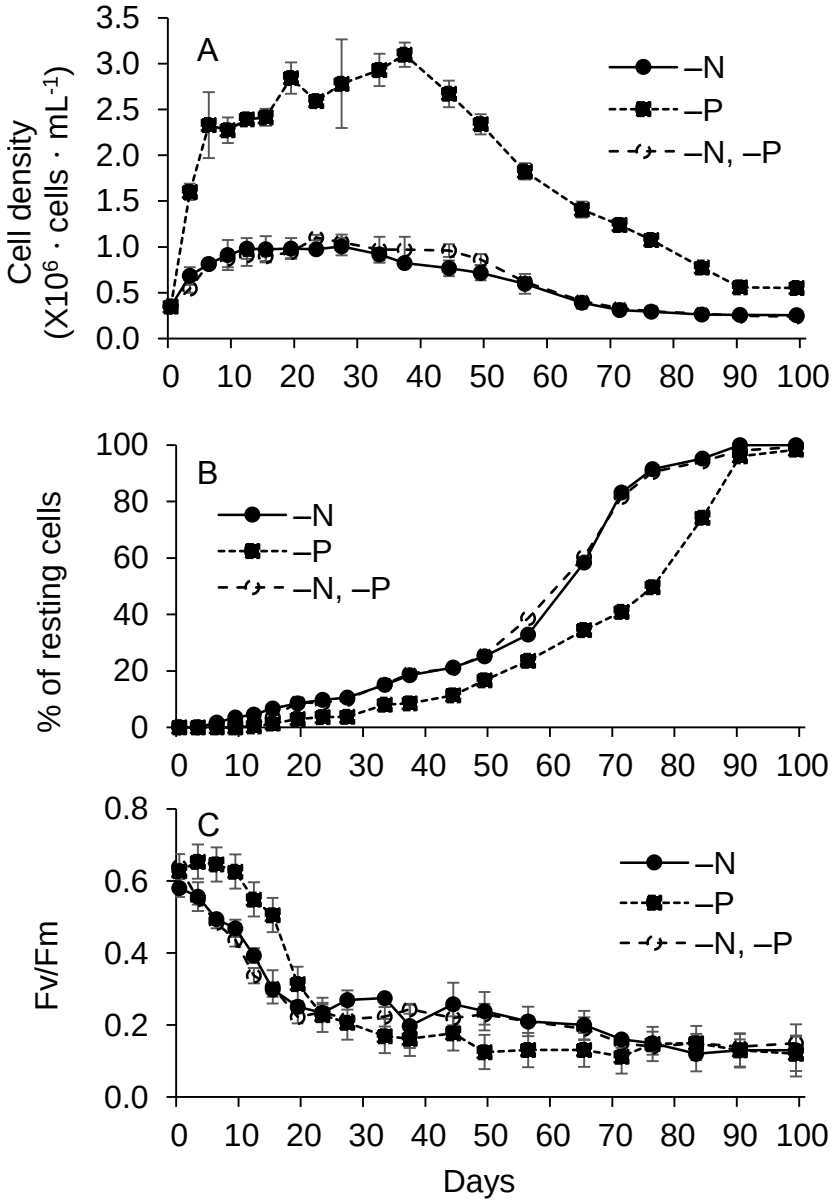



**Figure 5.** Responses of *Aureoumbra* to nutrient limitation. (A) cell density, (B) percent of resting cells, (C) $F_v/F_m$.

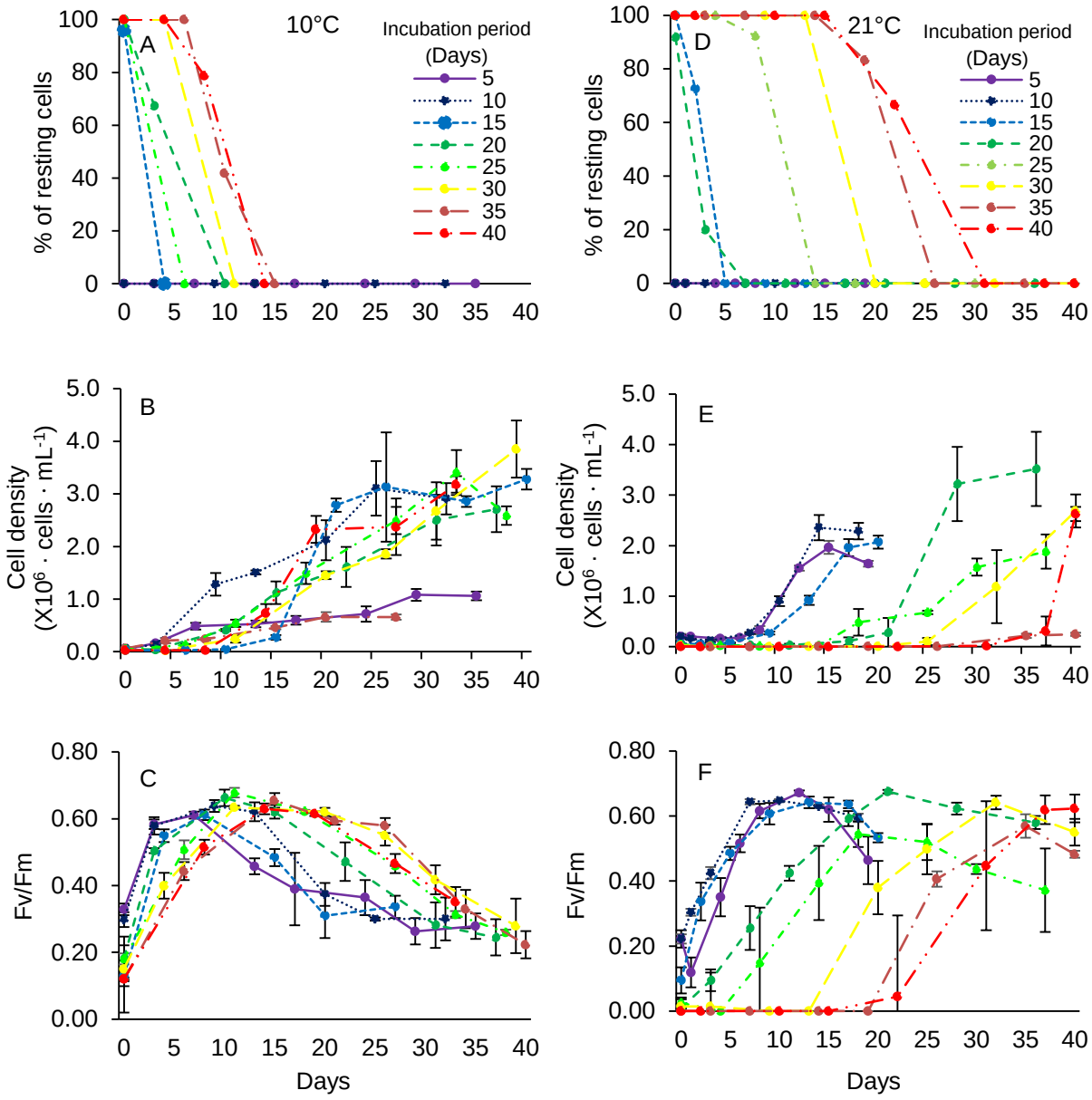



**Figure 6.** Responses of *Aureoumbra* cells upon exposure to light at 21°C after stored in the dark at 10°C (A) percent of resting cells, (B) cell density, and (C) $F_v/F_m$, and responses of *Aureoumbra* cells upon exposure to light at 21°C after stored in the dark at 21°C (D) percent of resting cells, (E) cell density, and (F) $F_v/F_m$.

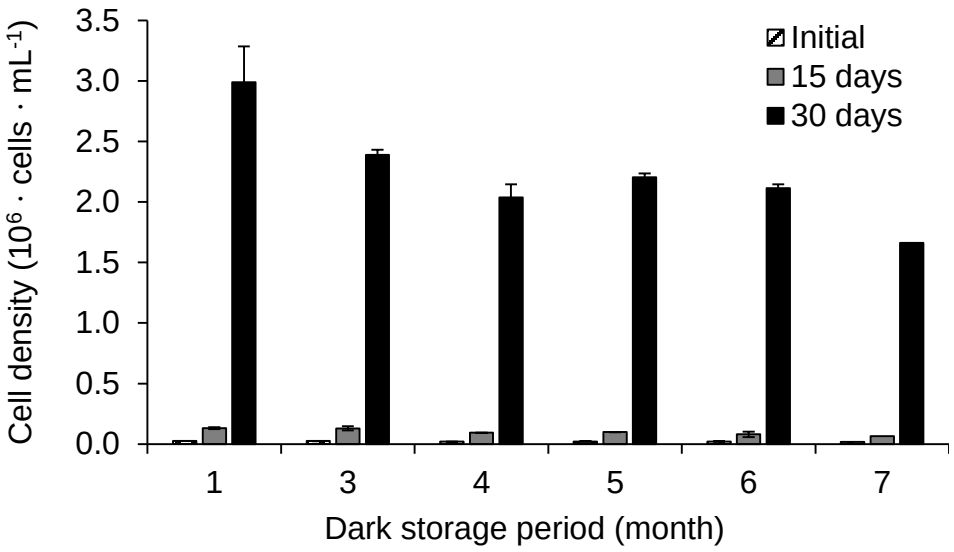



**Figure 7.** *Aureoumbra* cell density after cultures were stored in the dark at 10°C for seven months. Cell densities were evaluated at the start of the seven month experiment as well as 15 and 30 days after dark-stored cultures were transferred to 21°C with light.

# Chapter 4

## The brown tide algae, *Aureoumbra lagunensis* and *Aureococcus anophagefferens* (Pelagophyceae), allelopathically inhibit competing microalgae during harmful algal blooms

**Abstract**

Allelopathy is an important ecological strategy that can facilitate algal blooms caused by several classes of phytoplankton. Here, I describe the ability of the harmful pelagophytes, *Aureoumbra lagunensis* isolated from coastal waters of Cuba (CB), Florida (FL), and Texas (TX), USA and *Aureococcus anophagefferens* (CCMP1984) isolated from New York (NY) to inhibit the growth of other phytoplankton. The allelopathic effects of brown tide algae were broad and strong with both whole cells and culture filtrate causing up to a 30 - 96% reduction in cell abundance after 120 h in 9 of 10 phytoplankton strains originating from nine species and eight classes. The inhibition of target was dose-dependent with *Aureococcu*s or *Aureoumbra* cell densities exceeding 2.5 x $10^5$ cell $mL^{-1}$ causing significant reductions in the abundance of competing algae. Recently isolated strains of *Aureoumbra* (2012 - 2013) were more allelopathically potent than a strain isolated 20 years ago and culture filtrate from later growth stages was more potent than early growth stages. Hydrophobic resins (e.g. HP20, C18) significantly eliminated the effects of allelochemicals on eukaryotic, but not prokaryotic algae. Allelochemicals were heat-stable, but degraded at 22°C over two weeks. Filtrate from *Aureococcus* and *Aureoumbra* cultures and blooms was capable of significantly reducing the densities of natural assemblages of eukaryotic and prokaryotic phytoplankton from estuarine, bloom-prone waters in NY and FL, respectively. Collectively, these findings suggest that allelopathic inhibition of competing algae is an important process that facilitates the intensification and persistence of harmful brown tide blooms caused by *Aureococcus* and *Aureoumbra* across the globe.

## Introduction

Competition among co-occurring phytoplankton species for limited resources has shaped their evolution for billions of years (Tilman, 1977; Legrand et al., 2003). Allelopathy is the release of compounds from a plant that negatively affects the growth of co-existing species (Rice, 2013) and is an important survival strategy for many phytoplankton (Granéli and Hansen, 2006). Allelopathic chemicals associated with secondary metabolites (e.g. hemolytic compounds or toxins) can cause reductions in photosynthetic efficiency (Figueredo et al., 2007; Prince et al., 2008) and metabolism (Zheng et al., 2016), lysis of target algae (Igarashi et al., 1996; Rengefors and Legrand, 2001; Fistarol et al., 2003), target cell immobilization (Tillmann et al., 2007; Tillmann et al., 2009), and/or the formation of temporary cysts (Fistarol et al., 2004a). Allelopathic interactions between harmful microalgae and macroalgae have been considered a mechanism promoting bloom development and persistence (Prince et al., 2008; Tang and Gobler, 2010; Hattenrath-Lehmann and Gobler, 2011b) as a well as a strategy for mitigating some types of harmful algal blooms (Tang and Gobler, 2011; Tang et al., 2014). Many groups of phytoplankton are known to produce allelopathic chemicals including cyanobacteria (Suikkanen et al., 2005; Leflaive and Ten-Hage, 2007), dinoflagellates (Kubanek et al., 2008; Tang and Gobler, 2010; Hattenrath-Lehmann and Gobler, 2011b; Poulson-Ellestad et al., 2014), diatoms (Lim et al., 2014; Xu et al., 2015), prymnesiophytes (Schmidt and Hansen, 2001; Fistarol et al., 2003), and raphidophytes (Yamasaki et al., 2007; Fernández-Herrera et al., 2016). Studies of *Alexamdrium* spp. have demonstrated that allelopathic effects are not caused by saxitoxin (Tillmann and John, 2002; Hattenrath-Lehmann and Gobler, 2011b; Van de Waal et al., 2015), a well-known defense mechanism of this harmful alga (Fistarol et al., 2004b; Pavia et al., 2012; Selander et al., 2015).

Brown tides caused by the pelagophytes *Aureococcus anophagefferens* and *Aureoumbra lagunesis* have been disrupting ecosystems and fisheries around the world for more than three decades (Gobler and Sunda, 2012). *Aureoumbra* blooms have been causing food web disruption in Laguna Madre, Texas, USA for more than 25 years (Buskey et al., 2001), in the Indian River Lagoon, Florida, USA since 2012 (Gobler et al., 2013b), and in Guantanamo Bay, Cuba since 2013 (Koch et al., 2014). These brown tides have been shown to cause mortality of zooplankton, seagrass, shellfish, and polychaetes (Montagna et al., 1993; Onuf, 1996; Ward et al., 2000). Similarly, brown tides caused by *Aureococcus anophagefferens* have disrupted ecosystems on the East Coast of the US, in South Africa, and in China (Gobler et al., 2005; Probyn et al., 2010; Zhang et al., 2012) causing the poor growth, survival and reproduction of zooplankton (Lonsdale et al., 1996; Caron et al., 2004; Smith et al., 2008) and bivalves (Bricelj et al., 2001; Newell et al., 2009) as well as the destruction of seagrass meadows (Cosper et al., 1987).

During blooms, brown tide algae compete with cyanobacteria and other eukaryotic algae for dominance. As densities of brown tide algae peak in estuaries, the abundances of other phytoplankton diminish, leading to negative correlations between densities of this brown tide alga and phycocyanin-containing cyanobacteria (PC cyanobacteria; Fig. 1A, C) or other picoeukaryotes (Fig. 1B, D). Previously, brown tides have been shown to be promoted by the utilization of multiple forms of nitrogen (Berg et al., 1997; Mulholland et al., 2002; Muhlstein and Villareal, 2007), maximal growth under low light intensity (MacIntyre et al., 2004; Sunda and Hardison, 2007) and inhibition of zooplankton grazing (Buskey et al., 1997; Kang et al., 2015). Past field observations (Sieracki et al., 2004; Gobler et al., 2011, Gobler et al., 2013) coupled with preliminary co-culturing experiments with multiple strains of *Synechococcus* led me to question this conclusion and inspired this study.

For this investigation, I examined the allelopathic effects of the brown tide pelagophytes, *Aureococcus* and *Aureoumbra* on 10 strains of phytoplankton originating from eight individual algal classes as well as its effects on natural phytoplankton communities. Experiments were performed varying densities of donor and target species and using whole cells as well as culture filtrate. Experiments were performed to constrain the identity of the allelopathic compounds and the ecological relevance of allelopathy during brown tides. Collectively, experimental findings indicate allelopathy is likely an important mechanism contributing to the intensification and persistence of brown tides in coastal ecosystems.

## Materials and Methods

### *Cultures and culturing information*

Details regarding the donor and target algae tested in this study are provided in Table 1. Stock cultures of all species except for *Aureoumbra* were maintained in GSe medium (Doblin et al., 1999a) with salinity of 33 that was made by autoclaving and then filtering seawater using Stericup® Filter Units (0.2 µm). Because *Aureoumbra* growth is maximal on ammonium (Muhlstein and Villareal, 2007), this alga was maintained in modified GSe medium with nitrogen and phosphorus compounds replaced with 100 µm $NH_4^+$ and 6 µm $PO_4^-$. All cultures were incubated at 21°C with a 12:12 h light:dark cycle, illuminated by a bank of fluorescent lights.

For all experiments except for a direct-contact experiment described below, *Aureoumbra* and *Aureococcus* culture filtrate was used to study the allelopathic effects on target species. Filtrate is commonly used to perform allelopathy experiments (Fistarol et al., 2003; Hattenrath-Lehmann and Gobler, 2011b) and has the advantage of exploring the effects of extracellular

exudates on other phytoplankton while eliminating direct competition for resources between target and donor species. Cell-free medium was made by filtering cultures (cell density ~ 2.0 x $10^6$ cells $mL^{-1}$ in late exponential growth phase) through GFF glass fiber filters (diameter of 47mm) twice to assure all cells were removed. Prior to use, filtrate was examined on a CytoFLEX flow cytometer (Beckman Coulter, USA) to ensure the filtrate was indeed cell-free. Except for experiments testing strain variation in allelopathic effects, filtrate was made using a FL strain of *Aureoumbra* and a NY strain of *Aureococcus* (CCMP1984; Table 1). For experiments, triplicate KIMAX® 50 mL glass culture tubes were filled with 40 mL of target cells and various brown tide treatments described in detail below. A control was established in another triplicate set of tubes for each species by mixing target cells cultures with GSe medium. Nutrients were added at a GSe medium level (800 µL of GSe working stock) to the treatment to ensure nutrient conditions matched control levels. For all experiments, samples were incubated under the same conditions described above and cell density and photosynthetic efficiency ($F_v/F_m$) were monitored every 24h for 120h as described below.

*Allelopathic effects of* brown tide algae *on multiple target phytoplankton*

To explore the allelopathic effects of *Aureoumbra* and *Aureococcus* on the growth of potentially co-occurring algae, multiple classes of phytoplankton were incubated in filtrate of each pelagophyte. Nine target species tested in this study and their cell densities normalized by biovolume included *Chaetoceros calcitrans* (Bacillariophyceae; biovolume of 30 $\mu m^3$; cell density of 2.2 x $10^4$ cells $mL^{-1}$), *Chaetoceros muelleri* (Bacillariophyceae; 118 $\mu m^3$; 5.5 x $10^3$ cells $mL^{-1}$), *Synechococcus* sp. (CCMP1333 only, Cyanophyceae; 1.3 $\mu m^3$; 5.0 x $10^5$ cells $mL^{-1}$), *Prorocentrum minimum* (Dinophyceae; 878 $\mu m^3$; 7.4 x $10^2$ cells $mL^{-1}$), *Nannochloropsis oculata* (Eustigmatophyceae; 40 $\mu m^3$; 1.6 x $10^4$ cells $mL^{-1}$), *Isochrysis galbana* (Haptophyceae; 40 $\mu m^3$;

1.6 x $10^4$ cells $mL^{-1}$), *Heterosigma akashiwo* (Raphidophyceae; 452 $\mu m^3$; 1.4 x $10^3$ cells $mL^{-1}$), *Pavlova lutheri* (Pavlovophyceae; 73 $\mu m^3$; 8.9 x $10^3$ cells $mL^{-1}$) and *Testraselmis suecica* (Prasinophyceae; 300 $\mu m^3$; 2.2 x $10^3$ cells $mL^{-1}$). Details of all target algae are presented in Table 1. The allelopathic effects of pelagophyte filtrate on different strains of *Synechococcus* sp. were assessed by comparing 5.0 x $10^5$ cells $mL^{-1}$ of phycocyanin-containing coastal cyanobacteria (PC cyanobacteria; CCMP1333) and phycoerythrin-containing coastal cyanobacteria (PE cyanobacteria; RCC555) to *Aureoumbra* filtrate (Table 1).

*Variation in allelopathic effects*

To determine the clonal variation in allelopathic effects of pelagophyte filtrate, experiments were conducted with multiple strains of *Aureoumbra* cultures that were isolated from Florida (FL), Cuba (CU), and Texas (TX; Table 1). A model prokaryotic alga (*Synechococcus* sp. (CCMP1333)) and a model eukaryotic alga (*Chaetoceros calcitrans*) were added to filtrate of each *Aureoumbra* strain at a density of 5.0 x $10^5$ cells $mL^{-1}$ and 2.2 x $10^4$ cells $mL^{-1}$, respectively. The density-dependence of allelopathic effects of the pelagophytes on *Synechococcus* were also examined. *Synechococcus* cells (5.0 x $10^5$ cells $mL^{-1}$) were inoculated with varying concentrations of whole *Aureococcus* or *Aureoumbra* cultures that were from the same stock culture at exponential growth; 5.0 x $10^4$, 2.5 x $10^5$, 5.0 x $10^5$, 7.5 x $10^5$, and 1.0 x $10^6$ cells $mL^{-1}$. To determine if allelopathic potency of pelagophytes changed across different growth stages, a large volume (2,500 mL) of *Aureoumbra* stock culture (FL strain) was monitored daily and filtrate for experiments was obtained during early exponential (<0.8 x $10^6$ cells $mL^{-1}$), late exponential (2.5 x $10^6$ cells $m^{-1}$), and stationary growth (3.0 x $10^6$ cells $mL^{-1}$) of *Aureoumbra*. Experiments exploring the potency of these filtrates against *Synechococcus* sp. (CCMP1333) and *C. calcitrans* were performed as described above.

*Direct vs. Indirect contact*

To examine the importance of direct contact in facilitating the allelopathic effects of *Aureococcus* or *Aureoumbra* on other phytoplankton, experiments were conducted by exposing two model species (*Synechococcus* CCMP 1333 and *C. calcitrans*) to *Aureococcus* and *Aureoumbra* cultures and cell-free culture filtrate. A large volume of stock culture of brown tide algae (720 mL; ~2.0 x $10^6$ cells $mL^{-1}$ at exponential growth) was split into two. One culture was used to generate cell-free medium as described above for an indirect contact treatment and the other culture was used for a direct-contact treatment. *C. calcitrans* (2.2 x $10^4$ cells $mL^{-1}$) and *Synechococcus* (5.0 x $10^5$ cells $mL^{-1}$) were inoculated into 50 mL culture tubes that were filled with brown tide culture filtrate and pelagophyte cultures with cells, respectively. Controls of donor and target species were established to assess the growth of each species.

*Characterization of allelochemicals*

Two hydrophobic resins, Diaion® HP20 (SUPELCO, Bellefonte, PA, USA) and HF Bondesil C-18 (VARIAN Inc., Port City, CA, USA), were used determine if the allelochemicals produced by *Aureoumbra* were hydrophobic. Resins were activated according to manufacturer's protocols prior to use and 3 mL of activated resins were loaded into polyethylene columns. Filtrate from a large volume (400 mL) culture of *Aureoumbra* (FL strain) in late exponential phase growth was generated as described above. Filtrate from *Aureoumbra* culture was then passed through columns loaded with each resin by means of a Cole-Palmer peristaltic pump at a rate of 1 mL per minute and filtrate passing through the resins was collected. An experiment was performed where *C. calcitrans* and *Synechococcus* sp. (CCMP1333) were exposed to culture

filtrate, filtrate passed through each resin, and control conditions. Cultures were monitored as described above.

To assess the persistence and stability of allelochemicals over time, *Aureoumbra* filtrate obtained from a culture in late exponential growth was stored under different conditions: with a 1% antibiotic solution at 4 ºC, without antibiotics at 4 ºC, after boiling for an hour and storage of the filtrate at 4°C, with 1% antibiotics at 20 ºC, and without antibiotics at 20 ºC. After two weeks of storage under each condition, the eukaryote model *Chaetoceros calcitrans* was incubated in the filtrate stored under the five conditions using the approaches describe above.

*Allelopathic effects of pelagophyte filtrate on a natural phytoplankton assemblage*

An experiment was performed using water collected from Palm Bay, FL, USA (28.0658º N, -80.5806º W) on March of 2016 and shipped overnight to Southampton, NY, USA. Palm Bay, FL, is part of the Indian River Lagoon system that has been prone to brown tides since 2012 (Gobler et al., 2013; Kang et al., 2015), but had low levels of *Aureoumbra* during collection (<10,000 cells $mL^{-1}$ as determined using a polyclonal antibody; Koch et al., 2014). Similarly, water was collected from Great Peconic Bay, NY, USA (40.9461º N, -72.5022º W) during June of 2016 where *Aureococcus* densities were low (<$10^4$ cells $mL^{-1}$ as determined using a polyclonal antibody; Stauffer et al., 2008). This system had experienced *Aureococcus* blooms 20 years ago (Gobler et al., 2005). To create an additional treatment for the *Aureococcus* experiment, water was collected from the peak of *Aureococcus* bloom in Quantuck Bay, NY, USA (40.8081º N, -72.6203º W) in June 2016 during which *Aureococcus* cell densities were 2 x $10^6$ cells $mL^{-1}$. For experiments, triplicate 125 mL Nalgene PETG bottles were filled with 50% bay water and 50% filtrate (polycarbonate filters with pore size of 0.2 µm) of *Aureoumbra* or *Aureococcus* cultures

for experiments with water from FL and NY, respectively, or filtered *Aureococcus* bloom water. Controls were established by filling another triplicate bottles with 50% bay water and 50% filtered bay water (0.2 µm). Nutrients were added at a GSe media level (+Si) to all bottles ensure nutrient replete conditions. Samples were incubated and monitored for 72 h as described above.

*Measurements and data analysis*

Cell density, *in vivo* fluorescence, and photosynthetic efficiency ($F_v/F_m$) were measured during experiments. Cell density was quantified using a CytoFLEX flow cytometer based on fluorescence patterns and particle size (Collier, 2000). Photosynthetic efficiency 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 (Parkhill et al., 2001). Fluorescence readings were blank corrected using sterile GSe medium. Nutrient concentrations (Nitrate, ammonium, phosphate) at the final time point of all experiments were measured using QuikChem 8500 Series 2 flow-injection autoanalyzer (LACHAT Instruments, Loveland, CO, USA; Parsons, 2013) to ensure nutrient replete conditions were present while performing experiments. Dissolved inorganic nitrogen and phosphorus levels measured during experiments were always > 20 µM and > 2 µM, respectively, significantly above the half saturation constant for these nutrients from most phytoplankton (Smayda, 1997), assuring macronutrient replete growth during experiments. pH was measured during experiments using an Orion3 Star pH meter calibrated according to manufacturer's instructions and levels in control and treatment cultures were not significant different from each other. Growth rates were calculated using the formula $GR = \ln (B_t/B_0)/n$, where GR is the growth rate in each treatment, $B_t$ is cell density at the final, $B_0$ is cell density at initial, n is the

incubation time in days. Inhibition relative to control (%) was calculated using the formula $P = (T_f - C_f)/C_f * 100$, where P is the percent change relative to control (%) in each treatment, $T_f$ is cell density at the final in the treatment, $C_f$ is cell density at the final in control. Inhibition of the growth of competing algae by filtrate that was made from different growth stages of *Aureoumbra* culture was normalized by *Aureoumbra* cell densities (cells $mL^{-1}$) at each growth stage to ensure the effects of growth stage not culture abundance. Differences among levels of *Aureoumbra*, different growth stages, or among species were examined with T-test or One-Way ANOVAs followed by post-hoc Tukey multiple comparison tests.

**Results**

*Aureoumbra allelopathic effects on multiple classes of phytoplankton*

*Aureoumbra lagunensis* had strong allelopathic effects on multiple classes of phytoplankton examined in this study causing cell mortality, reduction in the photosynthetic efficiency ($F_v/F_m$), reductions in growth rate, and cell lysis. Filtrate of *Aureoumbra* cultures significantly inhibited the growth of nine target species, compared to controls (Fig. 2A). *Synechococcus* sp. (CCMP1333) was the most sensitive phytoplankton while *Tetrasalmis suecica*, a chlorophyte, was the least, with reductions in growth for all nine species examined ranging from 5 to 93% (Fig. 2A).

The other brown tide alga, *Aureococcus anophagefferens*, also had strong allelopathic effects on multiple classes of phytoplankton and caused similar morphological and physiological damage to target algae. Filtrate of *Aureococcus* cultures significantly inhibited the growth of seven of nine target species, compared to controls (Fig. 2A). *Synechococcus* sp. (CCMP1333; PC cyanobacteria) was the most sensitive while filtrate had no effect on the raphidophyte,

*Heterosigma akashiwo*, and slightly enhanced the growth of the chlorophyte, *Tetraselmis suecica*, with the inhibition of the seven vulnerable species ranging from 20 to 96% (Fig. 2A).

*Synechococcus* sp. showed a large reduction in photosynthetic efficiency (~50%) and while other algae showed small or no reduction (0 - 20%; Fig. 2B). The photosynthetic efficiency of *Nanochloropsis oculata* and *Pavlova lutheri* decreased significantly 1 h after initiation of experiments but gradually recovered over 24 – 48 h (data not shown). Upon exposure to *Aureoumbra* filtrate, there were substantial morphological changes in target cells that differed among phytoplankton species. Diatoms having a siliceous frustules (e.g. *Chaetoceros calcitrans, Chaetoceros muelleri*) lost their spines while several unarmored species (e.g. *Heterosigma akashiwo, Prorocentrum minimum*) were lysed or burst (Fig. 3). Given their extreme sensitivity to *Aureoumbra, Chatoceros calcitrans* and *Synechococcus* sp. (CCMP1333) were chosen as prokaryotic and eukaryotic model algae, respectively, for further experimentation.

*Variation in allelopathic effects of Aureoumbra – strain, density, growth-stage dependence*

The Cuba, FL, and TX strains of *Aureoumbra* all had strong allelopathic effects on target algae (Fig. 4). Upon exposure to filtrate of the TX strain for 120 h, cell density of the eukaryotic model species, *Chaetoceros* was suppressed by 58 ± 2.6% relative to control cell densities ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 4A) whereas *Chaetoceros* cultures exposed to filtrate of CB and FL strains were suppressed by a significantly greater amount, 82 ± 3.6% and 81 ± 2.4%, respectively ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 3A). In contrast, filtrate of all three *Aureoumbra* strains significantly inhibited the growth of *Synechococcus* sp. with cell densities of all treatments remaining at unchanged over 120 h of incubation time while cell

density of control grew more than six-fold and exceeded 3.0 x $10^6$ cells $mL^{-1}$ ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 4B).

The growth inhibition of *Synechococcus* by *Aureoumbra* was dose-dependent (Fig. 5A). For example, *Synechococcus* grew when *Aureoumbra* cell density was 5.0 x $10^4$ cells $mL^{-1}$, but experienced significant growth inhibition (40%) at 2.5 x $10^5$ cells $mL^{-1}$ and significant and consistent inhibition (60%) at 5.0 x $10^5$ cells $mL^{-1}$ ($p$<0.001; Tukey test; Fig 5A). The growth of *Aureoumbra* was largely unaffected by the presence of *Synechococcus* in the same treatments (data not shown). Similar to *Aureoumbra*, allelopathic effects of *Aureococcus* were dose-dependent. *Synechococcus* inhibition was not detected when *Aureococcus* cell densities were 5.0 x $10^4$ cells $mL^{-1}$, but the inhibition was significant (>20%; $p$<0.05; Tukey test; Fig 5B) at 2.5 x $10^5$ cells $mL^{-1}$ and significantly increased to 60 - 75% at cell densities exceeding 5.0 x $10^5$ cells $mL^{-1}$ ($p$<0.001; Tukey test; Fig 5B). The growth of *Aureococcus* was largely unaffected by the presence of *Synechococcus* in the same treatments (data not shown).

The strength of the allelopathic effects of *Aureoumbra* varied by growth stages. Cell density of *Chaetoceros* declined by 50% when exposed to filtrate from early exponential growth phase of *Aureoumbra* cultures but declined even further during exposure to filtrate from late exponential (63%) and stationary phase (84%; $p$<0.05; One-Way ANOVA; Tukey test; Fig. 6A). Similarly, *Aureoumbra* filtrate from early exponential growth inhibited *Synechococcus* sp. growth by 22%, whereas filtrate from late exponential and stationary growth were significantly stronger, yielding a 75% decline in *Synechococcus* sp. densities ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 6B). However, when the inhibition was normalized by cell density (cells $mL^{-1}$) of *Aureoumbra* cultures at each growth stage, *Chaetoceros* was more sensitive to filtrate from early exponential growth phase of *Aureoumbra* cultures (50%) than later phases (20% for late

exponential phase and 27% for stationary phase; $p<0.001$; One-Way ANOVA; Tukey test; Fig. 6C) while *Synechococcus* sp. was equally suppressed at all growth stages ($p>0.05$; Fig. 6D). These differences highlight the importance of cellular dose when considering the impacts of allelochemicals.

*Direct contact vs. Indirect contact*

Both *Aureoumbra* cells (direct contact) and filtrate (indirect contact) had strong allelopathic effects on *Chaetoceros* and *Synechococcus* (Fig. 7). Growth of *Chaetoceros* was almost completely and equally suppressed by *Aureoumbra* cells and filtrate during the 120 h experiment whereas control cell densities increased by nearly an order of magnitude ($p<0.001$; One-Way ANOVA; Tukey test; Fig. 7A). For *Synechococcus*, growth was significantly inhibited during exposure to *Aureoumbra* filtrate (80%) but direct exposure to cells resulted in a significantly lower final cell abundance, 10-fold decline in cell densities after 120h ($p<0.001$; Tukey test; Fig. 7B).

*Characterization of allelochemicals*

Two hydrophobic resins, HP20 and C18, were able to mitigate allelopathic effects of *Aureoumbra* on *Chaetoceros* and *Synechococcus* sp., although the effectiveness of the resins differed between the target species. The growth of both *Chaetoceros* and *Synechococcus* exposed to *Aureoumbra* filtrate that had passed through the HP20 resin was nearly identical to control treatments ($p>0.05$; Tukey test; Fig. 8A, B). While passing *Aureoumbra* filtrate through C18 resin also eliminated allelopathic effects on *Chaetoceros*, it did not do so for *Synechococcus* as its growth was still significantly reduced compared to the control treatment, even after passage through this resin ($p<0.001$; Tukey test; Fig. 8B).

Aging the *Aureoumbra* filtrate also mitigated its alleopathic effects. *Chaetoceros* cells in filtrate stored at 20°C with or without the addition of antibiotics for two weeks grew faster than cells in GSe medium with cell densities exceeding 3.0 x $10^5$ cells $mL^{-1}$ in these treatments at the end of experiments ($p$<0.05; Tukey test; Fig. 9). In contrast, when *Chaetoceros* was exposed to filtrate that was stored at 4ºC with antibiotics or boiled filtrate that was stored at 4ºC without antibiotics, *Chaetoceros* growth was completely inhibited during the 120 h experiment time ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 9).

*Allelopathic effects of brown tides on natural phytoplankton communities*

*Aureoumbra* filtrate was capable of suppressing the growth of wild populations of prokaryotic and eukaryotic phytoplankton from Indian River Lagoon, FL. The growth of PC cyanobacteria and PE cyanobacteria were reduced by 49 ± 11% ($p$<0.05; T-test) and 71 ± 3.6% ($p$<0.001; T-test) relative to the control treatment (Fig. 10A). Similarly, the growth rate of picoeukarytoes growing in *Aureoumbra* filtrate was reduced by 35 ± 4.7% ($p$<0.05; T-test; Fig. 10) whereas the growth rate of larger eukaryotes (>2 µm) were unaffected by the *Aureoumbra* filtrate (Fig. 10A).

Both *Aureococcus* culture filtrate and *Aureococcus* bloom water filtrate suppressed the growth of a natural phytoplankton community from Great Peconic Bay, NY. The growth of PE cyanobacteria was reduced by 82 ± 2.6% when exposed to *Aureococcus* culture filtrate and 92 ± 0.6% when exposed to *Aureococcus* bloom water filtrate compared to controls ($p$<0.001; Tukey tests; Fig. 10B). The growth of picoeukaryotes was inhibited by 54 ± 5.2% by *Aureococcus* culture filtrate ($p$<0.05; T-test) and by 65 ± 13% ($p$<0.001; Tukey tests) in *Aureococcus* bloom filtrate compared to controls (Fig. 10B).

## Discussion

This study describes the ability of brown tide algae, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, to allelopathically inhibit the growth of co-occurring phytoplankton. The allelopathic effects of brown tide algae were not species-specific, but rather broadly inhibited the growth of multiple classes of phytoplankton with inhibition of target algae being dependent on the dose, strain, and, to a lesser extent, the growth stage of the pelagophyte. This study represents the first report of allelochemicals in pelagophytes and, collectively, these findings provide novel insight regarding the ability of brown tides to extend bloom duration.

### *Inhibition of co-existing phytoplankton growth by allelopathic ability of brown tide algae*

#### Allelopathic effects of brown tide algae on multiple phytoplankton

*Aureococcus and Aureoumbra* were capable of inhibiting the growth of multiple phytoplankton strains and species belonging to different classes. Noticeable reductions in photosynthetic efficiency by *Aureoumbra* filtrate were also observed across several species including *Nanochloropsis oculata* and *Synechococcus* sp. suggesting that in some species allelochemicals were inhibiting the photosystems of target algae (Parkhill et al., 2001). Consistent with our findings, Prince et al. (2008) showed the photosynthetic efficiency of a target dinoflagellate, *Akashiwo sanguinea*, was significantly reduced after exposure to allelopathic extracts from the toxic dinoflagellate, *Karenia brevis*, although growth inhibition was not as strong as the photosynthetic efficiency inhibition in this case. Besides the growth and photosynthetic responses, morphological changes in target algae included the loss of spines for diatoms (e.g. *Chaetoceros calcitrans* and *Chaetoceros muelleri*) while some unarmored flagellates (e.g. *Heterosigma akashiwo* and *Prorocentrum minimum*) were lysed or burst,

occurrences consistent with prior investigations of the allelopathic effects of harmful algae (Gross, 2003; Granéli and Hansen, 2006).

Variation in allelopathic effects

Clonal variation in allelopathic effects of harmful algae is well known (Tillmann 2009, Hattenrath and Gobler 2011). For example, Hattenrath-Lehmann and Gobler (2011b) demonstrated clonal variation in lytic activity among *Alexandrium fundyense* strains isolated from New York (NY), Connecticut (CT), the Gulf of Maine, and the Bay of Fundy, with NY strains being the most potent and CT strain being the least. Tillman et al. (2009) demonstrated there can be substantial variation in allelopathic intensity among European strains of *Alexandrium* and that this variation occurs independent of the saxitoxin content of strains. In this study, *Synechococcus* sp. (CCMP1333) was vulnerable to all strains of *Aureoumbra* (FL, CB, and TX) whereas *Chaetoceros calcitrans* was less vulnerable to the TX strain compared to other strains (FL and CB). While the possibility of geographic or simple strain variation cannot be neglected (Tillmann et al., 2009; Hattenrath-Lehmann and Gobler, 2011b), previous studies of *Aureoumbra* resting cells demonstrated that TX strain transitioned more slowly from resting cells to vegetative cells than other strains (Kang et al., in press). That study concluded that newly established cultures (e.g. CB, FL, 2012-2013) may still exhibit phenotypic characteristics of cells that were first isolated whereas a strain isolated decades ago such as TX may have experienced genetic mutation or down-regulation of traits (Lakeman et al., 2009), thus, declining ecological fitness (in this case, allelopathic strength) relative to recently established cultures of CB and FL strains. Comparably, in the study of effects of *Aureococcus* isolates on bivalve feeding, Bricelj et al. (2001) have demonstrated that more recent isolates (e.g. CCMP1707 and CCMP1708 isolated in 1995) of *Aureococcus* were more potent and caused two orders of magnitude

reduction in clearance rates of juvenile mussels compared to control while an isolate which was established in 1986 had no effects on juvenile mussels. The reduction in the allelopathic potency of the TX strain suggests that the synthesis of allelochemicals may be dependent upon recent environmental cues such as exposure to other algae, that there is a physiological cost to the synthesis of these allelochemicals, and that *Aureoumbra* benefits from downregulating their production.

The lytic activity of pelagophyte cells was dose-dependent. *Aureococcus* and *Aureoumbra* cultures with cell densities above 2.5 x $10^5$ cells $mL^{-1}$ caused a high mortality (~60%) of *Synechococcus* sp. (CCMP1333) whereas the cultures with cell density at 5.0 x $10^4$ cells $mL^{-1}$ slightly enhanced the growth of *Synechococcus* sp. indicating a threshold level of allelochemicals is needed to inhibit competing algae. These findings are consistent with previous studies that have shown density-dependent effects of allelopathy by harmful algae (Poulson et al., 2010; Tang and Gobler, 2010; Hattenrath-Lehmann and Gobler, 2011b) and consistent with prior zooplankton grazer inhibition studies that have also shown zooplankton survival is reduced at *Aureoumbra* cell densities exceeding 2.5 x $10^5$ cells $mL^{-1}$ (Liu and Buskey, 2000a). Given that brown tides peak at cell densities of > $10^6$ cells $mL^{-1}$, these findings suggest allelochemicals will not affect the earlier stages of bloom initiation when cell densities increase from background to $10^5$ cells $mL^{-1}$ (Jonsson et al., 2009) but may play a key role in facilitating bloom intensification and persistence, which is noteworthy as brown tide can persist for months to years (Buskey 1997; Gobler and Sunda, 2012).

The role of allelopathy in brown tide persistence but not initiation is further supported by our comparison of cultures in different growth stages. Target algae were more vulnerable to the filtrate from cultures at late exponential or stationary stages that areconditions to peak or near

peak of a brown tide, relative to early exponential stage growth, conditions more similar bloom initiation. These findings additionally affirm that the degree of allelopathic potency depends on cell density and type of target algae. Once cell density was normalized, the growth stage differences were not apparent in *Synechococcus* sp. but *Chaetoceros calcitrans*.

Response of different types of *Synechococcus* sp. to brown tide allelopathy

Brown tides are known to co-exist and/or compete with picocyanobacteria (i.e. *Synechococcus* sp.) and are often succeeded by picocyanobacteria blooms (Sieracki et al., 2004; Kang et al., 2015). *Synechococcus* sp. was the more sensitive to brown tide allelochemicals than any other species of phytoplankton and wild populations of cyanobacteria were more vulnerable to pelagophyte allelochemicals than wild eukaryotic algae. These findings demonstrate allelopathy is most effective against the most common competitor of coastal pelagophytes and that post-brown tide cyanobacteria blooms may be the result of the alleviation of allelopathic inhibition.

There were differences in the vulnerability of *Synechococcus* strains to allelochemicals with a PC strain (CMMP1333) being more inhibited by brown tide culture filtrate than a PE strain (RC555) during laboratory experiments whereas among field populations, the PE cyanobacteria (likely comprised of multiple strains) growth was more inhibited compared to PC cyanobacteria. Prior work in FL lagoons indicated that PC cyanobacteria are indigenous and can form dense blooms whereas PE cyanobacteria belong to a clade comprised of individuals originating from open ocean regions (Berry et al., 2015), that there is more grazing pressure on PE cyanobacteria than PC cyanobacteria (Kang et al., 2015), and that PC cyanobacteria exist at higher levels than PE cyanobacteria during intense blooms (Kang et al., 2015). Collectively,

these findings suggest that allelopathic compounds released by pelagophytes and grazing pressure by zooplankton may have a synergistic effect on controlling abundance of PE cyanobacteria while abundance of PC cyanobacteria may be more controlled by allelopathic effects of pelagophytes and thus are slightly more competitive with brown tides.

*Characterization of allelochemicals*

In this study, both direct contact (whole cells) and indirect contact (filtrate of culture) with *Aureoumbra* yielded dramatic growth inhibition of *Chaetoceros calcitrans* and *Synechococcus* sp. (CCMP1333), implying that the agents that cause mortality of target cells are largely dissolved allelochemicals and not related to cell surfaces. Given that passing *Aureoumbra* filtrate through the hydrophobic resins (e.g. HP20, C18) nearly eliminated the allelopahtic effects on target species, the extracellular substances exuded by *Aureoumbra* were likely hydrophobic.

There were several lines of evidence to support the idea that *Aureoumbra* may produce multiple classes of allelochemicals. For example, the HP20 resin eliminated allelopathic effects on both *Synechococcus* and *Chaetoceros* but C18 resin did eliminate the effects for *Chaetoceros* but not *Synechococcus*. Further evidence of differential responses between this prokaryote and eukaryote came from the ability of direct contact with *Aureoumbra* to more strongly inhibit *Synechococcus* than filtrate of the same culture but there being no difference of filtrate or cell contact for *Chaetoceros*. Furthermore, early exponential phase filtrate was more inhibitory to *Chaetoceros* than late exponential and stationary phase filtrate, but the three types of filtrate were equally powerful against *Synechococcus*. *Synechococcus* experienced a large and prolonged reduction in the photosynthetic efficiency when exposed to allelochemicals, while

*Chaetoceros* and other eukaryotes did not. Collectively, these findings suggest that brown tide pelagophytes may produce multiple classes of allelochemicals that target different types of algae (Poulson et al., 2010) and/or are differentially potent against prokaryotic and eukaryotic algae.

Prior studies have striven to characterize and identify allelochemicals in different phytoplankton species (Ma et al., 2009; Yamasaki et al., 2009; Prince et al., 2010; Ma et al., 2011). Yamasaki et al. (2009) determined that allelochemicals from *Heterosigma akashiwo* were extracellular polysaccharide-protein complexes and demonstrated these compounds bind to target cell membranes with allelochemical-specific antibodies. Both brown tide species produce copious extracellular polysaccharides (DeYoe et al., 1997) and excrete more of these compounds when cells encounter stressful conditions (Liu and Buskey, 2000b, 2003). Allelochemicals produced by *Aureoumbra*, however, were heat-stable, losing no potency after boiling whereas extracellular polysaccharides are heat sensitive and high temperature can cause them to decrease in viscosity and molecular weight (Rederstorff et al., 2011). These findings indicate that allelochemicals may not be extracellular polysaccharides. The loss of allelochemical potency after two weeks at 20°C indicates that continual synthesis of allelochemicals is necessary to inhibit the target cell's growth. While antibiotics did not slow the degradation of the allelochemicals at 20°C, they prohibited some loss at 4°C suggesting that some of the allelochemical degradation was microbially-facilitated and that bacteria likely contributed to degradation of allelochemicals at 20°C since antibiotics degrade with time and do not inhibit all bacteria. Finally, the cell density of *Chaetoceros* grown in *Aureoumbra* filtrate that was stored at 20 ºC for two weeks was higher than the cell density in GSe medium, indicating that the degradation of allelochemical compounds may provide nutrient elements (e.g. carbon or nitrogen) and can eventually promote the growth of co-occurring algae.

*Ecological implication*

Brown tides have expanded across North America, Africa, and Asia during the past twenty years (Probyn et al., 2001; Zhang et al., 2012; Gobler et al., 2013b). In addition, once brown tides occur, they tend to recur annually and persist for longer than most algal blooms, often for months or even years (Buskey et al., 1997; Gobler and Sunda, 2012). The ability of brown tides to endure poor growth conditions and reform blooms annually has recently been attributed to their ability to form resting cells (Kang et al. in press). Other mechanisms have been also proposed to account for the extended duration of brown tides including resisting zooplankton grazing (Gobler et al., 2002b; Kang et al., 2015), maximal growth at low light levels (MacIntyre et al., 2004; Sunda and Hardison, 2007), and the ability to dominate algal assemblages when inorganic nutrient levels are low but organic nutrient levels are high (Berg et al., 2002; Mulholland et al., 2002; Muhlstein and Villareal, 2007). This study indicates that chemically-mediated interactions between brown tide pelagophytes and competing phytoplankton are likely a central, yet previously unrecognized mechanism that facilitates bloom persistence on an annual basis. Given the dose-dependent nature of pelagophyte allelochemicals, allelopathy may not promote bloom initiation, but likely becomes a prime mechanism supporting the intensification of blooms from $10^5$ to $10^6$ cell $mL^{-1}$, a typical peak cell density of brown tides.

Previously, brown tides have been hypothesized to be promoted by positive feedback whereby increasing cell densities suppress grazing of brown tide cells and the regeneration of inorganic nutrients, further favoring the dominance of brown tides (Sunda et al., 2006). The findings of this study both work within and expand the framework of positive feedback and the intensification of brown tides. Considering cells may lyse and burst upon exposure to brown tide allelochemicals, intra-cellular nutrients and organic matter that leaks through weakened

membranes or lysed cells of target algae may be rapidly incorporated into healthy brown tide cells during intense blooms, allowing more allelochemicals to be made and thus further suppressing competing algae. The continuance of this process with newly imported cells into an ecosystem via estuarine circulation may allow these blooms to persist for months in the case of *Aureococcus* which eventually ceases to bloom due to high summer temperature (Gobler et al., 2005) or years in the case of *Aureoumbra*, which is well adapted to high temperatures (Buskey et al., 1997; Sunda and Hardison, 2007).

Reciprocally, given that allelochemicals were dependent on cell density of pelagophytes and degraded allelochemicals promoted the growth of target cells, as brown tides decline, the degradation process may enable competing algae to be both released from allelochemical suppression and to access regenerated nutrients from allelochemicals. As such, allelopathy seems likely to be a centrally important process in facilitating the occurrence and persistence of intense and extended brown tides, as well as the ecological succession of phytoplankton following the end of these blooms.

## References


Berg, G.M., Glibert, P.M., Lomas, M.W., Burford, M.A., 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. Mar. Biol. 129 (2), 377-387.

Berg, G.M., Repeta, D.J., Laroche, J., 2002. Dissolved organic nitrogen hydrolysis rates in axenic cultures of Aureococcus anophagefferens (Pelagophyceae): Comparison with heterotrophic bacteria. Appl. Environ. Microbiol. 68 (1), 401-404.

Bricelj, V.M., MacQuarrie, S.P., Schaffner, R.A., 2001. Differential effects of Aureococcus anophagefferens isolates ("brown tide") in unialgal and mixed suspensions on bivalve feeding. Mar. Biol. 139 (4), 605-615.

Buskey, E.J., Liu, H.B., Collumb, C., Bersano, J.G.F., 2001. The decline and recovery of a persistent Texas brown tide algal bloom in the Laguna Madre (Texas, USA). Estuaries 24 (3), 337-346.

Buskey, E.J., Montagna, P.A., Amos, A.F., Whitledge, T.E., 1997. Disruption of grazer populations as a contributing factor to the initiation of the Texas brown tide algal bloom. Limnol. Oceanogr. 42 (5), 1215-1222.

Caron, D.A., Gobler, C.J., Lonsdale, D.J., Cerrato, R.M., Schaffner, R.A., Rose, J.M., Buck, N.J., Taylor, G., Boissonneault, K.R., Mehran, R., 2004. Microbial herbivory on the brown tide alga, *Aureococcus anophagefferens*: results from natural ecosystems, mesocosms and laboratory experiments. Harmful Algae 3 (4), 439-457.

Collier, J.L., 2000. Flow cytometry and the single cell in phycology. J. Phycol. 36 (4), 628-644.

Cosper, E.M., Dennison, W.C., Carpenter, E.J., Bricelj, V.M., Mitchell, J.G., Kuenstner, S.H., Colflesh, D., Dewey, M., 1987. Recurrent and Persistent Brown Tide Blooms Perturb Coastal Marine Ecosystem. Estuaries 10 (4), 284-290.

DeYoe, H.R., Stockwell, D.A., Bidigare, R.R., Latasa, M., Johnson, P.W., Hargraves, P.E., Suttle, C.A., 1997. Description and characterization of the algal species *Aureoumbra lagunensis* gen. et sp. nov. and referral of *Aureoumbra* and *Aureococcus* to the Pelagophyceae. J. Phycol. 33 (6), 1042-1048.

Doblin, M.A., Blackburn, S.I., Hallegraeff, G.M., 1999. Comparative study of selenium requirements of three phytoplankton species: Gymnodinium catenatum, Alexandrium minutum (Dinophyta) and Chaetoceros cf. tenuissimus (Bacillariophyta). J. Plankton Res. 21 (6), 1153-1169.

Fernández-Herrera, L.J., Band-Schmidt, C.J., López-Cortés, D.J., Hernández-Guerrero, C.J., Bustillos-Guzmán, J.J., Núñez-Vázquez, E., 2016. Allelopathic effect of Chattonella marina var. marina (Raphidophyceae) on Gymnodinium catenatum (Dinophycea). Harmful Algae 51, 1-9.

Figueredo, C.C., Giani, A., Bird, D.F., 2007. Does allelopathy contribute to *Cylindrospermopsis raciborskii* (Cyanobacteria) bloom occurrence and geographic expansion? J. Phycol. 43 (2), 256-265.

Fistarol, G.O., Legrand, C., Granéli, E., 2003. Allelopathic effect of *Prymnesium parvum* on a natural plankton community. Mar. Ecol. Prog. Ser. 255, 115-125.

Fistarol, G.O., Legrand, C., Rengefors, K., Graneli, E., 2004a. Temporary cyst formation in phytoplankton: a response to allelopathic competitors? Environ. Microbiol. 6 (8), 791-798.

Fistarol, G.O., Legrand, C., Selander, E., Hummert, C., Stolte, W., Granéli, E., 2004b. Allelopathy in Alexandrium spp.: effect on a natural plankton community and on algal monocultures. Aquat. Microb. Ecol. 35 (1), 45-56.

Gobler, C.J., Koch, F., Kang, Y., Berry, D.L., Tang, Y.Z., Lasi, M., Walters, L., Hall, L., Miller, J.D., 2013. Expansion of harmful brown tides caused by the pelagophyte, *Aureoumbra lagunensis* DeYoe et Stockwell, to the US East Coast. Harmful Algae 27, 29-41.

Gobler, C.J., Lonsdale, D.J., Boyer, G.L., 2005. A review of the causes, effects, and potential management of harmful brown tide blooms caused by *Aureococcus anophagefferens* (Hargraves et Sieburth). Estuaries 28 (5), 726-749.

Gobler, C.J., Renaghan, M.J., Buck, N.J., 2002. Impacts of nutrients and grazing mortality on the abundance of *Aureococcus anophagefferens* during a New York brown tide bloom. Limnol. Oceanogr. 47 (1), 129-141.

Gobler, C.J., Sunda, W.G., 2012. Ecosystem disruptive algal blooms of the brown tide species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*. Harmful Algae 14, 36-45.

Granéli, E., Hansen, P.J., 2006. Allelopathy in harmful algae: a mechanism to compete for resources?, Ecology of harmful algae. Springer, pp. 189-201.

Gross, E.M., 2003. Allelopathy of aquatic autotrophs. Crit. Rev. Plant Sci. 22 (3-4), 313-339.

Hattenrath-Lehmann, T.K., Gobler, C.J., 2011. Allelopathic inhibition of competing phytoplankton by North American strains of the toxic dinoflagellate, *Alexandrium fundyense*: Evidence from field experiments, laboratory experiments, and bloom events. Harmful Algae 11 (0), 106-116.

Igarashi, T., Satake, M., Yasumoto, T., 1996. Prymnesin-2: a potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. J. Am. Chem. Soc. 118 (2), 479-480.

Jonsson, P.R., Pavia, H., Toth, G., 2009. Formation of harmful algal blooms cannot be explained by allelopathic interactions. Proc. Natl. Acad. Sci. 106 (27), 11177-11182.

Kang, Y., Koch, F., Gobler, C.J., 2015. The interactive roles of nutrient loading and zooplankton grazing in facilitating the expansion of harmful algal blooms caused by the pelagophyte, Aureoumbra lagunensis, to the Indian River Lagoon, FL, USA. Harmful Algae 49, 162-173.

Koch, F., Kang, Y., Villareal, T.A., Anderson, D.M., Gobler, C.J., 2014. Novel immunofluorescence flow cytometry technique detects the expansion of harmful brown tides caused by *Aureoumbra lagunensis* to the Caribbean Sea. Appl. Environ. Microbiol. 80 (16), 4947-4957.

Kubanek, J., Naar, J., Myers, T.L., Prince, E.K., 2008. Competing phytoplankton undermines allelopathy of a bloom-forming dinoflagellate. Proceedings of the Royal Society B: Biological Sciences 275 (1652), 2733-2741.

Lakeman, M.B., von Dassow, P., Cattolico, R.A., 2009. The strain concept in phytoplankton ecology. Harmful Algae 8 (5), 746-758.

Leflaive, J., Ten-Hage, L., 2007. Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. Freshwat. Biol. 52 (2), 199-214.

Legrand, C., Rengefors, K., Fistarol, G.O., Graneli, E., 2003. Allelopathy in phytoplankton-biochemical, ecological and evolutionary aspects. Phycologia 42 (4), 406-419.

Lim, A.S., Jeong, H.J., Jang, T.Y., Jang, S.H., Franks, P.J.S., 2014. Inhibition of growth rate and swimming speed of the harmful dinoflagellate *Cochlodinium polykrikoides* by diatoms: Implications for red tide formation. Harmful Algae 37, 53-61.

Liu, H.B., Buskey, E.J., 2000a. The exopolymer secretions (EPS) layer surrounding *Aureoumbra lagunensis* cells affects growth, grazing, and behavior of protozoa. Limnol. Oceanogr. 45 (5), 1187-1191.

Liu, H.B., Buskey, E.J., 2000b. Hypersalinity enhances the production of extracellular polymeric substance (EPS) in the Texas brown tide alga, *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 36 (1), 71-77.

Liu, H.B., Buskey, E.J., 2003. Effects of media N: P ratio on extracellular polymeric substances (EPS) production of the brown tide-forming alga, *Aureoumbra lagunensis*. Plankton Biol. Ecol. 50 (2), 55-60.

Lonsdale, D.J., Cosper, E.M., Kim, W.S., Doall, M., Divadeenam, A., Jonasdottir, S.H., 1996. Food web interactions in the plankton of Long Island bays, with preliminary observations on brown tide effects. Marine Ecology-Progress Series 134 (1-3), 247-263.

Ma, H., Krock, B., Tillmann, U., Cembella, A., 2009. Preliminary characterization of extracellular allelochemicals of the toxic marine dinoflagellate Alexandrium tamarense using a Rhodomonas salina bioassay. Mar. Drugs 7 (4), 497-522.

Ma, H., Krock, B., Tillmann, U., Muck, A., Wielsch, N., Svatoš, A., Cembella, A., 2011. Isolation of activity and partial characterization of large non-proteinaceous lytic allelochemicals produced by the marine dinoflagellate *Alexandrium tamarense*. Harmful Algae 11, 65-72.

MacIntyre, H.L., Lomas, M.W., Cornwell, J., Suggett, D.J., Gobler, C.J., Koch, E.W., Kana, T.M., 2004. Mediation of benthic-pelagic coupling by microphytobenthos: an energy- and material-based model for initiation of blooms of *Aureococcus anophagefferens*. Harmful Algae 3 (4), 403-437.

Montagna, P.A., Stockwell, D.A., Kalke, R.D., 1993. Dwarf surfclam *Mulinia lateralis* (Say, 1822) populations and feeding during the Texas brown tide event J. Shellfish Res. 12 (2), 433-442.

Muhlstein, H.I., Villareal, T.A., 2007. Organic and inorganic nutrient effects on growth rate-irradiance relationships in the Texas brown-tide alga *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 43 (6), 1223-1226.

Mulholland, M.R., Gobler, C.J., Lee, C., 2002. Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. Limnol. Oceanogr. 47 (4), 1094-1108.

Newell, R.I.E., Tettelbach, S.T., Gobler, C.J., Kimmel, D.G., 2009. Relationships between reproduction in suspension-feeding hard clams Mercenaria mercenaria and phytoplankton community structure. Mar. Ecol. Prog. Ser. 387, 179-196.

Onuf, C.P., 1996. Seagrass responses to long-term light reduction by brown tide in upper Laguna Madre, Texas: Distribution and biomass patterns. Mar. Ecol. Prog. Ser. 138 (1-3), 219-231.

Parkhill, J.P., Maillet, G., Cullen, J.J., 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. 37 (4), 517-529.

Pavia, H., Baumgartner, F., Cervin, G., Enge, S., Kubanek, J., Nylund, G.M., Selander, E., Svensson, J.R., Toth, G.B., 2012. Chemical defences against herbivores. Chemical ecology in aquatic systems. Oxford University Press, New York, New York, USA, 210-235.

Poulson-Ellestad, K.L., Jones, C.M., Roy, J., Viant, M.R., Fernández, F.M., Kubanek, J., Nunn, B.L., 2014. Metabolomics and proteomics reveal impacts of chemically mediated competition on marine plankton. Proc. Natl. Acad. Sci. 111 (24), 9009-9014.

Poulson, K.L., Sieg, R., Prince, E.K., Kubanek, J., 2010. Allelopathic compounds of a red tide dinoflagellate have species-specific and context-dependent impacts on phytoplankton. Marine Ecology, Progress Series 416, 69-78.

Prince, E.K., Myers, T.L., Kubanek, J., 2008. Effects of harmful algal blooms on competitors: allelopathic mechanisms of the red tide dinoflagellate Karenia brevis. Limnol. Oceanogr. 53 (2), 531-541.

Prince, E.K., Poulson, K.L., Myers, T.L., Sieg, R.D., Kubanek, J., 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. Harmful Algae 10 (1), 39-48.

Probyn, T., Pitcher, G., Pienaar, R., Nuzzi, R., 2001. Brown tides and mariculture in Saldanha Bay, South Africa. Mar. Pollut. Bull. 42 (5), 405-408.

Probyn, T.A., Bernard, S., Pitcher, G.C., Pienaar, R.N., 2010. Ecophysiological studies on Aureococcus anophagefferens blooms in Saldanha Bay, South Africa. Harmful Algae 9 (2), 123-133.

Rederstorff, E., Fatimi, A., Sinquin, C., Ratiskol, J., Merceron, C., Vinatier, C., Weiss, P., Colliec-Jouault, S., 2011. Sterilization of exopolysaccharides produced by deep-sea bacteria: Impact on their stability and degradation. Mar. Drugs 9 (2), 224-241.

Rengefors, K., Legrand, C., 2001. Toxicity in *Peridinium aciculiferum*—an adaptive strategy to outcompete other winter phytoplankton? Limnol. Oceanogr. 46 (8), 1990-1997.

Rice, E.L., 2013. Allelopathy. Academic Press.

Schmidt, L.E., Hansen, P.J., 2001. Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: effect of cell concentration, growth phase and pH. Mar. Ecol. Prog. Ser. 216, 67-81.

Selander, E., Kubanek, J., Hamberg, M., Andersson, M.X., Cervin, G., Pavia, H., 2015. Predator lipids induce paralytic shellfish toxins in bloom-forming algae. Proc. Natl. Acad. Sci. 112 (20), 6395-6400.

Sieracki, M.E., Gobler, C.J., Cucci, T.L., Thier, E.C., Gilg, I.C., Keller, M.D., 2004. Pico- and nanoplankton dynamics during bloom initiation of *Aureococcus* in a Long Island, NY bay. Harmful Algae 3 (4), 459-470.

Smayda, T.J., 1997. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnol. Oceanogr. 42 (5), 1137-1153.

Smith, J.K., Lonsdale, D.J., Gobler, C.J., Caron, D.A., 2008. Feeding behavior and development of Acartia tonsa nauplii on the brown tide alga Aureococcus anophagefferens. J. Plankton Res. 30 (8), 937-950.

Stauffer, B.A., Schaffner, R.A., Wazniak, C., Caron, D.A., 2008. Immunofluorescence flow cytometry technique for enumeration of the brown-tide alga, *Aureococcus anophagefferens*. Appl. Environ. Microbiol. 74 (22), 6931-6940.

Suikkanen, S., Fistarol, G.O., Granéli, E., 2005. Effects of cyanobacterial allelochemicals on a natural plankton community. Mar. Ecol. Prog. Ser. 287, 1-9.

Sunda, W.G., Graneli, E., Gobler, C.J., 2006. Positive feedback and the development and persistence of ecosystem disruptive algal blooms. J. Phycol. 42 (5), 963-974.

Sunda, W.G., Hardison, D.R., 2007. Ammonium uptake and growth limitation in marine phytoplankton. Limnol. Oceanogr. 52 (6), 2496-2506.

Tang, Y.Z., Gobler, C.J., 2010. Allelopathic effects of Cochlodinium polykrikoides isolates and blooms from the estuaries of Long Island, New York, on co-occurring phytoplankton. Mar. Ecol. Prog. Ser. 406, 19-31.

Tang, Y.Z., Gobler, C.J., 2011. The green macroalga, Ulva lactuca, inhibits the growth of seven common harmful algal bloom species via allelopathy. Harmful Algae 10 (5), 480-488.

Tang, Y.Z., Kang, Y., Berry, D., Gobler, C.J., 2014. The ability of the red macroalga, Porphyra purpurea (Rhodophyceae) to inhibit the proliferation of seven common harmful microalgae. J. Appl. Phycol. 27, 531-544.

Tillmann, U., Alpermann, T.L., da Purificacao, R.C., Krock, B., Cembella, A., 2009. Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. Harmful Algae 8 (5), 759-769.

Tillmann, U., John, U., 2002. Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: an allelochemical defence mechanism independent of PSP-toxin content. Mar. Ecol. Prog. Ser. 230, 47-58.

Tillmann, U., John, U., Cembella, A., 2007. On the allelochemical potency of the marine dinoflagellate *Alexandrium ostenfeldii* against heterotrophic and autotrophic protists. J. Plankton Res. 29 (6), 527-543.

Tilman, D., 1977. Resource competition between plankton algae: an experimental and theoretical approach. Ecology 58 (2), 338-348.

Van de Waal, D.B., Tillmann, U., Martens, H., Krock, B., van Scheppingen, Y., John, U., 2015. Characterization of multiple isolates from an *Alexandrium ostenfeldii* bloom in The Netherlands. Harmful Algae 49, 94-104.

Ward, L.A., Montagna, P.A., Kalke, R.D., Buskey, E.J., 2000. Sublethal effects of Texas brown tide on *Streblospio benedicti* (Polychaeta) larvae. J. Exp. Mar. Biol. Ecol. 248 (1), 121-129.

Xu, N., Tang, Y.Z., Qin, J., Duan, S., Gobler, C.J., 2015. Ability of the marine diatoms Pseudo-nitzschia multiseries and P. pungens to inhibit the growth of co-occurring phytoplankton via allelopathy. Aquat. Microb. Ecol. 74, 29-41.

Yamasaki, Y., Nagasoe, S., Matsubara, T., Shikata, T., Shimasaki, Y., Oshima, Y., Honjo, T., 2007. Allelopathic interactions between the bacillariophyte *Skeletonema costatum* and the raphidophyte *Heterosigma akashiwo*. Mar. Ecol. Prog. Ser. 339, 83.

Yamasaki, Y., Shikata, T., Nukata, A., Ichiki, S., Nagasoe, S., Matsubara, T., Shimasaki, Y., Nakao, M., Yamaguchi, K., Oshima, Y., 2009. Extracellular polysaccharide-protein complexes of a harmful alga mediate the allelopathic control it exerts within the phytoplankton community. The ISME journal 3 (7), 808-817.

Zhang, Q.C., Qiu, L.M., Yu, R.C., Kong, F.Z., Wang, Y.F., Yan, T., Gobler, C.J., Zhou, M.J., 2012. Emergence of brown tides caused by *Aureococcus anophagefferens* Hargraves et Sieburth in China. Harmful Algae 19, 117-124.

Zheng, J.-W., Li, D.-W., Lu, Y., Chen, J., Liang, J.-J., Zhang, L., Yang, W.-D., Liu, J.-S., Lu, S.-H., Li, H.-Y., 2016. Molecular exploration of algal interaction between the diatom *Phaeodactylum tricornutum* and the dinoflagellate *Alexandrium tamarense*. Algal Research 17, 132-141.

**Table 1** Information of cultures examined in this study.

| Type | Class | Species | Clone/Strain | Origin | Cell volume ($\mu m^3$) |
|---|---|---|---|---|---|
| Donor | Pelagophyceae | *Aureoumbra lagunensis* | TX (CCMP1510) | Laguna Madre, TX, USA | 7.43 |
| | | *Aureoumbra lagunensis* | CB | Guantanamo bay, Cuba | 7.43 |
| | | *Aureoumbra lagunensis* | FL | Indian River Lagoon, FL, USA | 7.43 |
| | | *Aureococcus anophagefferens* | CCMP1984 | Great South Bay, NY, USA | 5.58 |
| Target | Bacillariophyceae | *Chaetoceros calcitrans* | - | - | 30 |
| | | *Chaetoceros muelleri* | - | - | 118 |
| | Cyanobacteria | *Synechococcus* sp. | CCMP1333 | Long Island Sound, CT, USA | 1.3 |
| | | *Synechococcus* sp. | RCC555 | Gulf of Aqaba, Red Sea | 1.3 |
| | Dinophyceae | *Prorocentrum minimum* | CCMP696 | East Messapequa, NY, USA | 878 |
| | Eustigmatophyceae | *Nannochloropsis oculata* | NO1NY | - | 40 |
| | Haptophyceae | *Isochrysis galbana* | IG1NY | - | 40 |
| | Raphidophyceae | *Heterosigma akashiwo* | HA2 | Hemsted Bay, NY, USA | 452 |
| | Pavlovophyceae | *Pavlova lutheri* | PL1NY | - | 73 |
| | Prasinophyceae | *Tetraselmis suecica* | TS1NY | - | 300 |

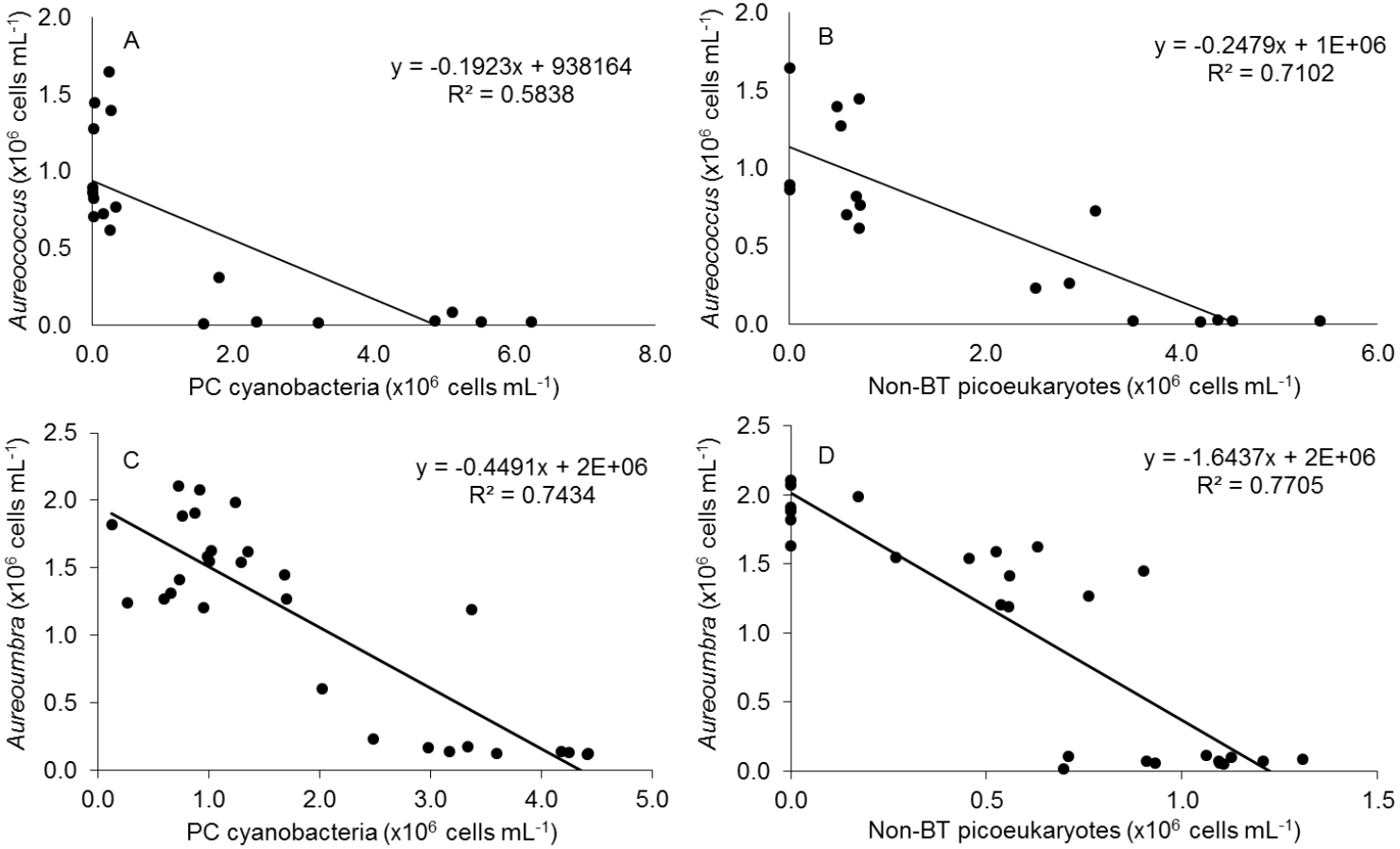



**Figure 1.** (A) Regression of cell density of *Aureococcus anophagefferens* and phycocyanin-containing (PC) cyanobacteria, and (B) *Aureococcus* and non-BT picoeukayrotes during brown tides in Quantuck Bay, NY and (C) *Aureoumbra lagunensis* and PC cyanobacteria and (D) *Aureoumbra* and non-BT picoeukaryotes during brown tides in Indian River Lagoon, FL.

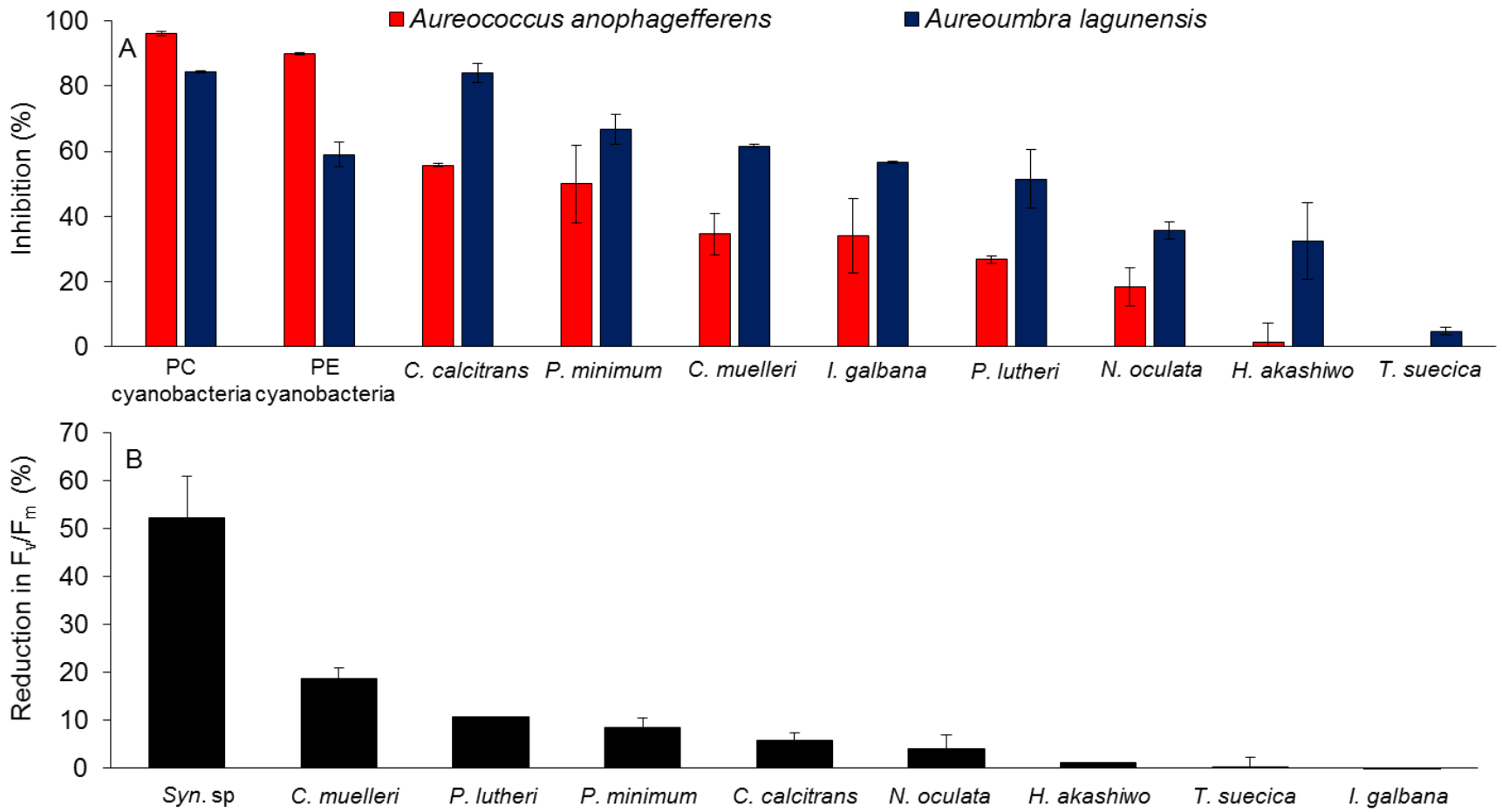



**Figure 2.** (A) Reduction in the photosynthetic efficiency ($F_v/F_m$) of multiple classes of phytoplankton after exposure to *Aureoumbra* filtrate for 120 h and (B) inhibition in the growth of competing micro algae after exposure to *Aureococcus* and *Aureoumbra* filtrate for 120 h. reduction in $F_v/F_m$ and inhibition represent percentage of change in treatment relative to control.

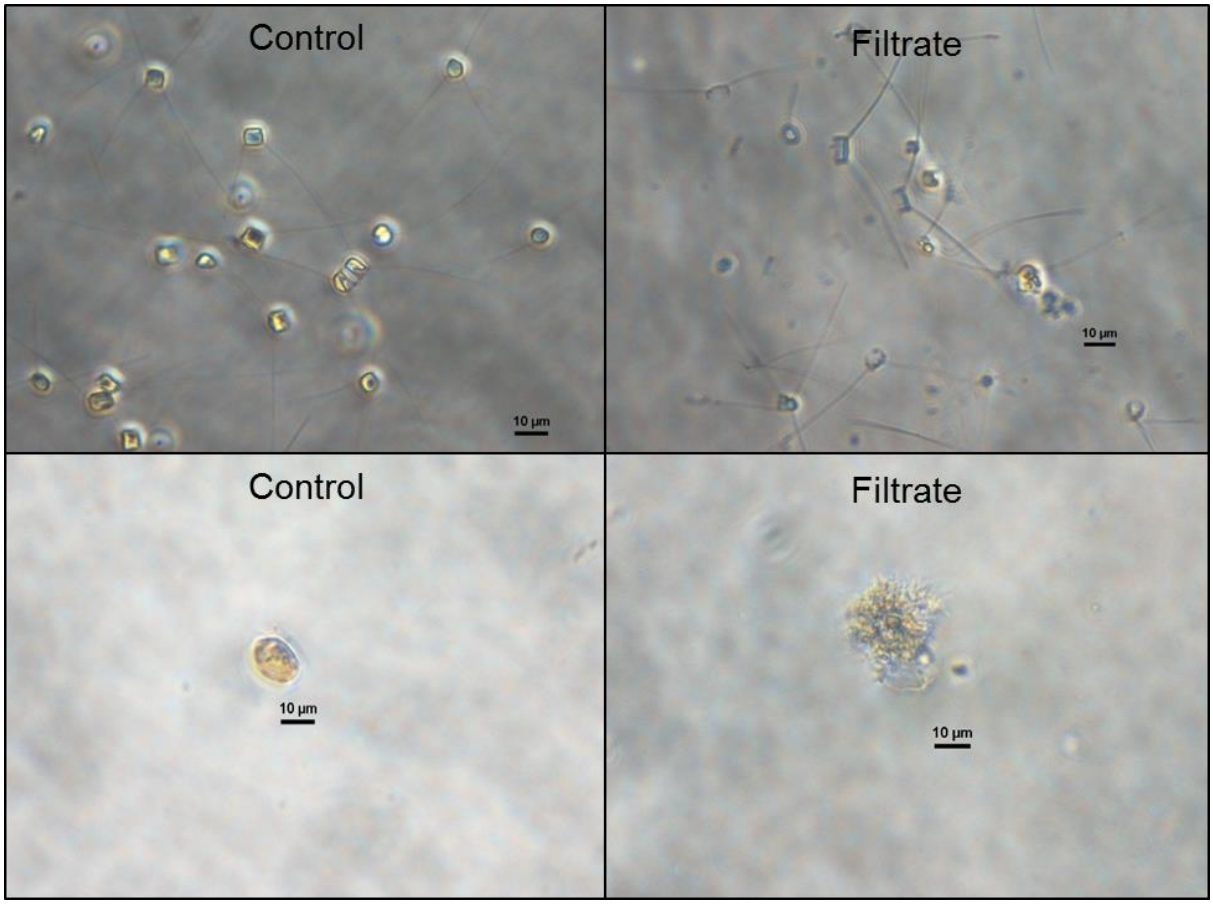



**Figure 3.** Morphological changes of target species before and after exposure to *Aureoumbra* filtrate for 120 h. (A, B) *Chaetoceros calcitrans* and (C, D) *Prorocentrum minium*..

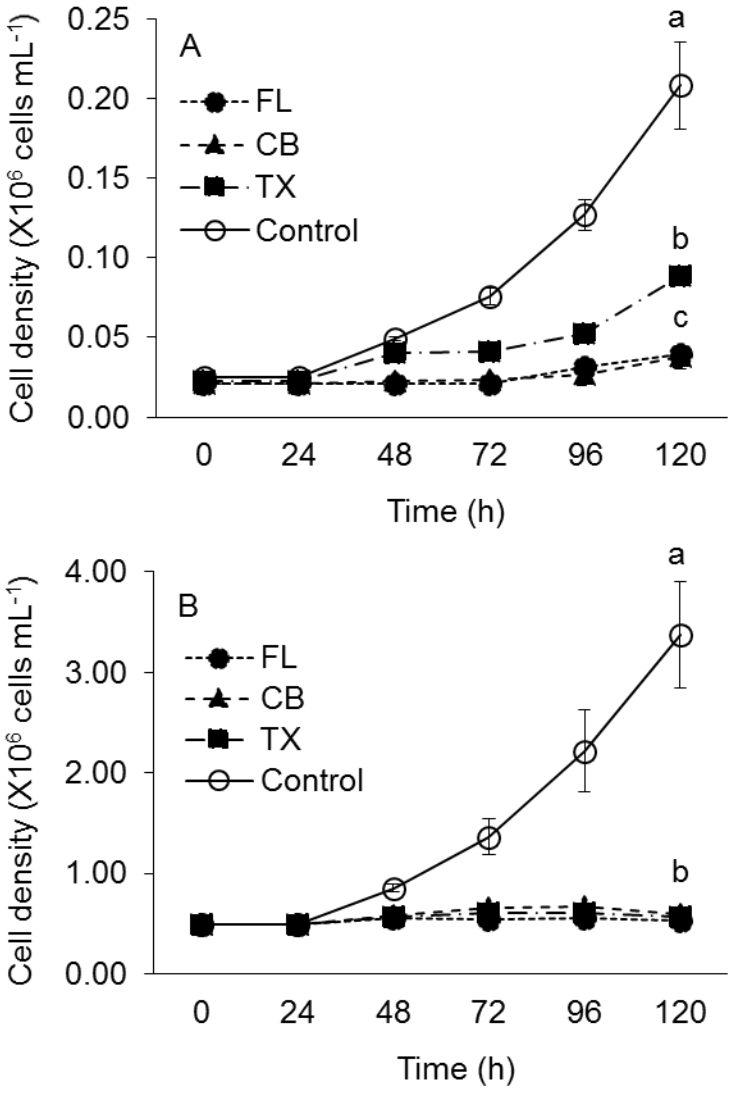



**Figure 4.** (A) Effects of filtrate from three clones of *Aureoumbra* on *Chaetoceros calcitrans* and (B) cell density of *Synechococcus* sp. (CCMP1333; B) during a 120 h experiment. One-Way ANOVA was performed with a post-hoc Tukey test. Letters indicate significant difference among treatments ($p<0.05$ for TX strain in Fig. 2A and $p<0.001$ for others).

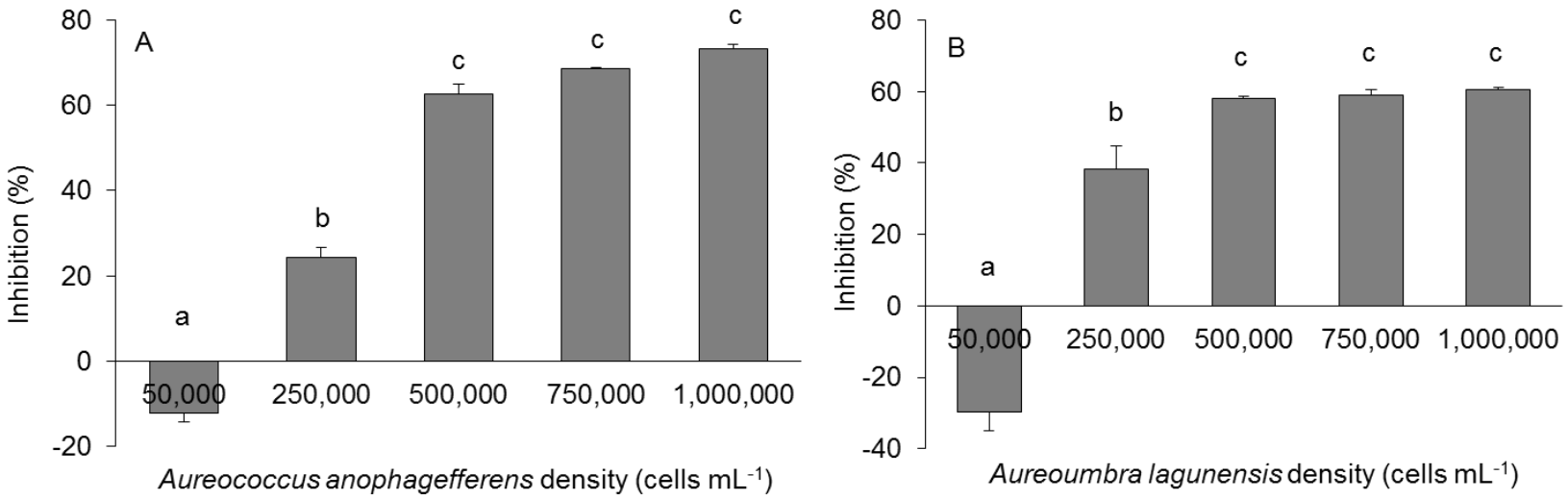



**Figure 5.** Dose-dependent mortality of *Synechococcus* sp. (CCMP1333) after exposure to varying density of (A) *Aureococcus* and (B) *Aureoumbra* for 120 h. Cell density of *Synechococcus* sp. was fixed as 500,000 cells $mL^{-1}$ across treatments while cell density of donors varied from 50,000 to 1,000,000 cells $mL^{-1}$. Data were analyzed using One-Way ANOVA followed by a post-hoc Tukey test. Letters indicate significant differences among treatments ($p<0.001$).

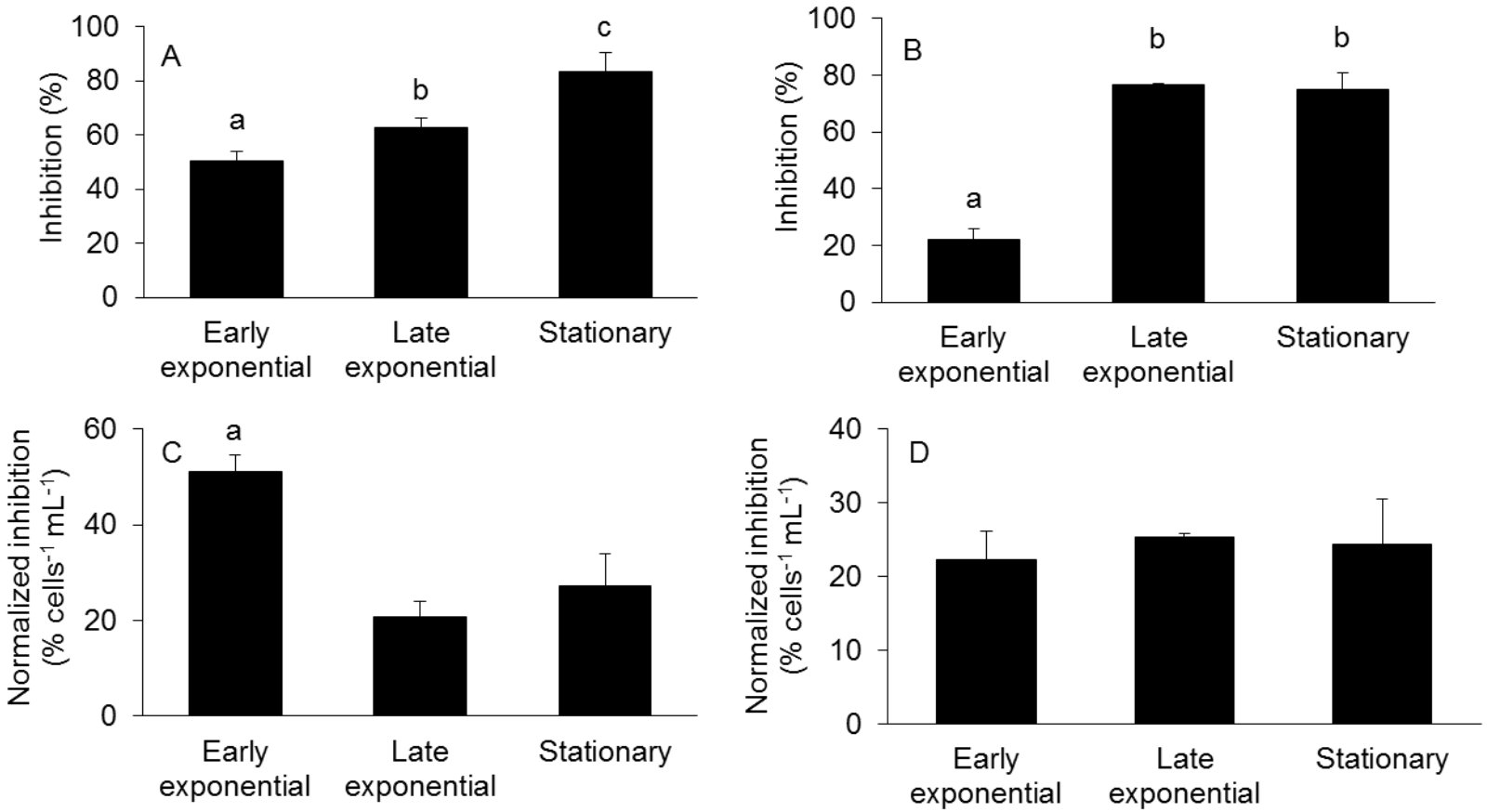



**Figure 6.** Inhibition (%) for (A) *Chaetoceros calcitrans* and (B) *Synechococcus* sp. and normazlied inhibition (% cells$^{-1}$ mL$^{-1}$) for (C) *Chaetoceros calcitrans* and (D) *Synechococcus* sp. after exposure to filtrate of *Aureoumbra lagunensis* for 120 h harvested at different growth stages during a course of growth. One-Way ANOVA was performed with a post-hoc Tukey test. Letters indicate significant differences among treatments ($p$<0.05 for Fig. 6A and $p$<0.001 for Fig. 6B, C).

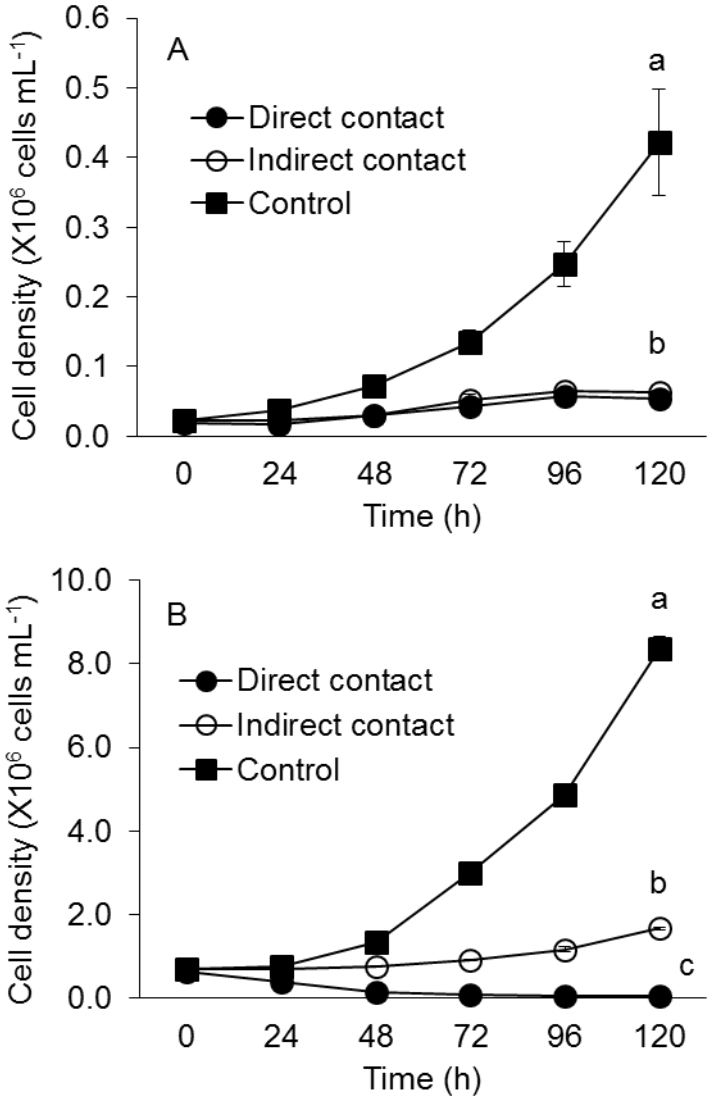



**Figure 7.** Comparison of allelopathic effects between direct and indirect contact. (A) *Chaetoceros calcitrans* and (B) *Synechococcus* sp. (CCMP1333). One-Way ANOVA was performed with a post-hoc Tukey test. Letters indicate significant difference of treatments compared to control ($p$<0.001).

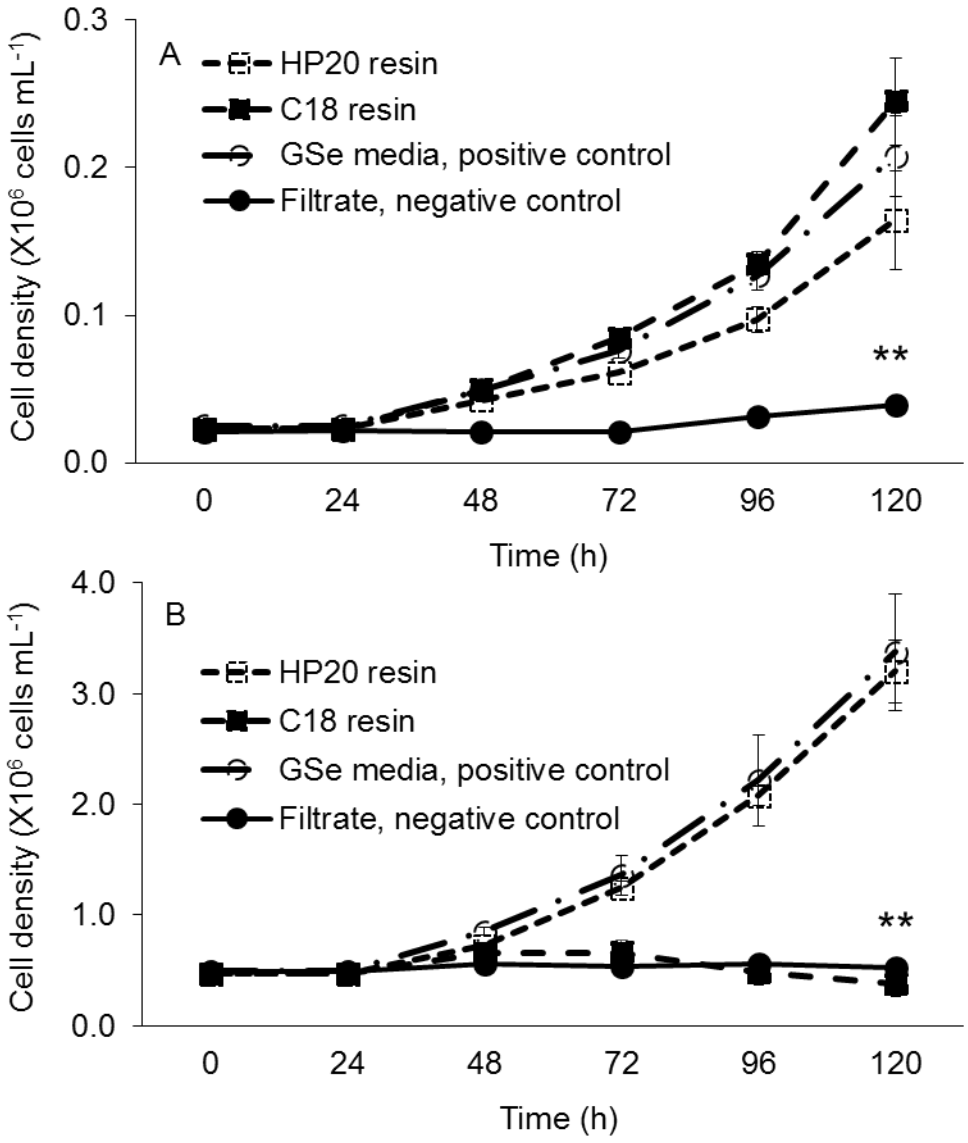



**Figure 8.** The ability of two hydrophobic resins HP20 and C18 to mitigate the allelopathic effects of *Aureoumbra* on (A) *Chaetoceros calcitrans* and (B) *Synechococcus* sp. (CCMP1333). Positive control indicates target species growing in GSe medium and negative control indicates target species growing in *Aureoumbra* filtrate. One-Way ANOVA was performed with a post-hoc Tukey test. Asterisks indicate significant difference among treatments compared to positive control (=GSe media; $p<0.001$).

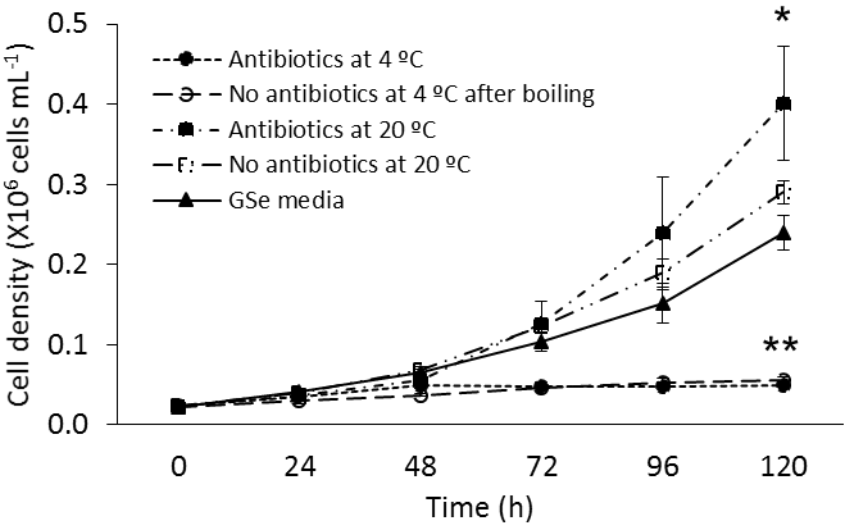



**Figure 9.** Cell density of *Chaetoceros calcitrans* (x$10^6$ cells $mL^{-1}$) after exposure to filtrate of *Aureoumbra lagunensis* for 120 h that were stored under different conditions for two weeks. One-Way ANOVA was performed with a post-hoc Tukey test. Asterisks indicate significant difference of treatment compared to control (=GSe media; * = $p$<0.05 and ** = $p$<0.001).

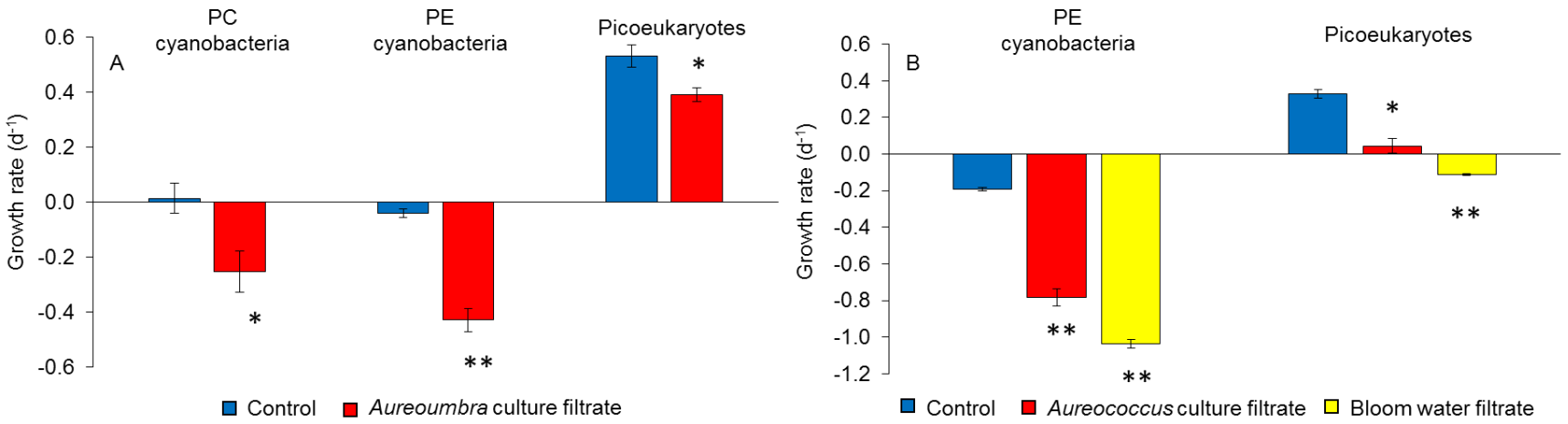



**Figure 10.** Growth rate ($d^{-1}$) of the major picoplankton community after exposure to (A) *Aureoumbra* and (B) *Aureococcus* filtrate for 72 h during experiments using non-bloom waters that were collected Indian River Lagoon, FL and Shinnecock Canal, NY, respectively. Bloom water filtrate was made using water collected from Quantuck Bay, NY. Abbreviations are as following: Aureoumbra culture filtrate = *Aureoumbra lagunensis* culture filtrate, *Aureococcus* culture filtrate = *Aureococcus anophagefferens* culture filtrate, PC cyano = phycocyanin-containing cyanobacteria, PE cyano = phycoerythrin-containing cyanobacteria, Pico-Euks = picoeukaryotes. T-test was performed between control and treatment of each picoplankton group. Asterisks indicate significant differences between control and treatment (* = $p<0.05$ and ** = $p<0.001$).

# Chapter 5

## Quantifying nitrogen assimilation rates of individual phytoplankton species and plankton groups during harmful algal blooms via sorting flow cytometry

**Abstract**

While $^{15}$N-labled nitrogen (N) compounds have been used to quantify N uptake rates by plankton communities for decades, accurately ascribing those rates to individual populations or species has been a challenge. Here I describe the development of method that applies sorting flow cytometry combined with species-specific immuno-detection of the harmful pelagophyte, *Aureococcus anophagefferens,* to contrast the nutritional ecology of this alga with other co-occurring picoplankton (picoeukaryotes, cyanobacteria, heterotrophic bacteria) during harmful brown tides. The washing of cells and levels of antibody used for cell detection were refined to yield 85 – 95% recovery of *A. anophagefferens* cells through the procedure and close agreement (85 – 101%) between plankton community $^{15}$N uptake quantified via traditional filtration and this novel sorting method. Sorting groups revealed that the $\delta^{15}$N values of *A. anophagefferens* and phycocyanin-containing cyanobacteria were more enriched (~10‰) than the values of other picoeukaryotes and heterotrophic bacteria that decreased to <0‰ after a brown tide declined. This suggests that non- *A. anophagefferens* picoeukarytoes and heterotrophic bacteria utilized isotopically lighter nitrogen sources such as recycled nutrients or fertilizer. *A. anophagefferens* utilized multiple forms of nitrogen (e.g. nitrate, ammonium, urea) during blooms and their uptake rates of ammonium and urea were high during experiments. However, *A. anophagefferens* displayed urea uptake rates on a per cell basis were significantly faster than other groups, affirming the nutritionally strategical uptake of urea and the importance of this N source in fueling brown tides. This study is the first to successfully sort a single algal species from a plankton community for the purposes of assessing nitrogen uptake and highlights a promising and powerful approach for investigating and contrasting the nutritional ecology of bloom-causing species and co-occurring plankton populations during harmful algal blooms.

## Introduction

During the past 25 years, flow cytometry has been used to identify and quantify pico- and nanoplankton in the ocean (Jacquet et al., 1998; Shi et al., 2011; Balzano et al., 2012). Flow cytometers pass liquid samples through a narrow capillary tube enclosed by outer sheath fluid, permitting the interrogation of individual cells via lasers for light excitation, fluorescence detectors, and light scattering detectors (Watson, 2004). For most biological oceanographic applications, individual cells (0.2 – 140 µm) are passed through a 488 nm blue argon-ion laser and 635 red diode laser beams and different plankton are identified and quantified based on light scattering (size) and fluorescent property (pigmentation) (Sosik et al., 2010). For example, intense orange fluorescence and red fluorescence emitted by *Synechococcus* due to possession of phycoerythrin and chlorophyll *a* are characteristics used to identify this group using flow cytometry (Olson et al., 1988) whereas *Prochlorococcus* are of a smaller size and possess relatively weak or absent orange fluorescence (Chisholm et al., 1988; Chisholm et al., 1992). Modern flow cytometers (e.g. CytoFLEX flow cytometer; Beckman Coulter, USA) can have multiple fluorescence channels that permit the differentiation of multiple types of small eukaryotic and prokaryotic phytoplankton based on their autofluorescence and can permit the detection of individual plankton species via the use of fluorochromes (Collier, 2000).

Fluorochrome-based detection of single species via flow cytometry can be highly useful for the quantification of picoplankton that are otherwise indistinguishable via microscopy or traditional flow cytometry. For example, species-specific antibodies have facilitated the immunological quantification of harmful, bloom-forming pelagophytes *A. anophagefferens* (Stauffer et al., 2008) and *Aureoumbra lagunensis* (Koch et al., 2014) via flow cytometry. Immunofluorescence flow cytometric detection of brown tide pelagophytes permits a more

accurate and rapid cell quantification method than traditional antibody approaches based on microscopy (Lopez-Barreiro, 1998) or even plate-reading spectrophotometers (Caron et al., 2003). Beyond cell quantification, flow cytometry has also emerged as a useful tool for cell sorting in microbiology (Davey and Kell, 1996; Brehm-Stecher and Johnson, 2004; Lomas et al., 2011).

Since nitrogen (N) is the most commonly limiting nutrient in the ocean, there is great interest in assessing the N sources being utilized by phytoplankton in marine ecosystems (Eppley et al., 1969; McCarthy et al., 1977; Antia et al., 1991). Historically, $^{15}$N-labled compounds have been a useful tool to quantify the rates of uptake and assimilation of N compounds in both laboratory and ecosystem studies (Dugdale and Wilkerson, 1986; Lomas and Glibert, 1999; Kudela and Cochlan, 2000; Mulholland et al., 2004). While ecosystem-based use of $^{15}$N-labled compounds can provide N uptake rates for entire plankton communities (Kokkinakis and Wheeler, 1987; Collos et al., 1997; Varela and Harrison, 1999) and even specific size classes within plankton communities (Probyn and Painting, 1985; Mulholland et al., 2002), understanding the individual plankton groups or species responsible for those rates can be a challenge (MacIsaac, 1978; Kang et al., 2015). The presence of detritus may preclude the precise determination of N assimilation by phytoplankton due to bacterial activity on the particles (Lipschultz, 1995) and the retention of bacteria on glass fiber filters that are used for most $^{15}$N approaches in aquatic studies can prohibit the precise measurement of uptake rates of phytoplankton only (Berg et al., 2001). Single-cell sorting using a flow cytometer can overcome some of these limitations and has recently become useful for assessing nutrient uptake and assimilation among picoplankton (Fawcett et al., 2011; Talarmin et al., 2011; Duhamel et al., 2012; Gomez-Pereira et al., 2013) especially to better characterize group-specific responses to

available nutrients. Furthermore, analyses of phytoplankton after incubation with $H^{14}CO_3^-$ have shown no statistically significant difference between unsorted and sorted cells (Rivkin et al., 1986) and the damage to cellular integrity from sorting procedure is generally minimal (Bradley et al., 2010a). Hence, sorting flow cytometry combined with isotopic labeling holds promise in quantifying accurate nutrient assimilation rates among individual plankton groups during algal blooms.

Brown tides caused by *A. anophagefferens* and *A. lagunensis* have impaired ecosystems in the Mid-Atlantic (Gobler and Sunda, 2012) and subtropical regions (Gobler et al., 2013b) of the US, respectively, for decades. Globally, these blooms have also occurred in China (Zhang et al., 2012), South Africa (Probyn et al., 2001), and Cuba (Koch et al., 2014) during the past 15 years. Brown tides have been shown to co-exist with multiple clades of picocyanobacteria whose abundance increases when brown tide abundance declines (Sieracki et al., 2004; Kang et al., 2015). For example, a brown tide that has persisted for most of 2016 (January through September) in the Banana and Indian River Lagoons of FL, USA, was characterized by a multi-month co-bloom of *A. lagunensis* and pico-cyanobacteria, with *A. lagunensis* often comprising ~95% of biovolume within the affected area and numerous fish kill reports (St. Johns River Water Management District, 2016). Furthermore, brown tides co-occur with picoeukaryotes and densities of heterotrophic bacteria during brown tides often exceed $10^7$ cells $mL^{-1}$ (Gobler et al., 2004; Sieracki et al., 2004). Brown tides are, therefore, not monospecific events.

This study was designed to evaluate the application of sorting flow cytometry coupled with N uptake by *A. anophagefferens* and co-occurring picoplankton during brown tides caused by *A. anophagefferens* in the lagoons of NY, USA. The use of a species-specific antibody permitted the sorting and isolation of the brown tide alga, while different groups of

cyanobacteria and picoeukaryotes were isolated based on size and fluorescence, and heterotrophic bacteria were isolated based on their size and DNA content using SYBR Green I. Experiments were performed to assess the efficacy and efficiency of the approach. The natural abundance of $^{15}N$ in each sorted population was quantified to assess the use of N from various sources. Experiments were performed with $^{15}N$-labled compounds (nitrate, ammonium, urea, glutamic acid) to quantify uptake rates of these compounds by each group. Collectively, the data suggest that this approach provides significant insight regarding the use and assimilation of various N sources by *A. anophagefferens* and co-occurring plankton, suggesting this is a promising application for understanding the nutritional ecology of HABs and their co-occurring plankton groups. This is the first published study to combine species-specific cell sorting and $^{15}N$ labeling to quantify nitrogen assimilation rates more specifically.

## Materials and Methods

### *Field surveys*

Samples were collected from Quantuck Bay (40.808ºN, -72.620ºW) in Long Island, New York, USA from spring through fall of 2014, and eastern Great South Bay (40.762 º N, -73.003ºW) during the spring and summer of 2015, using acid-washed and rinsed 20 L carboys that were transported to a laboratory of Stony Brook University in Southampton, USA. Three 5 mL samples were preserved to a final concentration of 1% formalin and 1% glutaraldehyde to quantify abundance of major phytoplankton populations and *A. anophagefferens*, respectively. The abundance of the major eukaryotic and prokaryotic communities including *A. anophagefferens*, cyanobacteria, and heterotrophic bacteria was enumerated using a Fluorescence Activated Cell Scan (FACScan; Becton, Dickinson and Company) flow cytometer

at the Flow Cytometry Facility Lab of Stony Brook University Hospital based on fluorescence patterns and particle size derived from side angle light scatter (Olson et al., 1991). Brown tide cells were enumerated on a flow cytometer after samples were incubated with monoclonal antibody conjugated to fluorescein isothiocyanate (FITC-MAb) for 30 min in the dark (Stauffer et al., 2008). Heterotrophic bacteria abundance was quantified using SYBR Green I (Jochem, 2001). Phycocyanin-containing cyanobacteria (PC cyanobacteria), phycoerythrin-containing cyanobacteria (PE cyanobacteria), and pico-eukaryotic communities were detected based on relative levels of chlorophyll *a* (Chl *a*) and phycoerythrin content. PC cyanobacteria were characterized by low content of phycoerythrin compared to PE cyanobacteria, which contain a high PE: Chl *a* ratio and picoeukaryotes were distinguished by their relatively small size, the presence of high Chl *a* and the absence of phycoerythrin (Berry et al., 2015). Non-brown tide picoeukaryotes (non-BT picoeukaryotes) were quantified by deducting brown tide abundance from the total picoeukaryote abundance. Dissolved nutrient samples were collected by filtering water through pre-combusted GF/F glass fiber filters (25 mm in diameter) and immediately stored at -20 ºC until analysis with QuikChem 8500 Series 2 flow-injection autoanalyzer (LACHAT Instruments, Loveland, CO, USA; Parsons, 2013). Dissolved free amino acids were measured with high performance liquid chromatography (HPLC) (Cowie and Hedges, 1992). To quantify the $\delta^{15}N$ values of major picoplankton groups 100 mL samples were preserved with buffered formalin at a final concentration of 1% and stored at -20 ºC until sorting analysis as described below.

*$^{15}N$ uptake experiments*

$^{15}N$ uptake experiments were performed within an hour of bloom water collection during brown tides in the summer and fall of 2014. To assess natural abundance of $^{15}N$ within each

phytoplankton group prior to experiments, three, 100 mL samples were immediately preserved with buffered formalin at a final concentration of 1% and stored at -20 ºC until sorting. For experiments, three sets of 12, 125 mL sterile Thermo Scientific Nalgene polycarbonate bottles were filled with 100 mL bloom water. $^{15}$N-labeled nitrogen sources (99% enriched $NO_3^-$, 99% enriched $NH_4^+$, 98% enriched urea, 98% enriched glutamic acid; Cambridge Isotope Laboratories, Andover, Massachusetts, USA) were amended at < 10% of ambient concentrations in triplicate to avoid disturbance of natural nutrient uptake kinetics in samples. The tracer addition was estimated from historic nutrient data and confirmed via measurements of $NO_3^-$, $NH_4^+$, urea, and glutamic acid. Prior to conducting experiments,natural abundance of $^{15}$N in experimental waters was determined by filtering 100 mL bloom water onto the pre-combusted GF/F and filters were stored at -20ºC until analysis. After a 60 min incubation, two sets of 12 samples were preserved with buffered formalin at a final concentration of 1% and stored at -20 ºC until analysis. Isotope dilution was not estimated, but the short incubation period minimized the likelihood of the isotope dilution altering uptake rates. For comparative purposes, of these two sets, one was sorted and the parallel set of stable-isotope enriched samples was filtered after preservation and storage at -20 ºC to quantify potential isotope loss from the concentration step and the storage period. The third set of parallel triplicate samples was filtered onto pre-combusted GF/F filters after incubated with $^{15}$N-labeled nitrogen sources to measure total uptake rates of plankton community in bloom water.

*Sorting major picoplankton populations*

For sorting plankton populations, frozen samples were thawed and concentrated to a final volume of < 3 mL by centrifuging twice at 2000 rpm for 15 min. A small volume (1-2 mL) of 0.01 M PBS-TWEEN 20, which has been shown to be effective at disaggregating *A.*

*anophagefferens* cells and other picoplankton (Stauffer et al., 2008; Koch et al., 2014), was then added to the concentrated samples to dislodge and disaggregate cells. Cells were sorted using BD FACSAria$^{TM}$ III at a Flow Cytometry Facility Lab of the Stony Brook University Hospital, Stony Brook, NY, USA. BD FACSAria$^{TM}$ III is designed for stable, high speed sorting of 15,000 cells $s^{-1}$. Samples were sorted in 'yield-purity' mode which maximizes the yield of sorted cells while maintaining a high purity of samples.

Frozen quality control samples were thawed and concentrated into the final volume of < 3 mL as described above but resuspended in filtered seawater to a final volume of 100 mL. Three samples for flow cytometric quantification of major phytoplankton were collected as described above from resuspended samples. A comparison of cell densities for each group in the initial whole water and in the processed sample provided a quantitative percent recovery for cells within each group and an indication of the extent to which processing the concentrated samples altered cell abundances.

Fluorescent antibodies for *A. anophagefferens* (Stauffer et al., 2008) were used to sort the brown tide alga. Samples were incubated with 428 µg of fluorescent antibody in 500 µL for 1 h and then washed by centrifuging the incubated samples for 10 min at 2000 rpm, removing the supernatant, and resuspending in flow cytometry grade (PBS, pH 7.4). Before samples were sorted, a second set of experimental, unwashed samples were treated with the same amount of antibody and the same duration of incubation to assess the necessity of the washing procedure to remove unbound antibody. An assessment of the optimal antibody addition was performed by adding 42.5 µg in 50 µL, 255 µg in 300 µL, and 425 µg in 500 µL to triplicate samples. SYBR Green I was used to sort the heterotrophic bacteria group and the washing step was found to be unnecessary for this group. Non-brown tide pico-eukaryotes and PC-cyanobacteria groups were

isolated based on Chl *a*, phycocyanin content, and size (*details in field surveys*). The purity of sorted plankton groups was assessed by analyzing sorted samples on a FACScan flow cytometer to confirm the presence of a single plankton group in all of the individually sorted plankton samples (*A. anophagefferens*, heterotrophic bacteria, non-brown tide picoeukaryotes, and picocyanobacteria). Sorted cell abundances of each group was at least $1.0 \times 10^6$ cells for *A. anophagefferens* and non-BT picoeukaryotes, $5.0 \times 10^5$ cells for PC cyanobacteria, and $1.0 \times 10^7$ cells for heterotrophic bacteria.

After each sample was sorted, sorted cells were immediately filtered onto pre-combusted GF/F filters and stored at -20 °C until isotopic analysis. Sorted heterotrophic bacteria cells were filtered onto silver membrane filters (0.2 µm in pore size, 25mm in diameter; Sterlitech Corporation, Kent, Washington, USA) to avoid a potential loss of cells (Gasol and Moran, 1999). Uptake rates were expressed on a per volume and a per cell basis in which case they were not dependent on quantitative recovery of cells.

*Isotopic analysis*

For analyses, filters were dried at 55 ºC for 48 h and sent to the U.C. Santa Cruz Stable Isotope Facility to measure isotopic signals of samples on a Finnigan Delta Plus XP isotope ratio mass spectrometer (Thermo Fisher Scientific). Prior to analysis, 0.53 µg of nitrogen carrier (= 100 µm $NH_4Cl$) was added to the filters to assure N masses were above the detection limit for reliable $^{15}N$ atom % enrichment values. The amount of N carrier was deducted from the total measured N mass to correct the addition.

*$\delta^{15}N$ signatures and uptake rates*

$\delta^{15}N$ values (‰) of natural abundance within sorted groups were calculated based on equations from Fry (2007).

$$\delta^{15}\mathrm{N}\ (‰) = \frac{R_{\mathrm{sample}} - \mathrm{R_{std}}}{\mathrm{R_{std}}} \text{ x } 1000$$

, where $\mathrm{R} = \frac{\mathrm{At\%^{15}N}}{\mathrm{At\%^{14}N}}$, $\mathrm{R_{sample}}$ is natural abundance in samples, and $\mathrm{R_{std}}$ is natural abundance standard (e.g. $\mathrm{At\%^{15}N} = 0.0036765$).

Absolute uptake rates (V; μmol N $L^{-1}$ $h^{-1}$) were calculated based on equations from Fry (2007).

$$\mathrm{V} = \frac{(^{15}\mathrm{N_S} - \mathrm{NA})}{(^{15}\mathrm{N_{Enr}} - \mathrm{NA})\mathrm{T}} \text{ x PN}$$

where NA is the natural abundance of $^{15}N$ in experimental samples, $^{15}N_{Enr}$ is the isotopic ratio of the labeled nitrogen source, $^{15}N_s$ is the atom% $^{15}N$ abundance of the sample at the end of the experiment, T is the incubation time, and PN is the particulate nitrogen in samples. For sorted samples, absolute uptake rates per cell (fmol N $cell^{-1}$ $L^{-1}$ $h^{-1}$) of each picoplankton group were calculated using PN divided by the number of sorted cells and absolute uptake rates per each group (μmol N $L^{-1}$ $h^{-1}$) were calculated using per cell PN by multiplying the total cell density (cells $L^{-1}$) of each group in untreated bloom water. As such, the yielded rates (i.e. absolute uptake rate per cell and absolute uptake rate per group) represented the cell-based absolute uptake rates in this study. Uptake rates were summed and converted into percentage to compare the percentage of uptake for each major picoplankton group.

*Statistical analysis*

One-way analysis of variance (ANOVA, $p<0.05$) with post-hoc Tukey tests were performed to assess statistically significant differences between total uptake rates and summation of each group's uptake rates for each experiment and between *A. anophagefferens* and other groups

for each experiment. Repeated measures ANOVA with a post-hoc Tukey test was performed to assess seasonal differences in $\delta^{15}N$ values among sorted groups.

## Results

### *Optimization of sorting with BD FACSAria III*

To increase the antibody-labeled *A. anophagefferens* detection by FITC (i.e. FL1) with the sorting flow cytometer, a washing step was applied to remove antibody in suspension via centrifugation. This procedure was critical as the unattached antibody in suspension blocked detection of the labeled cells on the flow cytometer whereas the procedure is not necessary for cell counts when the small amount of antibody (2 µL for 200 mL sample) is used. The washing step yielded a clearly separated group of *A. anophagefferens* whereas in samples that were not washed cells could not be distinguished from antibody in suspension (Fig. 1).

To find an optimal amount of antibody for sorting *A. anophagefferens* multiple tests were performed at varying amount of antibody of 42.5 µg, 255 µg, and 425 µg after incubation for the same duration (1 h) and the washing process. Adding more antibody provided clearer separation of *A. anophagefferens* cells from other plankton groups. *A. anophagefferens* was nearly indistinguishable when 42.5 µg antibody was added while the addition of 425 µg antibody provided clear separation of *A. anophagefferens* cells from other phytoplankton assemblages in the sample (Fig. 2).

Sorting samples on the yield-purity mode of the BD FACSAria™ III flow cytometer maximized the purity of sorted cells. Sorted samples had a single target group. For example, there was *A. anophagefferens* detected in *A. anophagefferens* sorted samples, which had relatively higher Chl. *a* content (Fig. 3A) compared to phycocyanin (Fig. 3B) and phycoerythrin (Fig. 3C)

and no other groups were detected in the same samples (Fig. 3) on the same acquisition setting that was used for sorting *A. anophagefferens*.

The potential biomass loss from concentrating samples via centrifugation and storage until analysis was measured by comparing cell density of unamended samples and that of concentrated samples, which were diluted to 100 mL with filtered seawater. Of the initial *A. anophagefferens* cell densities, 85 – 95% remained after the concentration step with cell densities ranging from 2.6 x $10^5$ to 4.1 x $10^5$ cells $mL^{-1}$, compared to the initial cell densities ranging from 3.1 x $10^5$ to 4.6 x $10^5$ cells $mL^{-1}$ (Fig. 4A). This small discrepancy, however, did not alter $^{15}N$ uptake rate calculations that were normalized by the cell density in the sorted samples.

Total community N uptake rates quantified via cell sorting were highly similar to and not significantly different from rates quantified via traditional filtration of whole water. The total absolute uptake rates of whole water were slightly higher by 15% and 1% during the June 27 and October 9, 2014 experiments compared to the summed rates of all sorted groups while the absolute uptake rate of whole water was lower by 1% during the October 2, 2014 experiment (Fig. 4B).

*Phytoplankton community dynamics and nutrient dynamics in 2014*

During the course of a brown tide in Quantuck Bay, NY, USA in 2014, cell densities of *A. anophagefferens* rose from 2.7 x $10^4$ cells $mL^{-1}$ on May 19 to 4.1 x $10^5$ cells $mL^{-1}$ on June 30. The bloom declined sharply after the peak and *A. anophagefferens* densities remained below 1.0 x $10^4$ cells $mL^{-1}$ from July 14 through August (Fig. 5). In early October, this bloom density sharply increased again in Quantuck Bay with a cell density of ~4.5 x $10^5$ cells $mL^{-1}$ (Fig. 5). Other picoplankton groups displayed an annual abundance patterns that differed from *A. anophagefferens* (Table 1). As the first brown tide declined, phycocyanin-containing

cyanobacteria (PC cyanobacteria) abundance increased steadily from 4.4 x $10^5$ cells $mL^{-1}$ on June 27 to ~7.0 x $10^6$ cells $mL^{-1}$ by August 10 (Table 1). Non-brown tide picoeukaryote (non-BT picoeuks) densities displayed bi-modal peaks, increasing through June to 2.3 x $10^6$ cells $mL^{-1}$ on June 27, then declining in early July, peaking once again at 3.6 x $10^6$ cells $mL^{-1}$ on August 7, and declining thereafter (Table 1). Similar to the non-BT picoeukaryotes, heterotrophic bacterial abundance increased in early summer to 9.5 x $10^7$ cells $mL^{-1}$ on July 3, decreased in early July, and increased again through August 7 (Table 1). When the brown tide reappeared in Quantuck Bay in October, abundance of non-BT picoeukaryotes and PC cyanobacteria were declining and an order of magnitude lower than their peak, summer abundances (Table 1).

Mean concentrations of nitrate and nitrite were low during 2014, ranging from 0.1 – 0.4 µM without a clear seasonal pattern (Table 2). Levels of ammonium were higher decreasing from 4.6 ± 1.0 µM in June to 1.2 ± 0.14 µM in October (Table 2). Urea levels were below 0.75 µM during the summer brown tide bloom, doubled to >1.5 µM in July and August, and then declined (Table 2). Phosphorus levels were consistently below 0.5 µM through early summer, reached the maximum of 0.84 ± 0.02 µM in July, and ranged from 0.3 – 0.5 µM thereafter (Table 2). Glutamic acid levels were below 0.1 µM through summer and fall (Table 2).

In the summer of 2015, brown tide occurred in eastern Great South Bay. *A. anophagefferens* densities reached up to 7.7 x $10^5$ cells $mL^{-1}$ on June 30 and declined by more than two-fold every week after the peak (Fig. 5). Densities of non-BT picoeukaryotes and PC cyanobacteria continuously increased to 5.1 x $10^6$ cells $mL^{-1}$ and 1.5 x $10^6$ cells $mL^{-1}$, respectively, in July (Table 1). Heterotrophic bacteria densities were 6.3 x $10^6$ cells $mL^{-1}$ on June 9 and increased by four-fold by mid July (2.4 x $10^7$ cells $mL^{-1}$; Table 1).

*$^{15}$N signatures and absolute uptake rates of $^{15}$N-labeled nitrogen compounds by the major plankton groups*

Natural abundance of $\delta^{15}N$ values differed among sorted plankton groups and between summer and fall. $\delta^{15}N$ of *A. anophagefferens* and PC cyanobacteria were 13 ± 4.9‰ and 9.8 ± 3.7‰, respectively during fall 2014 and 9.4 ± 2.7‰ and 8.7 ± 4.5‰ during summer 2015 ($p$>0.1; T-test; Fig. 6). For the entire study, the $\delta^{15}N$ of *A. anophagefferens* and PC cyanobacteria were 10.3 ± 3.7‰ and 9.0 ± 4.2‰, respectively ($p$>0.1; T-test; Fig. 6). While the $\delta^{15}N$ signatures of non-BT picoeukaryotes and heterotrophic bacteria were similar to *A. anophagefferens* and PC cyanobacteria during the fall of 2014 and first two weeks of June in 2015, there were seasonal changes as during the summer of 2015 $\delta^{15}N$ values declined from 7.5 ± 2.9 ‰ to -7.2 ± 7.7 ‰ for non-BT picoeukaryotes and from 11 ± 3.2 ‰ to 3.3 ± 6.7 ‰ for heterotrophic bacteria ($p$<0.05; Repeated measures ANOVA; Tukey Test; Fig. 6). These declines were coincident with the collapse of the brown tide in 2015 (Fig. 5, 6).

Absolute N uptake rates per cell (fmol N cell$^{-1}$ h$^{-1}$) revealed preferential uptake of specific N compounds by specific plankton groups. $^{15}$N-labeled ammonium dominated absolute uptake rates per cell of sorted groups except for heterotrophic bacteria for which $^{15}$N-labeled glutamic acid dominated uptake (Fig. 7, 8). *A. anophagefferens* (14.0 ± 2.9 fmol N cell$^{-1}$ h$^{-1}$) and non-BT picoeukaryote (13.9 ± 5.6 fmol N cell$^{-1}$ h$^{-1}$) cells has higher uptake rate per cell of $^{15}$N-labeled ammonium than other groups during the summer experiment ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 7A), but were less responsible for ammonium uptake in the fall (77% on October 2 and 49% on October 9, Fig. 7). $^{15}$N-labeled ammonium uptake rates of PC cyanobacteria cells significantly increased from 1.1 ± 0.4 fmol N cell$^{-1}$ h$^{-1}$ in the summer to 11 ± 1.7 fmol N cell$^{-1}$ h$^{-1}$ in the fall (Fig. 7A, C). Cell-based urea uptake was largely attributed to *A. anophagefferens* with rates increasing from 0.8 ± 0.5 fmol N cell$^{-1}$ h$^{-1}$ in the summer (Fig. 7A) to 3.2 ± 0.3 fmol N cell$^{-1}$

$h^{-1}$ on October 9 (Fig. 7C) as urea accounted for high fractions of total N uptake by *A. anophagefferens* (66% on October 2 and 37% on October 9; Fig. 8). Meanwhile, the $^{15}$N-labeled urea uptake rates per cell by other groups were consistently and significantly lower than the rates by *A. anophagefferens* ($p$<0.05; One-Way ANOVA; Tukey test; Fig. 7). For nitrate uptake rates, non-BT picoeukayrotes had the highest uptake rate of 1.4 ± 0.2 fmol N $cell^{-1}$ $h^{-1}$ in the summer ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 7) whereas nitrate uptake rate by PC cyanobacteria significantly increased in the fall and represented 30% of the total N uptake by PC cyanobacteria ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 7, 8). Heterotrophic bacteria had the highest glutamic acid uptake rate per cell with a maximal rate of 0.08 ± 0.007 fmol N $cell^{-1}$ $h^{-1}$ on October 9 ($p$<0.05; One-Way ANOVA; Tukey test; Fig. 7C) and being consistently responsible for more than 70% of the total N uptake by heterotrophic bacteria except for the uptake on October 9 (Fig. 8).

When considering contribution to whole population nutrient uptake rates, *A. anophagefferens* (1.4 ± 0.4 µmol N $L^{-1}$ $h^{-1}$) and non-BT picoeukaryote (2.6 ± 0.9 µmol N $L^{-1}$ $h^{-1}$) uptake rates of ammonium were up to 98% of the total ammonium uptake rates in the summer (Fig. 9A, 10) but declined to 83% on October 2 and 63% on October 9 (Fig. 10) while the contribution by PC cyanobacteria increased from 1.2% (0.05 ± 0.02 µmol N $L^{-1}$ $h^{-1}$) in the summer to up to 33% (0.63 ± 0.10 µmol N $L^{-1}$ $h^{-1}$) in the fall (Fig. 9, 10). *A. anophagefferens* (27%), non-BT picoeukaryotes (32%), and heterotrophic bacteria (36%) groups were comparably responsible for community urea uptake in the summer (Fig. 10) while *A. anophagefferens* dominated the urea uptake (80%) in the fall with an uptake rate of 0.4 ± 0.1 µmol N $L^{-1}$ $h^{-1}$ ($p$<0.001; One-Way ANOVA; Tukey test; Fig. 9, 10). While contributions of non-BT picoeukaryotes and heterotrophic bacteria decreased in the fall, PC cyanobacteria doubled the urea uptake rates from 0.04 ± 0.005

μmol N $L^{-1}$ $h^{-1}$ in the summer to 0.08 ± 0.03 μmol N $L^{-1}$ $h^{-1}$ in the fall (Fig. 9), comprising 21% of the urea uptake (Fig. 10). Non-BT picoeukaryote (95%) dominated the $^{15}$N-labeled nitrate uptake in the summer ($p$<0.001; One-Way ANOVA; Tukey test) but declined to ~50% in the fall ($p$<0.05; One-Way ANOVA; Tukey test; Fig. 10) while the contribution of heterotrophic bacteria to nitrate uptake increased by ~9-fold from the summer (4%) to the fall ($p$<0.05; One-Way ANOVA; Tukey test; 34%), which was accompanied by a reduced uptake of the glutamic acid by heterotrophic bacteria (87%) in the fall (Fig. 10).

**Discussion**

This study highlighted the utility of sorting flow cytometry to investigate the ecophysiology of *A. anophagefferens* and co-existing picoplankton groups. Nitrogen uptake rates of picoplankton groups and the brown tide pelagophyte, *A. anophagefferens* were quantified by sorting $^{15}$N-enriched cells. This approach represents a significant advance over prior approaches that separated cells by size-fractionated filtration (Mullholland et al. 2002) but could not distinguish among different types of picoplankton nor individual species. Beyond demonstrating the efficacy and efficiency of the approach, results indicated *A. anophagefferens* assimilated multiple N compounds when forming blooms. The method presented in this study represents an advance in the field of sorting flow cytometry as it demonstrates, for the first time, the ability to ascribe nutrient uptake rates to a single phytoplankton species within an assemblage and therefore represents a promising and powerful tool to study the nutritional ecology of phytoplankton in the field.

*The utility of sorting flow cytometry in HAB nutritional research*

$^{15}$N-labeled isotopes have been used to investigate N uptake and cycling in many ocean ecosystems (MacIsaac and Dugdale, 1969; Le Bouteiller, 1986; Mulholland et al., 2003) and during different types of harmful algal blooms (Mulholland et al., 2002; Kudela et al., 2008; Bronk et al., 2014; Hattenrath-Lehmann and Gobler, 2015; Kang et al., 2015). To generate a refined understanding of $^{15}$N uptake rates of different sized plankton populations, size-fractionated samples have been employed (Probyn and Painting, 1985; Mulholland et al., 2002). This, however, can be problematic when $^{15}$N uptake rates of different classes or types of plankton may co-occur within the same size fraction or when filters retain a significant amount of bacterial populations (Gasol and Moran, 1999). For example, although Mulholland et al. (2002) used filters to ascribe $^{15}$N-uptake rates in Quantuck Bay in the 1.2 – 5.0 µm size fraction to *A. anophagefferens*, these filters almost certainly also retained picoeukaryotes and picocyanobacteria.

To overcome these limitations, flow cytometric sorting has been utilized in many studies to measure N uptake measurement for multiple phytoplankton populations (Lipschultz, 1995; Casey et al., 2007; Michelou et al., 2007; Bradley et al., 2010a; Bradley et al., 2010b), particularly within coastal and estuarine systems where debris can interfere with measurements of uptake rates (Lipschultz, 1995; Bradley et al., 2010a; Bradley et al., 2010b). The flow cytometric sorting method has provided ecological insight regarding multiple questions that had long been unresolved. While the inability of *Prochlorococcus* to grow on nitrate had been hypothesized to shape the niche of these cyanobacteria (Bouman et al., 2006), a N uptake study with sorting flow cytometry has found that nitrate uptake by *Prochlorococcus* sp. can account for ~10 % of a total N uptake in the Sargasso Sea (Casey et al., 2007). Another case is phytoplankton competition with heterotrophic bacteria for small organic nitrogen compounds:

Bradley et al. (2010a) have shown that phytoplankton account for a significant fraction of dissolved free amino acid uptake within the Chesapeake Bay.

Despite such advantages, sorting flow cytometry has never been utilized in nutritional studies of HABs. While many HAB events are formed by a single-predominant species, some HABs co-occur (e.g. co-occurrence of *Alexandrium* and *Dinophysis* in Northport, NY; Hattenrath-Lehmann et al., 2013) and phytoplankton communities present during HABs are composed of multiple groups (Smayda, 1997; Sieracki et al., 2004; Kang et al., 2015). In the case of brown tides caused by *A. anophagefferens,* while they have been described as 'monospecific' (Cosper et al., 1987; Gobler and Sanudo-Wilhelmy, 2001b), heterotrophic bacteria abundances simultaneously increase during brown tides (Gobler and Sanudo-Wilhelmy, 2001a) and picocyanobacteria can be found at high densities during blooms (Sieracki et al., 2004). Thus, sorting flow cytometry is needed to accurately assign and quantify N uptake rates to individual plankton groups during brown tides.

*Sorting flow cytometry combined with an immunofluorescence detection technique*

To date, studies have estimated uptake rates of major elements by phytoplankton of different sizes (Lipschultz, 1995) or different groups (Talarmin et al., 2011) via sorting flow cytometry. Without modification, these prior approaches cannot isolate pelagophytes such as *A. anophagefferens* since they cannot be separated from other picoeukaryotes that can outnumber *A. anophagefferens* by several fold during modest blooms such as the ones studied here. For this reason, a species-specific antibody was utilized to separate *A. anophagefferens* from other plankton. Analyses of post-sorted samples on a standard flow cytometer proved that sorting populations using traditional flow cytometric metrics including FITC, Chl *a* content, size, and

the relative ratios of Chl *a*: PE or Chl *a*: PC successfully isolated each target group without mixing cells from other groups across sorted samples.

During the process of concentrating and washing cells for $^{15}N$ analysis I found that 5 – 15% of cells were lost, a percentage within the error estimates of many of the analyses reported herein including cell counts and $^{15}N$ content. Despite the minor cell loss (<15%) associated with storage, concentration, and/or washing, the sorted cells that survived the preparation and sorting process should have accurately represented the relative importance of each group in the N uptake during brown tide blooms. This is further supported by the observation that the $^{15}N$ uptake rates attained from the collection of whole water samples on glass fiber filters were highly similar to and never significantly different from the community $^{15}N$ uptake rates determined by the summation of rates among each individual plankton group. The similarity also supports that there was no large cells in samples because the presence of the large cells would have skewed the uptake results or cells larger than the maximum particle size (140 µm) that can pass through a capillary tube would have clogged the flow cytometer.

*Uptake rates of major plankton community during brown tides*

The $\delta^{15}N$ signatures of particulate organic matter have been used to trace N sources with low values (<3‰) being linked to N from fertilizer or atmospheric deposition and heavier values (>3‰) being associated with N from wastewater (Lapointe et al., 2004; Kendall and McDonnell, 2012). In this study, the $\delta^{15}N$ values of *A. anophagefferens* and PC cyanobacteria were always >5‰ except for June 15, 2015 and averaged ~10‰ suggesting that the growth of *A. anophagefferens* and PC cyanobacteria during these blooms might be derived from wastewater (Bianchi, 2006; Kendall and McDonnell, 2012), a finding consistent with prior conclusions that

wastewater is the primary source of N to Long Island's south shore bays (Kinney and Valiela, 2011; Stinnette, 2014). For parts of this study (fall 2014, spring 2015), the $^{15}N$ values of non-BT picoeukaryotes and heterotrophic bacteria were also isotopically heavy and consistent with a wastewater source of N. On average, non-BT picoeukaryotes (1.7 ± 9.6 ‰) were lighter in $\delta^{15}N$ than *A. anophagefferens* (10.3 ± 3.7 ‰), PC cyanobacteria (9.0 ± 4.2 ‰), and heterotrophic bacteria (5.2 ± 6.8 ‰) throughout the study, suggesting they utilized a different, lighter N sources. Given these non-BT picoeukaryotes were, on average, larger (>3µm) than *A. anophagefferens* (~2 - 3µm) and PC cyanobacteria (~1µm), these differences may be a function of size-based differences in nutrient assimilation kinetics (Hein et al., 1995). Differences among plankton groups were even more pronounced during the peak and collapse of brown tide in Great South Bay in 2015 as the values of non-BT picoeukaryotes and heterotrophic bacteria switched from positive to negative suggesting that those groups were exploiting a different nitrogen source perhaps associated with fertilizer (Bianchi, 2006; Kendall and McDonnell, 2012) potentially introduced into the bay via sudden precipitation (10 cm of rain) during June 2015 or lighter, recycled nitrogen associated with the bloom collapse (Clark et al., 2008; Fawcett et al., 2011), or both. Regardless, these findings indicate that sorting flow cytometry can be useful for contrasting the natural isotopic abundance of different plankton species and groups during HABs.

The isolation and analysis of *A. anophagefferens* cells from $^{15}N$-enrichment experiments via sorting flow cytometry revealed that these cells relied primarily on ammonium and urea as N sources during blooms, with ammonium uptake rates exceeding urea in summer but rates being similar in fall. These findings are consistent with the well-known ecological strategy of brown tides in exploiting recycled nutrients (Taylor et al., 2006; Gobler and Sunda, 2012) and

specifically consistent with Mulholland et al. (2004) who has shown ammonium was the principal nitrogen source used by plankton < 5µm in Quantuck Bay during a summer brown tide. By specifically isolating *A. anophagefferens* from other plankton groups, however, more definitive difference in brown tide uptake rates can be elucidated. For example, on a per cell basis, *A. anophagefferens* was able to assimilate urea significantly faster than all other plankton groups. Similarly, in two of three experiments, urea was the only compound *A. anophagefferens* took up faster than other groups at a population level during the October experiments. In contrast, other groups always assimilated nitrate, ammonium, and glutamic acid significantly faster than *A. anophagefferens*. These findings, therefore, indicate that *A. anophagefferens'* greatest nutritional advantage in terms of N comes from the exploitation of urea, and not ammonium and support previous observations that *A. lagunensis* is well adapted to low DIN (e.g. ammonium) levels during blooms (Nixon et al., 1994) with low $K_s$ and $V_{max}$ for ammonium (Lomas et al., 1996). This conclusion contrasts Mulholland et al. (2002) who concluded ammonium was the most important source of N for *Aureococcus*, a conclusion supported by the reliance on filters that also retained dense populations of cyanobacteria and picoeukaryotes that were also likely assimilating ammonium. In fact, during this study, uptake rates of all sorted groups showed ammonium was taken up at the fastest rate whereas isolation of each group demonstrated that the maximal affinity for each group differed from the total, community derived rates. This contrast emphasizes the power and utility of using species-specific cell sorting to identify the nutritional niche of individual phytoplankton species. While N uptake rates can be concentration-dependent, the concentrations of nitrogenous nutrients during these experiments were consistent with previously reported summer and fall brown tides (Lomas et al.,

1996; Mulholland et al., 2002; Gobler and Sunda, 2012), suggesting the findings presented here may be broadly applicable to brown tides.

Uptake rates at a population level were governed by total cell densities of each group in bloom waters. For example, high cell densities of *A. anophagefferens* during October experiments led to the similar degree of contribution to whole community's ammonium uptake in both summer and fall although per cell-basis uptake rates were lower during October experiments than summer. This was the same for urea uptake of *A. anophagefferens* which was highest on a per cell basis among plankton groups during the summer experiment, but relatively lower cell densities led to a lower contribution to the uptake rate at a population level. In a similar manner, the highest nitrate uptake rate per cell by PC cyanobacteria during the second October experiment did not yield the highest contribution of PC cyanobacteria to nitrate uptake rate at a population level, as the whole community's nitrate uptake was more dominated by abundant non-BT picoeukaryotes and heterotrophic bacteria (82%). Therefore, varying densities of major plankton groups will control N dynamics during the course of brown tides. This differential view between per cell and community-level N uptake imparts significant insight regarding the ecology of different clades of plankton and was afforded specifically by the sorting of these populations.

The use of sorting flow cytometry in this study provided the first ever, definitive evidence that *A. anophagefferens* populations concurrently assimilated multiple N compounds. During brown tides, a fraction of the *A. anophagefferens* population assimilated nitrate accounting for up to 15% of the populations' N uptake. This was not surprising since *A. anophagefferens* cultures (e.g. CCMP1984, CCMP1850) isolated from Long Island, NY, grow well on nitrate or ammonium (personal observation). In contrast to *A. anophagefferens*, another

brown tide pelagophyte, *A. lagunensis* is known for its inability to grow on nitrate (Deyoe and Suttle, 1994). While this study supports the hypothesis that DON utilization is the shared feature of brown tide-forming pelagophytes (Gobler and Sunda, 2012), the results also highlight a distinct difference between two species with respect to nitrate utilization.

Non-BT picoeukaryotes and cyanobacteria exploited multiple forms of nitrogen (e.g. nitrate, ammonium, urea) during blooms and accounted for ~65% of community ammonium uptake and ~35% of community urea uptake. PC cyanobacteria were responsible for ~20% of urea uptake in the fall evidencing of nutrient competition between this group and *A. anophagefferens*. Given the competition for this nutrient, the ability of *A. anophagefferens* to allelopathically inhibit these cyanobacteria (Chapter 4) may represent a strategy that facilitates the use of critical N sources such as urea.

While brown tides usually peak at > $10^6$ *A. anophagefferens* cells $mL^{-1}$ (Gobler and Sunda, 2012), brown tides observed during this study were less intense with the maximal cell density of 4.5 x $10^5$ cells $mL^{-1}$ in 2014. Even with these only moderate densities, sorting flow cytometry could still be utilized to effectively isolate N assimilation by *A. anophagefferens* and other populations and differentiate the nutritional ecology of various plankton groups. During more intense brown tides, this approach would be even easier with less sorting required to obtain signals.

This study demonstrated that sorting flow cytometry combined with an immunofluorescence detection technique was highly effective at assessing the N sources utilized by *A. anophagefferens* and other plankton groups during brown tides in a manner that prior studies could not. Similar approaches in the future might consider the use of other types of

species-specific probes that might provide such as fluorescent in-situ hybridization (Biegala et al., 2003; Amann and Fuchs, 2008) to isolate the uptake rates by individual species of harmful algae, phytoplankton, bacteria, archaea, or other plankton. Such approaches hold great promise for defining the precise nutritional ecology of specific wild plankton species that are well-sorted via flow cytometry (<140 μm in size).

## References


Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Reviews Microbiology 6 (5), 339-348.

Antia, N.J., Harrison, P.J., Oliveira, L., 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. Phycologia 30 (1), 1-89.

Balzano, S., Marie, D., Gourvil, P., Vaulot, D., 2012. Composition of the summer photosynthetic pico and nanoplankton communities in the Beaufort Sea assessed by T-RFLP and sequences of the 18S rRNA gene from flow cytometry sorted samples. The ISME journal 6 (8), 1480-1498.

Berg, G., Glibert, P., Jørgensen, N.G., Balode, M., Purina, I., 2001. Variability in inorganic and organic nitrogen uptake associated with riverine nutrient input in the Gulf of Riga, Baltic Sea. Estuaries 24 (2), 204-214.

Berry, D.L., Goleski, J.A., Koch, F., Wall, C.C., Peterson, B.J., Anderson, O.R., Gobler, C.J., 2015. Shifts in cyanobacterial strain dominance during the onset of harmful algal blooms in Florida Bay, USA. Microb. Ecol. 70 (2), 361-371.

Bianchi, T.S., 2006. Biogeochemistry of estuaries. Oxford University Press.

Biegala, I.C., Not, F., Vaulot, D., Simon, N., 2003. Quantitative assessment of picoeukaryotes in the natural environment by using taxon-specific oligonucleotide probes in association with tyramide signal amplification-fluorescence in situ hybridization and flow cytometry. Appl. Environ. Microbiol. 69 (9), 5519-5529.

Bouman, H.A., Ulloa, O., Scanlan, D.J., Zwirglmaier, K., Li, W.K., Platt, T., Stuart, V., Barlow, R., Leth, O., Clementson, L., 2006. Oceanographic basis of the global surface distribution of Prochlorococcus ecotypes. Science 312 (5775), 918-921.

Bradley, P.B., Lomas, M.W., Bronk, D.A., 2010a. Inorganic and organic nitrogen use by phytoplankton along Chesapeake Bay, measured using a flow cytometric sorting approach. Estuaries and Coasts 33 (4), 971-984.

Bradley, P.B., Sanderson, M.P., Frischer, M.E., Brofft, J., Booth, M.G., Kerkhof, L.J., Bronk, D.A., 2010b. Inorganic and organic nitrogen uptake by phytoplankton and heterotrophic bacteria in the stratified Mid-Atlantic Bight. Estuar. Coast. Shelf Sci. 88 (4), 429-441.

Brehm-Stecher, B.F., Johnson, E.A., 2004. Single-cell microbiology: tools, technologies, and applications. Microbiol. Mol. Biol. Rev. 68 (3), 538-559.

Bronk, D.A., Killberg-Thoreson, L., Sipler, R.E., Mulholland, M.R., Roberts, Q.N., Bernhardt, P.W., Garrett, M., O'Neil, J.M., Heil, C.A., 2014. Nitrogen uptake and regeneration (ammonium regeneration, nitrification and photoproduction) in waters of the West Florida Shelf prone to blooms of *Karenia brevis*. Harmful Algae (0).

Caron, D.A., Dennett, M.R., Moran, D.M., Schaffner, R.A., Lonsdale, D.J., Gobler, C.J., Nuzzi, R., McLean, T.I., 2003. Development and application of a monoclonal-antibody technique for counting *Aureococcus anophagefferens,* an alga causing recurrent brown tides in the Mid-Atlantic United States. Appl. Environ. Microbiol. 69 (9), 5492-5502.

Casey, J.R., Lomas, M.W., Mandecki, J., Walker, D.E., 2007. *Prochlorococcus* contributes to new production in the Sargasso Sea deep chlorophyll maximum. Geophys. Res. Lett. 34 (10).

Chisholm, S.W., Frankel, S.L., Goericke, R., Olson, R.J., Palenik, B., Waterbury, J.B., West-Johnsrud, L., Zettler, E.R., 1992. *Prochlorococcus marinus* nov. gen. nov. sp.: an oxyphototrophic marine prokaryote containing divinyl chlorophyll *a* and *b*. Arch. Microbiol. 157 (3), 297-300.

Chisholm, S.W., Olson, R.J., Zettler, E.R., Goericke, R., Waterbury, J.B., Welschmeyer, N.A., 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature 334, 340-343.

Clark, I., Timlin, R., Bourbonnais, A., Jones, K., Lafleur, D., Wickens, K., 2008. Origin and Fate of Industrial Ammonium in Anoxic Ground Water—$^{15}$N Evidence for Anaerobic Oxidation (Anammox). Ground Water Monit. Remediat. 28 (3), 73-82.

Collos, Y., Vaquer, A., Bibent, B., Slawyk, G., Garcia, N., Souchu, P., 1997. Variability in nitrate uptake kinetics of phytoplankton communities in a Mediterranean coastal lagoon. Estuarine Coastal and Shelf Science 44 (3), 369-375.

Cosper, E.M., Dennison, W.C., Carpenter, E.J., Bricelj, V.M., Mitchell, J.G., Kuenstner, S.H., Colflesh, D., Dewey, M., 1987. Recurrent and Persistent Brown Tide Blooms Perturb Coastal Marine Ecosystem. Estuaries 10 (4), 284-290.

Cowie, G.L., Hedges, J.I., 1992. Improved amino acid quantification in environmental samples: charge-matched recovery standards and reduced analysis time. Mar. Chem. 37 (3), 223-238.

Davey, H.M., Kell, D.B., 1996. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. Microbiological reviews 60 (4), 641-696.

Deyoe, H.R., Suttle, C.A., 1994. The inability of the Texas brown tide alga to use nitrate and the role of nitrogen in the initiation of a persistent bloom of this organism. J. Phycol. 30 (5), 800-806.

Dugdale, R.C., Wilkerson, F.P., 1986. The use of $^{15}$N of measure nitrogen uptake in eutrophic oceans - experimental considerations. Limnol. Oceanogr. 31 (4), 673-689.

Duhamel, S., Björkman, K.M., Karl, D.M., 2012. Light dependence of phosphorus uptake by microorganisms in the subtropical North and South Pacific Ocean. Aquat. Microb. Ecol. 67, 225-238.

Eppley, R.W., Rogers, J.N., Mccarthy, J.J., 1969. Half-Saturation Constants for Uptake of Nitrate and Ammonium by Marine Phytoplankton. Limnol. Oceanogr. 14 (6), 912-&.

Fawcett, S.E., Lomas, M., Casey, J.R., Ward, B.B., Sigman, D.M., 2011. Assimilation of upwelled nitrate by small eukaryotes in the Sargasso Sea. Nature Geoscience 4 (10), 717-722.

Fry, B., 2007. Stable isotope ecology. Springer Science & Business Media.

Gasol, J.M., Moran, X.A., 1999. Effects of filtration on bacterial activity and picoplankton community structure as assessed by flow cytometry. Aquat. Microb. Ecol. 16 (3), 251-264.

Gobler, C.J., Koch, F., Kang, Y., Berry, D.L., Tang, Y.Z., Lasi, M., Walters, L., Hall, L., Miller, J.D., 2013. Expansion of harmful brown tides caused by the pelagophyte, *Aureoumbra lagunensis* DeYoe et Stockwell, to the US East Coast. Harmful Algae 27, 29-41.

Gobler, C.J., Sanudo-Wilhelmy, S.A., 2001a. Effects of organic carbon, organic nitrogen, inorganic nutrients, and iron additions on the growth of phytoplankton and bacteria during a brown tide bloom. Mar. Ecol. Prog. Ser. 209, 19-34.

Gobler, C.J., Sanudo-Wilhelmy, S.A., 2001b. Temporal variability of groundwater seepage and brown tide blooms in a Long Island embayment. Marine Ecology-Progress Series 217, 299-309.

Gobler, C.J., Sunda, W.G., 2012. Ecosystem disruptive algal blooms of the brown tide species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*. Harmful Algae 14, 36-45.

Gomez-Pereira, P.R., Hartmann, M., Grob, C., Tarran, G.A., Martin, A.P., Fuchs, B.M., Scanlan, D.J., Zubkov, M.V., 2013. Comparable light stimulation of organic nutrient uptake by SAR11 and *Prochlorococcus* in the North Atlantic subtropical gyre. ISME J 7 (3), 603-614.

Hattenrath-Lehmann, T.K., Marcoval, M.A., Berry, D.L., Fire, S., Wang, Z., Morton, S.L., Gobler, C.J., 2013. The emergence of Dinophysis acuminata blooms and DSP toxins in shellfish in New York waters. Harmful Algae 26 (0), 33-44.

Hattenrath-Lehmann, T., Gobler, C.J., 2015. The contribution of inorganic and organic nutrients to the growth of a North American isolate of the mixotrophic dinoflagellate, Dinophysis acuminata. Limnol. Oceanogr.

Hein, M., Pedersen, M.F., Sand-Jensen, K., 1995. Size-dependent nitrogen uptake in micro-and macroalgae. Mar. Ecol. Prog. Ser. 118 (1), 247-253.

Jacquet, S., Lennon, J.-F., Marie, D., Vaulot, D., 1998. Picoplankton population dynamics in coastal waters of the northwestern Mediterranean Sea. Limnol. Oceanogr. 43 (8), 1916-1931.

Jochem, F.J., 2001. Morphology and DNA content of bacterioplankton in the northern Gulf of Mexico: analysis by epifluorescence microscopy and flow cytometry. Aquat. Microb. Ecol. 25 (2), 179-194.

Kang, Y., Koch, F., Gobler, C.J., 2015. The interactive roles of nutrient loading and zooplankton grazing in facilitating the expansion of harmful algal blooms caused by the pelagophyte, Aureoumbra lagunensis, to the Indian River Lagoon, FL, USA. Harmful Algae 49, 162-173.

Kendall, C., McDonnell, J.J., 2012. Isotope tracers in catchment hydrology. Elsevier.

Kinney, E.L., Valiela, I., 2011. Nitrogen loading to Great South Bay: land use, sources, retention, and transport from land to bay. J. Coast. Res. 27 (4), 672-686.

Koch, F., Kang, Y., Villareal, T.A., Anderson, D.M., Gobler, C.J., 2014. Novel immunofluorescence flow cytometry technique detects the expansion of harmful brown tides caused by *Aureoumbra lagunensis* to the Caribbean Sea. Appl. Environ. Microbiol. 80 (16), 4947-4957.

Kokkinakis, S.A., Wheeler, P.A., 1987. Nitrogen uptake and phytoplankton growth in coastal upwelling regions. Limnol. Oceanogr. 32 (5), 1112-1123.

Kudela, R.M., Cochlan, W.P., 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. Aquat. Microb. Ecol. 21 (1), 31-47.

Kudela, R.M., Lane, J.Q., Cochlan, W.P., 2008. The potential role of anthropogenically derived nitrogen in the growth of harmful algae in California, USA. Harmful Algae 8 (1), 103-110.

Lapointe, B.E., Barile, P.J., Matzie, W.R., 2004. Anthropogenic nutrient enrichment of seagrass and coral reef communities in the Lower Florida Keys: discrimination of local versus regional nitrogen sources. J. Exp. Mar. Biol. Ecol. 308 (1), 23-58.

Le Bouteiller, A., 1986. Environmental control of nitrate and ammonium uptake by phytoplankton in the Equatorial Atlantic Ocean. Mar. Ecol. Prog. Ser 30, 167-179.

Lipschultz, F., 1995. Nitrogen-specific uptake rates of marine phytoplankton isolated from natural populations of particles by flow cytometry. Marine ecology progress series. Oldendorf 123 (1), 245-258.

Lomas, M.W., Bronk, D.A., van den Engh, G., 2011. Use of flow cytometry to measure biogeochemical rates and processes in the ocean. Annual review of marine science 3, 537-566.

Lomas, M.W., Glibert, P.M., 1999. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. Limnol. Oceanogr. 44 (3), 556-572.

Lomas, M.W., Glibert, P.M., Berg, G.M., Burford, M., 1996. Characterization of nitrogen uptake by natural populations of Aureococcus anophagefferens (Chrysophyceae) as a function of incubation duration, substrate concentration, light, and temperature. J. Phycol. 32 (6), 907-916.

Lopez-Barreiro, T., 1998. Development of an antibody against the Texas brown tide (*Aureoumbra lagunensis*). Harmful Algae, 263-265.

MacIsaac, J.J., 1978. Diel cycles of inorganic nitrogen uptake in a natural phytoplankton population dominated by *Gonyaulax polyedra*.

MacIsaac, J.J., Dugdale, R.C., 1969. The kinetics of nitrate and ammonia uptake by natural populations of marine phytoplankton. Deep Sea Research and Oceanographic Abstracts 16 (1), 45-57.

McCarthy, J.J., Taylor, W.R., Taft, J.L., 1977. Nitrogenous nutrition of the plankton in the Chesapeake Bay. 1. Nutrient availability and phytoplankton preferences. Limnol. Oceanogr 22 (6), 996-1011.

Michelou, V.K., Cottrell, M.T., Kirchman, D.L., 2007. Light-stimulated bacterial production and amino acid assimilation by cyanobacteria and other microbes in the North Atlantic Ocean. Appl. Environ. Microbiol. 73 (17), 5539-5546.

Mulholland, M.R., Boneillo, G., Minor, E.C., 2004. A comparison of N and C uptake during brown tide (*Aureococcus anophagefferens*) blooms from two coastal bays on the east coast of the USA. Harmful Algae 3 (4), 361-376.

Mulholland, M.R., Gobler, C.J., Lee, C., 2002. Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. Limnol. Oceanogr. 47 (4), 1094-1108.

Mulholland, M.R., Lee, C., Glibert, P.M., 2003. Extracellular enzyme activity and uptake of carbon and nitrogen along an estuarine salinity and nutrient gradient. Mar. Ecol. Prog. Ser. 258, 3-17.

Nixon, S.W., Granger, S.L., Taylor, D.I., Johnson, P.W., Buckley, B.A., 1994. Subtidal Volume Fluxes, Nutrient Inputs and the Brown Tide—an Alternate Hypothesis. Estuar. Coast. Shelf Sci. 39 (3), 303-312.

Olson, R.J., Chisholm, S.W., Zettler, E.R., Armbrust, E.V., 1988. Analysis of Synechococcus pigment types in the sea using sigle and dual beam flow-cytometry. Deep-Sea Res. 35 (3), 425-440.

Olson, R.J., Chisholm, S.W., Zettler, E.R., Dusenberry, J.A., 1991. Advances in oceanography through flow cytometry., In: Demers, S. (Ed.), Individual Cell and Particle Analysis in Oceanography. Springer Verlag, pp. 352-402.

Probyn, T., Painting, S., 1985. Nitrogen uptake by size-fractionated phytoplankton populations in Antarctic surface waters. Limnol. Oceanogr. 30 (6), 1327-1332.

Probyn, T., Pitcher, G., Pienaar, R., Nuzzi, R., 2001. Brown tides and mariculture in Saldanha Bay, South Africa. Mar. Pollut. Bull. 42 (5), 405-408.

Rivkin, R.B., Phinney, D.A., Yentsch, C.M., 1986. Effects of flow cytometric analysis and cell sorting on photosynthetic carbon uptake by phytoplankton in cultures and from natural populations. Appl. Environ. Microbiol. 52 (4), 935-938.

Shi, X.L., Lepère, C., Scanlan, D.J., Vaulot, D., 2011. Plastid 16S rRNA gene diversity among eukaryotic picophytoplankton sorted by flow cytometry from the South Pacific Ocean. PLoS One 6 (4), e18979.

Sieracki, M.E., Gobler, C.J., Cucci, T.L., Thier, E.C., Gilg, I.C., Keller, M.D., 2004. Pico- and nanoplankton dynamics during bloom initiation of *Aureococcus* in a Long Island, NY bay. Harmful Algae 3 (4), 459-470.

Smayda, T.J., 1997. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnol. Oceanogr. 42 (5), 1137-1153.

Sosik, H.M., Olson, R.J., Armbrust, E.V., 2010. Flow cytometry in phytoplankton research, Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications. Springer, pp. 171-185.

Stauffer, B.A., Schaffner, R.A., Wazniak, C., Caron, D.A., 2008. Immunofluorescence flow cytometry technique for enumeration of the brown-tide alga, *Aureococcus anophagefferens*. Appl. Environ. Microbiol. 74 (22), 6931-6940.

Stinnette, I., 2014. Nitrogen Loading to the South Shore, Eastern Bays, NY: Sources, Impacts and Management Options (Master's thesis). Stony Brook University: School of Marine and Atmospheric Sciences. Stony Brook, NY.

Talarmin, A., Van Wambeke, F., Duhamel, S., Catala, P., Moutin, T., Lebaron, P., 2011. Improved methodology to measure taxon-specific phosphate uptake in live and unfiltered samples. Limnol. Oceanogr. Methods 9, 443-453.

Varela, D.E., Harrison, P.J., 1999. Seasonal variability in nitrogenous nutrition of phytoplankton assemblages in the northeastern subarctic Pacific Ocean. Deep-Sea Res. Part II-Top. Stud. Oceanogr. 46 (11-12), 2505-2538.

Watson, J.V., 2004. Introduction to flow cytometry. Cambridge University Press.

Zhang, Q.C., Qiu, L.M., Yu, R.C., Kong, F.Z., Wang, Y.F., Yan, T., Gobler, C.J., Zhou, M.J., 2012. Emergence of brown tides caused by *Aureococcus anophagefferens* Hargraves et Sieburth in China. Harmful Algae 19, 117-124.

**Table 1** Abundance (cells $mL^{-1}$) of major phytoplankton community and heterotrophic bacteria during $^{15}N$ uptake experiments using bloom waters from Quantuck Bay, NY in 2014, and Great South Bay in 2015. Abbreviations are as follows: non-BT picoeukarytoes = non-brown tide picoeukaryotes, PC cyanobacteria = phycocyanin-containing cyanobacteria. '-' indicates data is not available. Asterisk indicates no data available.

| Date | *Aureococcus anophagefferens* | Non-BT PicoEukaryotes | PC cyanobacteria | Heterogrophic bacteria |
|---|---|---|---|---|
| 5/19/2014 | 27,400 | * | * | * |
| 5/28/2014 | 13,800 | * | * | * |
| 6/4/2014 | 61,000 | * | * | * |
| 6/11/2014 | 341,100 | * | * | * |
| 6/17/2014 | 216,400 | 179,800 | 29,000 | 11,956,900 |
| 6/20/2014 | 231,300 | 632,400 | 71,400 | 24,124,800 |
| 6/27/2014 | 309,600 | 2,297,100 | 444,700 | 58,420,900 |
| 7/3/2014 | 225,200 | 1,635,100 | 995,600 | 95,377,400 |
| 7/10/2014 | 292,100 | 892,800 | 2,095,400 | 21,695,200 |
| 7/24/2014 | 43,100 | 1,990,400 | 4,942,400 | 35,447,300 |
| 8/7/2014 | 12,000 | 3,618,100 | 6,696,200 | 35,818,100 |
| 10/2/2014 | 448,000 | 698,300 | 588,300 | 3,415,500 |
| 10/9/2014 | 456,600 | 316,900 | 186,200 | 9,410,200 |
| 6/9/2015 | 278,300 | 44,100 | 604,100 | 6,259,600 |
| 6/15/2015 | 427,100 | 194,100 | 626,700 | 7,806,100 |
| 6/23/2015 | 618,400 | 252,900 | 712,600 | 10,144,600 |
| 6/30/2015 | 766,600 | 333,300 | 724,900 | 11,115,400 |
| 7/7/2015 | 311,800 | 1,798,700 | 1,398,600 | 22,587,100 |
| 7/14/2015 | 81,600 | 5,120,700 | 1,539,700 | 24,227,600 |

**Table 2** Nutrient levels (μM) in Quantuck Bay, NY during summer and fall, 2014. Asterisk indicates no data available.

| | $NO_2^- + NO_3^-$ | $NH_4^+$ | $PO_4^-$ | Urea | Glutamic acid |
|---|---|---|---|---|---|
| 6/17/2014 | 0.13 ± 0.07 | 4.6 ± 1.0 | 0.46 ± 0.01 | 0.54 ± 0.02 | * |
| 6/20/2014 | 0.18 ± 0.08 | 2.6 ± 0.72 | 0.44 ± 0.05 | 0.72 ± 0.11 | 0.06 |
| 6/27/2014 | 0.25 ± 0.05 | 3.0 ± 0.41 | 0.47 ± 0.01 | 0.44 ± 0.14 | 0.04 |
| 7/3/2014 | 0.48 ± 0.05 | 2.7 ± 0.35 | 0.65 ± 0.02 | 0.43 ± 0.15 | * |
| 7/10/2014 | 0.24 ± 0.10 | 2.1 ± 0.23 | 0.84 ± 0.02 | 0.50 ± 0.10 | * |
| 7/15/2014 | 0.19 ± 0.16 | 1.6 ± 0.11 | 0.44 ± 0.01 | 0.65 ± 0.09 | * |
| 7/24/2014 | 0.36 ± 0.12 | 1.9 ± 0.04 | 0.49 ± 0.02 | 1.6 ± 1.4 | * |
| 8/7/2014 | 0.41 ± 0.07 | 1.4 ± 0.16 | 0.32 ± 0.06 | 2.4 ± 2.0 | 0.09 |
| 10/2/2014 | 0.35 ± 0.12 | 1.2 ± 0.14 | 0.49 ± 0.02 | 0.35 ± 0.20 | 0.03 |
| 10/9/2014 | 0.33 ± 0.07 | 2.4 ± 1.5 | 0.50 ± 0.01 | 0.66 ± 0.09 | 0.09 |

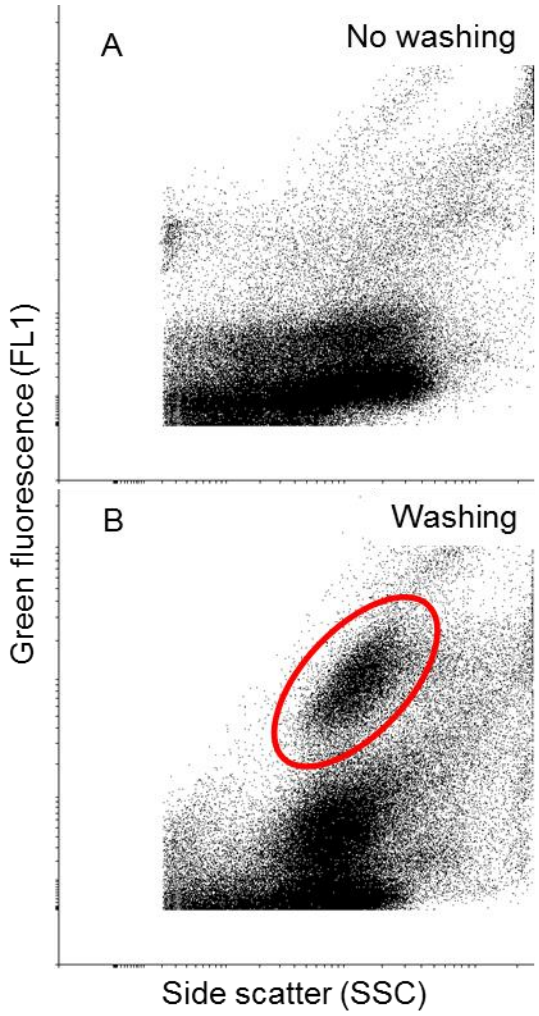



**Figure 1.** Data output from BD FACSAria III for a *Aureococcus anophagefferens* concentrated sorting sample (A) without and (B) with a washing process. Samples were compared on the same acquisition setting of a flow cytometer. Sample was enriched with $^{15}N$-labeled $NH_4^+$ and collected on June 27, 2014.

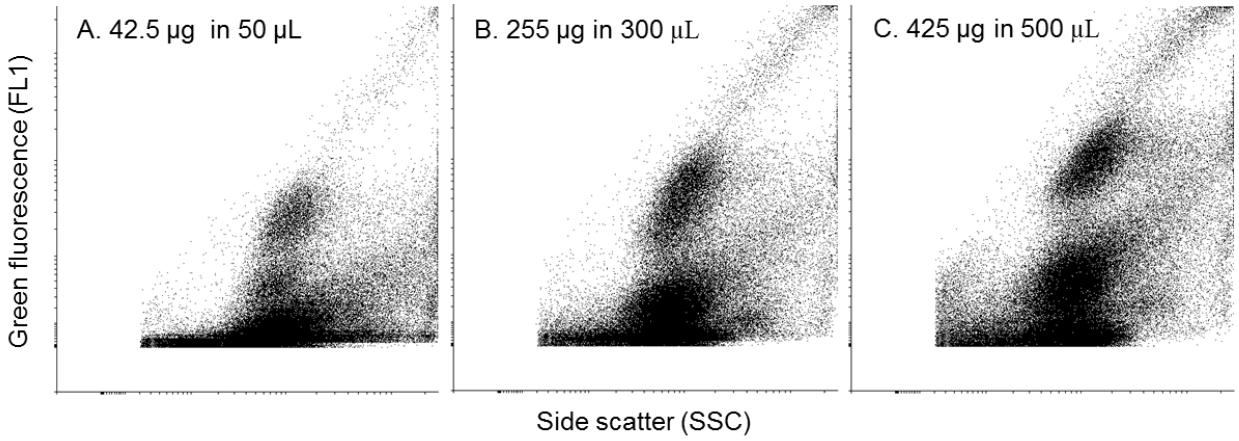



**Figure 2.** Flow cytometry data output of sorting sample after 1 hr incubation with different amounts of species-specific antibody. (A) 42.5 µg in 50 µL antibody addition, (B) 255 µg in 300 µL antibody addition, (C) 425 µg in 500 µL antibody addition. Samples were compared on the same acquisition setting of a flow cytometer.

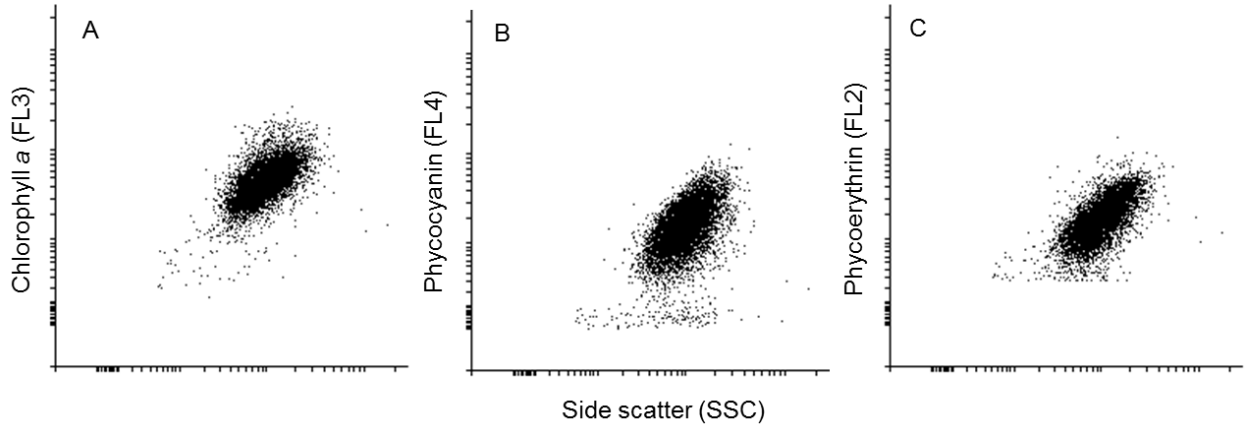



**Figure 3.** Flow cytometry data output of sorted *Aureococcus anophagefferens* cells with different pigment characteristics compared to size, which are the main parameters used to identify natural phytoplankton community with a flow cytometer. (A) Chlorophyll vs. size, (B) Phycocyanin vs. size, and (C) Phycoerythrin vs. size which are the main parameters used to identify natural phytoplankton community with a flow cytometer.

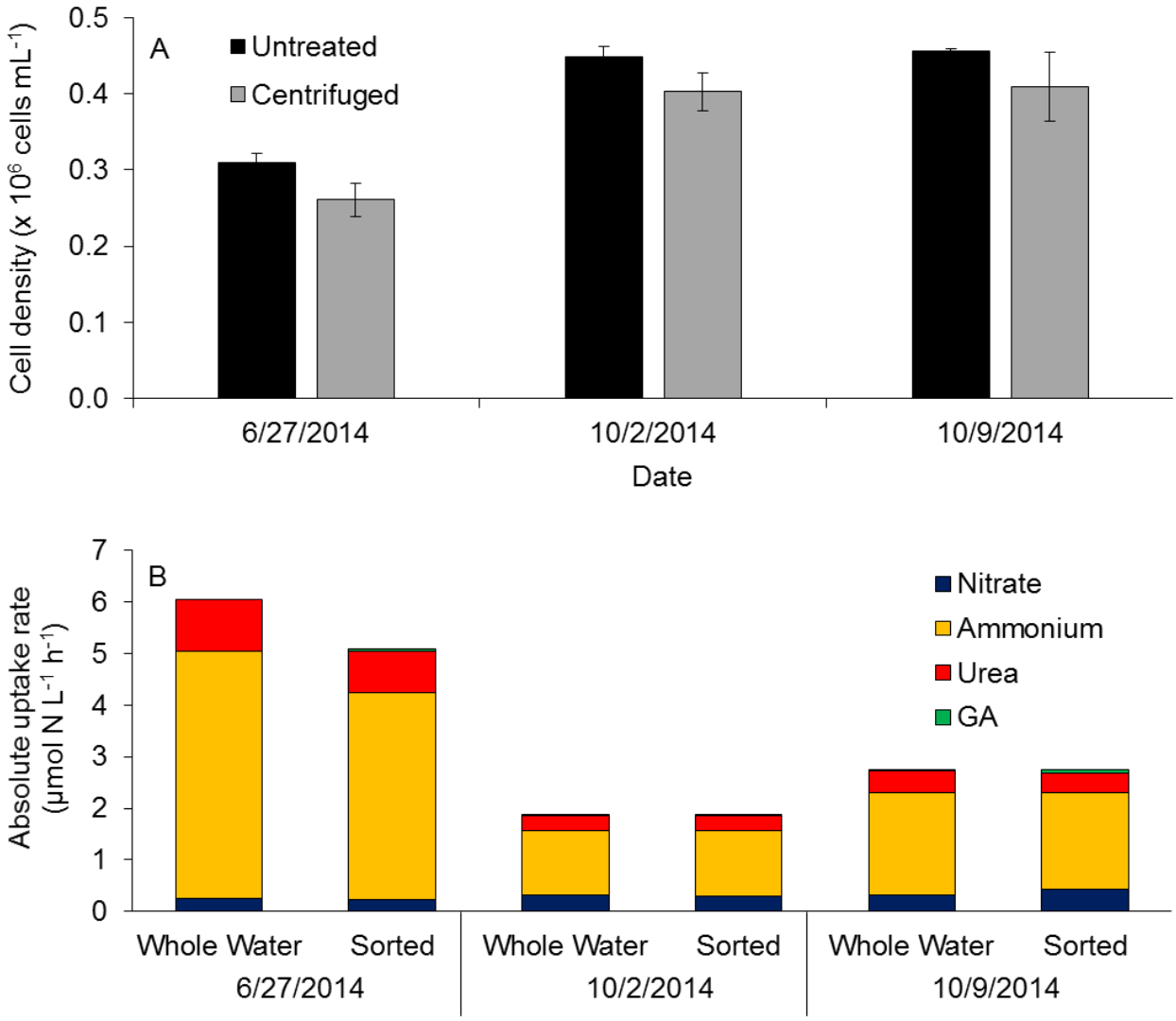



**Figure 4.** (A) Cell density of *Aureococcus* in untreated bloom samples and centrifuged and resuspended samples and (B) absolute uptake rates of the whole water and sum of all sorted groups during $^{15}N$ uptake experiments.

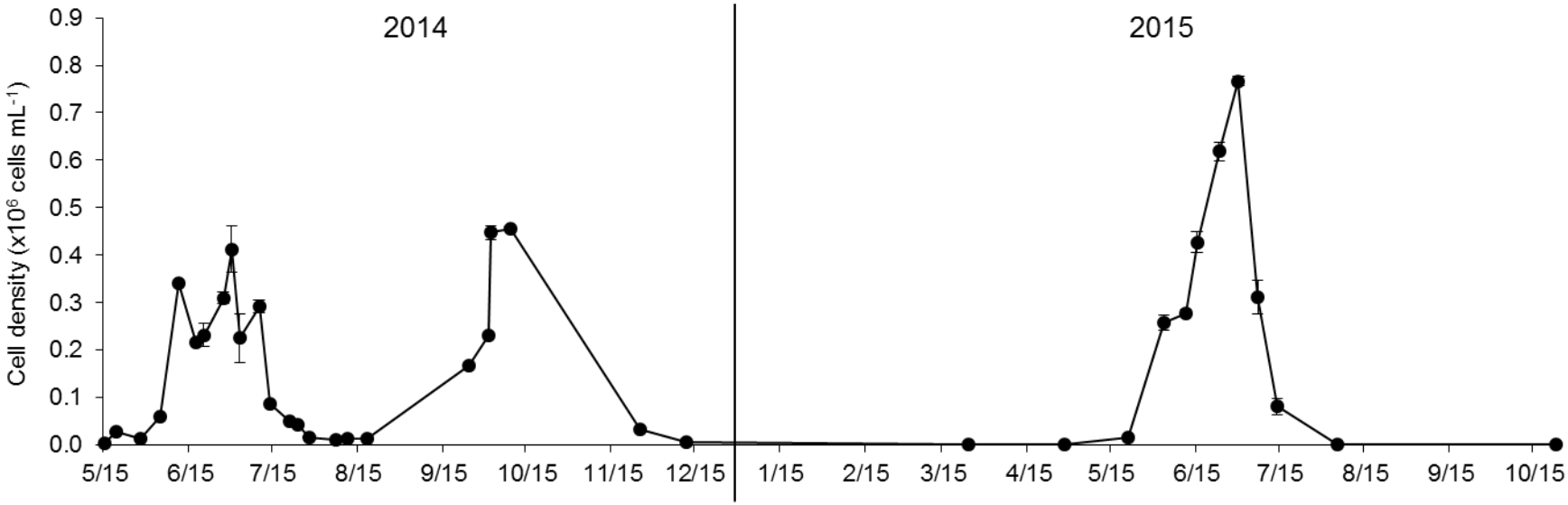



**Figure 5.** Brown tide dynamics in Quantuck Bay, NY in 2014 and Patchogue Bay, NY in 2015.

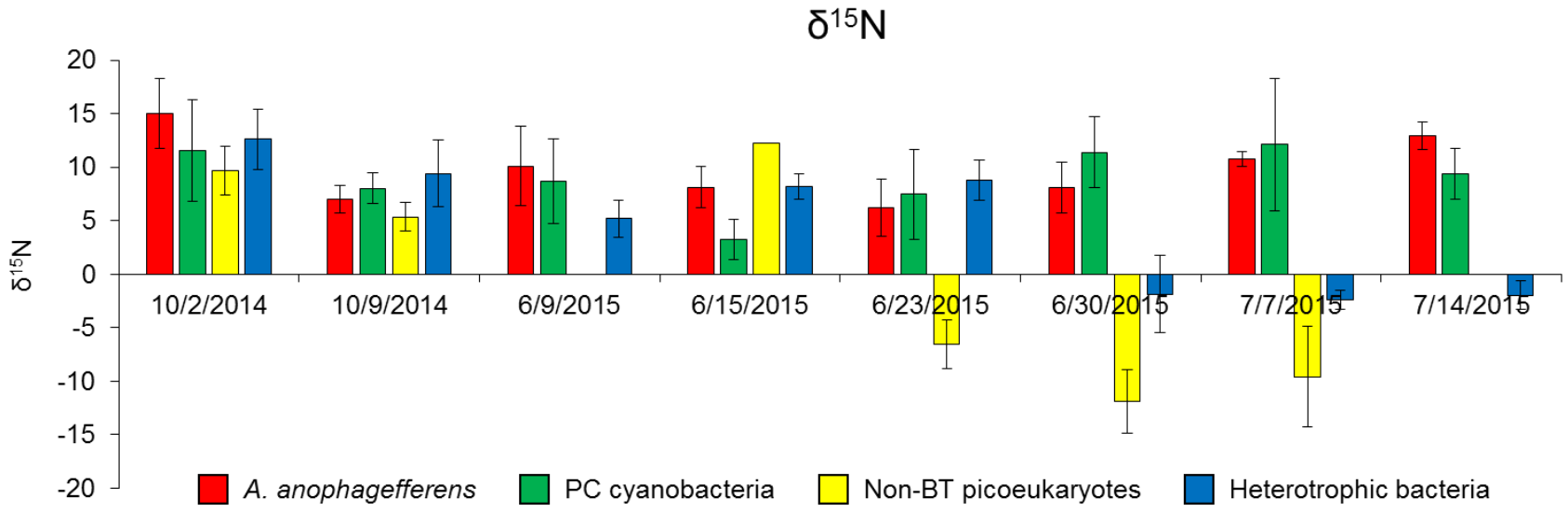



**Figure 6.** $\delta^{15}N$ values of major plankton groups during brown tide in the fall of 2014 and the summer of 2015. Abbreviations are as follows: *A. anopahgefferens* = *Aureococcus anophagefferens*, non-BT picoeuks = non-brown tide picoeukaryotes, pc cyanobacteria = phycocyanin-containing cyanobacteria. Denote that no data for non-BT picoeuks on June 9 and July 14, 2015.

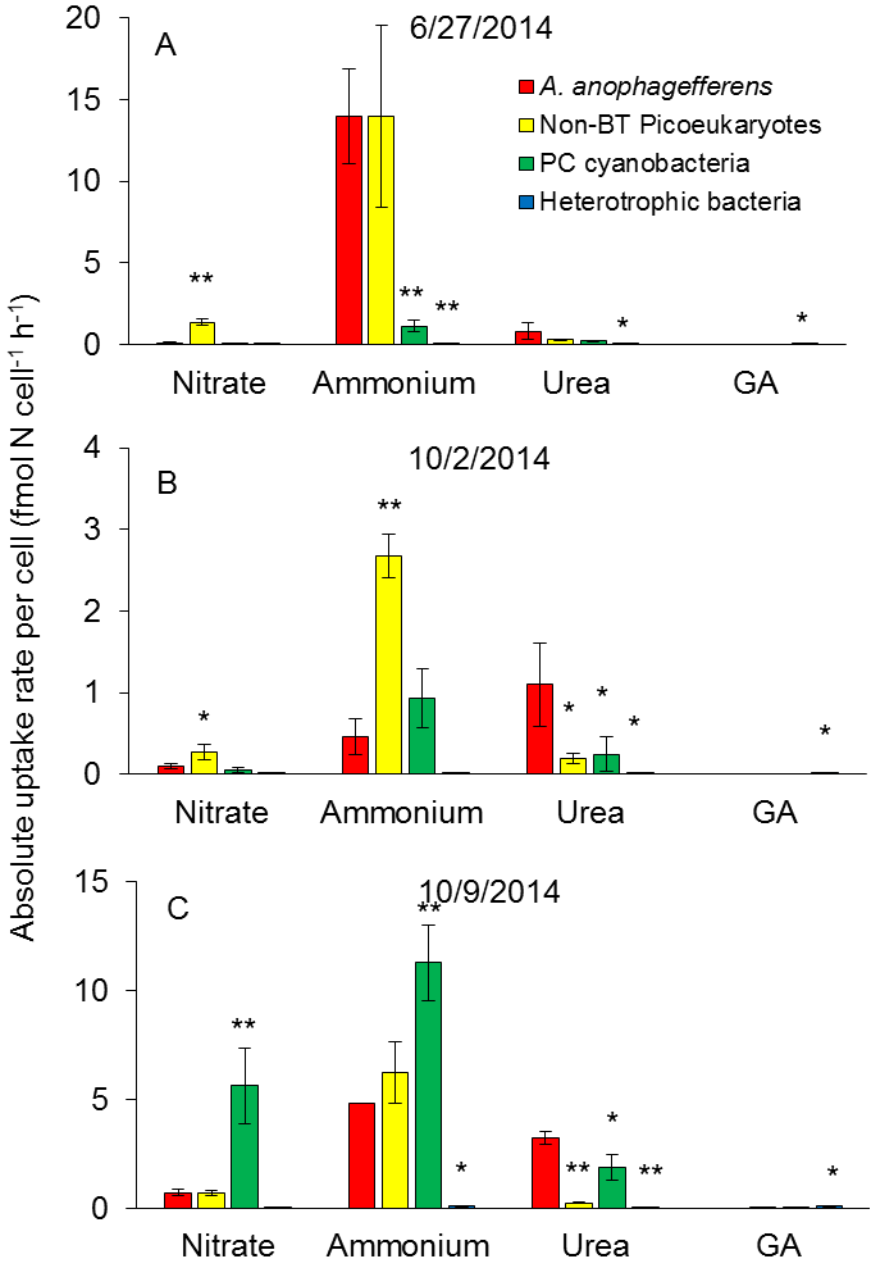



**Figure 7.** Absolute uptake rate per cell (fmol N cell$^{-1}$ h$^{-1}$) of major picoplankton groups. Abbreviations are as follows: *A. anopahgefferens* = *Aureococcus anophagefferens*, non-BT picoeuks = non-brown tide picoeukaryotes, PC cyanobacteria = phycocyanin-containing cyanobacteria. One-Way ANOVA was performed with a post-hoc Tukey test to assess the statistical difference between *Aureococcus* and other groups. Asterisks indicate significant difference among groups (* = $p$<0.05, ** = $p$<0.001).

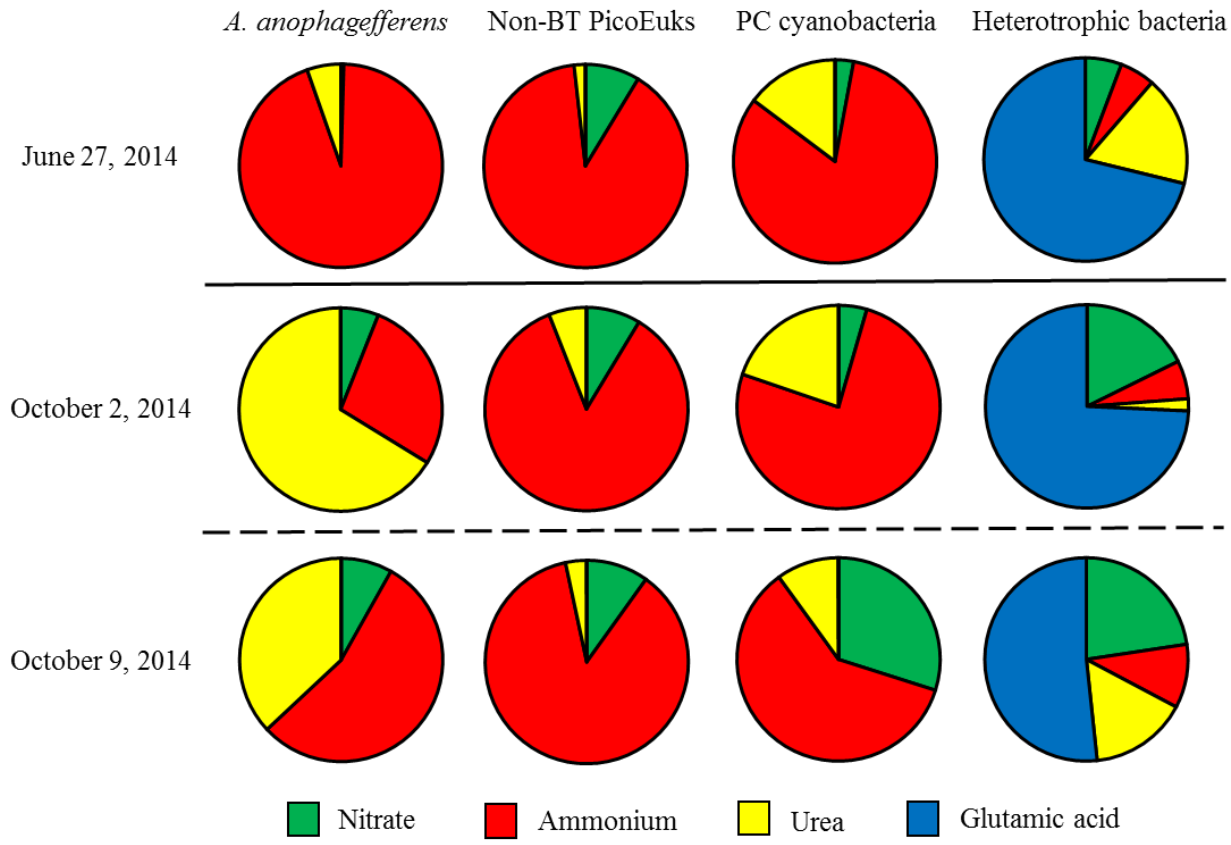



**Figure 8.** Proportion (%) of N uptake by major picoplankton groups. Abbreviations are as follows: *A. anopahgefferens* = *Aureococcus anophagefferens*, non-BT picoeuks = non-brown tide picoeukaryotes, PC cyanobacteria = phycocyanin-containing cyanobacteria. Solid line separates between summer and fall experiments and dashed line separates different experimental dates.

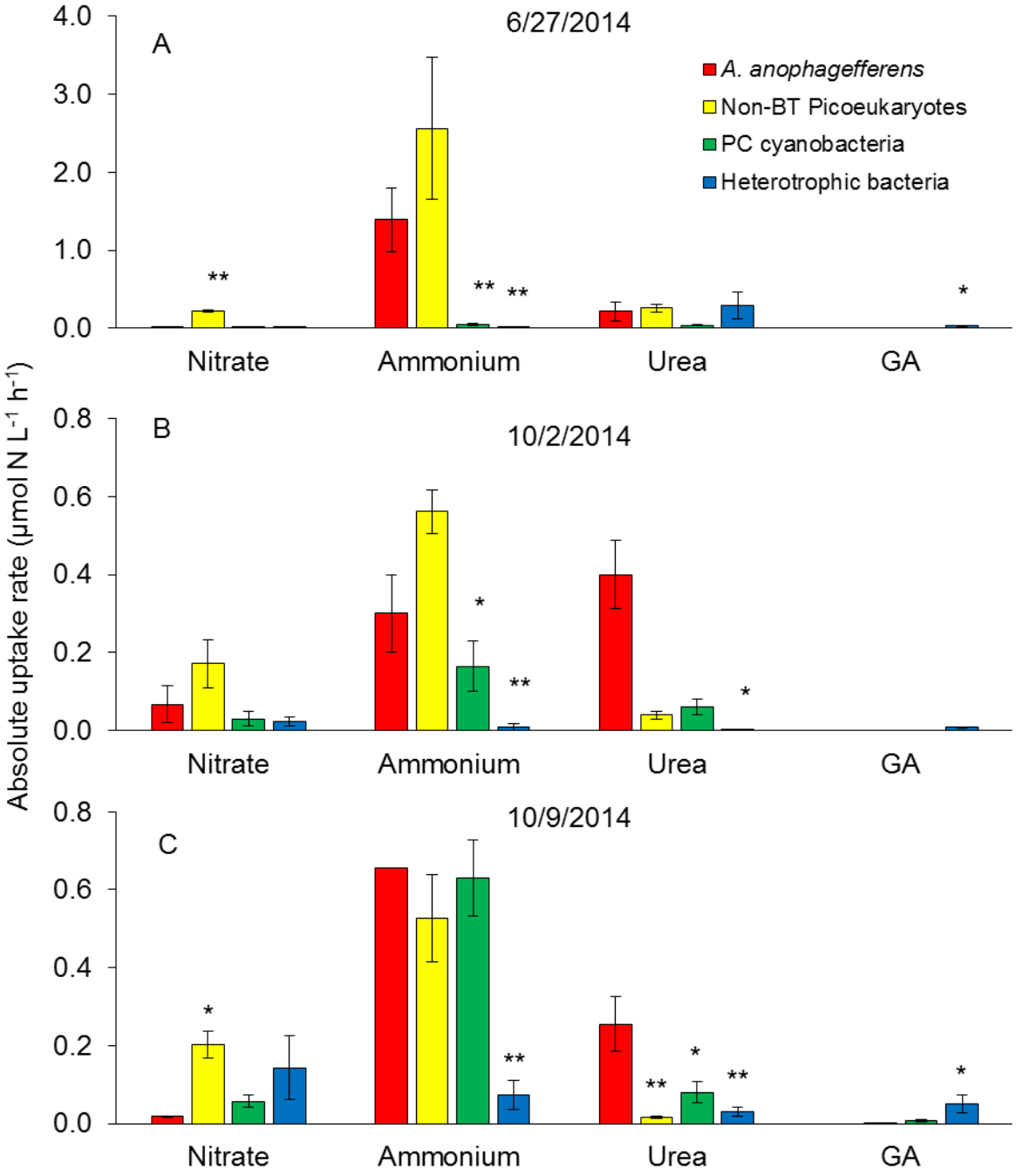



**Figure 9.** Absolute uptake rate (µmol N $L^{-1}$ $h^{-1}$) of major picoplankton groups. Abbreviations are as follows: *A. anopahgefferens* = *Aureococcus anophagefferens*, non-BT picoeuks = non-brown tide picoeukaryotes, pc cyanobacteria = phycocyanin-containing cyanobacteria. One-Way ANOVA was performed with a post-hoc Tukey test to assess the statistical difference between *Aureococcus* and other groups. Asterisks indicate significant difference among groups (* = $p<0.05$, ** = $p<0.001$).

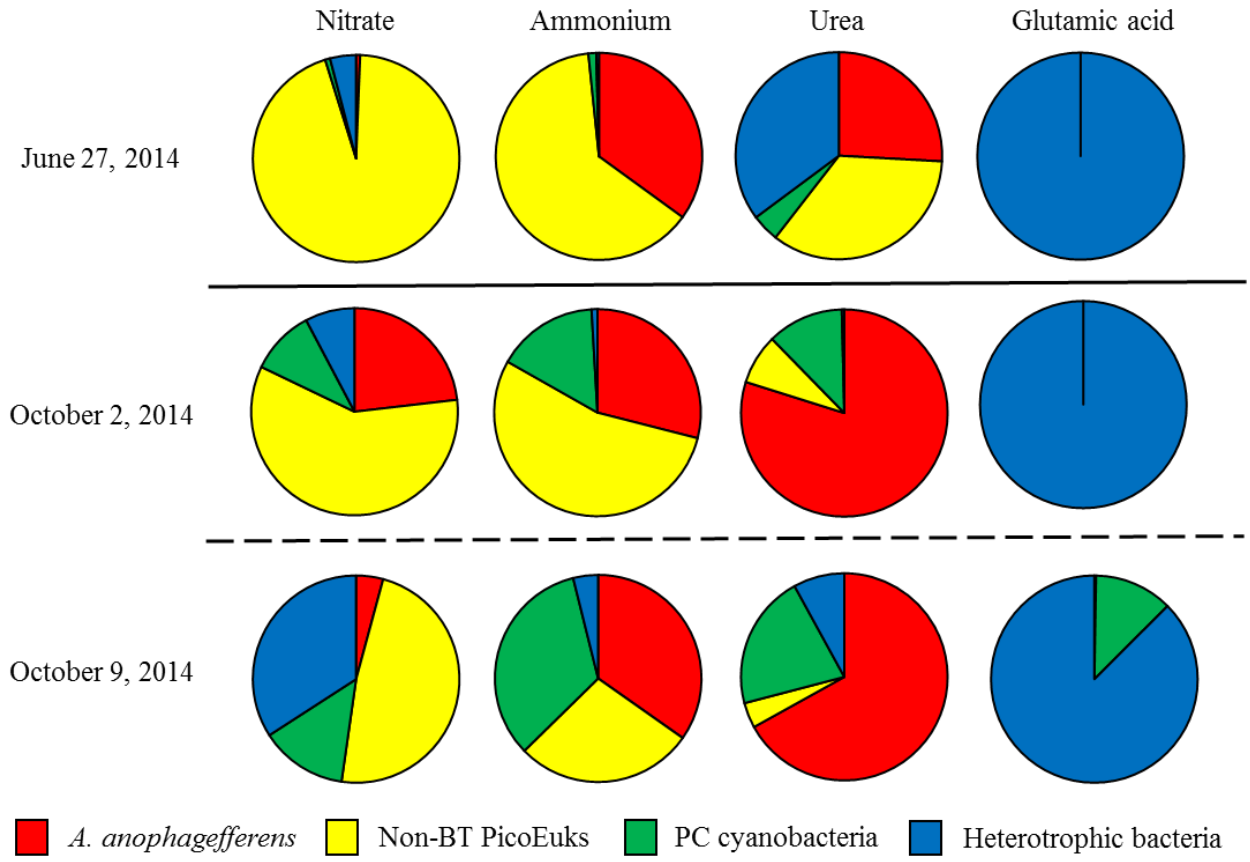



**Figure 10.** Percentage (%) of absolute uptake of major picoplankton groups. Abbreviations are as follows: *A. anopahgefferens* = *Aureococcus anophagefferens*, non-BT picoeuks = non-brown tide picoeukaryotes, pc cyanobacteria = phycocyanin-containing cyanobacteria. Solid line separates between summer and fall experiments and dashed line separates different experimental dates.

# Chapter 6

## Comparative transcriptomes of open ocean and coastal, bloom-forming pelagophytes in response to nutrient (N, P) limitation and light limitation

**Abstract**

Pelagophytes are important members of marine plankton communities, being dominant picophytoplankton at subsurface chlorophyll maximum in ocean ecosystems and the causative algae of harmful brown tides in estuaries. Still, the physiological pathways facilitating the ecological success of pelagophytes in these diverse ecosystems are poorly understood. Here, I used next generation sequencing to investigate the transcriptomic response of two coastal pelagophytes, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, and two open ocean pelagophytes, *Pelagococcus subviridis* and *Pelagomonas calceolata*, to conditions commonly found within the marine ecosystems where they thrive: low levels of nitrogen (N), phosphorus (P), or light. A total of 62,653 clusters were generated with 8,482 clusters for *Aureoumbra*, 15,578 clsuters for *Pelagomonas*, 17,033 clusters for *Pelagococcus*, and 21,560 clusters for *Aureococcus* and only a small amount of these clusters being shared among all pelagophytes (13% to 33%). Of these clusters, 8% were significantly and differentially expressed under low N, P, or light with the majority being associated with energy and lipid metabolism. Specific cluster sets that were differentially expressed under low N, P, and light included genes associated with cleavage of N from organic compounds, intracellular P recycling, lipid hydrolysis, and low-light adaptation. Orthologous comparisons highlighted distinct physiological adaptations among pelagophytes such as the expression of unique phosphate transporters by *Aureococcus* in low P, the use of intracellularly recycled phosphate originating from pyrophosphate and/or nucleoside triphosphate under low P in *Aureococcus* and *Pelagomonas*, the use of flavodoxins over ferredoxins under low light for *Pelagomonas*, and the upregulation of lysophospholipase under low light in *Pelagococcus*. Collectively, this study demonstrates that coastal and open ocean

pelagophytes share several common ecophysiological features that help them adapt to distinct marine habitats.

## Introduction

During the past two decades, whole genome sequencing has provided significant insight regarding the physiological potential of multiple species of phytoplankton (Palenik et al., 2003; Armbrust et al., 2004; Gobler et al., 2011; Lin et al., 2015). Since these genomes represent only the genetic potential that an organism may have, it remains to be understood which genes that are activated in response to environmentally relevant conditions (McLean, 2013). With development of high throughput sequencing techniques (i.e. next generation sequencing; NGS) and recent advances in bioinformatic approaches (Soneson and Delorenzi, 2013), the sequencing and quantification of messenger RNA transcript abundances, also known as transcriptomes, have been used to bridge the information gap between genomic potential and phytoplankton response to ecosystem conditions (Keeling et al., 2014; Mock et al., 2016).

Pelagophytes are important members of ocean plankton communities (Simon et al., 1994; Andersen et al., 1996) (John et al., 2007). Prior studies have used flow cytometric and electron microscopic analyses to demonstrate that the pelagophyte, *Pelagomonas* sp., is one of the most abundant picoeukaryotes in the North Atlantic and Pacific subtropical oceans (Simon et al., 1994). A more recent study utilized sorting flow cytometry and whole-genome sequencing to reveal that *Pelagomonas calceolata* is ubiquitously distributed across world oceans (Cuvelier et al., 2010; Worden et al., 2012). Dupont et al. (2014) sequenced metagenomes and metatranscriptomes of microbial communities in Eastern sub-tropical Pacific Ocean and found that *Pelagomonas calceolata* was highly abundant in the subsurface chlorophyll maximum

(SCM) being responsible for the majority of nitrogen-assimilation transcripts in the SCM (Dupont et al., 2014). In coastal zones, the bloom-forming pelagophytes, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, are notorious for their ability to form harmful brown tides that cause disruption and harm in coastal ecosystems (Gobler and Sunda, 2012). *Aureococcus* and *Aureoumbra* are the two most closely related pelagophytes and share a highly similar ecological niche (Gobler and Sunda 2012).

While coastal and open ocean ecosystems differ radically with respect to many environmental conditions, some characteristics of coastal zones mimic those of the subsurface chlorophyll maximum (i.e. low light, slightly elevated nutrient levels, mix of nitrogen sources; Cullen et al., 1982) where *Pelagomonas calceolata* dominates (Dimier et al., 2009; Li et al., 2013). In addition, brown tide blooms often occur during periods when dissolved inorganic nitrogen (DIN) and phosphorus (DIP) levels are low (Gobler et al., 2004a; Gobler et al., 2013a). This is contrast to other harmful algal blooms that are attributed to direct loading of inorganic nutrients (Heisler et al., 2008). Both *Aureococcus* and *Aureoumbra* can rely on dissolved organic nitrogen (DON) and phosphorus (Camacho et al.) for growth (Gobler et al., 2004a; Muhlstein and Villareal, 2007) and the levels of DON and DOP can significantly decrease during a course of brown tide, suggesting active degradation of compounds in these pools (Gobler et al., 2004a; Gobler et al., 2013a). The ability of *Aureococcus* and *Aureoumbra* to utilize DON and DOP has been highlighted in previous genomic, transcriptomic, and proteomic studies (Berg et al., 2008; Gobler et al., 2011; Wurch et al., 2011b; Sun et al., 2012; Wurch et al., 2013; Frischkorn et al., 2014).

For this chapter, I investigated the transcriptomic response of two coastal pelagophytes, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, and two open ocean pelagophytes, *Pelagococcus subviridis* and *Pelagomonas calceolata*, to conditions commonly found within the

ecosystems where they thrive: low levels of N, P, or light. To understand universal transcriptomic features among pelagophytes, I examined shared clusters among the pelagophytes under each condition. To understand how each pelagophyte responds to low N, P, or light conditions, I examined the transcripts of clusters that significantly increased or decreased in abundance in response to each condition.

## Methods and Materials

### *Culture maintenance*

Two costal pelagophytes, *Aureococcus anophagefferens* (CCMP1850 isolated from Great South Bay, NY, USA, 40.651°N, -73.152°, in 1998), *Aureoumbra lagunensis* (CCMP1510 isolated from Laguna Madre, TX, USA, 27.471°N, -97.320°, in 1992) and two oceanic pelagophytes, *Pelagococcus subviridis* (CCMP1429; isolated from the subarctic Pacific, 49.917° N, -145.117°, isolation date unknown) and *Pelagomonas calceolata* (CCMP1214, isolated from the sub-tropical Pacific, 30.833°N, -136.833°, in 1973) were used to perform experiments and for transciptome sequencing. Cultures were grown in modified GSe medium (Tang and Gobler, 2009) made with boiled and then filter-sterilized (0.22 µm) North Atlantic seawater (40.290N, -71.989) with a final salinity of 32.5 and amended with 60 µM $NH_4^+$, 5 µM $PO_4^-$. 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., USA) to minimize bacterial abundance. Cultures were grown with a 14:10 h light:dark cycle, illuminated by a bank of fluorescent lights (100 µmol quanta $\cdot m^{-2} \cdot s^{-1}$). *Aureococcus*, *Aureoumbra*, and *Pelagomonas* were grown at 21ºC whereas *Pelagococcus*, isolated from the subarctic Pacific, was grown at 14ºC.

*Experimental design*

To investigate responses to different environmental conditions, cultures were incubated under conditions of low P (1 µM $PO_4^-$), low N (30 µM $NH_4^+$), and low light (20 µE $m^{-2}$ $s^{-1}$) and control cultures were incubated with N, P (60 µM $NH_4^+$, 5 µM $PO_4^-$) and light (100 µE $m^{-2}$ $s^{-1}$). Triplicate, 450 mL cultures were inoculated at ~3.5 x $10^5$ cells $mL^{-1}$ and monitored daily at the same time each day to avoid diel variation in gene expression or physiology responses. *In vivo* fluorescence was measured on Turner Designs TD-700 fluorometer with EM filter of >665 nm and EX filter of 340-500 nm (Sunnyvale, CA, USA). Maximum quantum efficiency of photosystem II ($F_v/F_m$) of cultures 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 on the same fluorometer. All readings were blank corrected using culture media. The addition of DCMU blocks the electron transfer between photosystem I and photosystem II, thus maximize the fluorescence (Parkhill et al., 2001). $F_v/F_m$ has been utilized as an indicator of nutrient status with values of ~ 0.6 under nutrient replete conditions and decreasing to ~ 0.3 under nutrient deplete conditions (Parkhill et al., 2001; Harke and Gobler, 2013). The maximum quantum efficiency was calculated based on the equation below:

$$F_v/F_m = (F_m - F_i)/F_m$$

, where $F_m$ is the DCMU-enhanced *in vivo* fluorescence, $F_i$ is the normal *in vivo* fluorescence. Alkaline phosphatase activity (APA) was measured daily on the TD-700 fluorometer using 4-Methylumbelliferyl phosphate of 250 µM (Hoppe, 1983). Cell density was quantified with a 50 µm aperture after preserved with Lugol's iodine using a Beckman Multisizer 3 Coulter Counter

(Fullerton, CA, USA). Differences in growth rates, $F_v/F_m$, and APA among cultures were assessed via One-Way Analysis of Variance (Epifanova and Terskikh) and post-hoc Tukey tests.

*RNA extraction and cDNA sequencing*

Control condition cultures were harvested during exponential growth phase while low N and low P cultures were harvested when $F_v/F_m$ was reduced and APA was elevated relative to control cultures, respectively, and low light cells were harvested when cultures established a stable but slower growth rate compared to control cultures. Cell pellets of each culture were made by centrifuging for 10 min at 1300 x g at 21ºC or 14ºC for *Pelagococcus*. After supernatant removal, the pellets were resuspended into 2 mL micro-centrifuge tubes and centrifuged again. Concentrated cells were immediately frozen in liquid nitrogen and stored at -80ºC. Total processing time from experimental conditions to -80ºC was $\leq$ 20 min.

Total RNA of pooled replicates per treatment was extracted with an UltraClean™ plant RNA isolation kit (Mo Bio Laboratories Inc. CA) according to manufacturer's instructions. Any residual DNA was digested using an Ambion Turbo DNA-*free*™ kit according to manufacturer's instructions. RNA quantity was assessed with Invitrogen Qubit Q32855 and RNA quality was assessed with an Agilent 2100 Bionalayzer. As a part of the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (Keeling et al., 2014), sequencing was performed at National Center for Genome Resources (NCGR) using an Illumina TruSeq RNA Sample Preparation Kit to generate libraries using ~2 µg of RNA. Sequencing of 50 base pair paired-end reads from each library was performed on an Illumina HiSeq 2000 at the NCGR. Optimally, 2 Gbp of sequence was generated per library. Sequence data is available on iMicrobe server (http://data.imicrobe.us) with the identification numbers MMETSP0914 – MMETSP0917 for

*Aureococcus anophagefferens* (0914=replete control, 0915=low N, 0916=low P, 0917=low light), MMETSP0890 – MMETSP0893 for *Aureoumbra lagunensis* (0890=replete control, 0891=low N, 0892=low P, 0893=low light),, MMETSP0882 – MMETSP0885 for *Pelagococcus subviridis* (0882=replete control, 0883=low N, 0884=low P, 0885=low light),, MMETSP0886 – MMETSP0889 for *Pelagomonas calceolate* (0886=replete control, 0887=low N, 0888=low P, 0889=low light).

*De novo* assembly, annotations, OrthoMCL clustering, and expression analysis

*De novo* assembly of sequence data was performed to reconstruct transcriptomes using ABySS (Simpson et al., 2009). Assembly and quantification were conducted using NCGR's pipeline called BPA2.0 (Batch Parallel Assembly version 2.0). Low quality reads were trimmed using a String Graph Assembler with swinging average at Q15 (SGA; Simpson and Durbin, 2012). After reads shorter than 25 base pairs were discarded, preprocessed sequences were assembled using ABySS with 20 unique k-mer sizes between k=26 and k=50 (Simpson and Durbin, 2012). Then, the assembled reads (=unitigs) from all k-mer assemblies were combined and clustered at 98% sequence identity using CD-Hit (Li and Godzik, 2006). Then, CAP3 was used to assemble clustered contigs into larger sequences (Huang and Madan, 1999) and the resulting contigs were paired-end scaffolded using ABySS (Simpson et al., 2009). GapCloser, a part of SOAP *de novo* package, was used to close gaps created during scaffolding. Redundant sequences were removed using CD-Hit and contigs less than 150 bp were filtered out (Li and Godzik, 2006).

The Expressed Sequence Tag Scan (ESTScan) was used to predict coding sequences (Iseli et al., 1999). Sequence reads were aligned back to the predicted coding sequences using

Burrows-Wheeler Alignment tool (BWA; Li and Durbin, 2009). Peptide predictions over 30 amino acids in length were annotated. Hits against the UniProtKB/Swiss-Prot database were generated using BLASTp (Altschul et al., 1990). Coding sequences were also characterized using HMMER3 (Zhang and Wood, 2003) against Pfam-A (Bateman et al., 2004), TIGRFAM (Haft et al., 2001), and SUPERFAMILY (Gough et al., 2001) databases.

After assembly, predicted peptide sequences were clustered into orthologous groups using OrthoMCL (Li et al., 2003) by running all versus all BLASTP with default settings: an e-value cutoff of 1e-5 and an inflation value (-I) of 1.5. OrthoMCL generates clusters of peptides where each cluster is composed of orthologs or paralogs (Li et al., 2003) and has been used to provide orthologous groups across multiple taxa (Bender et al., 2014; Di Dato et al., 2015; Simmons et al., 2016) or strains (Alexander, 2016). OrthoMCL was performed via virtual workbench, the Discovery Environment of the iPlant Collaborative (https://de.iplantcollaborative.org/de/) (Stein, 2008; Oliver et al., 2013). Read counts of contigs for the entire set of OrthoMCL results were summed at each cluster level by each treatment and each species and Analysis of Sequence Counts (ASC) analyses were performed using the summed read counts from each species separately. Statistically significant differences in read count at cluster level in low nutrients (N, P) and low light treatments compared to control were determined by a fold-change greater than or equal to 2 and a posterior probability greater than 0.95 (Wu et al., 2010). Typically, sequencing of replicates is used to assess differential gene expression. However, in this study, three biological replicates were pooled and single sequence was analyzed. ASC was specifically selected to statistically assess differences in gene expression of treatments compared to the replete control as ASC is an empirical Bayesian method that has been used to explore differential gene expression in other marine phytoplankton

studies without sequenced replicates (Dyhrman et al., 2012; Konotchick et al., 2013; Frischkorn et al., 2014) and has been shown to identify genes with a log-fold change greater than 2 under such conditions (Wu et al., 2010). The sequencing depth (> 10 M reads per sample) used for this study enhances statistical power and precision and the likelihood of detecting significant changes in expression without replicates (Liu et al., 2014). $Log_2$ fold changes were calculated from the normalized read counts that were summed at the cluster level (reads or tags per million; TPM). Statistically significantly changes in expressed clusters were mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2006) using KEGG Automatic Annotation Server (http://genome.jp/tools/kaas/) on a complete or draft genome mode. Grouping clusters into multiple modules via KEGG database describes differences in the cluster distribution at a module level. Because the majority of clusters that are involved in nitrogen or phosphate metabolism do not have KEGG annotations (Marchetti et al., 2012; Alexander et al., 2015), annotations from NCGR pipeline and KEGG annotations were comparably used to characterize biochemical pathways. Clusters associated with phosphate metabolism were also assigned as energy metabolism that has nitrogen metabolism in KEGG database. Of the significantly expressed clusters, core clusters that were shared among all four pelagophytes were used to assess the common biochemical features among pelagophytes and the entire clusters of each species were analyzed to assess the response of each species to experimental conditions representing ecosystem conditions in which pelagophytes thrive. To compare functions of differentially expressed clusters, the clusters that were associated with light, N, P, selenocysteine, and transport-related metabolism were pooled from the total of differentially expressed clusters and partitioned into five functional groups. Selenocysteine and transport

groups were chosen because of their importance in pelagophyte growth (Gobler et al., 2013c) and nutrient assimilation (Berg et al., 2008; Wurch et al., 2011b).

## Results

### *Culture growth in different treatments*

Cells were harvested at a different time for each treatment and species to assure unlimited, exponential or resource limiting conditions were present for each culture. Low P cultures of *Aureococcus anophagefferens* were incubated three days later than other treatments that were harvested on day 9 (Fig. 1A) while low N cultures of *Aureoumbra lagunensis* were harvested on day 6, a day earlier than other treatments (Fig. 1B). Similarly, *Pelagococcus subviridis* was harvested on day 10 for low N and day 11 for other treatments (Fig. 1C). Low light cultures of *Pelagomonas calceolata* were incubated for 11 days and others were terminated on day 6 (Fig. 1D). At the time of harvest, growth rates of nutrient-replete cultures (=control) were significantly higher than those of other treatments ($p$<0.05; One-Way ANOVA; Tukey test) except for *Aureoumbra*, of which growth rates in low P were as high as control (Table 1). Further, the photosynthetic efficiency ($F_v/F_m$) of low N cultures was significantly lower than that of the control in each experiment ($p < 0.05$; One-Way ANOVA; Tukey test; Table 1). Alkaline phosphatase activity in low P cultures was significantly higher than control cultures on the day of harvest for all species ($p < 0.05$; One-Way ANOVA; Tukey test; Table 1) except for *A. anophagefferens*.

### *Contigs and clusters comparison*

A total of 62,653 clusters were generated from a total of 78,780 peptides predicted from contigs comprising 8,482 clusters for *Aureoumbra*, 15,578 clusters for *Pelagomonas*, 17,033

clusters for *Pelagococcus*, and 21,560 clusters for *Aureococcus* (Fig. 2A, B). The total clusters were clustered into three groups: core clusters found in all species, shared clusters found in at least two species, and unique clusters found in only one species (Fig. 2A). The core clusters in each species accounted for relatively small proportion of the total clusters ranging from 13% in *Aureococcus* to 33% in *Aureoumbra* while the proportion of shared and unique clusters were much higher (Fig. 2C). Of the total clusters in each species, the number of clusters having single-copy contigs that did not cluster with any other contigs was similar to the number of clusters having multi-copy contigs and being composed of paralogs (Fig. 3), except for *Aureoumbra* for which the number of clusters with single-copy contigs was three-fold lower (2,210) compared to that of clusters with multi-copy contigs (6,272) (Fig. 3).

*Significant changes in cluster abundance induced by resource limitation*

Raw read counts that were summed at the cluster level were analyzed using ASC to examine differentially expressed clusterscluster. Of the total clusters generated, 4,879 clusters were differentially expressed among treatments with 1,224 clusters being significantly increased and 3,655 clusters being significantly decreased in the summed readcounts relative to control (Fig. 4). Clusters displaying significantly higher abundance under treatment conditions were mostly detected in *Pelagomonas* while significantly decreased clusters were more dominant in other pelagophytes (Fig. 4). The treatment eliciting the largest number of clusters with a significant change in abundance varied among species being N for *Aureoumbra*, P for *Aureococcus*, and light for the open ocean pelagophytes, *Pelagomonas* and *Pelagococcus* (Fig. 4).

Clusters that did not mapped to any KEGG module comprised 83% of the total of differentially expressed clusters with 11% for *Aureococcus*, 18% for *Pelagococcus*, 19% for *Aureoumbra*, and 23% for *Pelagomonas* (Fig. 5A). The largest number of differentially expressed clusters was mapped to energy metabolism and genetic information processing modules (Fig 5A). Carbohydrate and lipid metabolism contributed to <3% of significantly expressed annotated clusters in most pelagophytes but accounted for 6% in *Pelagococcus* (Fig. 5A). Additionally, transport system accounted for slightly higher percentage of differentially expressed clusters in *Pelagococcus* (4%) relative to other species (2 – 3%; Fig. 5A).

The number of KEGG-annotated, differentially expressed clusters per treatment ranged from 59 – 187 in *Aureococcus* and *Pelagomonas*, but was smaller in *Aureoumbra* and *Pelagococcus* (9 – 16) with the exception of the low N treatment for *Aureoumbra* which had 163 (Fig. 5B). In a manner similar to the total clusters, the treatment eliciting the largest number of KEGG-annotated, differentially expressed clusters was N for *Aureoumbra*, P for *Aureococcus*, and light for the open ocean pelagophytes, *Pelagomonas* and *Pelagococcus* (Fig. 5B). As shown in the functional composition of significantly expressed clusters, clusters involved in energy metabolism and genetic information processing (420 clusters) were responsible for more than half of KEGG-annotated, differentially expressed clusters with energy metabolism-related clusters alone accounting for 28% of the total of KEGG-annotated, differentially expressed clusters (Fig 5B). Other categories with an above average number of KEGG-annotated, differentially expressed clusters per category included carbohydrate and lipid metabolism, and nucleotide and amino acid processing (Fig 5B).

*Differentially expressed functional clusters*

Of the total differentially expressed clusters (4,879 clusters) that include unique, shared, and core clusters, five functional groups (309 clusters) were generated by partitioning the clusters into light, N, P, selenocysteine, and transport-related groups (Fig. 6). Many of the functional clusters (71 clusters) that had highly significant and large increases in abundance were present in *Pelagomonas* while *Aureococcus* (67 clusters) and *Aureoumbra* (64 clusters) had more clusters that displayed significant decreases in abundance of the functional clusters (Fig. 6). Pelagophytes produced more clusters in response to N-limitation and light-limitation than P-limitation (Fig. 6). Among the five categories, N-, light-, and transport-related clusters were dominant with 57, 62 and 72 clusters being differentially expressed, respectively, compared to others (Fig. 6).

The N-related clusters included those encoding amino acid metabolism and N-relevant transporters as well as clusters associated with cleavage of organic nitrogen (e.g. urease, cathepsin, and arginase; Fig. 7A). Clusters encoding arginase, cathepsin and ureqase highly increased in abundance in low N for *Pelagomonas* (Fig. 7A). Concurrently, the abundance of some transporters associated with nitrogen assimilation were highly increased in low light or low P treatments (e.g. ammonium transporter in low P and nitrate transporter in low light; Fig. 7A).

Most P-related clusters were attributed to phosphate (19 clusters) and lipid metabolism (12 clusters) with a variety of phosphatases and phosphate transporters having significantly different expressions (Fig. 7B). Clusters associated with phosphate transporters were differentially upregulated in *Aureococcus* that was unique among the pelagophytes whereas clusters related to inorganic pyrophosphatase were highly expressed as a core or shared feature in response to P-limitation by *Aureococcus* and *Pelagomonas* (Fig. 7B). *Pelagomonas* also upregulated a nucleoside triphosphatase and arsenite methyltransferase under low P (Fig. 7B),

with the latter being known to methylate and detoxify aresenite that can accumulate under low P conditions (Frischkorn et al., 2014). In low light, *Pelagomonas* has two flavodoxin-associated clusters with significantly increased abundance and five ferredoxin clusters with significantly lower expression levels (Fig. 7C). In contrast, *Aureococcus* had two ferredoxin clusters with higher expression levels under low light (Fig. 7C). An cluster encoding lysophospholipase was upregulated under low light conditions for *Pelagococcus* but down regulated for other pelagophytes (Fig. 7C). Clusters encoding photosystem II proteins increased in abundance in response to light limitation for *Aureoumbra* (Fig. 7C). Methyltransferase-relevant clusters were a primary component of the selenocysteine-related group and largely displayed a significant decrease in abundance of some of those clusters across all treatments while thioredoxins also largely decreased in all treatments for all pelagophytes except *Pelagococcus* (Fig. 7D). Various types of transporters were significantly upregulated in *Pelagomonas* while they significantly decreased in other three pelagophytes in all treatments (Fig. 7E).

**Discussion**

In this study, I compared transcriptional profiles of four pelagophytes – *Aureococcus, Aureoumbra, Pelagococcus,* and *Pelgomonas* – under low nutrients (N, P) and low light conditions which are commonly found in the pelagic ecosystems where they exist and compete with other co-existing phytoplankton. The comparative transcriptomes provided insight regarding the functional traits that may be essential for pelagophytes to survive in such conditions. This study highlights the importance of clusters related to energy metabolism and lipid metabolism in the ecophysiology and survival of pelagophytes. Many functional clusters were commonly expressed among pelagophytes under similar conditions, indicating these gene

sets may be associated with pathways that permit pelagophytes to persist and, in some cases, dominate some marine ecosystems.

*Abundant core or shared clusters in Pelagophytes*

Clustering orthologous proteins has been used to assess functional traits shared among multiple phytoplankton strains (Alexander, 2016) or species (Bender et al., 2014; Di Dato et al., 2015; Simmons et al., 2016). In this study, the large number of annotated core and shared clusters clusteramong pelagophytes suggests that pelagophytes share in their pathway-level responses to nutrient limitation and light limitation. Although *Aureococcus* had the largest number of clusters, *Pelagomonas* possessed almost half of functional clusters that were differentially expressed under resource-limited conditions suggesting a broader physiological response in this alga to resource limitation, more restrictive conditions for *Pelagomonas* at the time of harvest, or a combination of these factors. Nonetheless, it is notable that *Aureococcus* and *Pelagomonas* had higher number ofcluster annotated clusters among four pelagophytes. These differences may reflect he ability to better recognize clusters as the whole genome of *Aureococcus* has been sequenced (Gobler et al., 2011) and transcriptomes of *Pelagomonas* have been previously generated (Worden et al., 2012; Dupont et al., 2014).

It is notable that the number of clusters (21,560) for *Aureococcus* in this study was greater than the number of genes (11,500) reported for its genome (Gobler et al., 2011). While the transcriptomes presented here are from a different strain (CCMP1850) than the strain with a sequenced genome (CCMP1984), a prior study has shown that ABySS transcriptome contigs contained multiple isoforms that may result in higher percentage (95%) of aligning *de novo* contigs to the genome scaffolds compared to genome models (Frischkorn et al., 2014). In

addition, other prior efforts mapping ABySS transcriptome contigs to the *Aureococcus* genome have revealed that many of these contigs map to the same gene (M. Harke, Columbia University, pers. comm.). Hence, it is possible that clusters with the single-copy contigcluster reported here may be isoforms of genes which is highly relevant, given that there are multiple copies of many individual genes in the *Aureococcus* genome (Gobler et al., 2011). For future analyses, it would be of interest to adjust the inflation parameter within the OrthoMCL program which affects cluster tightness with higher values yielding more sequences in fewer groups (Li et al., 2003).

*Clusters associated with organic nitrogen scavenging*

Low N conditions commonly led that *Pelagomonas* significantly increased abundance of clusters encoding genes related to the cleavage of organic nitrogen compounds including urease, arginase, cathepsin, and aminopeptidase, suggesting that the use of these enzymes is a survival strategy of *Pelagomonas* under low N conditions. By utilizing DON the coastal pelagophytes, *Aureococcus* and *Aureoumbra*, are able to bloom when DIN levels are low (Gobler et al., 2004a; Gobler et al., 2013a). My previous study of measurement of $^{15}N$ uptake rates per cell using sorting flow cytometry (Chapter 5) described that *Aureococcus* assimilates urea faster than other co-occurring picoplankton. The whole genome study of *Aureococcus* identified more genes associated with organic nitrogen metabolism in that alga compared to other phytoplankton (Gobler et al., 2011) and *Aureococcus* has significantly increased the expression of urea transporters under N-limitation (Berg et al., 2008; Wurch et al., 2011b). A proteomic study of *Aureoumbra* highlighted the utilization of regenerated, extracellular, organic nutrients (Sun et al., 2012). Those previous studies suggest that DON scavenging is an essential trait for both bloom-forming pelagophytes in facilitating brown tides. Importantly, prior transcriptomic studies of pelagophytes have established that ecologically important genes may not always be significantly

differentially expressed under specific environmental conditions especially when examined via ASC (Frischkorn et al., 2014) suggesting the lack of upregulation of specific clusters by some species may not mean those genes are ecologically unimportant. Regardless, the orthologous comparison performed here combined with prior studies (Berg et al., 2008; Gobler et al., 2011; Wurch et al., 2011b; Frischkorn et al., 2014) collectively emphasize the importance of urease and other genes associated with organic N transport and degradation in the nutritional ecophysiology of brown tide pelagophytes under low inorganic N conditions.

Of the pelagophytes studied here, *Pelagomonas* most strongly upregulated organic nitrogen degrading clusters including urease, arginase, cathepsin, and aminopeptidase under low N, a characteristic suggesting that the ability to break down organic nitrogen compounds is an important N-responsive feature for open ocean pelagophytes. Similarly, another dominant ocean picoplankton, *Prochlorococcus*, is known to assimilate DON at a high rate in open ocean ecosystems (Zubkov et al., 2003) where bacterially-mediated nutrient regeneration exceeds nitrate production (Azam et al., 1994). *Pelagomonas* is commonly found across oligotrophic open oceans and specifically at the subsurface chlorophyll maximum (SCM) (Simon et al., 1994; Andersen et al., 1996) and is responsible for most of nitrate transporter transcripts (>90%) within the SCM, whereas they express more stress-responsive genes in surface waters (Dupont et al., 2014), as nitrate is scarce (Johnson et al., 2010). My study implicated the ability of *Pelagomonas* to catabolize DON compounds under DIN limited conditions that prevail in surface ocean waters allowing it to compete with co-existing picoplankton (e.g. *Prochlorococcus*). This ability may allow *Pelagomonas* to vertically partition its physiological strategies within the euphotic zone with cells acclimating to low nitrate surface waters via use of DON and those within the SCM being highly reliant on nitrate.

*Clusters in response to low P*

Phosphate is present at a nanomolar concentration in oligotrophic oceans (Wu et al., 2000). It is an essential compound for microbial communities (Dyhrman et al., 2007) that may adapt to environmental variability with cell size-dependent phosphate uptake capabilities (Lomas et al., 2014). In contrast to the open ocean, phosphorus is relatively copious in shallow coastal waters due to benthic P-fluxes from sediments (Boynton and Kemp, 1985; Sundby et al., 1992) or rapid regeneration in the water column (Benitez-Nelson, 2000). Previous studies have shown that brown tides can, at times, be P-limited and may significantly reduce DOP levels during a peak of bloom (Gobler et al., 2004a). Laboratory growth experiments (Muhlstein and Villareal, 2007) as well as genomic and transcriptomic analyses (Gobler et al., 2011; Wurch et al., 2011b) have suggested that *Aureococcus* possess the ability to utilize P from a variety of organic compounds. In low P treatments, *Aureococcus* and *Pelagomonas* transcriptomes were characterized by up-regulation of clusters associated with orthophosphate assimilation with significant increases in the abundance of inorganic pyrophosphatases or phosphate transporters which have been previously shown to be indicative of P-limitation in *Aureococcus* (Wurch et al., 2011b; Frischkorn et al., 2014). Increasing phosphate uptake rate ($V_{max}$) in response to P deficiency is a common strategy for picoplankton (Zubkov et al., 2007; Lomas et al., 2014). In a particular study exploring the diversity of picoplankton phosphate uptake, Lomas et al. (2014) reported that $V_{max}$ was positively correlated with a cell size, consistent with greater uptake ability of picoeukaryotes than picocyanobacteria at a community level. The high $V_{max}$ with increasing P-limitation suggests an induction of phosphate transporters in cells, a finding consistent with the transcriptomic response of *Aureococcus*, which uniquely expressed clusters encoding phosphate transporter under low P conditions. Moreover, the affinity of phosphate transporters for

orthophosphate may have varied as differences in phosphate transport affinity have been described previously in proteomic response of *Aureococcus* to phosphorus deficiency (Wurch et al., 2011a) and in studies of other marine phytoplankton (Chung et al., 2003; Monier et al., 2012) and higher plants (Mitsukawa et al., 1997). Orthologs encoding phosphate transporters contained a single contig in each cluster, a finding that further suggests that multiple clusters encoding phosphate transporters in *Aureococcus* are more likely represent for different types of contigs rather than copies of the same contig.

Pyrophosphatases hydrolyze inorganic pyrophosphate and produce two molecules of phosphate (Chiou and Lin, 2011). In higher plants, high expression of inorganic pyrophosphatase genes is observed under P-deficient conditions (Hernández-Domíguez et al., 2012). In contrast to phosphate transporters that were distinctly upregulated in *Aureococcus* under low P conditions, multiple pelagophytes displayed increased abundance of clusters coding for inorganic pyrophosphatase implying the use of intracellularly recycled phosphate originating from pyrophosphate is a common molecular response that allows pelagophytes to survive extended periods of low P. The upregulation of a nucleoside triphosphatase specifically in *Pelagomonas* represents an additional intracellular P scavenging mechanism as this enzyme cleaves P from nucleoside-triphosphate. Finally, *Pelagomonas* upregulated an arsenite methyltransferase under low P. Since inorganic phosphate transporters cannot discriminate between phosphate and arsenate (Budd and Craig, 1981; Silver and Phung, 2005), eukaryotes have been shown to accumulate high levels of arsenate when P limited. Eukaryotes often reduce arsenate to arsenite intracellularly and use arsenite methyltransferase to make dimethylarsinate which is of a lower cellular toxicity than arsenate or arsenite (Zakharyan et al., 2005).

*Clusters associated with lipid metabolism in low light*

Low light conditions significantly altered the expression of clusters encoding genes related to lipid metabolism (e.g. lysophospholipase) and photosynthesis (e.g. flavodoxin and ferredoxin) in the pelagophytes. Lysophospholipases are located in cell membranes and cleave lysophospholipids to liberate amino acids that are then incorporated into cells (Pride et al., 2013). For most pelagophytes, these clusters were down regulated but *Pelagococcus* showed an increase in expression of a lysophospholipase-encoding cluster suggesting this pelagophyte may more efficiently rearrange and recycle cell membranes compared to other pelagophytes as a source of nutrition in response to low light conditions. While the biogeography of *Pelagococcus* has been poorly described, like *Pelagomonas*, this alga may also dominate the SCM (Dupont et al., 2014) where adapting to low light may be an important survival strategy.

Ferredoxin is an electron transport enzyme in many photosynthetic organisms delivering electrons generated via photosynthesis to metabolic processes (Hase et al., 2006) and can be replaced with the isofunctional flavodoxin which, unlike ferredoxin, does not require iron (Fe) as a co-factor (Sancho, 2006). Low light induced an increase in the number of clusters encoding ferredoxin in *Aureococcus* whereas *Pelagomonas* displayed a significant increase in flavodoxin cluster expression and significant decreases in ferredoxin clusters. Fe is more limited in open ocean than coastal waters (Boyd and Ellwood, 2010) and the enzymes associated with photosynthesis and low light adaptation including ferredoxin create a large Fe demand (Hase et al., 2006). As a coastal pelagophyte commonly exposed to high levels of Fe (Gobler et al., 2002a), *Aureococcus* may never be Fe-limited and rely on ferredoxin to adapt to low light. In contrast, as an open ocean pelagophyte whose pelagic habitats are low in Fe, an increase in abundance of flavodoxin in *Pelagomonas* under low light may assist their dominance in SCM while coping with the potential co-limitation of Fe and light.

*Clusters associated with ion transport and selenoproteins*

While clusters coding for various types of transporters were differentially expressed in all four pelagophytes, the largest response was in *Aureococcus* which displayed decreased cluster abundance across all treatments. A greater degree of differential expression of ion- and metal-related transporters in *Aureococcus* is not surprising as this alga is known to possess more genes coding for molybdenum, copper, and nickel-containing enzymes and related metal transporters compared to competing algae, a trait permitting *Aureococcus* to access abundant metals in coastal environments to form blooms (Gobler et al., 2011). The lowered expression of all of these transporters in *Aureococcus* is likely related to their slowed growth and thus lowered requirement for metals as well as the need to allocate energy to other, more vital cellular processes.

*Aureococcus* has been shown to contain the largest number of selenoproteins of any alga that has been sequenced so far (Gobler et al., 2013c) and many selenoproteins were differentially expressed by all of the pelagophytes except for *Pelagococcus* during this study. Thioredoxins are selenoproteins important for regulating cellular redox homeostasis (Stadtman, 1996; Kim et al., 2006; Lobanov et al., 2009) and while all three responding pelagophytes had thioredoxins with lower expression levels under resource-limiting conditions, *Aureococcus* and *Pelagomonas* also had some thioredoxin clusters with higher levels of expression under low light and low P, suggesting that these proteins were particularly important for combating cellular stress under these conditions.

In summary, nutrient and light limitation triggered a significant expression of clusters encoding functional genes associated with energy and lipid metabolism among four

pelagophytes. Specific cluster sets that were differentially expressed under low N, P, and light included genes associated with N cleavage, intracellular P recycling, lipid hydrolysis, and low light adaptation. Transcriptomic comparisons highlighted distinct features among pelagophytes such as unique expression of phosphate transporters by *Aureococcus* in low P. The ecophysiological traits important for *Pelagomonas* to adapt to its open ocean environment included the ability to exploit organic N under low N conditions, the use of intracellularly recycled phosphate originating from pyrophosphate and nucleoside triphosphate under low P conditions, and the use of flavodoxins over ferredoxins under low light. Collectively, this study demonstrates that coastal pelagophytes and open ocean pelagophytes utilized both shared and unique ecophysiological features to thrive within their preferred habitats.

## References


Alexander, H., 2016. Defining the ecological and physiological traits of phytoplankton across marine ecosystems. Massachusetts Institute of Technology and Woods Hole Oceanographic Institution.

Alexander, H., Jenkins, B.D., Rynearson, T.A., Dyhrman, S.T., 2015. Metatranscriptome analyses indicate resource partitioning between diatoms in the field. Proc. Natl. Acad. Sci. 112 (17), E2182-E2190.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403-410.

Andersen, R.A., Bidigare, R.R., Keller, M.D., Latasa, M., 1996. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans. Deep-Sea Res PT II 43 (2–3), 517-537.

Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S., Allen, A.E., Apt, K.E., Bechner, M., 2004. The genome of the diatom Thalassiosira pseudonana: ecology, evolution, and metabolism. Science 306 (5693), 79-86.

Azam, F., Smith, D., Steward, G., Hagström, Å., 1994. Bacteria-organic matter coupling and its significance for oceanic carbon cycling. Microb. Ecol. 28 (2), 167-179.

Bateman, A., Coin, L., Durbin, R., Finn, R.D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E.L., 2004. The Pfam protein families database. Nucleic Acids Res. 32 (suppl 1), D138-D141.

Bender, S.J., Durkin, C.A., Berthiaume, C.T., Morales, R.L., Armbrust, E.V., 2014. Transcriptional responses of three model diatoms to nitrate limitation of growth. Frontiers in Marine Science 1.

Benitez-Nelson, C.R., 2000. The biogeochemical cycling of phosphorus in marine systems. Earth-Sci. Rev. 51 (1), 109-135.

Berg, G.M., Shrager, J., Glockner, G., Arrigo, K.R., Grossman, A.R., 2008. Understanding nitrogen limitation in *Aureococcus anophagefferens* (Pelagophyceae) through cDNA and qRT-PCR analysis. J. Phycol. 44 (5), 1235-1249.

Boyd, P., Ellwood, M., 2010. The biogeochemical cycle of iron in the ocean. Nature Geoscience 3 (10), 675-682.

Boynton, W., Kemp, W.M., 1985. Nutrient regeneration and oxygen consumption by sediments along an estuarine salinity gradient. Marine ecology progress series. Oldendorf 23 (1), 45-55.

Budd, K., Craig, S.R., 1981. Resistance to arsenate toxicity in the blue-green alga Synechococcus leopoliensis. Canadian Journal of Botany 59 (8), 1518-1521.

Chiou, T.-J., Lin, S.-I., 2011. Signaling network in sensing phosphate availability in plants. Annu. Rev. Plant Biol. 62, 185-206.

Chung, C.-C., Hwang, S.-P.L., Chang, J., 2003. Identification of a high-affinity phosphate transporter gene in a prasinophyte alga, Tetraselmis chui, and its expression under nutrient limitation. Appl. Environ. Microbiol. 69 (2), 754-759.

Cullen, J.J., 1982. The deep chlorophyll maximum: comparing vertical profiles of chlorophyll *a*. Can. J. Fish. Aquat. Sci. 39 (5), 791-803.

Cuvelier, M.L., Allen, A.E., Monier, A., McCrow, J.P., Messie, M., Tringe, S.G., Woyke, T., Welsh, R.M., Ishoey, T., Lee, J.H., Binder, B.J., DuPont, C.L., Latasa, M., Guigand, C., Buck, K.R., Hilton, J., Thiagarajan, M., Caler, E., Read, B., Lasken, R.S., Chavez, F.P., Worden, A.Z., 2010. Targeted metagenomics and ecology of globally important uncultured eukaryotic phytoplankton. Proc. Natl. Acad. Sci. 107 (33), 14679-14684.

Di Dato, V., Musacchia, F., Petrosino, G., Patil, S., Montresor, M., Sanges, R., Ferrante, M.I., 2015. Transcriptome sequencing of three Pseudo-nitzschia species reveals comparable gene sets and the presence of Nitric Oxide Synthase genes in diatoms. Scientific reports 5.

Dimier, C., Brunet, C., Geider, R., Raven, J., 2009. Growth and photoregulation dynamics of the picoeukaryote *Pelagomonas calceolata* in fluctuating light. Limnol. Oceanogr. 54 (3), 823-836.

Dupont, C.L., McCrow, J.P., Valas, R., Moustafa, A., Walworth, N., Goodenough, U., Roth, R., Hogle, S.L., Bai, J., Johnson, Z.I., Mann, E., Palenik, B., Barbeau, K.A., Venter, J.C., Allen, A.E., 2014. Genomes and gene expression across light and productivity gradients in eastern subtropical Pacific microbial communities. ISME Journal 9 (5), 1076-1092.

Dyhrman, S.T., Ammerman, J.W., Van Mooy, B.A., 2007. Microbes and the marine phosphorus cycle.

Dyhrman, S.T., Jenkins, B.D., Rynearson, T.A., Saito, M.A., Mercier, M.L., Alexander, H., Whitney, L.P., Drzewianowski, A., Bulygin, V.V., Bertrand, E.M., Wu, Z.J., Benitez-Nelson, C., Heithoff, A., 2012. The transcriptome and proteome of the diatom *Thalassiosira pseudonana* reveal a diverse phosphorus stress response. Plos One 7 (3).

Frischkorn, K.R., Harke, M.J., Gobler, C.J., Dyhrman, S.T., 2014. *De novo* assembly of *Aureococcus anophagefferens* transcriptomes reveals diverse responses to the low nutrient and low light conditions present during blooms. Name: Frontiers in Microbiology 5, 375.

Gobler, C.J., Berry, D.L., Dyhrman, S.T., Wilhelm, S.W., Salamov, A., Lobanov, A.V., Zhang, Y., Collier, J.L., Wurch, L.L., Kustka, A.B., Dill, B.D., Shah, M., VerBerkmoes, N.C., Kuo, A., Terry, A., Pangilinan, J., Lindquist, E.A., Lucas, S., Paulsen, I.T., Hattenrath-Lehmann, T.K., Talmage, S.C., Walker, E.A., Koch, F., Burson, A.M., Marcoval, M.A., Tang, Y.Z., LeCleir, G.R., Coyne, K.J., Berg, G.M., Bertrand, E.M., Saito, M.A.,

Gladyshev, V.N., Grigoriev, I.V., 2011. Niche of harmful alga *Aureococcus anophagefferens* revealed through ecogenomics. Proc. Natl. Acad. Sci. 108 (11), 4352-4357.

Gobler, C.J., Boneillo, G.E., Debenham, C.J., Caron, D.A., 2004. Nutrient limitation, organic matter cycling, and plankton dynamics during an *Aureococcus anophagefferens* bloom. Aquat. Microb. Ecol. 35 (1), 31-43.

Gobler, C.J., Donat, J.R., Consolvo, J.A., Sañudo-Wilhelmy, S.A., 2002. Physicochemical speciation of iron during coastal algal blooms. Mar. Chem. 77 (1), 71-89.

Gobler, C.J., Koch, F., Kang, Y., Berry, D.L., Tang, Y.Z., Lasi, M., Walters, L., Hall, L., Miller, J.D., 2013a. Expansion of harmful brown tides caused by the pelagophyte, *Aureoumbra lagunensis* DeYoe et Stockwell, to the US east coast. Harmful Algae 27 (0), 29-41.

Gobler, C.J., Lobanov, A.V., Tang, Y.Z., Turanov, A.A., Zhang, Y., Doblin, M., Taylor, G.T., Sanudo-Wilhelmy, S.A., Grigoriev, I.V., Gladyshev, V.N., 2013b. The central role of selenium in the biochemistry and ecology of the harmful pelagophyte, Aureococcus anophagefferens. Isme Journal 7 (7), 1333-1343.

Gobler, C.J., Sunda, W.G., 2012. Ecosystem disruptive algal blooms of the brown tide species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*. Harmful Algae 14, 36-45.

Gough, J., Karplus, K., Hughey, R., Chothia, C., 2001. Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. J. Mol. Biol. 313 (4), 903-919.

Haft, D.H., Loftus, B.J., Richardson, D.L., Yang, F., Eisen, J.A., Paulsen, I.T., White, O., 2001. TIGRFAMs: a protein family resource for the functional identification of proteins. Nucleic Acids Res. 29 (1), 41-43.

Harke, M.J., Gobler, C.J., 2013. Global transcriptional responses of the toxic cyanobacterium, *Microcystis aeruginosa*, to nitrogen stress, phosphorus stress, and growth on organic matter. Plos One 8 (7), e69834-e69834.

Hase, T., Schürmann, P., Knaff, D.B., 2006. The interaction of ferredoxin with ferredoxin-dependent enzymes, Photosystem I. Springer, pp. 477-498.

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, D.A., Stoecker, D.K., Suddleson, M., 2008. Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8 (1), 3-13.

Hernández-Domíguez, E.E., Valencia-Turcotte, L.G., Rodríguez-Sotres, R., 2012. Changes in expression of soluble inorganic pyrophosphatases of Phaseolus vulgaris under phosphate starvation. Plant Sci. 187, 39-48.

Hoppe, H., 1983. Significance of exoenzymatic activities in the ecology of brackish water-measurements by means of methylumbelliferyl-substrates. Mar. Ecol. Prog. Ser. 11 (3), 299-308.

Huang, X., Madan, A., 1999. CAP3: A DNA sequence assembly program. Genome Res. 9 (9), 868-877.

Iseli, C., Jongeneel, C.V., Bucher, P., 1999. ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences, ISMB, pp. 138-148.

John, D.E., Wang, Z.A., Liu, X., Byrne, R.H., Corredor, J.E., López, J.M., Cabrera, A., Bronk, D.A., Tabita, F.R., Paul, J.H., 2007. Phytoplankton carbon fixation gene (RuBisCO) transcripts and air-sea $CO_2$ flux in the Mississippi River plume. The ISME journal 1 (6), 517-531.

Johnson, K.S., Riser, S.C., Karl, D.M., 2010. Nitrate supply from deep to near-surface waters of the North Pacific subtropical gyre. Nature 465 (7301), 1062-1065.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K.F., Itoh, M., Kawashima, S., Katayama, T., Araki, M., Hirakawa, M., 2006. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. 34 (suppl 1), D354-D357.

Keeling, P.J., Burki, F., Wilcox, H.M., Allam, B., Allen, E.E., Amaral-Zettler, L.A., Armbrust, E.V., Archibald, J.M., Bharti, A.K., Bell, C.J., 2014. The marine microbial eukaryote transcriptome sequencing project (MMETSP): illuminating the functional diversity of eukaryotic life in the oceans through transcriptome sequencing. PLoS Biol. 12 (6), e1001889.

Kim, H.-Y., Fomenko, D.E., Yoon, Y.-E., Gladyshev, V.N., 2006. Catalytic advantages provided by selenocysteine in methionine-S-sulfoxide reductases. Biochemistry 45 (46), 13697-13704.

Konotchick, T., Dupont, C.L., Valas, R.E., Badger, J.H., Allen, A.E., 2013. Transcriptomic analysis of metabolic function in the giant kelp, *Macrocystis pyrifera*, across depth and season. New Phytol. 198 (2), 398-407.

Li, B., Karl, D.M., Letelier, R.M., Bidigare, R.R., Church, M.J., 2013. Variability of chromophytic phytoplankton in the North Pacific Subtropical Gyre. Deep-Sea Res. Part II-Top. Stud. Oceanogr. 93, 84-95.

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25 (14), 1754-1760.

Li, L., Stoeckert, C.J., Roos, D.S., 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13 (9), 2178-2189.

Li, W., Godzik, A., 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22 (13), 1658-1659.

Lin, S., Cheng, S., Song, B., Zhong, X., Lin, X., Li, W., Li, L., Zhang, Y., Zhang, H., Ji, Z., 2015. The *Symbiodinium kawagutii* genome illuminates dinoflagellate gene expression and coral symbiosis. Science 350 (6261), 691-694.

Liu, Y., Zhou, J., White, K.P., 2014. RNA-seq differential expression studies: more sequence or more replication? Bioinformatics 30 (3), 301-304.

Lobanov, A.V., Hatfield, D.L., Gladyshev, V.N., 2009. Eukaryotic selenoproteins and selenoproteomes. Biochimica et Biophysica Acta (BBA)-General Subjects 1790 (11), 1424-1428.

Lomas, M.W., Bonachela, J.A., Levin, S.A., Martiny, A.C., 2014. Impact of ocean phytoplankton diversity on phosphate uptake. Proc. Natl. Acad. Sci. 111 (49), 17540-17545.

Marchetti, A., Schruth, D.M., Durkin, C.A., Parker, M.S., Kodner, R.B., Berthiaume, C.T., Morales, R., Allen, A.E., Armbrust, E.V., 2012. Comparative metatranscriptomics identifies molecular bases for the physiological responses of phytoplankton to varying iron availability. Proc. Natl. Acad. Sci. 109 (6), E317-E325.

McLean, T.I., 2013. "Eco-omics": A Review of the Application of Genomics, Transcriptomics, and Proteomics for the Study of the Ecology of Harmful Algae. Microb. Ecol., 1-15.

Mitsukawa, N., Okumura, S., Shirano, Y., Sato, S., Kato, T., Harashima, S., Shibata, D., 1997. Overexpression of an Arabidopsis thaliana high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limited conditions. Proc. Natl. Acad. Sci. 94 (13), 7098-7102.

Mock, T., Daines, S.J., Geider, R., Collins, S., Metodiev, M., Millar, A.J., Moulton, V., Lenton, T.M., 2016. Bridging the gap between omics and earth system science to better understand how environmental change impacts marine microbes. Global Change Biol. 22 (1), 61-75.

Monier, A., Welsh, R.M., Gentemann, C., Weinstock, G., Sodergren, E., Armbrust, E., Eisen, J.A., Worden, A.Z., 2012. Phosphate transporters in marine phytoplankton and their viruses: cross-domain commonalities in viral-host gene exchanges. Environ. Microbiol. 14 (1), 162-176.

Muhlstein, H.I., Villareal, T.A., 2007. Organic and inorganic nutrient effects on growth rate-irradiance relationships in the Texas brown-tide alga *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 43 (6), 1223-1226.

Oliver, S.L., Lenards, A.J., Barthelson, R.A., Merchant, N., McKay, S.J., 2013. Using the iPlant collaborative discovery environment. Current Protocols in Bioinformatics, 1.22. 21-21.22. 26.

Palenik, B., Brahamsha, B., Larimer, F.W., Land, M., Hauser, L., Chain, P., Lamerdin, J., Regala, W., Allen, E.E., McCarren, J., Paulsen, I., Dufresne, A., Partensky, F., Webb,

E.A., Waterbury, J., 2003. The genome of a motile marine Synechococcus. Nature 424 (6952), 1037-1042.

Parkhill, J.P., Maillet, G., Cullen, J.J., 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. 37 (4), 517-529.

Pride, A.C., Herrera, C.M., Guan, Z., Giles, D.K., Trent, M.S., 2013. The outer surface lipoprotein VolA mediates utilization of exogenous lipids by Vibrio cholerae. mBio 4 (3), e00305-00313.

Sancho, J., 2006. Flavodoxins: sequence, folding, binding, function and beyond. Cell. Mol. Life Sci. 63 (7-8), 855-864.

Silver, S., Phung, L.T., 2005. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Appl. Environ. Microbiol. 71 (2), 599-608.

Simmons, M.P., Sudek, S., Monier, A., Limardo, A.J., Jimenez, V., Perle, C.R., Elrod, V.A., Pennington, J.T., Worden, A.Z., 2016. Abundance and biogeography of picoprasinophyte ecotypes and other phytoplankton in the Eastern North Pacific Ocean. Appl. Environ. Microbiol. 82 (6), 1693-1705.

Simon, N., Barlow, R.G., Marie, D., Partensky, F., Vaulot, D., 1994. Characterization of oceanic photosynthetic picoeukaryotes by flow cytometry. J. Phycol. 30 (6), 922-935.

Simpson, J.T., Durbin, R., 2012. Efficient de novo assembly of large genomes using compressed data structures. Genome Res. 22 (3), 549-556.

Simpson, J.T., Wong, K., Jackman, S.D., Schein, J.E., Jones, S.J., Birol, I., 2009. ABySS: a parallel assembler for short read sequence data. Genome Res. 19 (6), 1117-1123.

Soneson, C., Delorenzi, M., 2013. A comparison of methods for differential expression analysis of RNA-seq data. BMC Bioinformatics 14 (1), 91.

Stadtman, T.C., 1996. Selenocysteine. Annu. Rev. Biochem. 65 (1), 83-100.

Stein, L.D., 2008. Towards a cyberinfrastructure for the biological sciences: progress, visions and challenges. Nature Reviews Genetics 9 (9), 678-688.

Sun, M.M., Sun, J., Qiu, J.W., Jing, H.M., Liu, H.B., 2012. Characterization of the proteomic profiles of the brown tide alga *Aureoumbra lagunensis* under phosphate- and nitrogen-limiting conditions and of its phosphate limitation-specific protein with alkaline phosphatase activity. Appl. Environ. Microbiol. 78 (6), 2025-2033.

Sundby, B., Gobeil, C., Silverberg, N., Alfonso, M., 1992. The phosphorus cycle in coastal marine sediments. Limnol. Oceanogr. 37 (6), 1129-1145.

Tang, Y., Gobler, C., 2009. Characterization of the toxicity of Cochlodinium polykrikoides isolates from Northeast US estuaries to finfish and shellfish. Harmful Algae 8 (3), 454-462.

Worden, A.Z., Janouskovec, J., McRose, D., Engman, A., Welsh, R.M., Malfatti, S., Tringe, S.G., Keeling, P.J., 2012. Global distribution of a wild alga revealed by targeted metagenomics. Curr. Biol. 22 (17), R675-R677.

Wu, J., Sunda, W., Boyle, E.A., Karl, D.M., 2000. Phosphate depletion in the western North Atlantic Ocean. Science 289 (5480), 759-762.

Wu, Z.J., Jenkins, B.D., Rynearson, T.A., Dyhrman, S.T., Saito, M.A., Mercier, M., Whitney, L.P., 2010. Empirical bayes analysis of sequencing-based transcriptional profiling without replicates. BMC Bioinformatics 11.

Wurch, L.L., Bertrand, E.M., Saito, M.A., Van Mooy, B.A.S., Dyhrman, S.T., 2011a. Proteome Changes Driven by Phosphorus Deficiency and Recovery in the Brown Tide-Forming Alga Aureococcus anophagefferens. Plos One 6 (12).

Wurch, L.L., Gobler, C.J., Dyhrman, S.T., 2013. Expression of a xanthine and phosphate transporter in cultures and field populations of the harmful alga *Aureococcus anophagefferens*: tracking nutritional deficiency during brown tides. Environ. Microbiol., 2444-2457.

Wurch, L.L., Haley, S.T., Orchard, E.D., Gobler, C.J., Dyhrman, S.T., 2011b. Nutrient-regulated transcriptional responses in the brown tide-forming alga *Aureococcus anophagefferens*. Environ. Microbiol. 13 (2), 468-481.

Zakharyan, R.A., Tsaprailis, G., Chowdhury, U.K., Hernandez, A., Aposhian, H.V., 2005. Interactions of sodium selenite, glutathione, arsenic species, and omega class human glutathione transferase. Chem. Res. Toxicol. 18 (8), 1287-1295.

Zhang, Z., Wood, W.I., 2003. A profile hidden Markov model for signal peptides generated by HMMER. Bioinformatics 19 (2), 307-308.

Zubkov, M.V., Fuchs, B.M., Tarran, G.A., Burkill, P.H., Amann, R., 2003. High rate of uptake of organic nitrogen compounds by Prochlorococcus cyanobacteria as a key to their dominance in oligotrophic oceanic waters. Appl. Environ. Microbiol. 69 (2), 1299-1304.

Zubkov, M.V., Mary, I., Woodward, E.M.S., Warwick, P.E., Fuchs, B.M., Scanlan, D.J., Burkill, P.H., 2007. Microbial control of phosphate in the nutrient-depleted North Atlantic subtropical gyre. Environ. Microbiol. 9 (8), 2079-2089.

**Table 1** Alkaline phosphatase activity (nmol $mL^{-1}$ $h^{-1}$), photosynthetic efficiency ($F_v/F_m$) and growth rate ($d^{-1}$) at the time of harvest. Asterisks indicate significant differences between control and treatment (* = $p<0.05$ and ** = $p<0.001$).

| Species | Alkaline phosphatase activity (nmol $mL^{-1}$ $h^{-1}$) | | $F_v/F_m$ | | | | Growth rate ($d^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Replete | Low P | Replete | Low N | Low P | Low Light | Replete | Low N | Low P | Low Light |
| *Aureococcus anophageffenres* | - | - | 0.58 ± 0.02 | 0.47 ± 0.00 ** | 0.51 ± 0.01 | 0.52 ± 0.01 | 0.44 ± 0.01 | 0.09 ± 0.02 | 0.04 ± 0.02 | 0.07 ± 0.02 |
| *Aureoumbra lagunensis* | 0.07 ± 0.03 | 0.20 ± 0.05 | 0.63 ± 0.02 | 0.57 ± 0.04 | 0.67 ± 0.04 | 0.61 ± 0.01 | 0.24 ± 0.03 | 0.09 ± 0.02 | 0.27 ± 0.02 | 0.19 ± 0.05 |
| *Pelagococcus subviridis* | 0.08 ± 0.04 | 0.15 ± 0.03 | 0.57 ± 0.04 | 0.52 ± 0.02 | 0.58 ± 0.03 | 0.59 ± 0.01 | 0.12 ± 0.09 | 0.01 ± 0.01 | 0.09 ± 0.06 | 0.01 ± 0.04 |
| *Pelagomonas calceolata* | 0.02 ± 0.001 | 0.07 ± 0.01 | 0.54 ± 0.03 | 0.48 ± 0.02* | 0.35 ± 0.02 | 0.57 ± 0.01 | 0.43 ± 0.01 | 0.23 ± 0.001 | 0.16 ± 0.11 | 0.18 ± 0.04 |

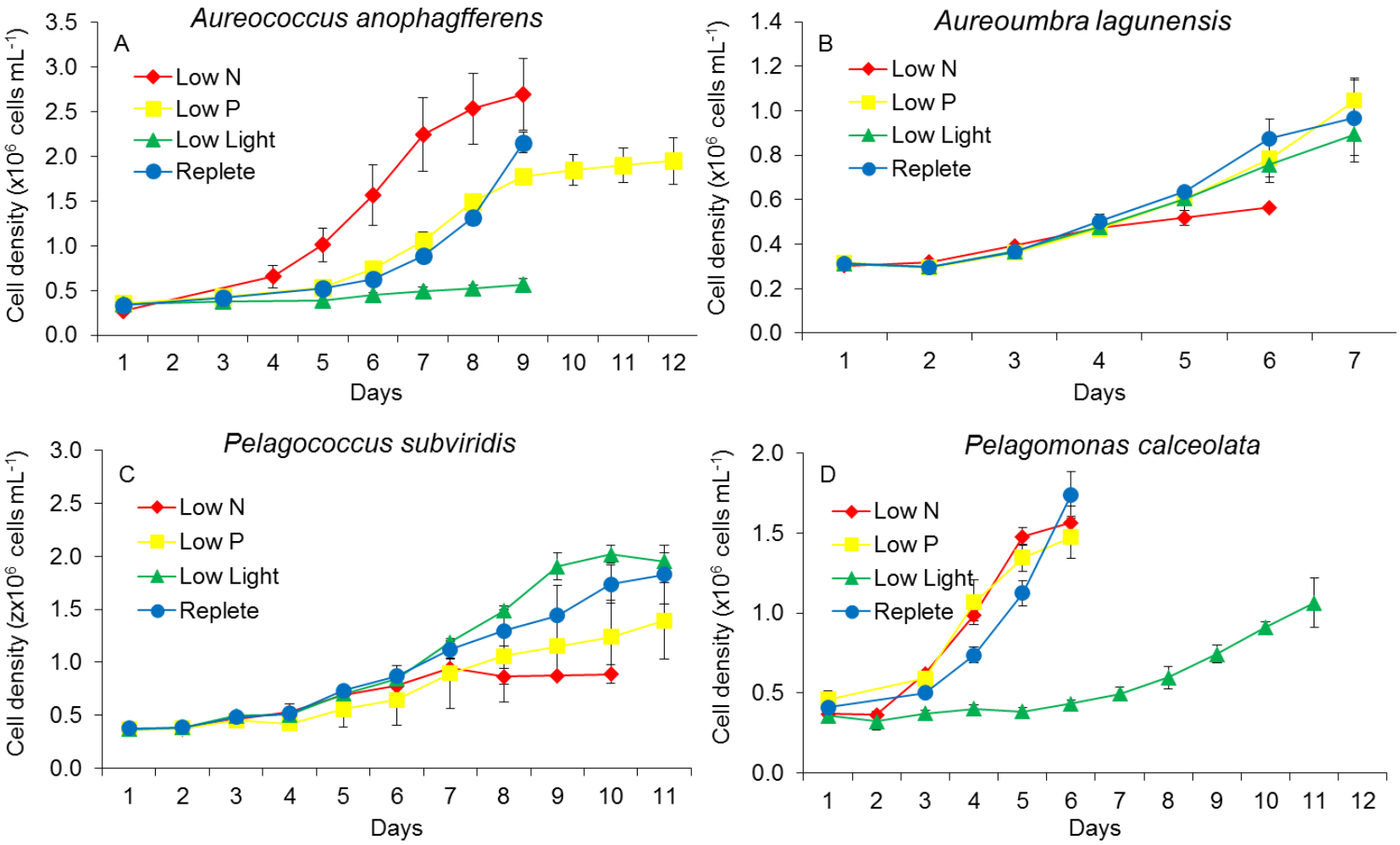
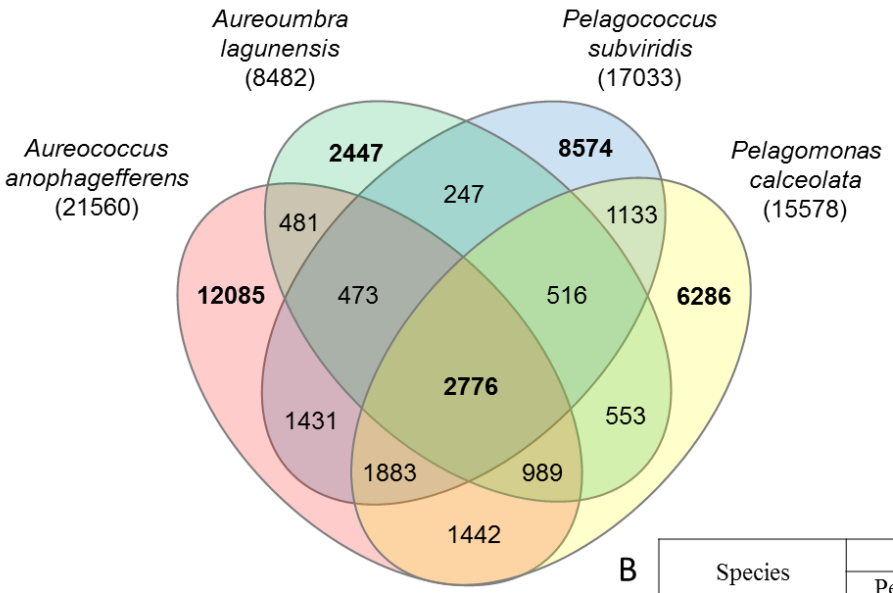



**Figure 1.** Cell density during experiments. Cells from each treatment and each species were harvested at different times indicated by the last time point of each line.

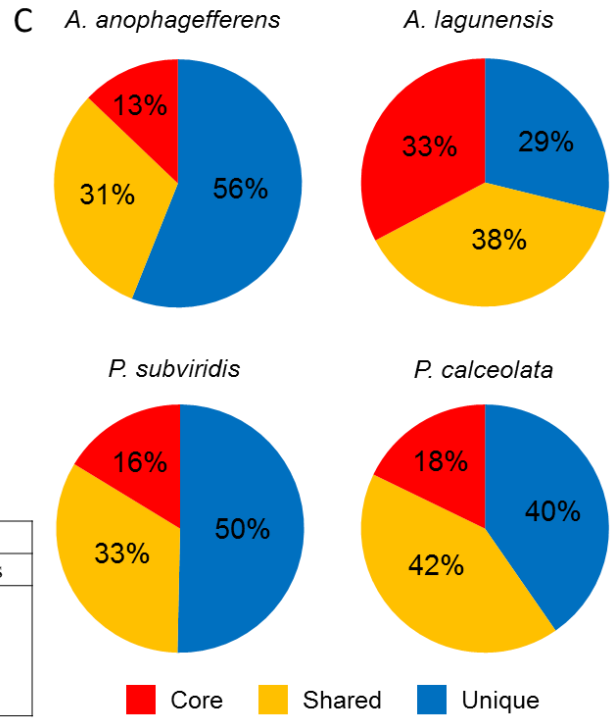
A




B

| Species | Total number | |
|---|---|---|
| | Peptides | Clusters |
| *Aureococcus* | 30301 | 21560 |
| *Aureoumbra* | 10284 | 8482 |
| *Pelagococcus* | 20101 | 17033 |
| *Pelagomonas* | 18094 | 15578 |

C




**Figuew 2.** (A)Venn diagram showing the number of core, shared, and unique clusters among the four pelagophytes, (B) the number of peptide sequences that were used for OrthoMCL analysis and the number of clusters after the analysis, and (C) proportion of core, shared, and unique clusters in each species. Core clusters were expressed in all four species, shared clusters were expressed in two or three of the four species, and unique clusters were expressed in a single species.

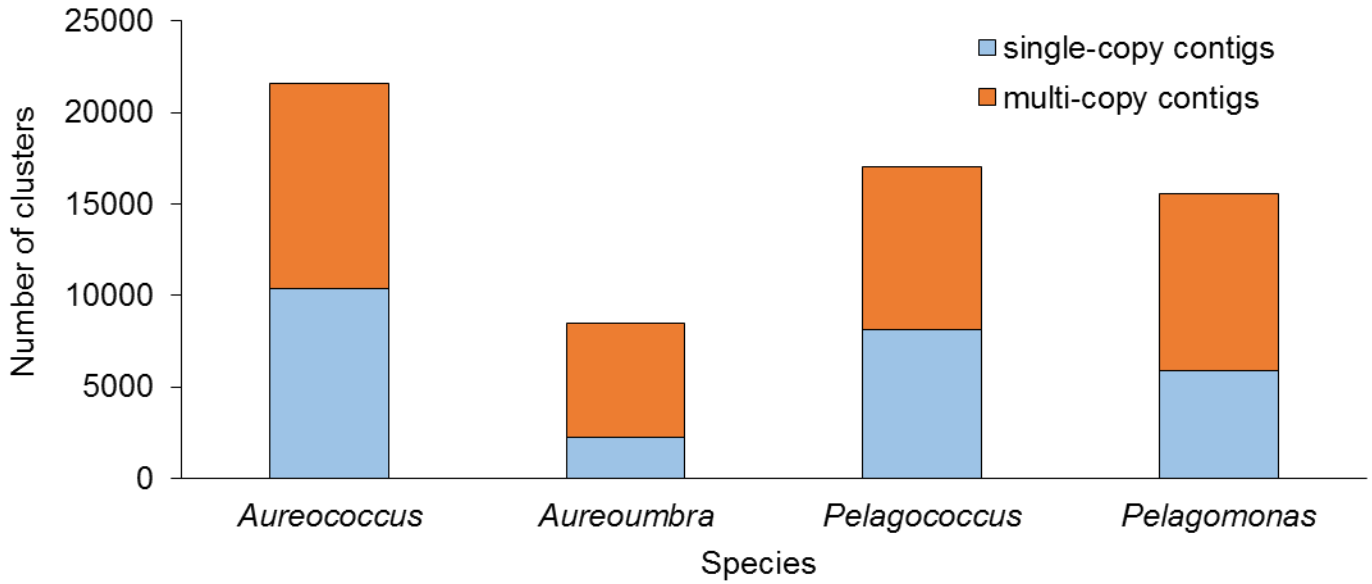



**Figure 3.** Number of clusters that have single-copy contigs or multi-copy contigs among total clusters. Clusters having single-copy contigs did not cluster with any other contigs and clusters having multi-copy contigs are composed of paralogs that likely arose from gene duplication.

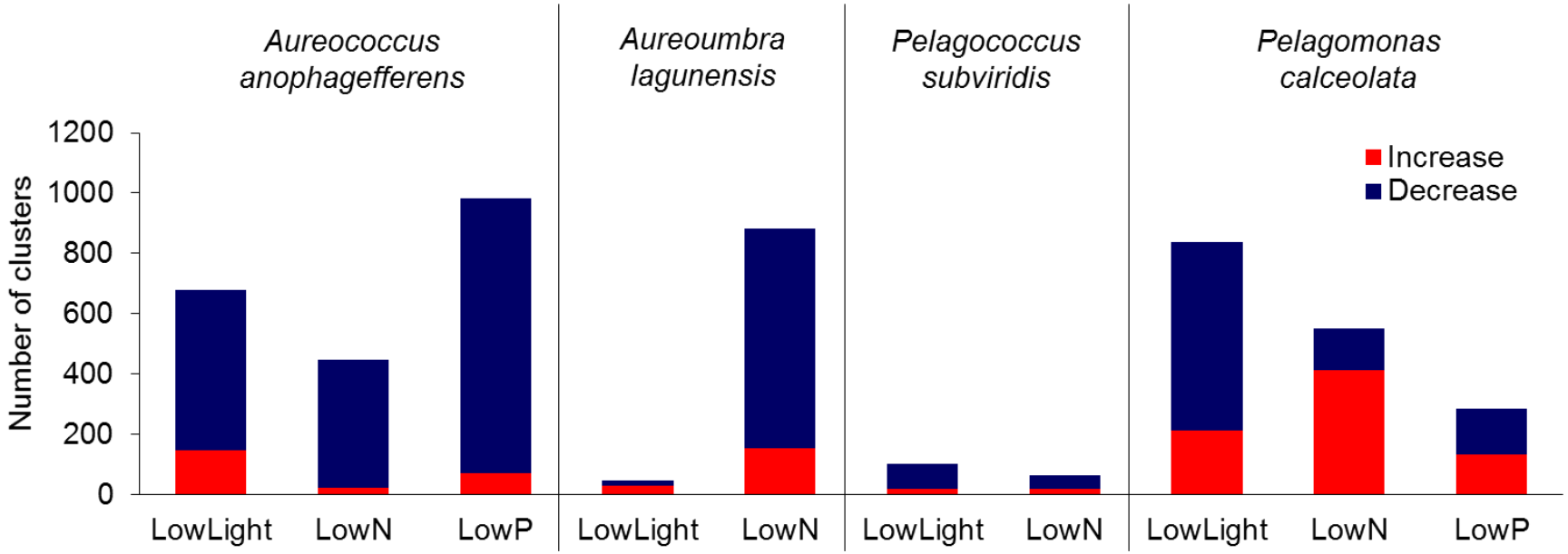



**Figure 4.** Statistically significantly increased or decreased clusters that were present in each treatment.

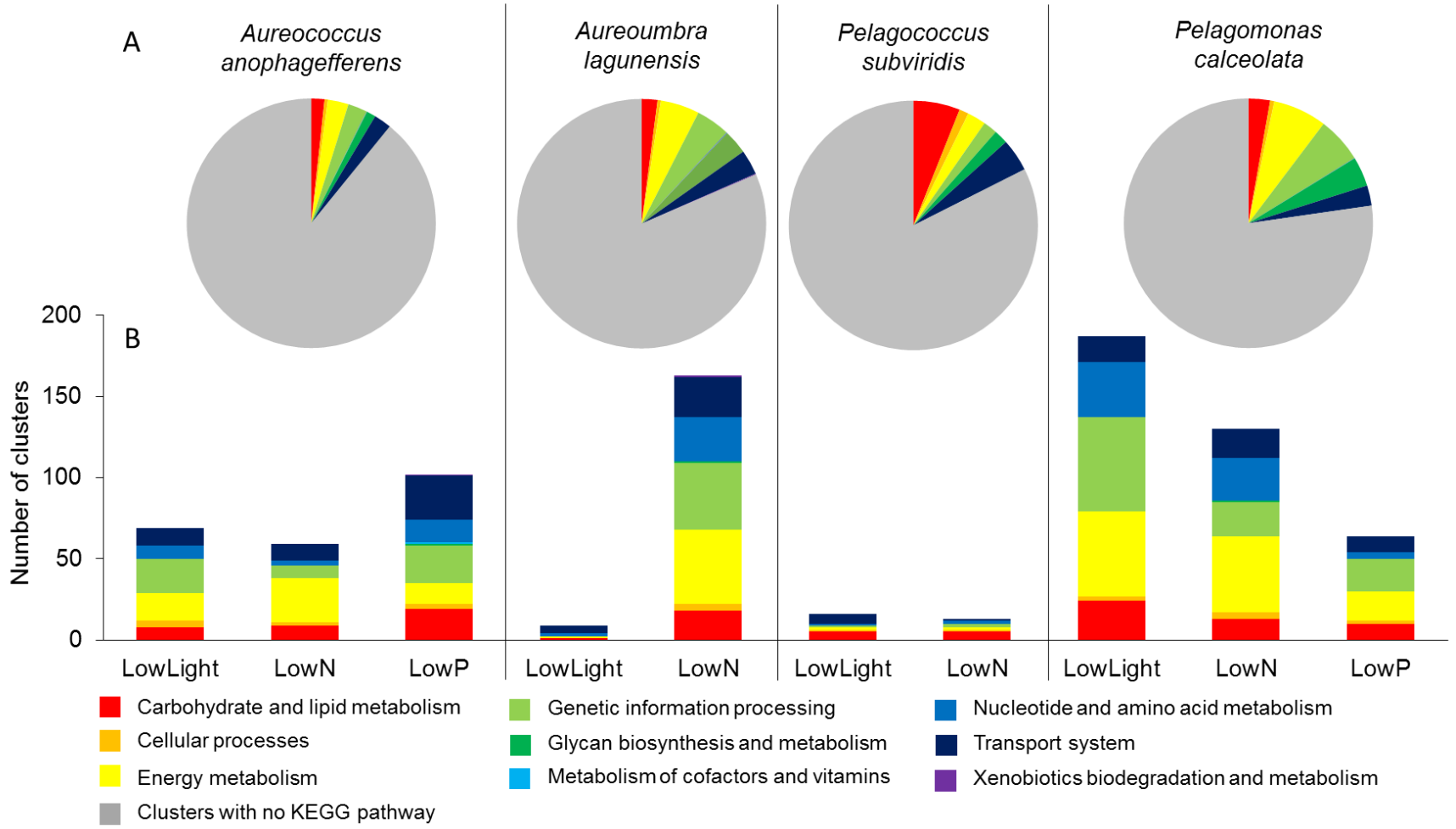



**Figure 5.** KEGG modules of differentially expressed clusters from each species. (A) Modules that were annotated based on KEGG database and (B) the number of significantly expressed clusters in each treatment and clusters that were not mapped to KEGG module were excluded.

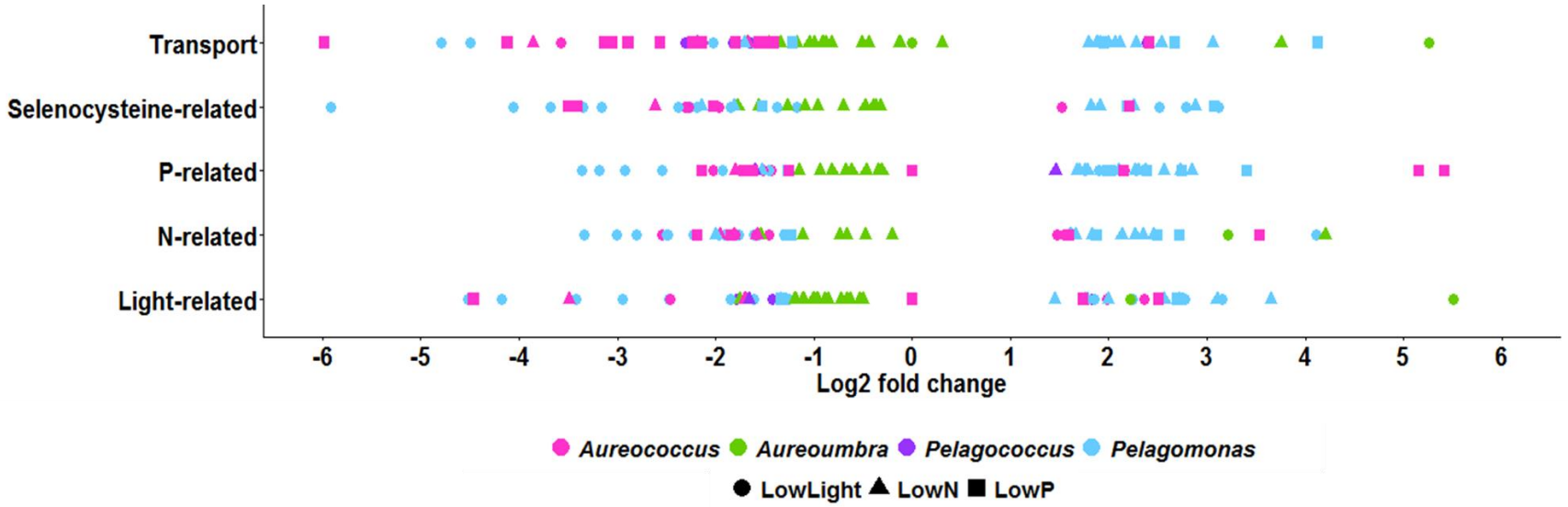



**Figure 6.** Functional clusters that were differentially expressed among the four species in the three treatments. Functional clusters were explored here based on their function relevant to treatments.

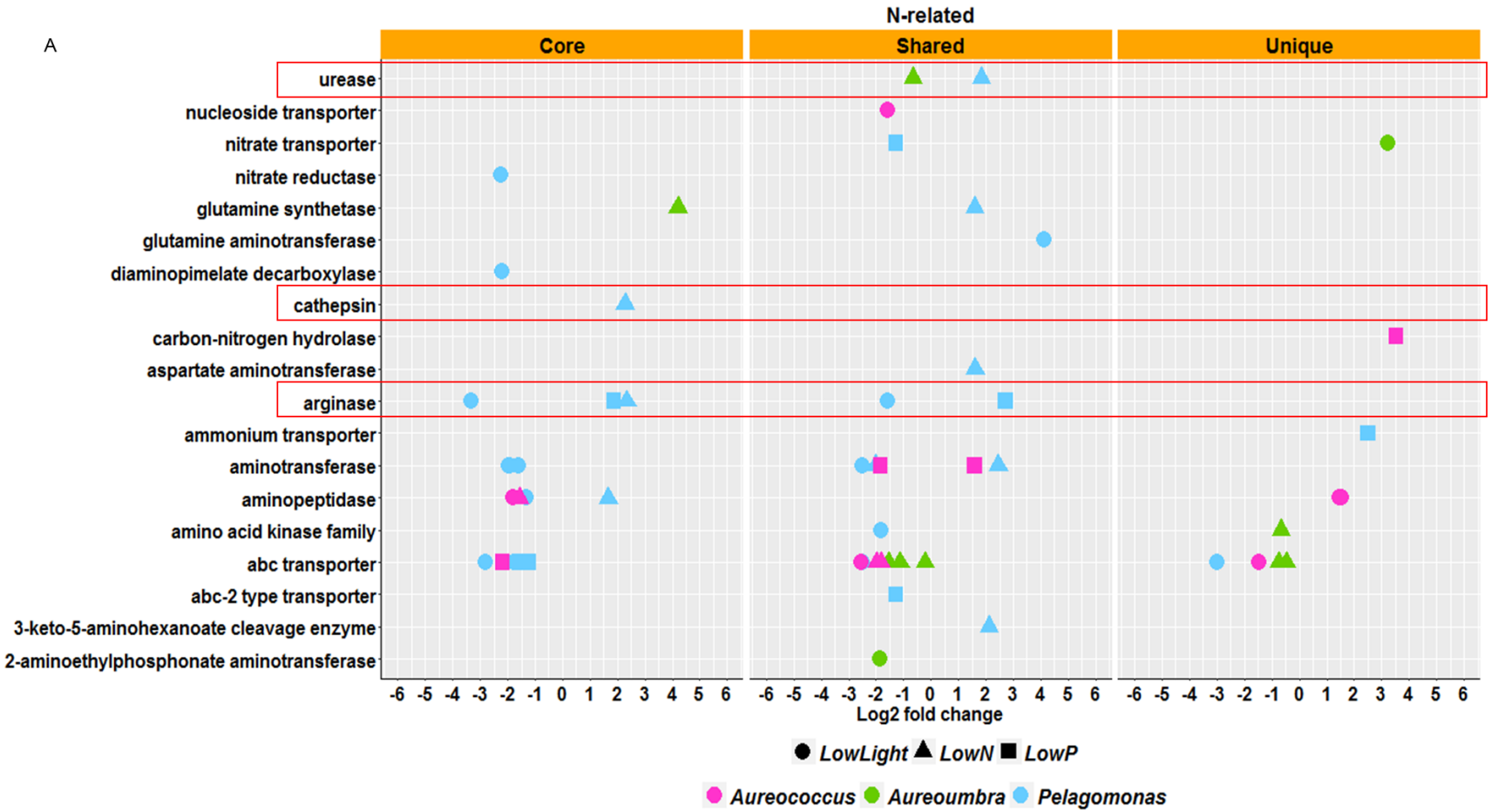



**Figure 7.** Functional clusters that were partitioned into five functional groups. (A) N-related group, (B) P-related group, (C) Light-related group, (D) Selenocystein-related group, and (E) Transporters. Red boxed indicate functional clusters that were described in this study.

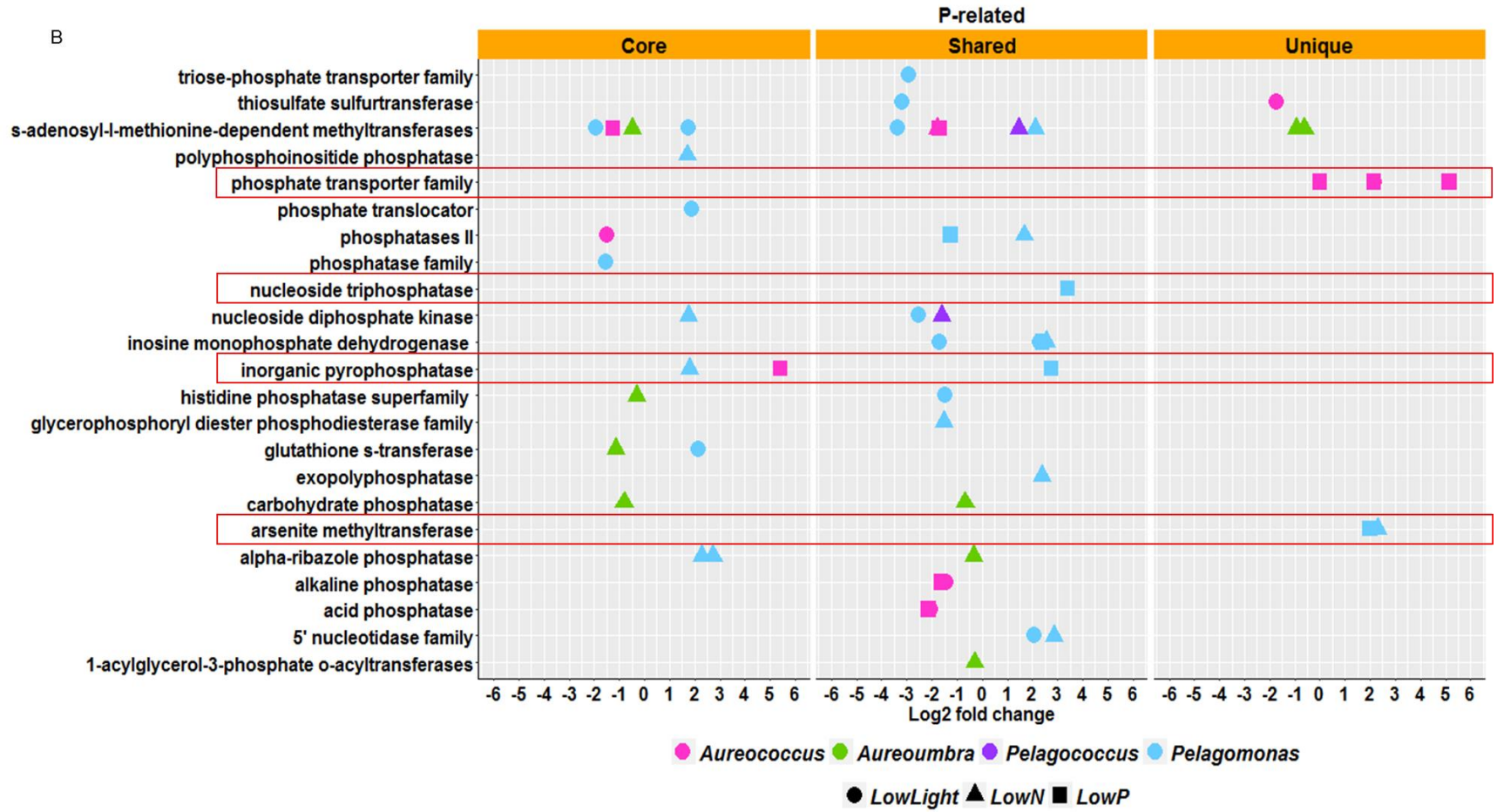



**Figure 7.** Continue (P-related).

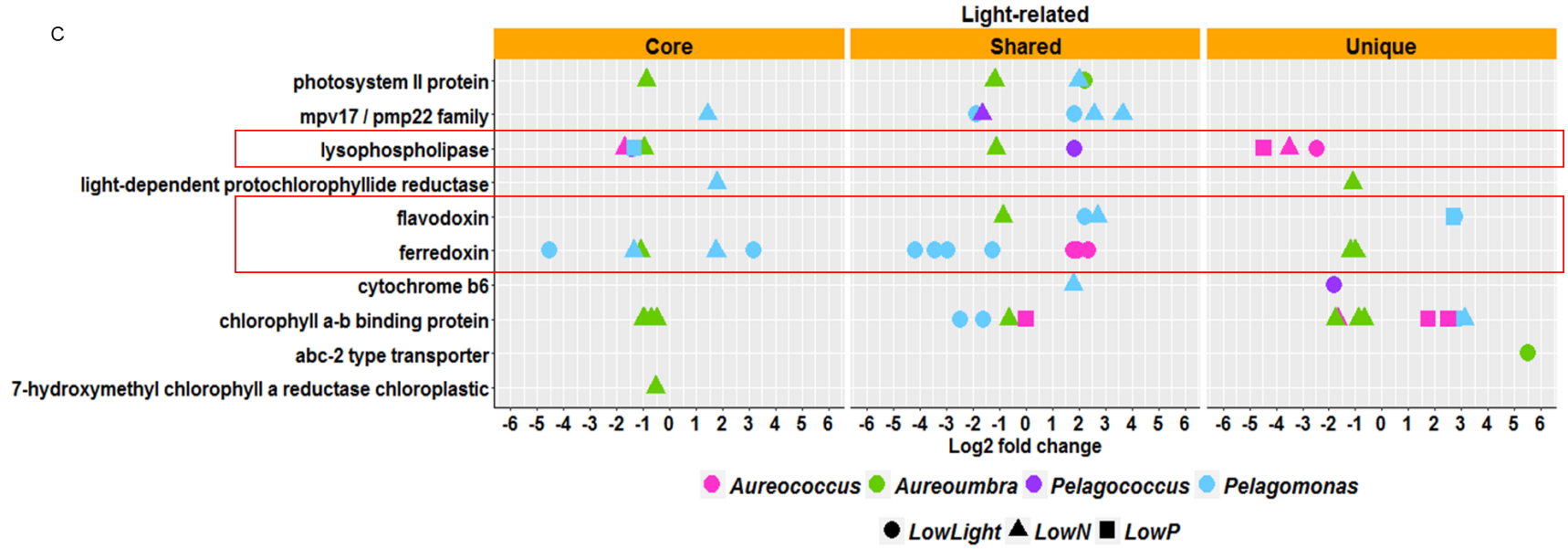



**Figure 7.** Continue (Light-related).

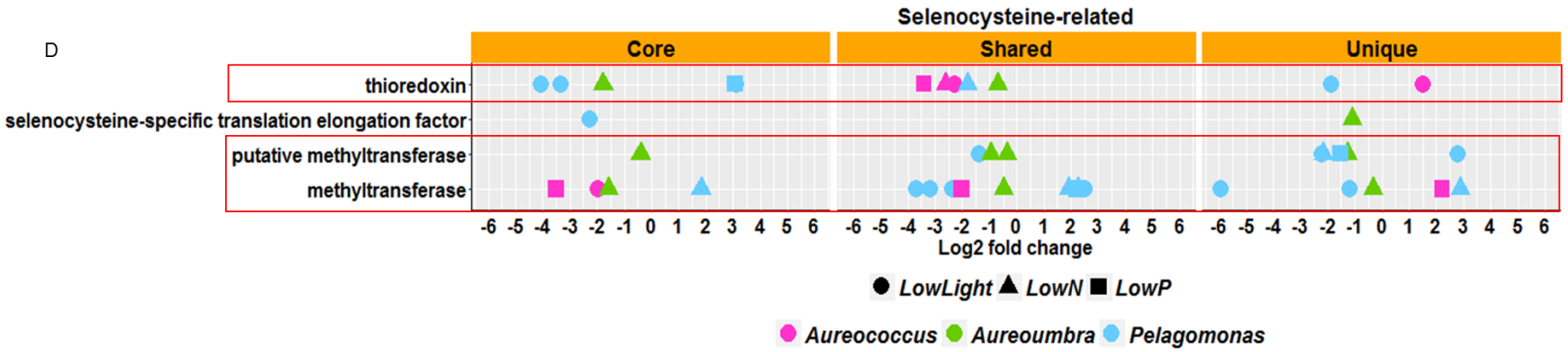



**Figure 7.** Continue (Selenocysteine-related).

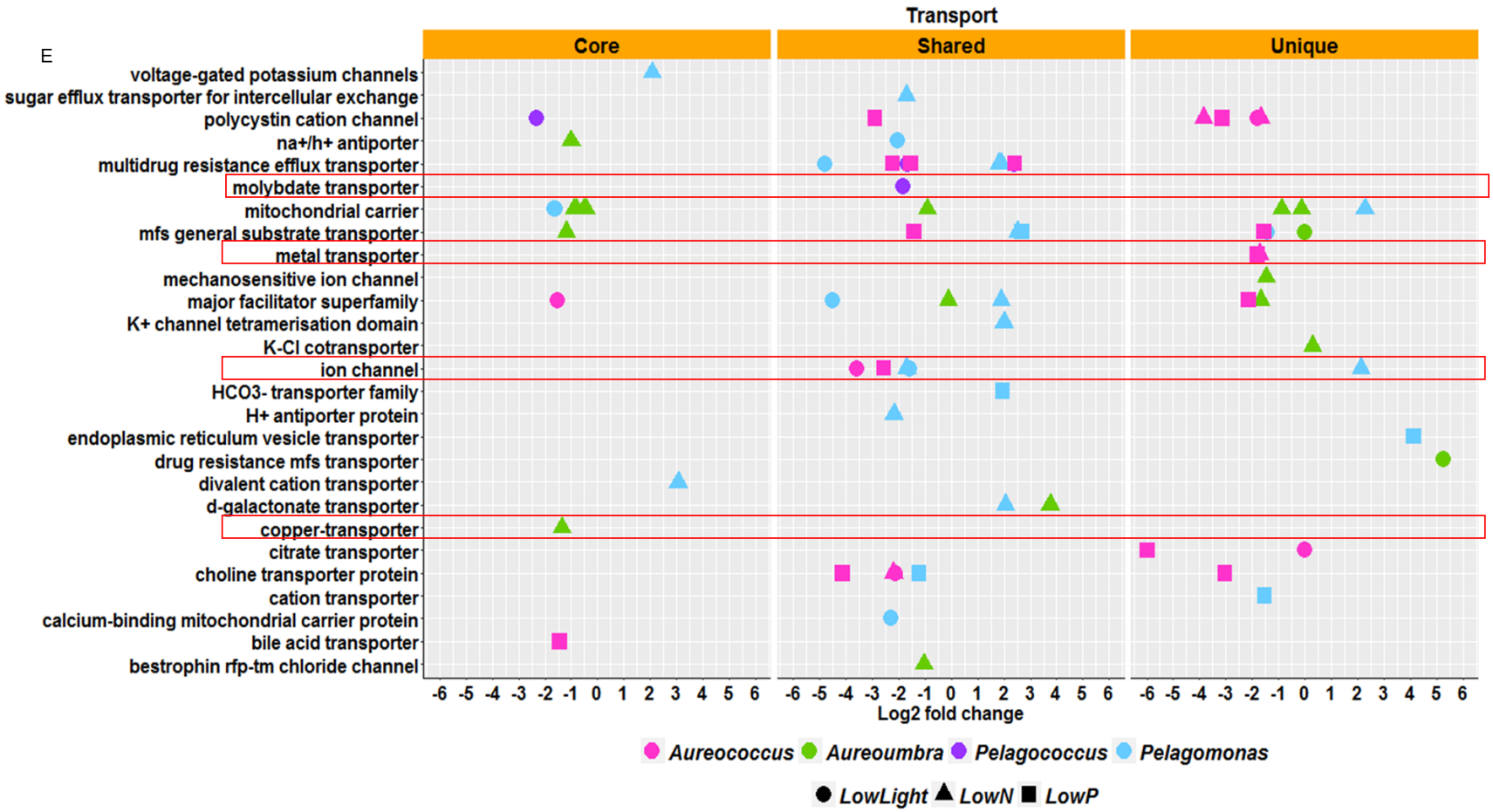



**Figure 7.** Continue (Transport system).

# Chapter 7

## Dissertation Summary

**Dissertation Summary**

Since the 1980s, oceanographic studies have focused on the role of picoplankton in food webs and biogeochemical cycles (Moon-van der Staay et al., 2001; Cuvelier et al., 2010; Massana, 2011) with the majority of this effort focused on the picoprokarytoes, *Synechococcus* and *Prochlorococcus* (Waterbury et al., 1986; Chisholm et al., 1992; Partensky et al., 1999; Rocap et al., 2002). Picoeukaryotes, in contrast, have been comparatively less studied, despite their likely importance in ocean ecology (Moon-van der Staay et al., 2001; Cuvelier et al., 2010; Massana, 2011). Field and genomic studies have identified pelagophytes as some of the most abundant picoeukaryotes in oligotrophic oceans, but ecological niche has not been well defined (Simon et al., 1994; Andersen et al., 1996; Worden et al., 2012; Dupont et al., 2014). In contrast, the importance of pelagophytes in coastal ecosystems has been well-described, with two pelagophytes, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, causing harmful brown tides in coastal waters and causing harm or mortality in marine organisms (Gobler and Sunda, 2012).

Ecological traits facilitating brown tides in estuaries include avoidance of zooplankton grazing (Buskey and Hyatt, 1995; Gobler et al., 2002b), achieving maximal growth rates at low light levels (Milligan and Cosper, 1997; Pustizzi et al., 2004), and DON assimilation (Berg et al., 2002; Mulholland et al., 2002; Muhlstein and Villareal, 2007). Ecosystem-based assessment of these factors, however, have been largely studied in NY and TX. No study to date has definitively ascribed brown tide alga-specific uptake rates in an ecosystem setting. Furthermore, other important ecological processes known to promote and sustain harmful algal blooms (HABs) such as allelopathic inhibition of co-existing phytoplankton and the formation of resting cells have never been assessed for the brown tide algae. Finally, accurate physiological

responses of pelagophytes to the dominant conditions (low nitrogen (N), phosphorus (P), and light) in oligotrophic, pelagic environments where the pelagophytes *Pelagomonas* and *Pelagococcus* dominate and turbid estuaries where *Aureococcus* and *Aureoumbra* form brown tides are not fully clear. This dissertation presents comparative studies of pelagophytes that assesses ecological processes that facilitate brown tides and the transcriptional responses of open ocean and coastal pelagophytes to low N, P, and light.

The first investigation of my dissertation explored the emergence of brown tides caused by *Aureoumbra lagunensis* outside of TX, USA, specifically within the northern Indian River Lagoon (Walther et al.), FL, USA. In Chapter 2, I presented 15 zooplankton grazer dilution experiments, 15 nutrient amendment experiments, and a series of $^{15}N$ assimilation experiments during three field surveys in 2012 and 2013 that were designed to assess the factors that facilitated the outbreak of these new brown tides. Experimental results indicated that *Aureoumbra* experienced low levels of zooplankton grazing during blooms, preferentially assimilated recycled nutrients (e.g. ammonium and urea), and outgrew competing cyanobacteria when ammonium levels were experimentally enriched. Collectively, the results demonstrated that the ability to disrupt pelagic grazing while exploiting reduced forms of N permitted *Aureoumbra* to form brown tides in this ecosystem.

Given the spread of *Aureoumbra* blooms to FL, USA, in 2012 (Gobler et al., 2013), and then to Cuba in 2013 (Koch et al., 2014), I next explored the ability of *Aureoumbra* to form resting cells in response to environmental stressors. The first brown tide in FL in 2012 persisted for six months, from May through November, and returned to this region the next spring, six months later. Since 2012, these brown tides have recurred each year. While dinoflagellates are known to form resting cysts and germinate to form new blooms under favorable growth

conditions, no resting stage has ever been described in any pelagophyte. In Chapter 3 of my dissertation, I explored the ability of *Aureoumbra* to form resting cells in response to stressful conditions such as high temperature, nutrient limitation, and darkness. Through multiple experiments conducted under resource limiting or high temperature conditions, *Aureoumbra* formed resting cells that were larger in size and biovolume, depleted in RNA, respired at slowed rates, and had lower levels of chlorophyll *a* compared to vegetative cells. Resting cells also developed intracellular, red accumulation bodies. Raman microspectrometry was used to demonstrate that resting cells were enriched in sterols but depleted in photosynthesis-related pigments (i.e. carotenoids) compared to vegetative cells. In addition, experiments demonstrated that *Aureoumbra* resting cells could revert back to vegetative cells after seven months of dark storage. Collectively, this chapter demonstrated that *Aureoumbra* forms resting cells that may contribute to the annual recurrence of blooms in estuaries and may facilitate the anthropogenic spread of this HAB to new ecosystems such as estuaries in FL and Cuba.

In Chapter 4, I explored another mechanism that may contribute toward the intensification and persistence of brown tides, specifically the allelopathic inhibition of other phytoplankton. Allelopathy is a mechanism that has been shown to promote many HABs (Granéli and Hansen, 2006) and brown tide is usually succeeded by cyanobacterial bloom (Sieracki et al., 2004; Chapter 2). For Chapter 4, I performed a series of experiments using 10 strains of phytoplankton including phycocyanin (PC)-containing *Synechococcus* as a prokaryotic model species and the diatom, *Chaetoceros calcitrans,* as a eukaryotic model species. The brown tide algae, *Aureococcus* and *Aureoumbra*, significantly suppressed the growth of 9 of the 10 phytoplankton. PC and PE cyanobacteria, the major competitors with brown tide algae, were the most sensitive phytoplankton to the allelochemicals. *Aureococcus* and *Aureoumbra* cultures

with cell densities >2.5 x $10^5$ cells $mL^{-1}$ induced strong allelopathic effects on target algae suggesting allelochemicals may promote bloom persistence and intensification but not bloom initiation. Given that brown tides persist for longer than most algal blooms, often for months or even years at a time (Buskey et al., 1997; Gobler and Sunda, 2012), I hypothesize that allelopathic inhibition of competing phytoplankton may contribute toward the expansion of brown tides if resting cells (Chapter 3) are anthropogenically transported to other regions via ship's ballast water or if the extended nature of blooms increases the likelihood of vegetative cell transport via physical advection.

While I have intensively used flow cytometry to enumerate picoplankton during my dissertation and performed multiple $^{15}$N-uptake experiments in Chapter 2 of my dissertation, for Chapter 5, I combined these approaches. While $^{15}$N-uptake rates of whole phytoplankton or size-fractionated phytoplankton communities have been measured for decades, species-specific $^{15}$N-uptake rates of individual phytoplankton species in an ecosystem setting have not been assessed. In Chapter 5, sorting flow cytometry was used in combination with species-specific immuno-detection of *Aureococcus* to contrast the nutritional ecology of this alga with other co-occurring picoplankton (picoeukaryotes, cyanobacteria, heterotrophic bacteria) during brown tides. While *Aureococcus* utilized multiple forms of nitrogen (e.g. nitrate, ammonium, urea) during blooms, *Aureococcus* cells assimilated urea significantly faster than other plankton groups providing clear evidence of the importance of urea in promoting brown tide blooms, a result that was consistent with an observation during Florida brown tides of *Aureoumbra* (Chapter 2) and prior studies of *Aureococcus*. Collectively, this chapter highlighted a powerful new approach for assessing nutritional ecology of harmful algae and co-occurring plankton populations.

While the first four chapters of my dissertation focused exclusively on the ecophysiological factors facilitating brown tides blooms in coastal waters, for my final research chapter (Chapter 6), I contrasted the ecophysiology of the coastal pelagophytes, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, with the open ocean pelagophytes, *Pelagococcus subviridis* and *Pelagomonas calceolata*. I specifically generated and contrasted the transcriptomes of these four pelgophytes under the habitat-relevant conditions such as low N, low P, low light, and replete conditions. This study demonstrated that pelagophytes expressed clusters associated with utilization of organic nitrogen, intracellular P recycling, lipid hydrolysis, and low light-adaptation in response to resource limitation. Not all transcriptomic patterns were universal among the pelagophytes as *Aureococcus* upregulated a unique set of phosphate transporters in response to low P and *Pelagomonas* upregulated clusters associated with the degradation of organic nitrogen compounds under low N and more strongly expressed flavodoxins over ferredoxins under low light. These findings in *Pelagomonas* were also consistent with findings in Chapter 2, 3, and 5 that highlighted the ability of bloom-forming, coastal pelagophytes to utilize organic N in low N environments. This study provided transcriptional evidence to support field observations and laboratory studies while providing new information regarding the physiological responses of open ocean pelagophytes to resource limitation.

My dissertation has generated new insights and understanding of the ecophysiological strategies that allow pelagophytes to thrive and persist in coastal and open ocean environments. Like any dissertation, however, there were aspects of my research that could have expanded upon or improved. During the investigation of brown tides in FL (Chapter 2), dilution experiments were performed to assess grazing pressure on brown tide populations and to

quantify grazing rates. Lacking, however, was a quantification of zooplankton (e.g. micro- and macrozooplankton) densities that may have provided a mechanistic understanding that could have accounted for the differential grazing pressure on *Aureoumbra* and co-occurring phytoplankton.

During the study of resting stage formation by *Aureoumbra* (Chapter 3), I was not able to search for resting cells in sediments of bloom-prone estuaries or assess the ability of another brown tide alga, *Aureococcus* to form resting cells. While the former potential study would have provided confirmation of the ecological importance of resting cell formation, the latter potential study seems like a logical follow-up study given *Aureococcus* can persist in the dark for 30 days (Popels and Hutchins, 2002).

In Chapter 4, strain variability in allelophathic effects of *Aureoumbra* was confirmed but the variability in *Aureococcus* was not assessed. Also, while I was able to document allelochemical degradation at 20°C in the dark, experiments to contrast light, UV, and dark degradation of allelochemicals were not performed. Further, some of the experiments designed to characterize the properties of allelochemicals were performed with filtrate from *Aureoumbra* cultures, but not with filtrate from *Aureococcus* cultures. Hence, there are obvious follow-up studies to be performed regarding the allelopathy of coastal pelagophytes.

In the study of nutritional assimilation of major picoplankton during brown tides formed by *Aureococcus* performed using sorting flow cytometry (Chapter 5), only three experiments were analyzed due to prohibitively high costs and time involved in performing analyses. It would have been of interest to understand how $^{15}$N-uptake rates of major picoplankton changed over the periods of bloom initiation, peak, and decline, although the requirement of elevated

*Aureococcus* biomass may have prevented such an investigation. It would have been specifically of interest to understand how the nutritional ecology of cyanobacteria changed as they were released from allelopathic inhibition and became the dominant phytoplankton as brown tide collapsed. Additionally, if size-fractionated $^{15}$Nuptake rates were measured along with the uptake rates of sorted plankton populations, it could have provided additional contrast of nitrogen assimilation by small and large plankton.

Lastly, during the transcriptomic sequencing performed in Chapter 6, RNA isolated from triplicate flasks was combined and sequenced rather than sequenced individually, a practice mandated by the Marine Microbial Eukaryote Transcriptome Sequencing Project which sequenced these samples. This lack of replication necessitated the use of the conservative ASC software to assess differential expression. It is likely that more genes would have been differentially expressed had triplicate transcriptomes been sequenced. Also in this chapter, the orthologous comparison showed some common transcriptomic response among four pelagophytes under resource limiting conditions at the cluster level which included multiple contigs. It is likely that differential gene expression would have been more frequently or significantly detected if comparisons of contig-level expression were made for the four pelagophytes.

In the future, it would be worth further investigating all of the points described above to support or refute hypotheses generated in each dissertation chapter. In the bigger picture, findings in this dissertation provide new directions for future research on pelagophytes. As an initial characterization of allelochemicals, I performed experiments that identified the heat-stable and hydrophobic nature of allelochemicals secreted by *Aureoumbra* and the degradation of these compounds over time at 20°C. Some logical follow-on work from these efforts would include a

precise chemical characterization of the compounds retained on the hydrophobic resins via mass spectrometry. An obvious goal would be to fully characterize and quantify the allelochemicals produced by coastal pelagophytes. Additional open questions regarding allelochemicals would include how environmental conditions alter the production of allelechemicals by pelagophytes. For example, how do changes in the source and concentration of N alter the production of allelochemicals? Different sources of nitrogen may favor production of a single or multiple classes of alleochemicals and may alter rates of allelochemical production. Another open question is the fate of nutrient elements from target species and the degraded allelochemicals. In Chapter 4, I hypothesized that regenerated nutrients from compromised target algae may be rapidly assimilated by heathy brown tide algae potentially creating a positive feedback loop for sustaining and promoting blooms. Labeling and tracking the elements within target algae in follow-up experiments could help test this hypothesis.

Given that global warming tends to latitudinally expand the distribution of phytoplankton poleward (Thomas et al., 2012), the northward expansion of *Aureoumbra* and *Aureococcus* blooms in the Northern Hemisphere may happen. Hence, while brown tides caused by *Aureococcus* have occurred along the Mid-Atlantic coast of USA from Virginia to Rhode Island for decades, one may expect their expansion further north into New England in the future. Similarly, one might anticipate the expansion of *Aureoumbra* northward toward the Mid-Atlantic region, particularly given its ability to form resting cells. With such future expansion, there may even be an ecosystem within which *Aureococcus* and *Aureoumbra* eventually co-occur in which case it would be of interest to assess competition between these two species.

Despite the mounting evidence that *Pelagomonas* is a globally important picoeukaryote (Simon et al., 1994; Andersen et al., 1996), little is known regarding its nutritional ecology.

Timmermans et al. (2005) performed experiments to assess the growth rate kinetics of *Pelagomonas* over a range of ammonium levels and found it had a high affinity for this nutrient. Given the dominance of *Pelagomonas* and other pelagophytes in the DCM, nitrate may be an equal or more important source of nitrogen for its growth (Cullen, 1982; Dupont et al., 2014). In addition, although the assimilation of dissolved organic nitrogen (DON) is a common feature of coastal pelagophytes (Berg et al., 1997; Muhlstein and Villareal, 2007) and my dissertation demonstrated that *Pelagmonas* used enzymes associated with organic nitrogen cleavage under N limiting conditions (Chapter 6), DON utilization of this oceanic pelagophyte species has not been studied in an ecosystem or laboratory setting.

Moreover, given the ability of pelagophytes to phagotrophically consume *Prochlolococcus* in oligotrophic oceans (Hartmann et al., 2012; Hartmann et al., 2013) quantifying such grazing rates as well as rates of bacterivory would provide key insight with regard to the relative importance of this process in the total nutrition of open ocean pelagophytes and the role of this group in microbial food webs. Finally, while coastal pelagophytes are poorly grazed by zooplankton (Chapter 2; Gobler and Sunda, 2012), the extent to which open ocean pelagophytes such as *Pelagomonas* resist grazing is unknown, but could be an important aspect of its ecological niche.

In conclusion, my dissertation has provided a better understanding of the mechanisms facilitating brown tides as well as the shared and unique transcriptional response among coastal and open ocean pelagophytes. My findings have highlighted the plasticity of pelagophyte physiology in adapting to dynamic marine environments regarding nutrient availability, zooplankton grazing, and competition. Beyond the development of a new application of sorting flow cytometry combined with immunofluorescence detection to assess the nutritional ecology

of HABs, I also discovered the ability of *Aureoumbra* to form resting cells and *Aureococcus* and *Aureoumbra* to produce allelochemicals. Collectively, my research has facilitated a deeper, more detailed understanding of the mechanisms facilitating brown tides both locally and globally and has developed ecological and molecular tools should help investigate ecophysiology of other HAB species in the future.

## References


Alexander, H., 2016. Defining the ecological and physiological traits of phytoplankton across marine ecosystems. Massachusetts Institute of Technology and Woods Hole Oceanographic Institution.

Alexander, H., Jenkins, B.D., Rynearson, T.A., Dyhrman, S.T., 2015. Metatranscriptome analyses indicate resource partitioning between diatoms in the field. Proc. Natl. Acad. Sci. 112 (17), E2182-E2190.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403-410.

Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Reviews Microbiology 6 (5), 339-348.

Andersen, R.A., Bidigare, R.R., Keller, M.D., Latasa, M., 1996. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans. Deep-Sea Res PT II 43 (2–3), 517-537.

Andersen, R.A., Saunders, G.W., Paskind, M.P., Sexton, J.P., 1993. Ultrastructure and 18S ribosomal RNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov. and the description of a new algal class, the Pelagophyceae classis nov. J. Phycol. 29 (5), 701-715.

Anderson, D.M., 1980. Effects of temperature conditioning on development and germination of *Gonyaulax tamarensis* (Dinophyceae). J. Phycol. 16 (2), 166-172.

Anderson, D.M., Burkholder, J.M., Cochlan, W.P., Glibert, P.M., Gobler, C.J., Heil, C.A., Kudela, R., Parsons, M.L., Rensel, J., Townsend, D., 2008. Harmful algal blooms and eutrophication: Examining linkages from selected coastal regions of the United States. Harmful Algae 8 (1), 39-53.

Anderson, D.M., Coats, D.W., Tyler, M.A., 1985. Encystment of the dinoflagellate *Gyrodinium uncatenum*: temperature and nutrient effects. J. Phycol. 21 (2), 200-206.

Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences. Estuaries 25 (4), 704-726.

Anderson, D.M., Taylor, C.D., Armbrust, E.V., 1987. The effects of darkness and anaerobiosis on dinoflagellate cyst germination. Limnol. Oceanogr 32 (2), 340-335.

Anderson, O.R., 1975. The ultrastructure and cytochemistry of resting cell formation in *Amphora coffaeformis* (Bacillariophyceae). J. Phycol. 11 (3), 272-281.

Anderson, O.R., 1976. Respiration and photosynthesis during resting cell formation in Amphora coffeaeformis (Ag.) Kutz. Limnol. Oceanogr. 21 (3), 452-456.

Antia, N.J., Harrison, P.J., Oliveira, L., 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. Phycologia 30 (1), 1-89.

Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S., Allen, A.E., Apt, K.E., Bechner, M., 2004. The genome of the diatom Thalassiosira pseudonana: ecology, evolution, and metabolism. Science 306 (5693), 79-86.

Azam, F., Smith, D., Steward, G., Hagström, Å., 1994. Bacteria-organic matter coupling and its significance for oceanic carbon cycling. Microb. Ecol. 28 (2), 167-179.

Baas-Becking, L.G.M., 1934. Geobiologie; of inleiding tot de milieukunde. WP Van Stockum & Zoon NV.

Baas Becking, L.G.M., 1934. Geobiologie of inleiding tot de Milieukunde. Van Stockum W. P. & Zoon N. V., The Hague, The Netherlands.

Balzano, S., Marie, D., Gourvil, P., Vaulot, D., 2012. Composition of the summer photosynthetic pico and nanoplankton communities in the Beaufort Sea assessed by T-RFLP and sequences of the 18S rRNA gene from flow cytometry sorted samples. The ISME journal 6 (8), 1480-1498.

Bateman, A., Coin, L., Durbin, R., Finn, R.D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E.L., 2004. The Pfam protein families database. Nucleic Acids Res. 32 (suppl 1), D138-D141.

Behrenfeld, M.J., 2010. Abandoning Sverdrup's Critical Depth Hypothesis on phytoplankton blooms. Ecology 91 (4), 977-989.

Bender, S.J., Durkin, C.A., Berthiaume, C.T., Morales, R.L., Armbrust, E.V., 2014. Transcriptional responses of three model diatoms to nitrate limitation of growth. Frontiers in Marine Science 1.

Benitez-Nelson, C.R., 2000. The biogeochemical cycling of phosphorus in marine systems. Earth-Sci. Rev. 51 (1), 109-135.

Berg, G., Glibert, P., Jørgensen, N.G., Balode, M., Purina, I., 2001. Variability in inorganic and organic nitrogen uptake associated with riverine nutrient input in the Gulf of Riga, Baltic Sea. Estuaries 24 (2), 204-214.

Berg, G.M., Glibert, P.M., Lomas, M.W., Burford, M.A., 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. Mar. Biol. 129 (2), 377-387.

Berg, G.M., Repeta, D.J., Laroche, J., 2002. Dissolved organic nitrogen hydrolysis rates in axenic cultures of Aureococcus anophagefferens (Pelagophyceae): Comparison with heterotrophic bacteria. Appl. Environ. Microbiol. 68 (1), 401-404.

Berg, G.M., Shrager, J., Glockner, G., Arrigo, K.R., Grossman, A.R., 2008. Understanding nitrogen limitation in *Aureococcus anophagefferens* (Pelagophyceae) through cDNA and qRT-PCR analysis. J. Phycol. 44 (5), 1235-1249.

Berges, J.A., Charlebois, D.O., Mauzerall, D.C., Falkowski, P.G., 1996. Differential effects of nitrogen limitation on photosynthetic efficiency of photosystems I and II in microalgae. Plant Physiol. 110 (2), 689-696.

Berry, D.L., Goleski, J.A., Koch, F., Wall, C.C., Peterson, B.J., Anderson, O.R., Gobler, C.J., 2015. Shifts in cyanobacterial strain dominance during the onset of harmful algal blooms in Florida Bay, USA. Microb. Ecol. 70 (2), 361-371.

Bersano, J.G.F., Buskey, E.J., Villareal, T.A., 2002. Viability of the Texas brown tide alga, *Aureoumbra lagunensis*, in fecal pellets of the copepod *Acartia tonsa*. Plankton Biol. Ecol. 49 (2), 88-92.

Bianchi, T.S., 2006. Biogeochemistry of estuaries. Oxford University Press.

Bibby, B.T., Dodge, J.D., 1972. The encystment of a freshwater dinoflagellate: a light and electron-microscopical study. Brit Phycol J. 7 (1), 85-100.

Biegala, I.C., Not, F., Vaulot, D., Simon, N., 2003. Quantitative assessment of picoeukaryotes in the natural environment by using taxon-specific oligonucleotide probes in association with tyramide signal amplification-fluorescence in situ hybridization and flow cytometry. Appl. Environ. Microbiol. 69 (9), 5519-5529.

Bloch, K., 1992. Sterol molecule: structure, biosynthesis, and function. Steroids 57 (8), 378-383.

Boissonneault-Cellineri, K.R., Mehta, M., Lonsdale, D.J., Caron, D.A., 2001. Microbial food web interactions in two Long Island embayments. Aquat. Microb. Ecol. 26 (2), 139-155.

Bouman, H.A., Ulloa, O., Scanlan, D.J., Zwirglmaier, K., Li, W.K., Platt, T., Stuart, V., Barlow, R., Leth, O., Clementson, L., 2006. Oceanographic basis of the global surface distribution of Prochlorococcus ecotypes. Science 312 (5775), 918-921.

Boyd, P., Ellwood, M., 2010. The biogeochemical cycle of iron in the ocean. Nature Geoscience 3 (10), 675-682.

Boyd, P.W., Rynearson, T.A., Armstrong, E.A., Fu, F., Hayashi, K., Hu, Z., Hutchins, D.A., Kudela, R.M., Litchman, E., Mulholland, M.R., Passow, U., Strzepek, R.F., Whittaker, K.A., Yu, E., Thomas, M.K., 2013. Marine phytoplankton temperature versus growth responses from polar to tropical waters - outcome of a scientific community - wide study. Plos One 8 (5), e63091.

Boynton, W., Kemp, W.M., 1985. Nutrient regeneration and oxygen consumption by sediments along an estuarine salinity gradient. Marine ecology progress series. Oldendorf 23 (1), 45-55.

Bradley, P.B., Lomas, M.W., Bronk, D.A., 2010a. Inorganic and organic nitrogen use by phytoplankton along Chesapeake Bay, measured using a flow cytometric sorting approach. Estuaries and Coasts 33 (4), 971-984.

Bradley, P.B., Sanderson, M.P., Frischer, M.E., Brofft, J., Booth, M.G., Kerkhof, L.J., Bronk, D.A., 2010b. Inorganic and organic nitrogen uptake by phytoplankton and heterotrophic bacteria in the stratified Mid-Atlantic Bight. Estuar. Coast. Shelf Sci. 88 (4), 429-441.

Bravo, I., Figueroa, R.I., 2014. Towards an ecological understanding of dinoflagellate cyst functions. Microorganisms 2 (1), 11-32.

Brehm-Stecher, B.F., Johnson, E.A., 2004. Single-cell microbiology: tools, technologies, and applications. Microbiol. Mol. Biol. Rev. 68 (3), 538-559.

Bricelj, V.M., Lonsdale, D.J., 1997. *Aureococcus anophagefferens*: Causes and ecological consequences of brown tides in US mid-Atlantic coastal waters. Limnol. Oceanogr. 42 (5), 1023-1038.

Bricelj, V.M., MacQuarrie, S.P., Schaffner, R.A., 2001. Differential effects of Aureococcus anophagefferens isolates ("brown tide") in unialgal and mixed suspensions on bivalve feeding. Mar. Biol. 139 (4), 605-615.

Bronk, D.A., Killberg-Thoreson, L., Sipler, R.E., Mulholland, M.R., Roberts, Q.N., Bernhardt, P.W., Garrett, M., O'Neil, J.M., Heil, C.A., 2014. Nitrogen uptake and regeneration (ammonium regeneration, nitrification and photoproduction) in waters of the West Florida Shelf prone to blooms of *Karenia brevis*. Harmful Algae (0).

Budd, K., Craig, S.R., 1981. Resistance to arsenate toxicity in the blue-green alga Synechococcus leopoliensis. Canadian Journal of Botany 59 (8), 1518-1521.

Burkholder, J.M., Glibert, P.M., 2009. The importance of intraspecific variability in harmful algae-Preface to a collection of topical papers. Harmful Algae 8 (5), 744-745.

Burkill, P.H., Mantoura, R.F.C., Llewellyn, C.A., Owens, N.J.P., 1987. Microzooplankton grazing and selectivity of phytoplankton in coastal waters. Mar. Biol. 93 (4), 581-590.

Buskey, E.J., 2008. How does eutrophication affect the role of grazers in harmful algal bloom dynamics? Harmful Algae 8 (1), 152-157.

Buskey, E.J., Deyoe, H., Jochem, F.J., Villareal, T.A., 2003. Effects of mesozooplankton removal and ammonium addition on planktonic trophic structure during a bloom of the Texas 'brown tide': a mesocosm study. J. Plankton Res. 25 (2), 215-228.

Buskey, E.J., Hyatt, C.J., 1995. Effects of the Texas (USA) brown tide alga on planktonic grazers. Mar. Ecol. Prog. Ser. 126 (1-3), 285-292.

Buskey, E.J., Liu, H.B., Collumb, C., Bersano, J.G.F., 2001. The decline and recovery of a persistent Texas brown tide algal bloom in the Laguna Madre (Texas, USA). Estuaries 24 (3), 337-346.

Buskey, E.J., Montagna, P.A., Amos, A.F., Whitledge, T.E., 1997. Disruption of grazer populations as a contributing factor to the initiation of the Texas brown tide algal bloom. Limnol. Oceanogr. 42 (5), 1215-1222.

Buskey, E.J., Stockwell, D.A., 1993. Effects of a persistent "brown tide" on zooplankton populations in the Laguna Madre of South Texas, Toxic phytoplankton blooms in the sea. Proc. 5th Int. Conf. on Toxic Marine Phytoplankton. Elsevier, pp. 659-665.

Buskey, E.J., Wysor, B., Hyatt, C., 1998. The role of hypersalinity in the persistence of the Texas 'brown tide' in the Laguna Madre. J. Plankton Res. 20 (8), 1553-1565.

Calbet, A., Landry, M.R., 2004. Phytoplankton growth, microzooplankton grazing, and carbon cycling in marine systems. Limnol. Oceanogr. 49 (1), 51-57.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., Madden, T.L., 2009. BLAST+: architecture and applications. BMC Bioinformatics 10 (1), 421.

Campbell, L., Vaulot, D., 1993. Photosynthetic picoplankton community structure in the subtropical North Pacific Ocean near Hawaii (station ALOHA). Deep Sea Research Part I: Oceanographic Research Papers 40 (10), 2043-2060.

Caron, D.A., Dennett, M.R., Moran, D.M., Schaffner, R.A., Lonsdale, D.J., Gobler, C.J., Nuzzi, R., McLean, T.I., 2003. Development and application of a monoclonal-antibody technique for counting *Aureococcus anophagefferens*, an alga causing recurrent brown tides in the Mid-Atlantic United States. Appl. Environ. Microbiol. 69 (9), 5492-5502.

Caron, D.A., Gobler, C.J., Lonsdale, D.J., Cerrato, R.M., Schaffner, R.A., Rose, J.M., Buck, N.J., Taylor, G., Boissonneault, K.R., Mehran, R., 2004. Microbial herbivory on the brown tide alga, *Aureococcus anophagefferens*: results from natural ecosystems, mesocosms and laboratory experiments. Harmful Algae 3 (4), 439-457.

Casey, J.R., Lomas, M.W., Mandecki, J., Walker, D.E., 2007. *Prochlorococcus* contributes to new production in the Sargasso Sea deep chlorophyll maximum. Geophys. Res. Lett. 34 (10).

Chapman, D., Dodge, J., Heaney, S., 1982. Cyst formation in the freshwater dinoflagelalte *Ceratium hirundinella* (Dinophyceae). J. Phycol. 18 (1), 121-129.

Chiou, T.-J., Lin, S.-I., 2011. Signaling network in sensing phosphate availability in plants. Annu. Rev. Plant Biol. 62, 185-206.

Chisholm, S.W., Frankel, S.L., Goericke, R., Olson, R.J., Palenik, B., Waterbury, J.B., West-Johnsrud, L., Zettler, E.R., 1992. *Prochlorococcus marinus* nov. gen. nov. sp.: an oxyphototrophic marine prokaryote containing divinyl chlorophyll *a* and *b*. Arch. Microbiol. 157 (3), 297-300.

Chisholm, S.W., Olson, R.J., Zettler, E.R., Goericke, R., Waterbury, J.B., Welschmeyer, N.A., 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature 334, 340-343.

Chung, C.-C., Hwang, S.-P.L., Chang, J., 2003. Identification of a high-affinity phosphate transporter gene in a prasinophyte alga, Tetraselmis chui, and its expression under nutrient limitation. Appl. Environ. Microbiol. 69 (2), 754-759.

Clark, I., Timlin, R., Bourbonnais, A., Jones, K., Lafleur, D., Wickens, K., 2008. Origin and Fate of Industrial Ammonium in Anoxic Ground Water—$^{15}$N Evidence for Anaerobic Oxidation (Anammox). Ground Water Monit. Remediat. 28 (3), 73-82.

Collier, J.L., 2000. Flow cytometry and the single cell in phycology. J. Phycol. 36 (4), 628-644.

Collos, Y., Vaquer, A., Bibent, B., Slawyk, G., Garcia, N., Souchu, P., 1997. Variability in nitrate uptake kinetics of phytoplankton communities in a Mediterranean coastal lagoon. Estuarine Coastal and Shelf Science 44 (3), 369-375.

Cosper, E.M., Dennison, W.C., Carpenter, E.J., Bricelj, V.M., Mitchell, J.G., Kuenstner, S.H., Colflesh, D., Dewey, M., 1987. Recurrent and persistent brown tide blooms perturb coastal marine ecosystem. Estuaries 10 (4), 284-290.

Cowie, G.L., Hedges, J.I., 1992. Improved amino acid quantification in environmental samples: charge-matched recovery standards and reduced analysis time. Mar. Chem. 37 (3), 223-238.

Cullen, J.J., 1982. The deep chlorophyll maximum: comparing vertical profiles of chlorophyll *a*. Can. J. Fish. Aquat. Sci. 39 (5), 791-803.

Cuvelier, M.L., Allen, A.E., Monier, A., McCrow, J.P., Messie, M., Tringe, S.G., Woyke, T., Welsh, R.M., Ishoey, T., Lee, J.H., Binder, B.J., DuPont, C.L., Latasa, M., Guigand, C., Buck, K.R., Hilton, J., Thiagarajan, M., Caler, E., Read, B., Lasken, R.S., Chavez, F.P., Worden, A.Z., 2010. Targeted metagenomics and ecology of globally important uncultured eukaryotic phytoplankton. Proc. Natl. Acad. Sci. 107 (33), 14679-14684.

Dale, B., 1983. Dinoflagellate resting cysts: "benthic plankton", In: Fryxell, G.A. (Ed.), Survival strategies of the algae. Cambridge University Press, Cambridge, p. 144.

Davey, H.M., Kell, D.B., 1996. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. Microbiological reviews 60 (4), 641-696.

Dawes, E.A., Senior, P.J., 1973. The role and regulation of energy reserve polymers in micro-organisms, In: Rose, A.H., Tempest, D.W. (Eds.), Adv. Microb. Physiol. Academic Press, London and New York, pp. 135-266.

De Gelder, J., De Gussem, K., Vandenabeele, P., Moens, L., 2007. Reference database of Raman spectra of biological molecules. Journal of Raman Spectroscopy 38 (9), 1133-1147.

Dehning, I., Tilzer, M.M., 1989. Survival of *Scenedesmus acuminatus* (Chlorophyceae) in darkness. J. Phycol. 25 (3), 509-514.

Dempster, E.L., Pryor, K.V., Francis, D., Young, J.E., Rogers, H.J., 1999. Rapid DNA extraction from ferns for PCR-based analyses. BioTechniques 27 (1), 66-68.

Deonarine, S.N., Gobler, C.J., Lonsdale, D.J., Caron, D.A., 2006. Role of zooplankton in the onset and demise of harmful brown tide blooms (*Aureococcus anophagefferens*) in US mid-Atlantic estuaries. Aquat. Microb. Ecol. 44 (2), 181-195.

DeYoe, H.R., Buskey, E.J., Jochem, F.J., 2007. Physiological responses of *Aureoumbra lagunensis* and *Synechococcus* sp to nitrogen addition in a mesocosm experiment. Harmful Algae 6 (1), 48-55.

DeYoe, H.R., Stockwell, D.A., Bidigare, R.R., Latasa, M., Johnson, P.W., Hargraves, P.E., Suttle, C.A., 1997. Description and characterization of the algal species *Aureoumbra lagunensis* gen. et sp. nov. and referral of *Aureoumbra* and *Aureococcus* to the Pelagophyceae. J. Phycol. 33 (6), 1042-1048.

Deyoe, H.R., Suttle, C.A., 1994. The inability of the Texas brown tide alga to use nitrate and the role of nitrogen in the initiation of a persistent bloom of this organism. J. Phycol. 30 (5), 800-806.

Di Dato, V., Musacchia, F., Petrosino, G., Patil, S., Montresor, M., Sanges, R., Ferrante, M.I., 2015. Transcriptome sequencing of three Pseudo-nitzschia species reveals comparable gene sets and the presence of Nitric Oxide Synthase genes in diatoms. Scientific reports 5.

Dimier, C., Brunet, C., Geider, R., Raven, J., 2009. Growth and photoregulation dynamics of the picoeukaryote *Pelagomonas calceolata* in fluctuating light. Limnol. Oceanogr. 54 (3), 823-836.

Doblin, M.A., Blackburn, S.I., Hallegraeff, G.M., 1999a. Comparative study of selenium requirements of three phytoplankton species: Gymnodinium catenatum, Alexandrium minutum (Dinophyta) and Chaetoceros cf. tenuissimus (Bacillariophyta). J. Plankton Res. 21 (6), 1153-1169.

Doblin, M.A., Blackburn, S.I., Hallegraeff, G.M., 1999b. Growth and biomass stimulation of the toxic dinoflagellate *Gymnodinium catenatum* (Graham) by dissolved organic substances. J. Exp. Mar. Biol. Ecol. 236 (1), 33-47.

Doblin, M.A., Popels, L.C., Coyne, K.J., Hutchins, D.A., Cary, S.C., Dobbs, F.C., 2004. Transport of the harmful bloom alga *Aureococcus anophagefferens* by oceangoing ships and coastal boats. Appl. Environ. Microbiol. 70 (11), 6495-6500.

Dortch, Q., Roberts, T.L., Clayton Jr, J.R., Ahmed, S.I., 1983. RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. Mar. Ecol. Prog. Ser. 13 (1), 61-71.

Dugdale, R.C., Wilkerson, F.P., 1986. The use of $^{15}N$ of measure nitrogen uptake in eutrophic oceans - experimental considerations. Limnol. Oceanogr. 31 (4), 673-689.

Duhamel, S., Björkman, K.M., Karl, D.M., 2012. Light dependence of phosphorus uptake by microorganisms in the subtropical North and South Pacific Ocean. Aquat. Microb. Ecol. 67, 225-238.

Dupont, C.L., McCrow, J.P., Valas, R., Moustafa, A., Walworth, N., Goodenough, U., Roth, R., Hogle, S.L., Bai, J., Johnson, Z.I., Mann, E., Palenik, B., Barbeau, K.A., Venter, J.C., Allen, A.E., 2014. Genomes and gene expression across light and productivity gradients in eastern subtropical Pacific microbial communities. ISME Journal 9 (5), 1076-1092.

Dwyer, L., Forsyth, P., 1998. Economic significance of cruise tourism. Ann. Tourism Res. 25 (2), 393-415.

Dyhrman, S.T., Ammerman, J.W., Van Mooy, B.A., 2007. Microbes and the marine phosphorus cycle.

Dyhrman, S.T., Jenkins, B.D., Rynearson, T.A., Saito, M.A., Mercier, M.L., Alexander, H., Whitney, L.P., Drzewianowski, A., Bulygin, V.V., Bertrand, E.M., Wu, Z.J., Benitez-Nelson, C., Heithoff, A., 2012. The transcriptome and proteome of the diatom *Thalassiosira pseudonana* reveal a diverse phosphorus stress response. Plos One 7 (3).

Epifanova, O.I., Terskikh, V., 1969. On the resting periods in the cell life cycle Cell Proliferation 2 (1), 75-93.

Eppley, R.W., 1972. Temperature and phytoplankton growth in the sea. Fish. Bull 70 (4), 1063-1085.

Eppley, R.W., Rogers, J.N., Mccarthy, J.J., 1969. Half-saturation constants for uptake of nitrate and ammonium by marine phytoplankton. Limnol. Oceanogr. 14 (6), 912-&.

Erdner, D.L., Anderson, D.M., 2006. Global transcriptional profiling of the toxic dinoflagellate *Alexandrium fundyense* using massively parallel signature sequencing. BMC Genomics 7 (1), 88.

Falkowski, P.G., Sukenik, A., Herzig, R., 1989. Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). II Relative abundance of chloroplast proteins. J. Phycol. 25 (3), 471-478.

Fawcett, S.E., Lomas, M., Casey, J.R., Ward, B.B., Sigman, D.M., 2011. Assimilation of upwelled nitrate by small eukaryotes in the Sargasso Sea. Nature Geoscience 4 (10), 717-722.

Fenchel, T., 2008. The microbial loop–25 years later. J. Exp. Mar. Biol. Ecol. 366 (1), 99-103.

Fernández-Herrera, L.J., Band-Schmidt, C.J., López-Cortés, D.J., Hernández-Guerrero, C.J., Bustillos-Guzmán, J.J., Núñez-Vázquez, E., 2016. Allelopathic effect of Chattonella marina var. marina (Raphidophyceae) on Gymnodinium catenatum (Dinophycea). Harmful Algae 51, 1-9.

Figueredo, C.C., Giani, A., Bird, D.F., 2007. Does allelopathy contribute to *Cylindrospermopsis raciborskii* (Cyanobacteria) bloom occurrence and geographic expansion? J. Phycol. 43 (2), 256-265.

Figueroa, R.I., Bravo, I., Garcés, E., 2005. Effects of nutritional factors and different parental crosses on the encystment and excystment of *Alexandrium catenella* (Dinophyceae) in culture. Phycologia 44 (6), 658-670.

Fistarol, G.O., Legrand, C., Granéli, E., 2003. Allelopathic effect of *Prymnesium parvum* on a natural plankton community. Mar. Ecol. Prog. Ser. 255, 115-125.

Fistarol, G.O., Legrand, C., Rengefors, K., Graneli, E., 2004a. Temporary cyst formation in phytoplankton: a response to allelopathic competitors? Environ. Microbiol. 6 (8), 791-798.

Fistarol, G.O., Legrand, C., Selander, E., Hummert, C., Stolte, W., Granéli, E., 2004b. Allelopathy in Alexandrium spp.: effect on a natural plankton community and on algal monocultures. Aquat. Microb. Ecol. 35 (1), 45-56.

Frischkorn, K.R., Harke, M.J., Gobler, C.J., Dyhrman, S.T., 2014. *De novo* assembly of *Aureococcus anophagefferens* transcriptomes reveals diverse responses to the low nutrient and low light conditions present during blooms. Name: Frontiers in Microbiology 5, 375.

Fry, B., 2007. Stable isotope ecology. Springer Science & Business Media.

Fu, F., Yu, E., Garcia, N.S., Gale, J., Luo, Y., Webb, E.A., Hutchins, D.A., 2014. Differing responses of marine $N_2$ fixers to warming and consequences for future diazotroph community structure. Aquat. Microb. Ecol. 72 (1), 33-46.

Fu, F.X., Tatters, A.O., Hutchins, D.A., 2012. Global change and the future of harmful algal blooms in the ocean. Mar. Ecol. Prog. Ser. 470, 207-233.

Gallegos, C.L., 1989. Microzooplankton grazing on phytoplankton in the Rhode River, Maryland - Nonlinear feeding kinetics. Mar. Ecol. Prog. Ser. 57 (1), 23-33.

Garces, E., Zingone, A., Reguera, B., 2001. Life histories of microalgal species causing harmful blooms. Calvi, Majorca, Spain: European Commission Directorate General Science, Research and Development.

Gasol, J.M., Moran, X.A., 1999. Effects of filtration on bacterial activity and picoplankton community structure as assessed by flow cytometry. Aquat. Microb. Ecol. 16 (3), 251-264.

Geider, R., Macintyre, Graziano, L., McKay, R.M., 1998. Responses of the photosynthetic apparatus of *Dunaliella tertiolecta* (Chlorophyceae) to nitrogen and phosphorus limitation. Eur. J. Phycol. 33 (4), 315-332.

Geider, R.J., Roche, J., Greene, R.M., Olaizola, M., 1993. Response of the photosynthetic apparatus of *Phaeodactylum tricornutum* (Bacillariophyceae) to nitrate, phosphate, or iron starvation1. J. Phycol. 29 (6), 755-766.

Giner, J.-L., Li, X., Boyer, G.L., 2001. Sterol composition of *Aureoumbra lagunensis*, the Texas brown tide alga. Phytochemistry 57 (5), 787-789.

Giner, J.L., Boyer, G.L., 1998. Sterols of the brown tide alga *Aureococcus anophagefferens*. Phytochemistry 48 (3), 475-477.

Giner, J.L., Zhao, H., Boyer, G.L., Satchwell, M.F., Andersen, R.A., 2009. Sterol chemotaxonomy of marine pelagophyte algae. Chem. Biodivers. 6 (7), 1111-1130.

Glibert, P.M., Anderson, D.M., Gentien, P., Graneli, E., Sellner, K.G., 2005. The global, complex phenomena of harmful algal blooms. Oceanography 18 (2), 132-141.

Gobler, C., Talmage, S., 2013. Short-and long-term consequences of larval stage exposure to constantly and ephemerally elevated carbon dioxide for marine bivalve populations. Biogeosciences 10 (4), 2241-2253.

Gobler, C.J., Berry, D.L., Dyhrman, S.T., Wilhelm, S.W., Salamov, A., Lobanov, A.V., Zhang, Y., Collier, J.L., Wurch, L.L., Kustka, A.B., Dill, B.D., Shah, M., VerBerkmoes, N.C., Kuo, A., Terry, A., Pangilinan, J., Lindquist, E.A., Lucas, S., Paulsen, I.T., Hattenrath-Lehmann, T.K., Talmage, S.C., Walker, E.A., Koch, F., Burson, A.M., Marcoval, M.A., Tang, Y.Z., LeCleir, G.R., Coyne, K.J., Berg, G.M., Bertrand, E.M., Saito, M.A., Gladyshev, V.N., Grigoriev, I.V., 2011. Niche of harmful alga *Aureococcus anophagefferens* revealed through ecogenomics. Proc. Natl. Acad. Sci. 108 (11), 4352-4357.

Gobler, C.J., Boneillo, G.E., Debenham, C.J., Caron, D.A., 2004a. Nutrient limitation, organic matter cycling, and plankton dynamics during an *Aureococcus anophagefferens* bloom. Aquat. Microb. Ecol. 35 (1), 31-43.

Gobler, C.J., Deonarine, S., Leigh-Bell, J., Gastrich, M.D., Anderson, O.R., Wilhelm, S.W., 2004b. Ecology of phytoplankton communities dominated by *Aureococcus*

*anophagefferens*: the role of viruses, nutrients, and microzooplankton grazing. Harmful Algae 3 (4), 471-483.

Gobler, C.J., Donat, J.R., Consolvo, J.A., Sañudo-Wilhelmy, S.A., 2002a. Physicochemical speciation of iron during coastal algal blooms. Mar. Chem. 77 (1), 71-89.

Gobler, C.J., Koch, F., Kang, Y., Berry, D.L., Tang, Y.Z., Lasi, M., Walters, L., Hall, L., Miller, J.D., 2013a. Expansion of harmful brown tides caused by the pelagophyte, *Aureoumbra lagunensis* DeYoe et Stockwell, to the US east coast. Harmful Algae 27 (0), 29-41.

Gobler, C.J., Koch, F., Kang, Y., Berry, D.L., Tang, Y.Z., Lasi, M., Walters, L., Hall, L., Miller, J.D., 2013b. Expansion of harmful brown tides caused by the pelagophyte, *Aureoumbra lagunensis* DeYoe et Stockwell, to the US East Coast. Harmful Algae 27, 29-41.

Gobler, C.J., Lobanov, A.V., Tang, Y.Z., Turanov, A.A., Zhang, Y., Doblin, M., Taylor, G.T., Sanudo-Wilhelmy, S.A., Grigoriev, I.V., Gladyshev, V.N., 2013c. The central role of selenium in the biochemistry and ecology of the harmful pelagophyte, Aureococcus anophagefferens. Isme Journal 7 (7), 1333-1343.

Gobler, C.J., Lonsdale, D.J., Boyer, G.L., 2005. A review of the causes, effects, and potential management of harmful brown tide blooms caused by *Aureococcus anophagefferens* (Hargraves et Sieburth). Estuaries 28 (5), 726-749.

Gobler, C.J., Renaghan, M.J., Buck, N.J., 2002b. Impacts of nutrients and grazing mortality on the abundance of *Aureococcus anophagefferens* during a New York brown tide bloom. Limnol. Oceanogr. 47 (1), 129-141.

Gobler, C.J., Sanudo-Wilhelmy, S.A., 2001a. Effects of organic carbon, organic nitrogen, inorganic nutrients, and iron additions on the growth of phytoplankton and bacteria during a brown tide bloom. Mar. Ecol. Prog. Ser. 209, 19-34.

Gobler, C.J., Sanudo-Wilhelmy, S.A., 2001b. Temporal variability of groundwater seepage and brown tide blooms in a Long Island embayment. Marine Ecology-Progress Series 217, 299-309.

Gobler, C.J., Sunda, W.G., 2012. Ecosystem disruptive algal blooms of the brown tide species, *Aureococcus anophagefferens* and *Aureoumbra lagunensis*. Harmful Algae 14, 36-45.

Goleski, J.A., Koch, F., Marcoval, M.A., Wall, C.C., Jochem, F.J., Peterson, B.J., Gobler, C.J., 2010. The role of zooplankton grazing and nutrient loading in the occurrence of harmful cyanobacterial blooms in Florida Bay, USA. Estuaries and Coasts 33 (5), 1202-1215.

Gomez-Pereira, P.R., Hartmann, M., Grob, C., Tarran, G.A., Martin, A.P., Fuchs, B.M., Scanlan, D.J., Zubkov, M.V., 2013. Comparable light stimulation of organic nutrient uptake by SAR11 and *Prochlorococcus* in the North Atlantic subtropical gyre. ISME J 7 (3), 603-614.

Gough, J., Karplus, K., Hughey, R., Chothia, C., 2001. Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. J. Mol. Biol. 313 (4), 903-919.

Granéli, E., Hansen, P.J., 2006. Allelopathy in harmful algae: a mechanism to compete for resources?, Ecology of harmful algae. Springer, pp. 189-201.

Granéli, E., Turner, J.T., 2006. Ecology of harmful algae. Springer.

Gross, E.M., 2003. Allelopathy of aquatic autotrophs. Crit. Rev. Plant Sci. 22 (3-4), 313-339.

Haft, D.H., Loftus, B.J., Richardson, D.L., Yang, F., Eisen, J.A., Paulsen, I.T., White, O., 2001. TIGRFAMs: a protein family resource for the functional identification of proteins. Nucleic Acids Res. 29 (1), 41-43.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32 (2), 79-99.

Hallegraeff, G.M., 1998. Transport of toxic dinoflagellates via ships' ballast water: bioeconomic risk assessment and efficacy of possible ballast water management strategies. Mar. Ecol. Prog. Ser. 168 (297-309), 10-53.

Hallegraeff, G.M., 2010. Ocean climate change, phytoplankton community responses and harmful algal blooms: a formidable predictive challenge. J. Phycol. 46 (2), 220-235.

Hallegraeff, G.M., Bolch, C.J., 1991. Transport of toxic dinoflagellate cysts via ships' ballast water. Mar. Pollut. Bull. 22 (1), 27-30.

Hargraves, P., 1983. Diatom resting spores: significance and strategies, In: Fryxell, G.A. (Ed.), Survival strategies of the algae. Cmbridge University Press, Cambridge, p. 144.

Harke, M.J., Gobler, C.J., 2013. Global transcriptional responses of the toxic cyanobacterium, *Microcystis aeruginosa*, to nitrogen stress, phosphorus stress, and growth on organic matter. Plos One 8 (7), e69834-e69834.

Harke, M.J., Gobler, C.J., Shumway, S.E., 2011. Suspension feeding by the Atlantic slipper limpet (*Crepidula fornicata*) and the northern quahog (*Mercenaria mercenaria*) in the presence of cultured and wild populations of the harmful brown tide alga, *Aureococcus anophagefferens*. Harmful Algae 10 (5), 503-511.

Hartmann, M., Grob, C., Tarran, G.A., Martin, A.P., Burkill, P.H., Scanlan, D.J., Zubkov, M.V., 2012. Mixotrophic basis of Atlantic oligotrophic ecosystems. Proc. Natl. Acad. Sci. 109 (15), 5756-5760.

Hartmann, M., Zubkov, M.V., Scanlan, D.J., Lepère, C., 2013. In situ interactions between photosynthetic picoeukaryotes and bacterioplankton in the Atlantic Ocean: evidence for mixotrophy. Environmental microbiology reports 5 (6), 835-840.

Hase, T., Schürmann, P., Knaff, D.B., 2006. The interaction of ferredoxin with ferredoxin-dependent enzymes, Photosystem I. Springer, pp. 477-498.

Hattenrath-Lehmann, T.K., Gobler, C.J., 2011a. Allelopathic inhibition of competing phytoplankton by North American strains of the toxic dinoflagellate, *Alexandrium fundyense*: Evidence from field experiments, laboratory experiments, and bloom events. Harmful Algae 11, 106-116.

Hattenrath-Lehmann, T.K., Gobler, C.J., 2011b. Allelopathic inhibition of competing phytoplankton by North American strains of the toxic dinoflagellate, *Alexandrium fundyense*: Evidence from field experiments, laboratory experiments, and bloom events. Harmful Algae 11 (0), 106-116.

Hattenrath-Lehmann, T.K., Marcoval, M.A., Berry, D.L., Fire, S., Wang, Z., Morton, S.L., Gobler, C.J., 2013. The emergence of Dinophysis acuminata blooms and DSP toxins in shellfish in New York waters. Harmful Algae 26 (0), 33-44.

Hattenrath-Lehmann, T., Gobler, C.J., 2015. The contribution of inorganic and organic nutrients to the growth of a North American isolate of the mixotrophic dinoflagellate, Dinophysis acuminata. Limnol. Oceanogr.

Hein, M., Pedersen, M.F., Sand-Jensen, K., 1995. Size-dependent nitrogen uptake in micro-and macroalgae. Mar. Ecol. Prog. Ser. 118 (1), 247-253.

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, D.A., Stoecker, D.K., Suddleson, M., 2008. Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8 (1), 3-13.

Hernández-Domíguez, E.E., Valencia-Turcotte, L.G., Rodríguez-Sotres, R., 2012. Changes in expression of soluble inorganic pyrophosphatases of Phaseolus vulgaris under phosphate starvation. Plant Sci. 187, 39-48.

Hinkle, S.R., Böhlke, J.K., Fisher, L.H., 2008. Mass balance and isotope effects during nitrogen transport through septic tank systems with packed-bed (sand) filters. Sci. Total Environ. 407 (1), 324-332.

Hoegh-Guldberg, O., Bruno, J.F., 2010. The impact of climate change on the world's marine ecosystems. Science 328 (5985), 1523-1528.

Hoppe, H., 1983. Significance of exoenzymatic activities in the ecology of brackish water-measurements by means of methylumbelliferyl-substrates. Mar. Ecol. Prog. Ser. 11 (3), 299-308.

Huang, W.E., Li, M., Jarvis, R.M., Goodacre, R., Banwart, S.A., 2010. Chapter 5 - Shining light on the microbial world: the application on Raman Microspectroscopy, In: Allen, I.L., Sima, S., Geoffrey, M.G. (Eds.), Adv. Appl. Microbiol. Academic Press, Burlington, pp. 153-186.

Huang, X., Madan, A., 1999. CAP3: A DNA sequence assembly program. Genome Res. 9 (9), 868-877.

Hutchinson, G.E., 1961. The paradox of the plankton. The American Naturalist 95 (882), 137-145.

Igarashi, T., Satake, M., Yasumoto, T., 1996. Prymnesin-2: a potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. J. Am. Chem. Soc. 118 (2), 479-480.

Irigoien, X., Flynn, K.J., Harris, R.P., 2005. Phytoplankton blooms: a 'loophole' in microzooplankton grazing impact? J. Plankton Res. 27 (4), 313-321.

Iseli, C., Jongeneel, C.V., Bucher, P., 1999. ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences, ISMB, pp. 138-148.

Itakura, S., Nagasaki, K., Yamaguchi, M., Imai, I., 1996. Cyst formation in the red tide flagellate *Heterosigma akashiwo* (Raphidophyceae). J. Plankton Res. 18 (10), 1975-1979.

Jacquet, S., Lennon, J.-F., Marie, D., Vaulot, D., 1998. Picoplankton population dynamics in coastal waters of the northwestern Mediterranean Sea. Limnol. Oceanogr. 43 (8), 1916-1931.

Jochem, F.J., 1999. Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. Mar. Biol. 135 (4), 721-728.

Jochem, F.J., 2001. Morphology and DNA content of bacterioplankton in the northern Gulf of Mexico: analysis by epifluorescence microscopy and flow cytometry. Aquat. Microb. Ecol. 25 (2), 179-194.

John, D.E., Wang, Z.A., Liu, X., Byrne, R.H., Corredor, J.E., López, J.M., Cabrera, A., Bronk, D.A., Tabita, F.R., Paul, J.H., 2007. Phytoplankton carbon fixation gene (RuBisCO) transcripts and air-sea $CO_2$ flux in the Mississippi River plume. The ISME journal 1 (6), 517-531.

Johnson, K.S., Riser, S.C., Karl, D.M., 2010. Nitrate supply from deep to near-surface waters of the North Pacific subtropical gyre. Nature 465 (7301), 1062-1065.

Jones, M.N., 1984. Nitrate reduction by shaking with cadmium - alternative to cadmium columns. Water Res. 18 (5), 643-646.

Jonsson, P.R., Pavia, H., Toth, G., 2009. Formation of harmful algal blooms cannot be explained by allelopathic interactions. Proc. Natl. Acad. Sci. 106 (27), 11177-11182.

Kana, T.M., Glibert, P.M., 1987. Effects of irradiances up to 2000 μE $m^{-2}$ $s^{-1}$ on marine *Synechococcus* WH7803.1. Growth, pigmentation and cell composition. Deep-Sea Res. 34 (4), 479-495.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K.F., Itoh, M., Kawashima, S., Katayama, T., Araki, M., Hirakawa, M., 2006. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. 34 (suppl 1), D354-D357.

Kang, Y., Koch, F., Gobler, C.J., 2015. The interactive roles of nutrient loading and zooplankton grazing in facilitating the expansion of harmful algal blooms caused by the pelagophyte, Aureoumbra lagunensis, to the Indian River Lagoon, FL, USA. Harmful Algae 49, 162-173.

Kaplan-Levy, R.N., Hadas, O., Summers, M.L., Rücker, J., Sukenik, A., 2010. Akinetes: dormant cells of cyanobacteria, In: Lubzens, E., Cerda, J., Clark, M. (Eds.), Dormancy and resistance in harsh environments. Springer, Berlin Heidelberg, pp. 5-27.

Keeling, P.J., Burki, F., Wilcox, H.M., Allam, B., Allen, E.E., Amaral-Zettler, L.A., Armbrust, E.V., Archibald, J.M., Bharti, A.K., Bell, C.J., 2014. The marine microbial eukaryote transcriptome sequencing project (MMETSP): illuminating the functional diversity of eukaryotic life in the oceans through transcriptome sequencing. PLoS Biol. 12 (6), e1001889.

Kendall, C., McDonnell, J.J., 2012. Isotope tracers in catchment hydrology. Elsevier.

Kim, H.-Y., Fomenko, D.E., Yoon, Y.-E., Gladyshev, V.N., 2006. Catalytic advantages provided by selenocysteine in methionine-S-sulfoxide reductases. Biochemistry 45 (46), 13697-13704.

Kim, J.-H., Park, B.S., Wang, P., Kim, J.H., Youn, S.H., Han, M.-S., 2015. Cyst morphology and germination in *Heterosigma akashiwo* (Raphidophyceae). Phycologia 54 (5), 435-439.

Kinney, E.L., Valiela, I., 2011. Nitrogen loading to Great South Bay: land use, sources, retention, and transport from land to bay. J. Coast. Res. 27 (4), 672-686.

Kirchman, D.L., Keil, R.G., Wheeler, P.A., 1989. The effect of amino acids on ammonium utilization and regeneration by heterotrophic bacteria in the subarctic Pacific. Deep-Sea Res. 36 (11), 1763-1776.

Koch, F., Kang, Y., Villareal, T.A., Anderson, D.M., Gobler, C.J., 2014. Novel immunofluorescence flow cytometry technique detects the expansion of harmful brown tides caused by *Aureoumbra lagunensis* to the Caribbean Sea. Appl. Environ. Microbiol. 80 (16), 4947-4957.

Koch, F., Sañudo-Wilhelmy, S.A., Fisher, N.S., Gobler, C.J., 2013. Effect of vitamins B and B 12 on bloom dynamics of the harmful brown tide alga, *Aureococcus anophagefferens* (Pelagophyceae). Limnol. Oceanogr 58 (5), 1761-1774.

Kokkinakis, S.A., Wheeler, P.A., 1987. Nitrogen uptake and phytoplankton growth in coastal upwelling regions. Limnol. Oceanogr. 32 (5), 1112-1123.

Kolber, Z., Zehr, J., Falkowski, P., 1988. Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in photosystem II. Plant Physiol. 88 (3), 923-929.

Konotchick, T., Dupont, C.L., Valas, R.E., Badger, J.H., Allen, A.E., 2013. Transcriptomic analysis of metabolic function in the giant kelp, *Macrocystis pyrifera*, across depth and season. New Phytol. 198 (2), 398-407.

Kubanek, J., Naar, J., Myers, T.L., Prince, E.K., 2008. Competing phytoplankton undermines allelopathy of a bloom-forming dinoflagellate. Proceedings of the Royal Society B: Biological Sciences 275 (1652), 2733-2741.

Kudela, R.M., Cochlan, W.P., 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. Aquat. Microb. Ecol. 21 (1), 31-47.

Kudela, R.M., Gobler, C.J., 2012. Harmful dinoflagellate blooms caused by *Cochlodinium* sp.: Global expansion and ecological strategies facilitating bloom formation. Harmful Algae 14 (0), 71-86.

Kudela, R.M., Lane, J.Q., Cochlan, W.P., 2008. The potential role of anthropogenically derived nitrogen in the growth of harmful algae in California, USA. Harmful Algae 8 (1), 103-110.

Kulandaivelu, G., Senger, H., 1976. Changes in the reactivity of the photosynthetic apparatus in heterotrophic ageing cultures of *Scenedesmus obliquus*. Physiol. Plant. 36 (2), 157-164.

Lakeman, M.B., von Dassow, P., Cattolico, R.A., 2009. The strain concept in phytoplankton ecology. Harmful Algae 8 (5), 746-758.

Landry, M.R., Barber, R.T., Bidigare, R.R., Chai, F., Coale, K.H., Dam, H.G., Lewis, M.R., Lindley, S.T., McCarthy, J.J., Roman, M.R., Stoecker, D.K., Verity, P.G., White, J.R., 1997. Iron and grazing constraints on primary production in the central equatorial Pacific: An EqPac synthesis. Limnol. Oceanogr. 42 (3), 405-418.

Landry, M.R., Kirshtein, J., Constantinou, J., 1995. A refined dilution technique for measuring the community grazing impact of microzooplankton, with experimental tests in the central equatorial Pacific. Mar. Ecol. Prog. Ser. 120 (1-3), 53-63.

Lapointe, B.E., Barile, P.J., Matzie, W.R., 2004. Anthropogenic nutrient enrichment of seagrass and coral reef communities in the Lower Florida Keys: discrimination of local versus regional nitrogen sources. J. Exp. Mar. Biol. Ecol. 308 (1), 23-58.

Lapointe, B.E., Herren, L.W., Debortoli, D.D., Vogel, M.A., 2015. Evidence of sewage-driven eutrophication and harmful algal blooms in Florida's Indian River Lagoon. Harmful Algae 43 (0), 82-102.

Lapointe, B.E., Krupa, S., 1995. Jupiter Creek septic tank water quality investigation. Final Report to the Loxahatchee River Environmental Control District. Jupiter, FL.

Le Bouteiller, A., 1986. Environmental control of nitrate and ammonium uptake by phytoplankton in the Equatorial Atlantic Ocean. Mar. Ecol. Prog. Ser 30, 167-179.

Leflaive, J., Ten-Hage, L., 2007. Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. Freshwat. Biol. 52 (2), 199-214.

Legrand, C., Rengefors, K., Fistarol, G.O., Graneli, E., 2003. Allelopathy in phytoplankton-biochemical, ecological and evolutionary aspects. Phycologia 42 (4), 406-419.

Letelier, R.M., Karl, D.M., Abbott, M.R., Bidigare, R.R., 2004. Light driven seasonal patterns of chlorophyll and nitrate in the lower euphotic zone of the North Pacific Subtropical Gyre. Limnol. Oceanogr 49 (2), 508-519.

Li, B., Karl, D.M., Letelier, R.M., Bidigare, R.R., Church, M.J., 2013. Variability of chromophytic phytoplankton in the North Pacific Subtropical Gyre. Deep-Sea Res. Part II-Top. Stud. Oceanogr. 93, 84-95.

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25 (14), 1754-1760.

Li, L., Stoeckert, C.J., Roos, D.S., 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13 (9), 2178-2189.

Li, W., Godzik, A., 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22 (13), 1658-1659.

Li, W., Rao, S., Harrison, W., Smith, J., Cullen, J., Irwin, B., Platt, T., 1983. Autotrophic picoplankton in the tropical ocean. Science 219 (4582), 292-295.

Li, W.K.W., Beaumont, A.R., Hunter, J.R., 1994. Primary production of prochlorophytes, cyanobacteria, and eucaryotic ultraphytoplankton: measurements from flow cytometric sorting. Mar. Biol 114, 85-95.

Lim, A.S., Jeong, H.J., Jang, T.Y., Jang, S.H., Franks, P.J.S., 2014. Inhibition of growth rate and swimming speed of the harmful dinoflagellate *Cochlodinium polykrikoides* by diatoms: Implications for red tide formation. Harmful Algae 37, 53-61.

Lin, S., Cheng, S., Song, B., Zhong, X., Lin, X., Li, W., Li, L., Zhang, Y., Zhang, H., Ji, Z., 2015. The *Symbiodinium kawagutii* genome illuminates dinoflagellate gene expression and coral symbiosis. Science 350 (6261), 691-694.

Lipschultz, F., 1995. Nitrogen-specific uptake rates of marine phytoplankton isolated from natural populations of particles by flow cytometry. Marine ecology progress series. Oldendorf 123 (1), 245-258.

Liu, H.B., Buskey, E.J., 2000a. The exopolymer secretions (EPS) layer surrounding *Aureoumbra lagunensis* cells affects growth, grazing, and behavior of protozoa. Limnol. Oceanogr. 45 (5), 1187-1191.

Liu, H.B., Buskey, E.J., 2000b. Hypersalinity enhances the production of extracellular polymeric substance (EPS) in the Texas brown tide alga, *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 36 (1), 71-77.

Liu, H.B., Buskey, E.J., 2003. Effects of media N: P ratio on extracellular polymeric substances (EPS) production of the brown tide-forming alga, *Aureoumbra lagunensis*. Plankton Biol. Ecol. 50 (2), 55-60.

Liu, H.B., Laws, E.A., Villareal, T.A., Buskey, E.J., 2001. Nutrient-limited growth of *Aureoumbra lagunensis* (Pelagophyceae), with implications for its capability to outgrow other phytoplankton species in phosphate-limited environments. J. Phycol. 37 (4), 500-508.

Liu, Y., Zhou, J., White, K.P., 2014. RNA-seq differential expression studies: more sequence or more replication? Bioinformatics 30 (3), 301-304.

Lobanov, A.V., Hatfield, D.L., Gladyshev, V.N., 2009. Eukaryotic selenoproteins and selenoproteomes. Biochimica et Biophysica Acta (BBA)-General Subjects 1790 (11), 1424-1428.

Loeblich, A.R.I., Loeblich, L.A., 1984. Dinoflagellate Cysts, In: Spector, D.L. (Ed.), Dinoflagellates. Academic Press, London, p. 560.

Lomas, M.W., Bonachela, J.A., Levin, S.A., Martiny, A.C., 2014. Impact of ocean phytoplankton diversity on phosphate uptake. Proc. Natl. Acad. Sci. 111 (49), 17540-17545.

Lomas, M.W., Bronk, D.A., van den Engh, G., 2011. Use of flow cytometry to measure biogeochemical rates and processes in the ocean. Annual review of marine science 3, 537-566.

Lomas, M.W., Glibert, P.M., 1999. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. Limnol. Oceanogr. 44 (3), 556-572.

Lomas, M.W., Glibert, P.M., Berg, G.M., Burford, M., 1996. Characterization of nitrogen uptake by natural populations of Aureococcus anophagefferens (Chrysophyceae) as a function of incubation duration, substrate concentration, light, and temperature. J. Phycol. 32 (6), 907-916.

Lonsdale, D.J., Cosper, E.M., Kim, W.S., Doall, M., Divadeenam, A., Jonasdottir, S.H., 1996. Food web interactions in the plankton of Long Island bays, with preliminary observations on brown tide effects. Marine Ecology-Progress Series 134 (1-3), 247-263.

Lopez-Barreiro, T., 1998. Development of an antibody against the Texas brown tide (*Aureoumbra lagunensis*). Harmful Algae, 263-265.

Ma, H., Krock, B., Tillmann, U., Cembella, A., 2009. Preliminary characterization of extracellular allelochemicals of the toxic marine dinoflagellate Alexandrium tamarense using a Rhodomonas salina bioassay. Mar. Drugs 7 (4), 497-522.

Ma, H., Krock, B., Tillmann, U., Muck, A., Wielsch, N., Svatoš, A., Cembella, A., 2011. Isolation of activity and partial characterization of large non-proteinaceous lytic allelochemicals produced by the marine dinoflagellate *Alexandrium tamarense*. Harmful Algae 11, 65-72.

MacIntyre, H.L., Lomas, M.W., Cornwell, J., Suggett, D.J., Gobler, C.J., Koch, E.W., Kana, T.M., 2004. Mediation of benthic-pelagic coupling by microphytobenthos: an energy- and material-based model for initiation of blooms of *Aureococcus anophagefferens*. Harmful Algae 3 (4), 403-437.

MacIsaac, J.J., 1978. Diel cycles of inorganic nitrogen uptake in a natural phytoplankton population dominated by *Gonyaulax polyedra*.

MacIsaac, J.J., Dugdale, R.C., 1969. The kinetics of nitrate and ammonia uptake by natural populations of marine phytoplankton. Deep Sea Research and Oceanographic Abstracts 16 (1), 45-57.

Malzahn, A., Clemmesen, C., Rosenthal, H., 2003. Temperature effects on growth and nucleic acids in laboratory-reared larval coregonid fish. Mar. Ecol. Prog. Ser. 259, 285-293.

Marchetti, A., Schruth, D.M., Durkin, C.A., Parker, M.S., Kodner, R.B., Berthiaume, C.T., Morales, R., Allen, A.E., Armbrust, E.V., 2012. Comparative metatranscriptomics identifies molecular bases for the physiological responses of phytoplankton to varying iron availability. Proc. Natl. Acad. Sci. 109 (6), E317-E325.

Massana, R., 2011. Eukaryotic picoplankton in surface oceans. Annu. Rev. Microbiol. 65, 91-110.

Matsuoka, K., Fukuyo, Y., 2000a. Technical guide for modern dinoflagellate cyst study. WESTPAC-HAB, Japan Society for the Promotion of Science, Tokyo, Japan.

Matsuoka, K., Fukuyo, Y., 2000b. Technical guide for modern dinoflagellate cyst study. WESTPAC-HAB, Japan Society for the Promotion of Science, Tokyo, Japan.

McCarthy, J.J., Taylor, W.R., Taft, J.L., 1977. Nitrogenous nutrition of the plankton in the Chesapeake Bay. 1. Nutrient availability and phytoplankton preferences. Limnol. Oceanogr 22 (6), 996-1011.

McLean, T.I., 2013. "Eco-omics": A Review of the Application of Genomics, Transcriptomics, and Proteomics for the Study of the Ecology of Harmful Algae. Microb. Ecol., 1-15.

Michelou, V.K., Cottrell, M.T., Kirchman, D.L., 2007. Light-stimulated bacterial production and amino acid assimilation by cyanobacteria and other microbes in the North Atlantic Ocean. Appl. Environ. Microbiol. 73 (17), 5539-5546.

Milligan, A.J., Cosper, E.M., 1997. Growth and photosynthesis of the 'brown tide' microalga *Aureococcus anophagefferens* in subsaturating constant and fluctuating irradiance. Mar. Ecol. Prog. Ser. 153, 67-75.

Mitsukawa, N., Okumura, S., Shirano, Y., Sato, S., Kato, T., Harashima, S., Shibata, D., 1997. Overexpression of an Arabidopsis thaliana high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limited conditions. Proc. Natl. Acad. Sci. 94 (13), 7098-7102.

Mock, T., Daines, S.J., Geider, R., Collins, S., Metodiev, M., Millar, A.J., Moulton, V., Lenton, T.M., 2016. Bridging the gap between omics and earth system science to better understand how environmental change impacts marine microbes. Global Change Biol. 22 (1), 61-75.

Modigh, M., Franzè, G., 2009. Changes in phytoplankton and microzooplankton populations during grazing experiments at a Mediterranean coastal site. J. Plankton Res. 31 (8), 853-864.

Monier, A., Welsh, R.M., Gentemann, C., Weinstock, G., Sodergren, E., Armbrust, E., Eisen, J.A., Worden, A.Z., 2012. Phosphate transporters in marine phytoplankton and their viruses: cross-domain commonalities in viral-host gene exchanges. Environ. Microbiol. 14 (1), 162-176.

Montagna, P.A., Stockwell, D.A., Kalke, R.D., 1993. Dwarf surfclam *Mulinia lateralis* (Say, 1822) populations and feeding during the Texas brown tide event J. Shellfish Res. 12 (2), 433-442.

Moon-van der Staay, S.Y., De Wachter, R., Vaulot, D., 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature 409 (6820), 607-610.

Moore, S.K., Trainer, V.L., Mantua, N.J., Parker, M.S., Laws, E.A., Backer, L.C., Fleming, L.E., 2008. Impacts of climate variability and future climate change on harmful algal blooms and human health. Environ. Health 7 (Suppl 2), S4.

Moustafa, A., Evans, A.N., Kulis, D.M., Hackett, J.D., Erdner, D.L., Anderson, D.M., Bhattacharya, D., 2010. Transcriptome profiling of a toxic dinoflagellate reveals a gene-rich protist and a potential impact on gene expression due to bacterial presence. PLoS One 5 (3), e9688.

Movasaghi, Z., Rehman, S., Rehman, I.U., 2007. Raman spectroscopy of biological tissues. Applied Spectroscopy Reviews 42 (5), 493-541.

Muhlstein, H.I., Villareal, T.A., 2007. Organic and inorganic nutrient effects on growth rate-irradiance relationships in the Texas brown-tide alga *Aureoumbra lagunensis* (Pelagophyceae). J. Phycol. 43 (6), 1223-1226.

Mulholland, M.R., Boneillo, G., Minor, E.C., 2004. A comparison of N and C uptake during brown tide (*Aureococcus anophagefferens*) blooms from two coastal bays on the east coast of the USA. Harmful Algae 3 (4), 361-376.

Mulholland, M.R., Gobler, C.J., Lee, C., 2002. Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. Limnol. Oceanogr. 47 (4), 1094-1108.

Mulholland, M.R., Lee, C., Glibert, P.M., 2003. Extracellular enzyme activity and uptake of carbon and nitrogen along an estuarine salinity and nutrient gradient. Mar. Ecol. Prog. Ser. 258, 3-17.

Newell, R.I.E., Tettelbach, S.T., Gobler, C.J., Kimmel, D.G., 2009. Relationships between reproduction in suspension-feeding hard clams Mercenaria mercenaria and phytoplankton community structure. Mar. Ecol. Prog. Ser. 387, 179-196.

Nixon, S.W., Granger, S.L., Taylor, D.I., Johnson, P.W., Buckley, B.A., 1994. Subtidal volume fluxes, nutrient inputs and the brown tide—an alternate hypothesis. Estuar. Coast. Shelf Sci. 39 (3), 303-312.

NOAA, 2013. Marine mammal unusual mortality event (MMUME).

Oliver, S.L., Lenards, A.J., Barthelson, R.A., Merchant, N., McKay, S.J., 2013. Using the iPlant collaborative discovery environment. Current Protocols in Bioinformatics, 1.22. 21-21.22. 26.

Olli, K., 1996. Resting cyst formation of *Eutreptiella gymnastica* (Euglenophyceae) in the northern coatal Baltic Sea. J. Phycol. 32 (4), 535-542.

Olson, R.J., Chisholm, S.W., Zettler, E.R., Armbrust, E.V., 1988. Analysis of Synechococcus pigment types in the sea using sigle and dual beam flow-cytometry. Deep-Sea Res. 35 (3), 425-440.

Olson, R.J., Chisholm, S.W., Zettler, E.R., Armbrust, E.V., 1990. Pigments, size, and distributions of *Synechococcus* in the North Atlantic and Pacific Oceans. Limnol. Oceanogr. 35 (1), 45-58.

Olson, R.J., Chisholm, S.W., Zettler, E.R., Dusenberry, J.A., 1991. Advances in oceanography through flow cytometry., In: Demers, S. (Ed.), Individual Cell and Particle Analysis in Oceanography. Springer Verlag, pp. 352-402.

Olson, R.J., Zettler, E.R., Anderson, O.K., 1989. Discrimination of eukaryotic phytoplankton cell - types from light scatter and autofluorescence properties measured by flow cytometry. Cytometry 10 (5), 636-643.

Onuf, C.P., 1996. Seagrass responses to long-term light reduction by brown tide in upper Laguna Madre, Texas: Distribution and biomass patterns. Mar. Ecol. Prog. Ser. 138 (1-3), 219-231.

Orcutt, K.M., Lipschultz, F., Gundersen, K., Arimoto, R., Michaels, A.F., Knap, A.H., Gallon, J.R., 2001. A seasonal study of the significance of $N_2$ fixation by *Trichodesmium* spp. at the Bermuda Atlantic Time-series Study (BATS) site. Deep-Sea Res. Part II-Top. Stud. Oceanogr. 48 (8-9), 1583-1608.

Palenik, B., Brahamsha, B., Larimer, F.W., Land, M., Hauser, L., Chain, P., Lamerdin, J., Regala, W., Allen, E.E., McCarren, J., Paulsen, I., Dufresne, A., Partensky, F., Webb, E.A., Waterbury, J., 2003. The genome of a motile marine Synechococcus. Nature 424 (6952), 1037-1042.

Parkhill, J.P., Maillet, G., Cullen, J.J., 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. 37 (4), 517-529.

Parsons, T.R., Maita, Y., Lalli, C.M., 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Oxford.

Partensky, F., Hess, W., Vaulot, D., 1999. *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiol. Mol. Biol. Rev. 63 (1), 106-127.

Pasciak, W.J., Gavis, J., 1974. Transport limitation of nutrient uptake in phytoplankton. Limnol. Oceanogr. 19 (6), 881-888.

Pavia, H., Baumgartner, F., Cervin, G., Enge, S., Kubanek, J., Nylund, G.M., Selander, E., Svensson, J.R., Toth, G.B., 2012. Chemical defences against herbivores. Chemical ecology in aquatic systems. Oxford University Press, New York, New York, USA, 210-235.

Phlips, E.J., Badylak, S., Christman, M.C., Lasi, M.A., 2010. Climatic trends and temporal patterns of phytoplankton composition, abundance, and succession in the Indian River Lagoon, Florida, USA. Estuaries and Coasts 33 (2), 498-512.

Phlips, E.J., Badylak, S., Lasi, M.A., Chamberlain, R., Green, W.C., Hall, L.M., Hart, J.A., Lockwood, J.C., Miller, J.D., Morris, L.J., 2014. From red tides to green and brown tides: bloom dynamics in a restricted subtropical lagoon under shifting climatic conditions. Estuaries and Coasts 38 (3), 886-904.

Phlips, E.J., Badylak, S., Youn, S., Kelley, K., 2004. The occurrence of potentially toxic dinoflagellates and diatoms in a subtropical lagoon, the Indian River Lagoon, Florida, USA. Harmful Algae 3 (1), 39-49.

Platt, T., Rao, D.S., Irwin, B., 1983. Photosynthesis of picoplankton in the oligotrophic ocean.

Pomeroy, L.R., Williams, P.J.I., Azam, F., Hobbie, J.E., 2007. The Microbial Loop. Oceanography 20 (2), 28-33.

Popels, L.C., Hutchins, D.A., 2002. Factors affecting dark survival of the brown tide alga *Aureococcus anophagefferens* (Pelagophyceae). J. Phycol. 38 (4), 738-744.

Popels, L.C., MacIntyre, H.L., Warner, M.E., Zhang, Y.H., Hutchins, D.A., 2007. Physiological responses during dark survival and recovery in *Aureococcus anophagefferens* (Pelagophyceae). J. Phycol. 43 (1), 32-42.

Poulson-Ellestad, K.L., Jones, C.M., Roy, J., Viant, M.R., Fernández, F.M., Kubanek, J., Nunn, B.L., 2014. Metabolomics and proteomics reveal impacts of chemically mediated competition on marine plankton. Proc. Natl. Acad. Sci. 111 (24), 9009-9014.

Poulson, K.L., Sieg, R., Prince, E.K., Kubanek, J., 2010. Allelopathic compounds of a red tide dinoflagellate have species-specific and context-dependent impacts on phytoplankton. Marine Ecology, Progress Series 416, 69-78.

Price, N.M., Harrison, P.J., 1987. Comparison of methods for the analysis of dissolved urea in seawater. Mar. Biol. 94 (2), 307-317.

Pride, A.C., Herrera, C.M., Guan, Z., Giles, D.K., Trent, M.S., 2013. The outer surface lipoprotein VolA mediates utilization of exogenous lipids by Vibrio cholerae. mBio 4 (3), e00305-00313.

Prince, E.K., Myers, T.L., Kubanek, J., 2008. Effects of harmful algal blooms on competitors: allelopathic mechanisms of the red tide dinoflagellate Karenia brevis. Limnol. Oceanogr. 53 (2), 531-541.

Prince, E.K., Poulson, K.L., Myers, T.L., Sieg, R.D., Kubanek, J., 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. Harmful Algae 10 (1), 39-48.

Probyn, T., Painting, S., 1985. Nitrogen uptake by size-fractionated phytoplankton populations in Antarctic surface waters. Limnol. Oceanogr. 30 (6), 1327-1332.

Probyn, T., Pitcher, G., Pienaar, R., Nuzzi, R., 2001. Brown tides and mariculture in Saldanha Bay, South Africa. Mar. Pollut. Bull. 42 (5), 405-408.

Probyn, T.A., Bernard, S., Pitcher, G.C., Pienaar, R.N., 2010. Ecophysiological studies on Aureococcus anophagefferens blooms in Saldanha Bay, South Africa. Harmful Algae 9 (2), 123-133.

Pustizzi, F., MacIntyre, H., Warner, M.E., Hutchins, D.A., 2004. Interaction of nitrogen source and light intensity on the growth and photosynthesis of the brown tide alga *Aureococcus anophagefferens*. Harmful Algae 3 (4), 343-360.

Rederstorff, E., Fatimi, A., Sinquin, C., Ratiskol, J., Merceron, C., Vinatier, C., Weiss, P., Colliec-Jouault, S., 2011. Sterilization of exopolysaccharides produced by deep-sea bacteria: Impact on their stability and degradation. Mar. Drugs 9 (2), 224-241.

Rengefors, K., Karlsson, I., Hansson, L.A., 1998. Algal cyst dormancy: a temporal escape from herbivory. P. Roy. Soc. B-Biol. Sci. 265 (1403), 1353-1358.

Rengefors, K., Legrand, C., 2001. Toxicity in *Peridinium aciculiferum*—an adaptive strategy to outcompete other winter phytoplankton? Limnol. Oceanogr. 46 (8), 1990-1997.

Rice, E.L., 2013. Allelopathy. Academic Press.

Richardson, T.L., Jackson, G.A., 2007. Small phytoplankton and carbon export from the surface ocean. Science 315 (5813), 838-840.

Rivkin, R.B., Phinney, D.A., Yentsch, C.M., 1986. Effects of flow cytometric analysis and cell sorting on photosynthetic carbon uptake by phytoplankton in cultures and from natural populations. Appl. Environ. Microbiol. 52 (4), 935-938.

Rocap, G., Distel, D.L., Waterbury, J.B., Chisholm, S.W., 2002. Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. Appl. Environ. Microbiol. 68 (3), 1180-1191.

Sakamoto, C., Karl, D., Jannasch, H., Bidigare, R., Letelier, R., Johnson, K., 2002. Nitrate variability measured in situ on the HALE ALOHA open ocean mooring: Influence of mesoscale eddy activity on phytoplankton community dynamics, AGU Fall Meeting Abstracts, p. 0198.

Sancho, J., 2006. Flavodoxins: sequence, folding, binding, function and beyond. Cell. Mol. Life Sci. 63 (7-8), 855-864.

Sandgren, C.D., 1981. Characteristics of sexual and asexual resting cyst (statospore) formation in *Dinobryon cylindricum* Imhof (Chrysophyta). J. Phycol. 17 (2), 199-210.

Sandgren, C.D., 1983. Survival strategies of chrysophycean flagellates: Reproduction and the formation of resistant resting cysts, In: Fryxell, G.A. (Ed.), Survival Strategies of the Algae. Cambridge University Press, Cambridge, pp. 23-49.

Schmidt, L.E., Hansen, P.J., 2001. Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: effect of cell concentration, growth phase and pH. Mar. Ecol. Prog. Ser. 216, 67-81.

Selander, E., Kubanek, J., Hamberg, M., Andersson, M.X., Cervin, G., Pavia, H., 2015. Predator lipids induce paralytic shellfish toxins in bloom-forming algae. Proc. Natl. Acad. Sci. 112 (20), 6395-6400.

Shi, X.L., Lepère, C., Scanlan, D.J., Vaulot, D., 2011. Plastid 16S rRNA gene diversity among eukaryotic picophytoplankton sorted by flow cytometry from the South Pacific Ocean. PLoS One 6 (4), e18979.

Sicko-Goad, L., Stoermer, E., Kociolek, J., 1989. Diatom resting cell rejuvenation and formation: time course, species records and distribution. J. Plankton Res. 11 (2), 375-389.

Sieracki, M.E., Gobler, C.J., Cucci, T.L., Thier, E.C., Gilg, I.C., Keller, M.D., 2004. Pico- and nanoplankton dynamics during bloom initiation of *Aureococcus* in a Long Island, NY bay. Harmful Algae 3 (4), 459-470.

Silver, S., Phung, L.T., 2005. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Appl. Environ. Microbiol. 71 (2), 599-608.

Simmons, M.P., Sudek, S., Monier, A., Limardo, A.J., Jimenez, V., Perle, C.R., Elrod, V.A., Pennington, J.T., Worden, A.Z., 2016. Abundance and biogeography of picoprasinophyte ecotypes and other phytoplankton in the Eastern North Pacific Ocean. Appl. Environ. Microbiol. 82 (6), 1693-1705.

Simon, N., Barlow, R.G., Marie, D., Partensky, F., Vaulot, D., 1994. Characterization of oceanic photosynthetic picoeukaryotes by flow cytometry. J. Phycol. 30 (6), 922-935.

Simpson, J.T., Durbin, R., 2012. Efficient de novo assembly of large genomes using compressed data structures. Genome Res. 22 (3), 549-556.

Simpson, J.T., Wong, K., Jackman, S.D., Schein, J.E., Jones, S.J., Birol, I., 2009. ABySS: a parallel assembler for short read sequence data. Genome Res. 19 (6), 1117-1123.

Smayda, T.J., 1997. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnol. Oceanogr. 42 (5), 1137-1153.

Smayda, T.J., 2007. Reflections on the ballast water dispersal—harmful algal bloom paradigm. Harmful Algae 6 (4), 601-622.

Smayda, T.J., 2008. Complexity in the eutrophication-harmful algal bloom relationship, with comment on the importance of grazing. Harmful Algae 8 (1), 140-151.

Smith, A.E., Morris, I., 1980. Synthesis of lipid during photosynthesis by phytoplankton of the Southern Ocean. Science 207 (4427), 197-199.

Smith, J.K., Lonsdale, D.J., Gobler, C.J., Caron, D.A., 2008. Feeding behavior and development of Acartia tonsa nauplii on the brown tide alga Aureococcus anophagefferens. J. Plankton Res. 30 (8), 937-950.

Smith, N.P., 1993. Tidal and nontidal flushing of Florida Indian River Lagoon. Estuaries 16 (4), 739-746.

Soneson, C., Delorenzi, M., 2013. A comparison of methods for differential expression analysis of RNA-seq data. BMC Bioinformatics 14 (1), 91.

Sosik, H.M., Olson, R.J., Armbrust, E.V., 2010. Flow cytometry in phytoplankton research, Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications. Springer, pp. 171-185.

Stadtman, T.C., 1996. Selenocysteine. Annu. Rev. Biochem. 65 (1), 83-100.

Stauffer, B.A., Schaffner, R.A., Wazniak, C., Caron, D.A., 2008. Immunofluorescence flow cytometry technique for enumeration of the brown-tide alga, *Aureococcus anophagefferens*. Appl. Environ. Microbiol. 74 (22), 6931-6940.

Stein, L.D., 2008. Towards a cyberinfrastructure for the biological sciences: progress, visions and challenges. Nature Reviews Genetics 9 (9), 678-688.

Stinnette, I., 2014. Nitrogen Loading to the South Shore, Eastern Bays, NY: Sources, Impacts and Management Options (Master's thesis). Stony Brook University: School of Marine and Atmospheric Sciences. Stony Brook, NY.

Stoecker, D.K., Capuzzo, J.M., 1990. Predation on protozoa: its importance to zooplankton. J. Plankton Res. 12 (5), 891-908.

Strom, S.L., 2008. Microbial ecology of ocean biogeochemistry: a community perspective. Science 320 (5879), 1043-1045.

Suikkanen, S., Fistarol, G.O., Granéli, E., 2005. Effects of cyanobacterial allelochemicals on a natural plankton community. Mar. Ecol. Prog. Ser. 287, 1-9.

Sun, M.M., Sun, J., Qiu, J.W., Jing, H.M., Liu, H.B., 2012. Characterization of the proteomic profiles of the brown tide alga *Aureoumbra lagunensis* under phosphate- and nitrogen-limiting conditions and of its phosphate limitation-specific protein with alkaline phosphatase activity. Appl. Environ. Microbiol. 78 (6), 2025-2033.

Sunda, W., Hardison, D., 2010. Evolutionary tradeoffs among nutrient acquisition, cell size, and grazing defense in marine phytoplankton promote ecosystem stability. Mar. Ecol. Prog. Ser. 401, 63-76.

Sunda, W.G., Graneli, E., Gobler, C.J., 2006. Positive feedback and the development and persistence of ecosystem disruptive algal blooms. J. Phycol. 42 (5), 963-974.

Sunda, W.G., Hardison, D.R., 2007. Ammonium uptake and growth limitation in marine phytoplankton. Limnol. Oceanogr. 52 (6), 2496-2506.

Sunda, W.G., Shertzer, K.W., 2012. Modeling ecosystem disruptive algal blooms: positive feedback mechanisms. Mar. Ecol. Prog. Ser. 447, 31-47.

Sundby, B., Gobeil, C., Silverberg, N., Alfonso, M., 1992. The phosphorus cycle in coastal marine sediments. Limnol. Oceanogr. 37 (6), 1129-1145.

Talarmin, A., Van Wambeke, F., Duhamel, S., Catala, P., Moutin, T., Lebaron, P., 2011. Improved methodology to measure taxon-specific phosphate uptake in live and unfiltered samples. Limnol. Oceanogr. Methods 9, 443-453.

Tang, Y., Gobler, C., 2009. Characterization of the toxicity of Cochlodinium polykrikoides isolates from Northeast US estuaries to finfish and shellfish. Harmful Algae 8 (3), 454-462.

Tang, Y.Z., Gobler, C.J., 2010. Allelopathic effects of Cochlodinium polykrikoides isolates and blooms from the estuaries of Long Island, New York, on co-occurring phytoplankton. Mar. Ecol. Prog. Ser. 406, 19-31.

Tang, Y.Z., Gobler, C.J., 2011. The green macroalga, Ulva lactuca, inhibits the growth of seven common harmful algal bloom species via allelopathy. Harmful Algae 10 (5), 480-488.

Tang, Y.Z., Gobler, C.J., 2012. The toxic dinoflagellate *Cochlodinium polykrikoides* (Dinophyceae) produces resting cysts. Harmful Algae 20, 71-80.

Tang, Y.Z., Kang, Y., Berry, D., Gobler, C.J., 2014. The ability of the red macroalga, Porphyra purpurea (Rhodophyceae) to inhibit the proliferation of seven common harmful microalgae. J. Appl. Phycol. 27, 531-544.

Taylor, G.T., Gobler, C.J., Sanudo-Wilhelmy, S.A., 2006. Speciation and concentrations of dissolved nitrogen as determinants of brown tide Aureococcus anophagefferens bloom initiation. Marine Ecology-Progress Series 312, 67-83.

Thomas, M.K., Kremer, C.T., Klausmeier, C.A., Litchman, E., 2012. A global pattern of thermal adaptation in marine phytoplankton. Science 338 (6110), 1085-1088.

Tillmann, U., Alpermann, T.L., da Purificacao, R.C., Krock, B., Cembella, A., 2009. Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. Harmful Algae 8 (5), 759-769.

Tillmann, U., John, U., 2002. Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: an allelochemical defence mechanism independent of PSP-toxin content. Mar. Ecol. Prog. Ser. 230, 47-58.

Tillmann, U., John, U., Cembella, A., 2007. On the allelochemical potency of the marine dinoflagellate *Alexandrium ostenfeldii* against heterotrophic and autotrophic protists. J. Plankton Res. 29 (6), 527-543.

Tilman, D., 1977. Resource competition between plankton algae: an experimental and theoretical approach. Ecology 58 (2), 338-348.

Timmermans, K.R., van der Wagt, B., Veldhuis, M.J.W., Maatman, A., de Baar, H.J.W., 2005. Physiological responses of three species of marine pico-phytoplankton to ammonium, phosphate, iron and light limitation. J. Sea Res. 53 (1–2), 109-120.

Valderrama, J.C., 1981. The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar. Chem. 10 (2), 109-122.

Van de Waal, D.B., Tillmann, U., Martens, H., Krock, B., van Scheppingen, Y., John, U., 2015. Characterization of multiple isolates from an *Alexandrium ostenfeldii* bloom in The Netherlands. Harmful Algae 49, 94-104.

Varela, D.E., Harrison, P.J., 1999. Seasonal variability in nitrogenous nutrition of phytoplankton assemblages in the northeastern subarctic Pacific Ocean. Deep-Sea Res. Part II-Top. Stud. Oceanogr. 46 (11-12), 2505-2538.

Villareal, T., Mansfield, A., Buskey, E., 1998. Growth and chemical composition of the Texas brown tide forming pelagophyte *Aureoumbra lagunensis*. Harmful algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Vigo, Spain, 359-362.

Villareal, T.A., Chirichella, T., Buskey, E.J., 2002. Regional distribution of the Texas Brown Tide (*Aureoumbra lagunensis*) in the Gulf of Mexico. Harmful Algae 2004, 374-376.

Volkman, J., 2003. Sterols in microorganisms. Appl. Microbiol. Biotechnol. 60 (5), 495-506.

von Dassow, P., Montresor, M., 2010. Unveiling the mysteries of phytoplankton life cycles: patterns and opportunities behind complexity. J. Plankton Res.

Wall, D., Dale, B., 1969. The 'hystrichosphaerid' resting spore of the dinoflagellate *Pyrodinium bahamense*, plate, 1906. J. Phycol. 5 (2), 140-149.

Walther, G.-R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T.J., Fromentin, J.-M., Hoegh-Guldberg, O., Bairlein, F., 2002. Ecological responses to recent climate change. Nature 416 (6879), 389-395.

Ward, L.A., Montagna, P.A., Kalke, R.D., Buskey, E.J., 2000. Sublethal effects of Texas brown tide on *Streblospio benedicti* (Polychaeta) larvae. J. Exp. Mar. Biol. Ecol. 248 (1), 121-129.

Waterbury, J.B., Watson, S.W., Valois, F.W., Franks, D.G., 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. Can Bull Fish Aquat Sci 214 (71), 120.

Watson, J.V., 2004. Introduction to flow cytometry. Cambridge University Press.

Wilken, S., Huisman, J., Naus-Wiezer, S., Van Donk, E., 2013. Mixotrophic organisms become more heterotrophic with rising temperature. Ecol. Lett. 16 (2), 225-233.

Wood, R.E., 2000. Caribbean cruise tourism: globalization at sea. Ann. Tourism Res. 27 (2), 345-370.

Worden, A.Z., Janouskovec, J., McRose, D., Engman, A., Welsh, R.M., Malfatti, S., Tringe, S.G., Keeling, P.J., 2012. Global distribution of a wild alga revealed by targeted metagenomics. Curr. Biol. 22 (17), R675-R677.

Wu, J., Sunda, W., Boyle, E.A., Karl, D.M., 2000. Phosphate depletion in the western North Atlantic Ocean. Science 289 (5480), 759-762.

Wu, Z.J., Jenkins, B.D., Rynearson, T.A., Dyhrman, S.T., Saito, M.A., Mercier, M., Whitney, L.P., 2010. Empirical bayes analysis of sequencing-based transcriptional profiling without replicates. BMC Bioinformatics 11.

Wurch, L.L., Bertrand, E.M., Saito, M.A., Van Mooy, B.A.S., Dyhrman, S.T., 2011a. Proteome Changes Driven by Phosphorus Deficiency and Recovery in the Brown Tide-Forming Alga Aureococcus anophagefferens. Plos One 6 (12).

Wurch, L.L., Gobler, C.J., Dyhrman, S.T., 2013. Expression of a xanthine and phosphate transporter in cultures and field populations of the harmful alga *Aureococcus anophagefferens*: tracking nutritional deficiency during brown tides. Environ. Microbiol., 2444-2457.

Wurch, L.L., Haley, S.T., Orchard, E.D., Gobler, C.J., Dyhrman, S.T., 2011b. Nutrient-regulated transcriptional responses in the brown tide-forming alga *Aureococcus anophagefferens*. Environ. Microbiol. 13 (2), 468-481.

Xu, N., Tang, Y.Z., Qin, J., Duan, S., Gobler, C.J., 2015. Ability of the marine diatoms Pseudo-nitzschia multiseries and P. pungens to inhibit the growth of co-occurring phytoplankton via allelopathy. Aquat. Microb. Ecol. 74, 29-41.

Yamasaki, Y., Nagasoe, S., Matsubara, T., Shikata, T., Shimasaki, Y., Oshima, Y., Honjo, T., 2007. Allelopathic interactions between the bacillariophyte *Skeletonema costatum* and the raphidophyte *Heterosigma akashiwo*. Mar. Ecol. Prog. Ser. 339, 83.

Yamasaki, Y., Shikata, T., Nukata, A., Ichiki, S., Nagasoe, S., Matsubara, T., Shimasaki, Y., Nakao, M., Yamaguchi, K., Oshima, Y., 2009. Extracellular polysaccharide-protein complexes of a harmful alga mediate the allelopathic control it exerts within the phytoplankton community. The ISME journal 3 (7), 808-817.

Zakharyan, R.A., Tsaprailis, G., Chowdhury, U.K., Hernandez, A., Aposhian, H.V., 2005. Interactions of sodium selenite, glutathione, arsenic species, and omega class human glutathione transferase. Chem. Res. Toxicol. 18 (8), 1287-1295.

Zhang, Q.C., Qiu, L.M., Yu, R.C., Kong, F.Z., Wang, Y.F., Yan, T., Gobler, C.J., Zhou, M.J., 2012. Emergence of brown tides caused by *Aureococcus anophagefferens* Hargraves et Sieburth in China. Harmful Algae 19, 117-124.

Zhang, Z., Wood, W.I., 2003. A profile hidden Markov model for signal peptides generated by HMMER. Bioinformatics 19 (2), 307-308.

Zheng, J.-W., Li, D.-W., Lu, Y., Chen, J., Liang, J.-J., Zhang, L., Yang, W.-D., Liu, J.-S., Lu, S.-H., Li, H.-Y., 2016. Molecular exploration of algal interaction between the diatom *Phaeodactylum tricornutum* and the dinoflagellate *Alexandrium tamarense*. Algal Research 17, 132-141.

Zubkov, M.V., Fuchs, B.M., Tarran, G.A., Burkill, P.H., Amann, R., 2003. High rate of uptake of organic nitrogen compounds by Prochlorococcus cyanobacteria as a key to their dominance in oligotrophic oceanic waters. Appl. Environ. Microbiol. 69 (2), 1299-1304.

Zubkov, M.V., Mary, I., Woodward, E.M.S., Warwick, P.E., Fuchs, B.M., Scanlan, D.J., Burkill, P.H., 2007. Microbial control of phosphate in the nutrient-depleted North Atlantic subtropical gyre. Environ. Microbiol. 9 (8), 2079-2089.