THE ASCIDIAN *STYELA PLICATA* AS A POTENTIAL BIOREMEDIATOR AGAINST THE BROWN TIDE PELAGOPHYTES *AUREOUMBRA LAGUNENSIS* AND *AUREOCOCCUS ANOPHAGEFFERENS*

by

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A Thesis Submitted to the Faculty of

The Charles E. Schmidt College of Science

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Florida Atlantic University

Boca Raton, FL

December 2015

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ACKNOWLEDGEMENTS

I would like to express my humblest gratitude to the following institutions and individuals without whom this project could not have been accomplished. Firstly, the Link Foundation and Harbor Branch Foundation whose funding was vital in my purchasing research materials and providing facilities in which to start and complete my research. Secondly, my graduate chair and committee Drs. James X Hartmann, John Scarpa, Brian Lapointe, and Paul Hargraves, who continued to challenge and push my abilities, and contributed to my becoming a better writer and scientist. To Courtney McArthur, Sarabeth George, and Naomi Huntley who provided much needed support in the lab. To Yoonja Kahn and Dr. Chris Gobler at Stony Brook University for providing algae cultures, answering many, many emails, and providing feedback on culturing technique. Dr. Ray Waldner, whose continued guidance, mentorship, and friendship I cherish. To Casey Schroer, who is my constant rock and source of laughter during times of stress. Finally, to each of my following co-workers, friends, and family, who provided constant support and encouraged me to pursue my ambitions: Eric Anderson, Julie Mitchell, Ginger Rigdon, Brandon Justice, Lory Gort, Eddie Barrow, Rachel Hartnett, Crystal Mas, JoBeth Simon, Katie Hynes, and my parents Lisa Elefante and Frederick Klarmann.

ABSTRACT

Author: Phyllis A. Klarmann

Title: The Ascidian *Styela plicata* As a Potential Bioremediator of the Brown Tide Pelagophytes *Aureoumbra lagunensis* and *Aureococcus anophagefferens*

Institution: Florida Atlantic University

Thesis Advisor: James X. Hartmann, Ph.D.

Degree: Master of Science

Year: 2015

A brown tide bloom of the pelagophyte *Aureoumbra lagunensis* caused significant impacts to north Indian River Lagoon (IRL) in 2012-2013, including seagrass die-offs, fish kills, and reduced growth and grazing of ecologically important bivalves. There is potential for another pelagophyte, *Aureococcus anophagefferens*, to expand into this system. Filtration rates (FR) of the pleated tunicate *Styela plicata* exposed to *Aureoumbra lagunensis* and *Aureococcus anophagefferens* were measured against exposure to a control alga (*Tisochrysis lutea*) in order to determine its potential use as a bioremediator against these harmful algal blooms (HABs). In addition, whether *S. plicata* might serve as a vector of HABs was studied by culturing fecal deposits. Short-term exposure to HABs significantly reduced FR, whereas long-term exposure indicates comparable cell removal compared to the control. Vector potential of *S. plicata* was inconclusive. Results warrant further research to determine whether *S. plicata* can acclimate or respond to HAB conditions over time.

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1 INTRODUCTION

1.1 The Indian River Lagoon and Drivers of Ecosystem Damage

The Indian River Lagoon (IRL) is a diverse coastal estuary located along the Treasure Coast of Florida, USA, and spans six counties (from north to south: Volusia, Brevard, Indian River, St. Lucie, Martin, and northern Palm Beach County). The IRL is uniquely positioned geographically and straddles temperate and sub-tropical ecotones, which contribute to its high biodiversity (Swain, 1995). The IRL is interconnected with the Mosquito Lagoon (ML) and Banana River Lagoon (BRL) to the north and the Saint Lucie Estuary (SLE) to the south. Together these systems are regarded as part of the Greater Indian River Lagoon system (Sime, 2005), and are collectively referred to as the "IRL" unless otherwise specified to discuss geographic location and spatial extent. In 1987, the Surface Water Improvement and Management (SWIM) Act designated the IRL as a "priority water body in need of restoration and special protection" (Steward et al., 2002). In 1991 it became part of the National Estuary Program (Indian River Lagoon National Estuary Program, 1996). Ecological attributes of the IRL include important communities such as submerged aquatic vegetation (SAV; e.g. seagrass), oyster, fish, and benthic communities (Sime, 2005). These communities are inextricably linked and the impacts on one could have cascading effects on another. For example, with loss and decline in the health and abundance of SAV beds, the location, abundance, and species composition of fisheries are in turn affected (Sime, 2005).

Anthropogenic disturbances can lead to ecosystem changes (Lapointe et al., 2012). Currently, urban land use, development, and water management systems are the primary external drivers that lead to ecological stress in the IRL (Sime, 2005). Sea level rise is an additional driver (Sime, 2005), and large data sets composed of time-series climatic data have been helpful in understanding estuarine ecosystem dynamics (Phlips et al., 2015). Sime (2005) indicated the major anthropogenic change made to the IRL and its watershed are related to freshwater discharge: high flows during the wet season, inadequate flows during the dry season, and the associated impacts of the distribution and quantity of water at local discharge locations to coastal estuaries such as the IRL and the Atlantic ocean.

In order to develop working ecological hypotheses for the IRL, Sime (2005) developed a conceptual ecological model for the IRL that highlighted six primary stressors of the system: altered hydrology; altered estuarine salinity; input and elevated levels of nutrients and dissolved organic matter; input of contaminants; boating and fishing pressure; and physical alteration to the system (i.e., development). One such consequence of these stressors is eutrophication, which is a process initiated by the input of nutrients, usually nitrogen and phosphorous, into an aquatic system that leads to spikes in algal growth. In the National Estuarine Eutrophication Assessment report (Bricker et al., 1999), the IRL is cited as moderately eutrophic, and conditions are expected to worsen within the lagoon by the year 2020. Reduction of anthropogenic nutrient loads at the source is a prescribed remedy for eutrophication (National Resource Council, 2001), which eliminates “nutrient pollution” and thus prevents anthropogenically-driven algal blooms, some of which are harmful.

Harmful algal blooms (HABs) have potential to damage ecosystems, ecosystem services, and occasionally pose a threat to public health depending on the species (Van Dolah, 2000; Landsberg et al., 2002; Phlips et al., 2004; Davis et al., 2009). Harmful algal blooms are each driven by different stressors and have a variety of impacts on the systems in which they occur. An increase in biomass of the red tide dinoflagellate *Pyrodinium bahamense* var. *bahamense* and the diatom *Pseudo-nitzschia calliantha*, which produce the toxins saxitoxin and domoic acid, respectively, were correlated with spikes of heavy rainfall and freshwater discharge following two years of drought in the IRL (Phlips et al., 2004; Badylak et al., 2006; Phlips et al., 2011). Saxitoxin causes paralytic shellfish poisoning, and have resulted in hospitalizations caused by the ingestion of contaminated pufferfish from local IRL waters (Landsberg et al., 2002). Further, it is hypothesized that *Pyrodinium bahamense* can reduce the top-down effects of filter-feeding "grazers" (Phlips et al., 2006). Freshwater inputs and nutrient pollution into the SLE are linked to blooms of *Microcystis aeruginosa*, a cyanobacterium that produces the hepatotoxin microcystin, which caused local waterways to be closed to swimming and fishing in 2013 (Phlips et al., 2011; Lapointe et al., 2012). Some species of *Prorocentrum* produce okadaic acid, a tumor promoter implicated in cases of fibropapillomatosis of the threatened green sea turtle *Chelonia mydas* (Landsberg et al., 1999; Capper et al., 2013) and diarrhetic shellfish poisoning in humans (Van Dolah, 2000).

The IRL "super bloom" that occurred in 2011 included a seven-month long green tide bloom (Pedinophyceae, probably a member of the genus *Resultor*; Lapointe et al., 2015) in the northern IRL (NIRL) and ML (Kamerosky et al., 2015; Phlips et al., 2015). This event was followed by drought and hypersaline conditions, which initiated brown

tide blooms of the pelagophyte *Aureoumbra lagunensis* in the NIRL (Gobler and Sunda, 2012; Gobler et al., 2013). As a result of light attenuation (Gobler and Sunda, 2012), 19,000 hectares of seagrass (approximately 60% of total seagrass cover within the lagoon) was lost between the Ponce de Leon and Ft. Pierce inlets (St. Johns River Water Management District, 2013). Additionally, anoxic conditions caused by the pelagophyte bloom resulted in fish kills (Gobler et al., 2013), and bivalves and other grazing organisms suffered massive die-offs throughout the system (Gobler and Sunda, 2012; Corcoran et al., 2013; Gobler et al., 2013).

1.2 Pelagophyte Blooms of the Indian River Lagoon

The "super bloom" that occurred in the NIRL and ML in 2011-2013 began with a green tide bloom of *Pedinophyceae* sp., followed by the brown tide bloom of the pelagophyte *Aureoumbra lagunensis* Stockwell, DeYoe, Hargraves et Johnson (DeYoe et al., 1997). Another brown tide pelagophyte species *Aureococcus anophagefferens* Hargraves et Sieburth (DeYoe et al., 1997) blooms in the northeastern United States and South Africa (Phlips et al., 2011; Gobler and Sunda, 2012), and persists in low concentrations in coastal estuaries along the east coast of the United States (Sieburth et al., 1988; Gobler et al., 2013). There is a potential threat for *Aureococcus anophagefferens* to expand to the IRL, as it has been detected in water samples as far south as north Florida (Popels et al., 2003). Its presence in mid-Atlantic water samples may suggest its potential to expand via oceanic sources (Popels et al., 2003). Both *Aureoumbra lagunensis* and *Aureococcus anophagefferens* blooms have expanded in range (Gobler et al., 2013); a trend in these and other HAB bloom expansions may be

linked to climate change, ballast water transport, eutrophication, and improved detection capabilities (Gobler et al., 2013).

There is evidence of toxic activity against zooplankton and bivalves by pelagophytes (Sieburth et al., 1988; Gainey an Shumway, 1991; Robbins et al., 2010), but no extracellular toxins have been isolated from either species. Rather, they may cause toxic effects due to their structure (e.g. mucilaginous secretions), accumulated biomass, or a combination of these factors (Anderson et al., 2002). Liu and Buskey (2000) hypothesized that a mucus layer of exopolymeric secretions (EPS) outside of the cell wall of *Aureoumbra lagunensis* discouraged grazing of microzooplankton on this alga. Discouraged grazing may help to maintain an ongoing bloom (Liu and Buskey, 2000). *Aureococcus anophagefferens* also possesses an EPS layer that may serve the same function (Gobler et al., 2013). The putative toxin in the EPS of both *Aureoumbra lagunensis* and *Aureococcus anophagefferens* interfered with the ciliary beating required for movement of captured particles to the gut in bivalves (Gainey and Shumway, 1991; Robbins et al., 2010), and adhered to cilia on the surface of protozoa, affecting both motility and feeding (Liu and Buskey, 2000). Even when ingested, the EPS may shield pelagophyte cells from digestion (Bersano et al., 2002). Evidence for both feeding and growth inhibition was demonstrated when Bricelj et al. (2004) exposed both the hard clam (*Mercenaria mercenaria*) and the blue mussel (*Mytilus edulis*) to toxic and non-toxic strains of *Aureococcus anophagefferens*. Growth and feeding were affected by the toxic strain when compared to the non-toxic strain, the latter of which was fed upon at comparable rates as other algal species (Bricelj et al., 2004).

Prior to the *Aureoumbra lagunensis* bloom in the IRL, blooms of this species had only been documented in Baffin Bay and Laguna Madre, Texas, USA. In Laguna Madre,

an *Aureoumbra lagunensis* bloom lasted almost eight years (Buskey et al., 1997; DeYoe et al., 1997; Gobler and Sunda, 2012). Similar to the IRL bloom, it was driven by a period of extensive drought and increased salinities; other factors such as two freeze-events exacerbated the blooms by causing mass losses of phytoplankton and subsequently a pulse in available ammonia (Gobler and Sunda, 2012). Pelagophytes require ammonia as a nitrogen source and outcompete other phytoplankton at low-nutrient levels (Sunda and Hardison, 2007, 2010; Gobler and Sunda, 2012; Gobler et al., 2013). *Aureoumbra lagunensis* has gained notoriety within the scientific community and more studies are being conducted as a result of the duration and intensity of the Laguna Madre bloom. Its expansion into new coastal estuaries warrants further research.

The wide-spread effects that pelagophyte blooms have on the ecology of estuarine systems are associated with light attenuation and the subsequent death of seagrass beds (Gobler and Sunda, 2012). Loss of seagrass may result in substantial ecological and economic impacts (SJRWMD, 2013). Large scale reductions in seagrass beds of the species *Zostera marina* resulted following an *Aureococcus anophagefferens* bloom in the eastern and southern reaches of Long Island, New York bays (Cosper et al., 1987) and impacted important habitat for the bay scallop *Argopecten irradians* (Gobler and Sunda, 2012). Consequently, the bay scallop industry collapsed in eastern Long Island. *Aureoumbra lagunensis* blooms impacted local fisheries in the IRL due to the loss of seagrass; it has been estimated that its fisheries lose $235 to $470 million each year when such blooms occur (SJRWMD, 2013). In addition, Shumway (1990) has extensively reviewed the impact of toxic algal blooms on aquaculture and the shellfish industry with recommendations for coping with the global problem.

The formation and persistence of pelagophyte blooms are somewhat complex and encompass bottom-up effects of nutrient availability, top-down effects by grazers, and additional physical factors such as salinity and flushing of the system (Sieburth et al., 1988; Buskey et al., 1997; Bricelj et al., 2001; Gobler and Sunda, 2012). Some of the causative agents for brown tides are arguably anthropogenic (e.g., nutrient pollution, Lapointe et al., 2015), but natural climatic changes may also influence their formation (Phlips et al., 2015). For example, a drought causing hypersaline conditions probably exacerbated the 2012-2013 *A. lagunensis* bloom in the NIRL and ML because this species can outcompete other phytoplankton at higher salinities (Liu and Buskey, 2000; Gobler et al., 2013). Additionally, its requirement for ammonia as its nitrogen source, which was provided by the decomposition of phytoplankton in the preceding bloom, further allowed *Aureoumbra lagunensis* to outcompete other phytoplankton and persist when nitrates were unavailable (Gobler et al., 2013). Further concern for the potential threat of *Aureococcus anophagefferens* blooms to expand their range to the IRL is because it has similar nutrient requirements and can likewise adapt to low-light and high salinity conditions (DeYoe et al., 1997; Gobler and Sunda, 2012).

The NIRL *Aureoumbra lagunensis* bloom in 2012-2013 was responsible for an ecosystem "tipping point"; the major loss of seagrass caused a trophic cascade resulting in several Unusual Mortality Events (UME) of Atlantic bottlenose dolphin (*Tursiops truncatus*; approximately 100 were found dead), West Indian manatee (*Trichechus manatus*; ~335 dead), and brown pelicans (*Pelecanus occidentalis*; ~250 dead) (Florida Fish and Wildlife Conservation Commission, unpublished data, as cited in Lapointe et al., 2015). The loss of available seagrass habitat led to the reduction of pigfish (*Orthopristis chrysoptera*) and its predator the spotted seatrout (*Cynoscion nebulosis*), the latter being a

preferred food source for dolphins in the IRL (Petersen et al., 2013, as cited in Lapointe et al., 2015). In addition, as seagrass died, the drift macroalgae *Gracilaria tikvahiae* increased and became a supplementary food source for manatees. This macroalgae is believed to produce cyanogenic glycosides (Peter Moeller et al., unpublished data, as cited in Lapointe et al., 2015), which may have been the driving force behind the manatee UME and a potential threat to human health as well (Seigler, 1991; Noguchi et al., 1994). While blooms of benthic macroalgae have been common within seagrass habitats of the IRL for decades (Benz et al., 1979), this "tipping point" caused by *Aureoumbra lagunensis* was unprecedented and highlighted the seriousness of a bloom's ability to disrupt entire ecosystems, damage the integrity of local and State economies, and threaten human health.

Other stressors on the system caused by *Aureoumbra lagunensis* blooms include hypoxic conditions that lead to productivity losses (30 fish kills occurred in Mosquito Lagoon in 2012, Gobler et al., 2013), mass die-offs and reduced growth of bivalves (Sieburth et al., 1988; Gobler et al., 2013), and discouraged grazing in filter-feeding organisms such as microzooplankton and mollusks (Robbins et al., 2010; Bricelj et al., 2001; Gobler and Sunda, 2012; Corcoran et al., 2013; Gobler et al., 2013). Blooms will also reduce the abundance of microzooplankton and important benthic grazers, such as occurred with the bivalve *Mulinia lateralis* in Baffin Bay, Texas, USA (Buskey et al., 1997).

1.3 Methods for Bioremediation of Water Quality Degradation and HABs

One approach for addressing environmental degradation in the IRL is the use of filter-feeding organisms for bioremediation. Bioremediation is generally defined as the

use of certain biological agents […] to remove or neutralize contaminants from polluted soil or water (Bioremediation, n.d.). While bioremediation is commonly used to clean up soil and groundwater using microbes that can metabolize and break down contaminants like polynuclear aromatic hydrocarbons (PAHs, Wilson and Jones, 1993), there are other methods which may fit the general definition of "bioremediation". Additional applications include a diverse number of "bioremediators" (i.e., the organism removing the contaminant), the contaminant being removed, and the environment in which bioremediation can be applied. In aquatic and marine applications, bioremediators may include filter-feeding invertebrates (e.g. bivalves, zooplankton), algae (e.g., macroalgae; Troell et al., 1997; Marinho-Soriano et al., 2011), and higher plants (e.g., transgenic plants; Krämer and Chardonnens, 2001). Although they are not necessarily defined as "bioremediation" in all contexts, additional methods are also quite common in aquaculture systems, and often include integrated systems with multiple trophic levels for the removal of particulates and nutrients within these systems (Ryther et al., 1975).

The suite of applications for bioremediation is diverse and may include removing (1) nutrients, as evident by the denitrification abilities of bivalves (Kellogg et al., 2013); (2) pesticides, trace elements, and other chemicals (e.g. Wilson and Jones, 1993; Krämer and Chardonnens, 2001; Brogan and Relyea, 2013); (3) bacterial blooms (e.g. Draughon et al., 2010), (4) algae blooms (e.g. de Bernardi and Giussani, 1990; Gulati, 1990; Lang et al., 1990; Draughon, 2010), and (5) turbidity (e.g. Cerco and Noel, 2007; Draughon, 2010; Draughon et al., 2010). Each bioremediator has pros and cons and should be evaluated based upon the desired effect.

Research on bivalves demonstrated their ability to remove nutrients and suspended particles from water. Kellogg et al. (2013) found that a restored Eastern oyster

reef (*Crassostrea virginica*) in Chesapeake Bay was able to increase denitrification efficiencies by 15 ± 2% at the restored site. Nitrogen and phosphorous were sequestered and assimilated into oyster shells and tissues with average standing stock of nitrogen and phosphorus greater than the control site by 95 ± 14.5 g N m^{-2} and 15 ± 2.5 g P m^{-2}. While Kellogg et al. (2013) conducted this study for oyster reef restoration and not "bioremediation" per se, specifically targeting nutrient and particulate removal by these organisms may serve a bioremediative function as part of the larger context of habitat restoration. *Crassostrea virginica* is ubiquitous in coastal estuaries along the east coast of the United States, including the Indian River Lagoon (Grizzle, 1990; Boudreaux et al., 2006). Intertidal and sub-tidal reefs of *C. virginica* are ecologically and commercially important. As ecosystem engineers this species provides a highly heterogeneous habitat compared to others found in soft-bottom systems like the IRL (Boudreaux et al., 2006). An oyster restoration model by Cerco and Noel (2007) extrapolated that a tenfold increase in existing oyster biomass in the Chesapeake Bay would reduce system-wide summer surface chlorophyll by approximately 1 mg m^{-3} (8% reduction in light attenuation) and add 2100 kg C to summer SAV biomass (20% increase in SAV). The benefits of *C. virginica* to biodiversity and water quality, and by extension ecosystem health, are apparent and might speak to its potential as a bioremediator against nutrient pollution and suspended particles including bacteria and HABs.

There are, as with any potential bioremediation method, some drawbacks to consider using *C. virginica* and other bivalves as bioremediators. There is concern that production of pseudofeces, mucus-bound inedible matter expelled by bivalves as a result of selective sorting, may decrease water clarity because the pseudofeces are easily re-suspended and may provide a source of organic enrichment when disturbed, leading to

phytoplankton productivity (Orlova et al., 2004). Further, while bivalves remove particulate material, they also excrete inorganic nutrients which may stimulate primary production of phytoplankton (Kohata et al., 2003; Orlova et al., 2004). Asmus and Asmus (1991) found that excretions from a mussel (*Mytilus edulis*) bed stimulated higher primary productivity than what was removed; the authors argued that in light of algal blooms initiated by anthropogenic nutrient loading, phytoplankton removal by mussel beds are unlikely to mitigate the blooms due to re-mineralization of nutrients back into the system by mussels. Thus, mussel beds may further induce continued growth of phytoplankton (Asmus and Asmus, 1991). Finally, the effectiveness of particulate removal by bivalves will vary depending on the size of the particle. Bivalves are capable of particle selection based on size (Defossez and Hawkins, 1997) and composition (Kennedy et al., 1996; Ward et al., 1998; Ward & Shumway, 2004), with oyster particle retention dropping by 20% when particle size falls below 6 µm (Stuart and Klumpp, 1984). Draughon et al. (2010) suggested that this particle selectivity may make bivalve grazing ineffective against some particles. This may have consequences for their bioremediative potential against bacteria and picoplanktonic harmful algae.

Other than filtration efficiency and particle retention, the toxicity of some bacteria or algae will affect the ability of a filter feeder to feed upon it. Harmful algal blooms have detrimental effects on bivalves by reduction of filtration rates (Tracey et al., 1988; Phlips et al., 2011; Gobler & Sunda, 2012; Gobler et al., 2013), damage to the bivalve gut (Alexander et al., 2008), and reduction in larval development which lead to smaller adults on average (Gobler et al., 2013). Following the 2012-2013 *Aureoumbra lagunensis* bloom in the IRL, several studies were conducted that demonstrate the alga's impact on bivalves. Gobler et al. (2013) demonstrated that filtration rates of both the hard clam

(*Mercenaria mercenaria*) and *C. virginica* were significantly reduced when fed IRL bloom water containing *Aureoumbra lagunensis* at densities of 10^6 cells mL^{-1} (89% and >93%, respectively). Similar results were observed when these bivalves were exposed to lab-grown cultures of *Aureoumbra lagunensis* at cell densities of 10^5 and 10^6 cells mL^{-1} (86-92% in the 10^5 treatment and 97-98% in the 10^6 treatment, respectively).

Bivalves may not be useful in the reduction of pelagophyte blooms because pelagophyte algae have been shown to adversely affect a few species of bivalve present in the IRL. Due to the particle selectivity of oysters and the negative effects HABs have on these and other potential bioremediators, alternative filter-feeding animals might be considered for this purpose. Draughon (2010) proposed the use of the pleated tunicate *Styela plicata* (Lesueur) as a potential bioremediator of bacterial and harmful algal bloom contamination. *Styela plicata* is a solitary ascidian and is one of the most commonly introduced tunicate species worldwide (Baker et al., 2004; Pineda et al., 2013). There is no consensus regarding the native range for *S. plicata*, although it is generally believed to be from the northwest Pacific (Carlton and Ruckelshaus, 1997; Lambert and Lambert, 1998; Lambert, 2001; Pineda et al., 2011, 2013). Bingham (1992) and Carlton and Ruckelshaus (1997) list *S. plicata* as an introduced species in Florida. It is widespread and abundant in the IRL near inlets and into the interior of the lagoon (Mook 1983). While it may be considered a highly biofouling organism, it is substrate limited (Dayton, 1971; Mook, 1983; Svane & Petersen, 2001; Stachowicz et al., 2002). It has a life span of approximately one year (Kott, 1972), although others contend *S. plicata* may live as long as two to three years (Lambert and Lambert, 1998). *Styela plicata* is known to be tolerant of polluted waters (Naranjo et al., 1996). Because of the global distribution and abundance of *S. plicata* in coastal systems, it is an important organism for ecological

studies (Sumerel, 2009). Tunicates are considered indiscriminate feeders, although they have the ability to cease feeding if no suitable or desirable food is present (MacGinitie, 1939; Petersen, 2007). It is not currently known what specific effects HABs might have on *S. plicata* or whether this species can efficiently filter harmful algal species. Draughon et al. (2010) reported that mean hourly filtration rates of *S. plicata* exposed to 10^5 cells mL^{-1} and 10^6 cells mL^{-1} *E. coli* was 4654 mL hr^{-1} and 2296 mL hr^{-1}, respectively. Draughon et al. (2010) extrapolated that 200 average-sized (~40g in weight) *S. plicata* would clear *E. coli* at 10^6 cells mL^{-1} from 13,000 L day^{-1} Therefore, 1,500–2,000 *S. plicata* placed in selected aquatic areas could reduce bacterial and algal counts to "safe limits" (Draughon et al., 2010). *S. plicata* not only removed *E. coli*, but killed all cells, rendering them unable to replicate (Draughon, 2010). This suggests that there is merit in investigating whether *S. plicata* is able to effectively filter other species of bacteria and microalgae (i.e., HABs such as *Aureoumbra lagunensis* from IRL waters).

There are some potential caveats which may cause some concern using *S. plicata* (or other ascidian tunicates) en masse for bioremediation. For example, Rosa et al. (2013) explored whether ascidians might serve as vectors of harmful algal introductions in aquaculture, including the species *Styela clava*, which shares its Genus with *S. plicata*. Using common biofouling ascidians (*S. clava, Ciona intestinalis, Molgula manhattensis, Botrylloides violaceus, Didemnum vexillum,* and *Botryllus schlosseri*), they exposed ascidians to cultured harmful algae (*Prorocentrum minimum, Alexandrium fundyense, A. monilatum, Karenia brevis, Aureococcusanophagefferens,* and *Heterosigma akashiwo*) at simulated bloom concentrations ranging from 10^2 to 10^6 cells mL^{-1} depending on HAB species. Results indicated that nearly all HAB cells remained viable post-digestion from at least one species of tunicate (after having been exposed to HABs for 24 hours), and

were able to re-establish populations within 48 hours of feces deposition. This study has consequences for bioremediation as well, especially if tunicates are transported between sites after having been deployed in bloom conditions. Further, stakeholder buy-in to using large aggregates of tunicates *in situ* may not be supported by those who consider these bio-fouling organisms as pests.

Considering the ability of *S. plicata* to filter large volumes of water, in addition to its ability to efficiently feed upon harmful fecal coliform bacteria (i.e. *E. coli*; Draughon, 2010), it is worth considering that it might be able to feed actively on other harmful cells like HABs. Therefore, *Aureoumbra lagunensis* and *Aureococcus anophagefferens* were selected as: 1) they are relatively easy species to culture and handle in a biological laboratory as they do not produce toxins; and 2) the recent incidence of *Aureoumbra lagunensis* blooms in the NIRL and ML, and the unknown probability for *Aureococcus anophagefferens* to expand southward into central and south-Florida coastal estuaries, allows for real-world context. Further, despite the tunicate's potential to feed on HABs, whether or not viable HAB cells would be present in tunicate fecal deposits and therefore serve as a vector of said cells was studied.

2 MATERIALS & METHODS

2.1 Experimental Overview

This study constitutes a series of experiments that examined the potential application of the pleated tunicate *Styela plicata* as a bioremediator against the HABs *Aureoumbra lagunensis* and *Aureococcus anophagefferens*. First, filtration rates (FR) and nutrient removal of *S. plicata* exposed to each HAB species at bloom densities were measured and compared to a nutritious control alga, *Tisochrysis lutea* Bendif et Probert (Bendif et al., 2013). Second, FR of *S. plicata* exposed to different densities of *A. lagunensis* was examined to determine the threshold density at which FR was not changed. Third, FR of *S. plicata* exposed to different initial densities of *A. lagunensis* and then exposed to bloom densities was examined to determine if "acclimation density" had an effect on FR.. Lastly, fecal deposits were collected from FR experiments with *A. lagunensis* and cultured to determine if viable HAB cells were present.

If *S. plicata* demonstrated no significant difference in filtration rates of HABs when compared to the control alga, then this ascidian has an advantage over bivalves in the removal of these pelagophyte algae. However, if filter-feeding by this ascidian is adversely affected by the experimental HABs, the utilization of *S. plicata* as a bioremediator may not be as beneficial for removal of HABs as proposed by Draughon (2010). Further, despite its possible bioremediative potential, presence of viable cells in feces that may allow subsequent re-population to bloom densities would argue against using this tunicate for improving HAB-impacted water quality.

2.2 Tunicate Specimen Collection and Holding

The methods of Draughon et al. (2010) were followed with some modifications. *Styela plicata* were collected from the ship channel at Harbor Branch Oceanographic Institute (HBOI, 27°32'1.84" N, 80°21'0.20" W), Florida Atlantic University, Fort Pierce, Florida. Tunicates were removed from sub-tidal twisted nylon ropes in the channel. At the time of collection, salinity and temperature were recorded using a YSI 85 meter (YSI Incorporated, Yellow Springs, Ohio). The tunicates were transported from the field in 12 L seawater (salinity 29-31) to the HBOI aquaculture laboratory within 10-20 minutes after collection. They were placed in a shallow 344 L tank (305 cm-long, 60 cm-wide, 45 cm-deep) filled with ~30 cm-deep filtered sea water (FSW; 1 µm filtered, UV-irradiated, salinity 29-30.7). Tunicates were allowed to acclimate for two to four hours prior to cleaning, which included the removal of commensal or other macro-fouling organisms and debris using forceps and a soft pipe-cleaner brush.

Tunicates were held for approximately 48-72 hours prior to experimentation and fed the microalgae *Tisochrysis lutea* (previously known as *Isochrysis* sp. [clone T-Iso]; Bendif et al., 2013) at a density of 1,000 cells mL^{-1} day^{-1} to minimize gut contents. Individuals with open siphons and consistent coloration over the holding period were selected for the filtration experiments. In order to prevent movement of an unattached individual during the experiment, the selected specimens were secured to individual plastic grids (10 cm x 10 cm with 1.6 cm openings) by lassoing monofilament line around the body, which was carefully placed as not to interfere with the lobes of the incurrent and excurrent siphons. Recovery of the tunicates was rapid as specimens were observed to open their siphons within minutes of attachment and placement back into water.

2.3 Microalgae Cultures

Cultures of *Tisochrysis lutea* for utilization as a control species were grown in f/2 medium (Florida Aqua Farms, Inc., Dade City, Florida) in the HBOI algae culture facility at ambient temperature (~25 **°C**) under 24 hr fluorescent light. *Tisochrysis lutea* was chosen because it is of comparable cell size to the experimental species (4-6 µm). Cultures were provided by John Scarpa, HBOI, Florida Atlantic University, Florida. Cultures of *Aureoumbra lagunensis* (strain CCMP 1510, originally isolated from Bird Island Station, Laguna Madre, Texas) and *Aureococcus anophagefferens* (strain CCMP 1984, originally isolated from Great South Bay, Long Island, New York) were provided by Chris Gobler, Stony Brook University, New York and were cultured separately in 0.22 µm filter-sterilized h/2 medium. This growth medium is a derivative of L1 medium that includes 500 mM NH_4Cl (National Center for Marine Algae and Microbiota, East Boothbay, Maine) and silicate stock solution omitted. One% penicillin/streptomycin (10,000 units mL^{-1}; Hyclone, Logan, Utah) was added to starter cultures to minimize bacterial growth (Y. Kahn, Stony Brook University, pers. comm.). Pelagophyte cultures were exposed to a 14:10 hr light:dark cycle of fluorescent light for the first filtration rate experiment using *Aureoumbra lagunensis*. Due to equipment and space availability conflicts, in subsequent experiments the cultures were grown under 24-hour light; this change in photoperiod did not appear to have any effect on growth rate. As cell density increased, transfers were made to larger-volume containers (i.e., 6L of heat-sterilized FSW with sterile f/2 nutrient stock added for *T. lutea*; and h/2 nutrient stock added to separate cultures of *Aureoumbra lagunensis* and *Aureococcus anophagefferens*), aerated with CO_2-enriched air), and at the previously-described photoperiods for each species.

2.4 Filtration Rate of *Styela plicata* Exposed to *Aureoumbra lagunensis*

The filtration rate of *S. plicata* exposed to the HAB *Aureoumbra lagunensis* was compared to the filtration rate of tunicates exposed to *Tisochrysis lutea* (control). Twenty individual tunicates (16.75-30.83g whole animal wet weight (WAWW)) were placed in separate 7.5 L beakers with 6 L of FSW (1 µm filtered, UV-irradiated, salinity 32.1). Beakers were arranged as an unbalanced, randomized block design (Figure 1) within 305 cm-long, shallow (45 cm deep) tanks filled with 30 cm FSW as a water bath. There were 10 experimental beakers for each algae species treatment, and four beakers for each algae species (without the inclusion of a tunicate) to account for sedimentation or adherence of cells to the beaker walls.

Before algae were added, *A. lagunensis* cultures were passed through a sieve with 20 µm pores in order to break up cell clumps and remove flocculent, which accumulated in the culture containers. Although *T. lutea* cultures did not produce any flocculent, these cultures were also passed through the sieve to help break up cell clumps. Cell counts were performed on filtered cultures, and algae were added to each beaker to a density of 5.0×10^5 cells mL^{-1} and mixed to homogenize. An initial 10 mL sample was taken (Hour 0), and subsequent samples were taken every hour for five hours. Samples were fixed with 1 mL 10% buffered formalin and stored at 4 ^oC until cell counts were performed; the dilution caused by adding formalin was corrected for during sample analysis. Beakers were moderately aerated to provide circulation to minimize HAB sedimentation, especially of *A. lagunensis* because this species is non-motile. Salinity (32.1), pH (7.44-7.97), and temperature (28.8 ^oC) were measured using the YSI 85 meter in each beaker

at Hour 0, Hour 2, and Hour 5 of the experimental period and remained constant throughout.

Following the experiment, tunicate specimens were removed from their respective beakers and placed on a paper towel to air-dry as in Draughon (2010). Specimens were weighed , placed in individual aluminum trays, dried in an oven at 95 ^{o}C for 48 hours, and held at 40 ^{o}C until whole animal dry weight (WADW) was measured, which was within a couple of hours following the end of the 48-hour drying time.

Cell counts were performed with the aid of a hemocytometer and phase contrast microscope. Quadruplicate cell counts were performed for each hourly sample and averaged for the calculation of filtration rate as described later. Filtration rate was calculated as in Draughon et al. (2010) to allow for direct comparison. In brief, Coughlan's formula (Coughlan, 1969), a version of Quayle's formula modified for the adjustment of particle sedimentation, was used to calculate filtration rate (FR):

$$FR = (V_0 + V_t)\,(\ln\,(S^E{}_0/S^E{}_t) - \ln(S^{C2}{}_0/S^{C2}{}_t))/(2nt)$$

where FR = filtration rate in mL hr^{-1}; V_0 = volume of water remaining in experimental beaker after withdrawal of sample at T_0; V_t =volume of water in experimental beaker before withdrawal of sample at time t; n = number of animals; t = duration of experiment; $S^E{}_0$, $S^E{}_t$ = suspension loads in cells or events mL^{-1} in experimental beaker at T_0 and t; $S^{C2}{}_0$, $S^{C2}{}_t$ = suspension loads in cells or events mL^{-1} in control beaker at T_0 and t.

There is inconsistency within the literature regarding the use and definitions of "filtration rate", "clearance rate", "pumping rate", and other terms (Riisgård, 2001). Throughout this thesis, "filtration rate" will be defined as "the volume of water cleared of a particular particle (i.e., algal cells) over time" in order to maintain consistency and allow comparisons to Draughon et al. (2010). If the authors of any cited literature use

different terms to describe, what in this thesis is defined as “filtration rate”, then the term will be changed to “filtration rate”. In addition, as there are inconsistencies in units used for reporting “filtration rate”, this thesis will maintain the cited authors’ original units unless otherwise specified.

The *a priori* null hypothesis (H_0) was “no significant difference exists between individual hourly or individual mean filtration rates of *S. plicata* for algae species”. The response variable “filtration rate” was analyzed by repeated measures ANOVA (RMANOVA; critical alpha = 0.05) for individual hourly FR between subjects and within subjects for each treatment; and FR was analyzed by two-way ANOVA (critical alpha = 0.05) for overall mean FR between treatments. Statistical analyses were conducted in R version 3.2.2. (R Core Team, 2015).

2.5 Filtration Rate of *Styela plicata* Exposed to *Aureococcus anophagefferens*

The previous experimental design was replicated to determine the filtration rate of *S. plicata* exposed to the HAB *Aureococcus anophagefferens* and compared to the filtration rate of tunicates exposed to *Tisochrysis lutea* (control). Salinity (29.7), pH (7.29-8.0), and temperature (28.0-28.3 ^{o}C) were measured in each beaker at Hour 0, Hour 2, and Hour 5 of the experimental period and remained constant within each of the beakers.

The *a priori* H_0 was “no significant difference exists between individual hourly or individual mean filtration rates of *S. plicata* for algae species”. The response variable “filtration rate” was analyzed by repeated measures ANOVA (RMANOVA; critical alpha = 0.05) for individual hourly FR between subjects and within subjects for each treatment; and FR was analyzed by two-way ANOVA (critical alpha = 0.05) for overall mean FR

between treatments. Statistical analyses were conducted in R version 3.2.2. (R Core Team, 2015).

2.6 Filtration Rate of *Styela plicata* Exposed to Different Initial Densities of *Aureoumbra lagunensis*

Styela plicata specimens were exposed to different initial cell densities (10^3, 10^4, and 10^5 cells mL^{-1}) of *Aureoumbra lagunensis* or *Tisochrysis lutea* (control) to determine if cell density had an effect on filtration rate. Each treatment was conducted in triplicate along with controls for sedimentation for each treatment. Individual tunicates (10.85-28.66g WAWW) were placed in 7.5 L beakers with 6 L of FSW (1 µm filtered, UV-irradiated, salinity ~30) and algae added to meet the treatment densities noted above. Beakers were arranged as a balanced, randomized block design within 305 cm-long shallow tanks which served as a water bath.

Culturing algae and subsequent processing for the FR experiment were performed as described in the initial two experiments. Sampling times of zero and 24 hours, instead of hourly as in the previous experiment, were employed. *Styela* possesses primitive phagocytic immune cells that are capable of responding to microbes through pattern recognition receptors by synthesizing and releasing antibiotics known as styelins (Lee et al., 1997; Taylor et al., 2000). However, the response is not immediate and thus the *S. plicata* specimens were exposed to the different densities of *A. lagunensis* over the full 24 hour period to allow the primitive immune response to respond and hence possibly affect FR. In the initial FR experiments, there was a significant difference between FR of *S. plicata* provided *T. lutea* and *A. lagunensis* (see Results), which may have been an artifact of the short experimental time (although there are likely additional compounding

factors (e.g. nutritional value of algae [see Discussion]). Hour 0 and Hour 24 water samples were collected and processed as described in the previous experiments.

Salinity (29.9-30.4), pH (7.26-7.99), and temperature (26.6-29.9 ^{o}C) was measured in each beaker at Hour 0 and Hour 24 of the experimental period. While temperatures within beakers and their respective blocks (water baths) were equal during each measurement, overall temperature dropped as much as 3 ^{o}C after 24 hours. This was not unexpected as the experiment was run overnight and the water baths cooled as the ambient temperature of the room cooled.

Cell counts were performed with the aid of a hemocytometer and phase-contrast microscopy. Quadruplicate cell counts were performed for each sample and averaged for the calculation of filtration rate (described earlier).

The *a priori* H_0 was "no significant difference exists between individual average FR for algae species or algal density". The response variable "filtration rate" was analyzed by two-way ANOVA (critical alpha = 0.05) for overall mean FR between treatments. Statistical analyses were conducted in R version 3.2.2. (R Core Team, 2015).

2.7 Determination of Viable Harmful Algae in Fecal Samples from *S. plicata*

The methods for this experiment were modified from Hégeret et al. (2008) and Rosa et al. (2013). The material collected for this experiment was from the one designed to measure filtration of *Aureoumbra lagunensis* at different initial densities (see section 2.7). In that experiment, *S. plicata* specimens were exposed in triplicate to three densities of *Aureoumbra lagunensis* (10^5 cells mL^{-1}, 10^4 cells mL^{-1}, and 10^3 cells mL^{-1}) for 24 hours. Fecal samples from the individual tunicates in the exposure beakers were collected at Hour 24 using sterile graduated pipets and then transferred and temporarily stored in

glass test tubes with 1 mL FSW until they could be processed (described later). While care was taken not to transfer exposure water (i.e., the water in which the tunicates were submerged and algae were added) with the fecal deposit samples, it is possible that undigested cells from the water could have attached to fecal deposits. This would cause a false-positive result for cell viability in feces after the processed fecal samples were cultured. Therefore, as a control, 1 mL of exposure water was collected from each replicate beaker with a sterile graduated pipet and transferred to separate test tubes. These exposure water controls were processed separately from the fecal samples, as described later.

Following fecal sample collection, each of the tunicate specimens exposed to *A. lagunensis* (n=9) was removed from its experimental beaker, rinsed thoroughly with FSW, and placed in separate 6 L beakers of FSW. After another 24 hours, fecal deposits and exposure water controls were collected from each of the containers as described previously, and the tunicates were rinsed and placed in a clean container of FSW. A final set of fecal deposits and exposure water controls were collected after another 24 hours, thus providing three sets of fecal deposits collected at 24, 48, and 72 hours post-HAB exposure.

Prior to processing, subsamples of collected feces were observed using blue fluorescence microscopy (emission at 515 nm; excitation at 490 nm; Olympus BX51 microscope; Olympus, Waltham, Massachusetts) to detect potentially viable cells, in which active chlorophyll *a* (chl-*a*) would fluoresce as red in color. The remainder of fecal samples were vortexed in 1 mL heat-sterilized FSW, and transferred into separate 10 mL silicate glass test tubes containing 5 mL heat-sterilized FSW. Test tubes were maintained at ambient room temperature **(**~25 ^{o}C**)** under 24 fluorescent light for four

weeks. Separate test tubes were prepared and inoculated with 1 mL of exposure water (i.e., control) into 5 mL of heat-sterilized FSW and maintained under the same conditions as the "fecal" test tubes to observe for cell replication.

Water samples from "fecal" and control test tubes were examined microscopically with the aid of a hemocytometer and phase contrast microscopy approximately every four-to-five days for four weeks. Re-establishment of an algal bloom population was defined as when cell density in the test tube was equal to or exceeded 5.0×10^5 cells mL^{-1}, which mimics bloom conditions (Gobler et al., 2013). Frequency of bloom formation was recorded.

3 RESULTS

3.1 Filtration Rate of *Styela plicata* Exposed to *Aureoumbra lagunensis*

The hourly average filtration rates (FR) for individual tunicates exposed to the harmful algal species *Aureoumbra lagunensis* ranged from -282 mL hr^{-1} to 542 mL hr^{-1}, whereas tunicates exposed to the control non-harmful algal species *Tisochrysis lutea* had FRs from 285 mL hr^{-1} to 2648 mL hr^{-1}. Calculations of FR in the *A. lagunensis* treatments do not include values obtained from sedimentation controls. Over the five-hour period, the cell densities of *A. lagunensis* in the controls for sedimentation fluctuated around a mean of 4.9 x 10^5 cells mL^{-1} (Figure 2A); whereas the cell densities of *T. lutea* in the controls for sedimentation gradually decreased from a mean of 4.9 x 10^5 cells mL^{-1} at Hour 0 to a mean of 4.0 x 10^5 cells mL^{-1} at Hour 5 (Figure 2B). Mean FR for *S. plicata* exposed to either *A. lagunensis* or *T. lutea* were significantly different (repeated-measures ANOVA [RMANOVA] $p < 0.0001$) with means of 128 ± 226 mL hr^{-1} and 1634 ± 797 mL hr^{-1}, respectively; there was greater than an order of magnitude decrease in FR of *S. plicata* in the presence of the harmful algae compared to the healthy algal control.

The data may also be expressed in terms of the decrease in cell density by *S. plicata* over time. Exposure of *S. plicata* to *A. lagunensis* showed no marked decrease in algal cell density over time and "leveling off" by Hour 3 around ~5.0 x 10^5 cells mL^{-1} (Figure 3). Meanwhile, replicates in the *T. lutea* treatments demonstrated a gradual

decrease in cell density over time from 5.4 x 10^5 cells mL^{-1} at Hour 0 to 1.6 x 10^5 cells mL^{-1} at Hour 5 (Figure 3).

3.2 Filtration Rate of *Styela plicata* Exposed to *Aureococcus anophagefferens*

The hourly average filtration rates (FR) for individual tunicates exposed to the harmful algal species *Aureococcus anophagefferens* ranged from 343 mL hr^{-1} to 593 mL hr^{-1} while. *T. lutea* ranged from 600 mL hr^{-1} to 2482 mL hr^{-1}. Calculations of FR in both HAB and *T. lutea* control treatments do not include values obtained from sedimentation controls. Over the five-hour period, the cell densities of *A. anophagefferens* in the controls for sedimentation fluctuated around a mean of 5.0 x 10^5 cells mL^{-1} (Figure 4A); and the cell densities of *T. lutea* in the controls for sedimentation fluctuated around a mean of 4.8 x 10^5 cells mL^{-1} (Figure 4B). The mean FRs of *S. plicata* exposed to *A. anophagefferens* and *T. lutea* were significantly different (RMANOVA $p < 0.0001$) with means of 485 ± 278 mL hr^{-1} and 1650 ± 733 mL $hr^{-}1$, respectively.

The data may also be expressed in terms of the decrease in cell density by *S. plicata* over time. Exposure of *S. plicata* to *A. anophagefferens* resulted in a slight decrease in cell density over time from 5.6 x 10^5 cells mL^{-1} at Hour 0 to 4.3 x 10^5 cells mL^{-1} at Hour 5. However, the decrease in *T. lutea* cell density over time was of greater magnitude from 5.0 x 10^5 cells mL^{-1} at Hour 0 to 1.2 x 10^5 cells mL^{-1} at Hour 5 (Figure 5).

3.3 *Post-hoc* Analysis of Filtration Rate as a Function off Specimen Size

In order to determine if the marked differences in FR between individuals from either preceding experiment were due to differences in specimen size, an analysis of FR

of *S. plicata* exposed to *T. lutea* as a function of weight was conducted. Regression analysis indicated there was no relationship between FR of *S. plicata* exposed to *T. lutea* and whole animal wet weight (WAWW) in either of the previously described FR experiments, as indicated by weak correlations ($r^2 = 0.0537$, Pearson r = 0.2317; and $r^2 = 0.0834$, Pearson r = 0.2888, respectively). No relationship tests between FR of *S. plicata* exposed to brown tide cultures and WAWW were conducted due to the apparent effects of the HABs on FR. For example, in the *Aureoumbra lagunensis* FR experiment, the specimen with the lowest mean FR when exposed to *T. lutea* (#12, 611 mL hr^{-1}) weighed 23.9 grams, whereas the specimen with the highest mean FR when exposed to *T. lutea* (#13, 2975 mL hr^{-1}) weighed 16.8 grams (Table 1). Similarly, in the *Aureococcus anophagefferens* FR experiment, the specimen with the lowest mean FR when exposed to *T. lutea* (#33, 600 mL hr^{-1}) weighed 14.7 grams, whereas the specimen with the highest mean FR when exposed to *T. lutea* (#37, 2482 mL hr^{-1}) weighed 17.0 grams (Table 2). These data exhibit the variability of FR between individuals.

3.4 Filtration Rate of *Styela plicata* Exposed to Different Initial Densities of *Aureoumbra lagunensis*

It was re-evaluated *post hoc* that in order to calculate FR over the 24 hour period, more samples would be required over time. Without multiple samples taken throughout the 24 hour period, it cannot be assessed whether the specimens were continually feeding, or if the specimen had ceased feeding at any point. It is likely that these counts under-estimate the FR of *S. plicata* because the samples were not collected continuously over time, but at Hour 0 and Hour 24 only. In lieu of FR, percent decrease in algal density by *S. plicata* was measured.

The mean percent decrease of *A. lagunensis* by *S. plicata* were 92, 99, and 77% in treatments with starting cell densities of 5.0 x 10^3 cells mL^{-1}, 5.0 x 10^4 cells mL^{-1}, and 5.0 x 10^5 cells mL^{-1}, respectively (Table 3). Comparatively, mean percent decrease of *T. lutea* by *S. plicata* were 73, 92, and 88% in treatments with starting cell densities of 5.0 x 10^3 cells mL^{-1}, 5.0 x 10^4 cells mL^{-1}, and 5.0 x 10^5 cells mL^{-1}, respectively. Analysis of variance (ANOVA) of percent decrease of algal cells between *S. plicata* exposed to different initial densities of each alga, as well as between individuals exposed to either alga of the same initial cell density, were not significant ($p > 0.10$).

3.5 Determination of Viable Harmful Algae in Fecal Samples from *S. plicata*

Fecal samples observed microscopically from *S. plicata* specimens exposed to different initial densities of *A. lagunensis* (10^3 cells mL^{-1}, 10^4 cells mL^{-1}, and 10^5 cells mL^{-1}) and collected 24, 42, and 72 hours after exposure indicated potentially viable *A. lagunensis* cells in some samples (Table 4). For the 10^3 cells mL^{-1} treatments, viable cells were found in at least two of three samples for each collection time. One of three samples was found for all collection times for the 10^4 cells mL^{-1} treatments. Finally, for the 10^5 cells mL^{-1} treatments, all samples at 24 hours had viable cells, but none were found in the 48 hour and 72 hour samples.

Over the 4-week fecal culture monitoring period, 19 of the 27 cultures had cell densities exceeding 5.0 x 10^5 cells mL^{-1}. Thirteen of the 19 cultures with re-established cell densities (a “bloom”) exceeded the threshold that originated from fecal samples or exposure water collected 24 hours post-exposure from 10^4 cells mL^{-1} and 10^5 cells mL^{-1} treatments (Table 5). After accounting for the exposure water controls, only 2 of the 19 fecal cultures bloomed without its control also developing a bloom. Fecal cultures that

developed a bloom did so as early as eight days following inoculation; however, their respective controls did as well. Only one fecal culture developed an algal bloom from samples collected 48-hour and 72-hour post-HAB exposure. No fecal cultures from 10^3 cells mL^{-1} treatments produced an algal bloom over the four-week observation period, except for one of the water control cultures 24 days after inoculation.

4 DISCUSSION

The Greater Indian River Lagoon (IRL) is a coastal estuary of ecological and economic significance (Indian River Lagoon National Estuary Program, 1996; Sime, 2005; St. Johns River Water Management District, 2013). Its proximity to developed coastlines and its connectivity to inland watersheds have resulted in a number of anthropogenic drivers of ecosystem damage and decline in habitat quality and health (Sime, 2005; Lapointe et al., 2012). Bottom-up processes of nutrient pollution have led to the classification of the IRL as a moderately-eutrophic system (Bricker et al., 1999). Eutrophic systems are plagued with harmful algal blooms (HABs), which are functionally diverse in their effects on biotic and abiotic processes (Van Dolah, 2000; Landsberg et al., 2002; Phlips et al., 2004; Davis et al., 2009). Within the last few years, the first incidence of a bloom of the pelagophyte *Aureoumbra lagunensis* was recorded in the northern IRL (N-IRL) and Mosquito Lagoon (ML) with observed loss of seagrass (St. Johns River Water Management District, 2013), decline in filter-feeding invertebrates (Gobler et al., 2013) and fish (Lapointe et al., 2015), and unusually-high deaths of birds and marine mammals (Florida Fish and Wildlife Conservation Commission, unpublished data, as cited in Lapointe et al., 2015). This ecosystem "tipping point" (Lapointe et al., 2015) has increased the demand for research to understand the effects of blooms on the ecosystem and means to mitigate environmental degradation to prevent further damage. The National Resource Council (2001) recommended reducing anthropogenic nutrient loads to remedy eutrophication, while others focus on localized bioremediation against

harmful bacterial and algal blooms using filter-feeding invertebrates (Draughon, 2010). There is promise in using the pleated tunicate *Styela plicata* to effectively bioremediate against fecal coliform bacteria (Draughon, 2010). Therefore, utilizing this species as a tool to remediate pelagophyte algal blooms was examined.

Firstly, the null hypotheses which stated there would be no significant difference in filtration rate (FR) of *S. plicata* exposed to the HABs *Aureoumbra lagunensis* or *Aureococcus anophagefferens* when compared to the healthy control alga *Tisochrysis lutea* were rejected. Secondly, the null hypothesis that there would be no significant difference in percent decrease of *A. lagunensis* and *T. lutea* cells by *S. plicata* at different initial cell densities could not be rejected. Lastly, the analysis of *S. plicata* fecal deposits following exposure to *A. lagunensis* at different initial cell densities, and the subsequent monitoring of prepared cultures of fecal deposits over four weeks, in order to determine potential vector transmission of this HAB by tunicates were inconclusive.

Based on the FR determination experiments of *S. plicata* exposed to pelagophytes over the short-term (i.e. five hours), the measured FR does not suggest this tunicate can bioremediate against these HABs. Mean filtration rate (FR) of *S. plicata* exposed to *Aureoumbra lagunensis* (128 ± 226 mL hr^{-1}) was greater than an order of magnitude less than the mean FR when exposed to the green microalga *Tisochrysis lutea* (1634 ± 797 mL hr^{-1}). Mean FR of *S. plicata* exposed to *Aureococcus anophagefferens* (485 ± 278 mL hr^{-1}) was greater than an order of magnitude less than the mean FR when exposed to *T. lutea* (1650 ± 733 mL hr^{-1}). This reduction in filtration rate is comparable to FR data collected by Gobler et al. (2013), who also observed an order of magnitude decline in FR of the hard clam (*Mercenaria mercenaria*) and the eastern oyster (*Crassostrea virginica*) when exposed to *A. lagunensis*. Filtration rate of the eastern oyster *Crassostrea virginica*

exposed to *A. lagunensis* and *T. lutea* were 0.18 ± 0.07 L g dry wt^{-1} hr^{-1} and 1.27 ± 0.46 L g dry wt^{-1} hr^{-1}, respectively (Gobler et al., 2013).

Filtration rate measurements from my thesis were converted to the units used to calculate FR in Gobler et al. (2013) for comparison: mean FR when *S. plicata* exposed to *A. lagunensis* and *T. lutea* are 0.05 ± 0.09 L g dry wt^{-1} hr^{-1} and 0.87 ± 0.37 L g dry wt^{-1} hr^{-1}, respectively (Table 6). Comparatively, converted measurements of mean FR of *S. plicata* exposed to *Aureococcus anophagefferens* and *T. lutea* from this thesis were 485 ± 278 mL hr^{-1} and 1650 ± 733 mL hr^{-1}, respectively. Bricelj et al. (2001) exposed *M. mercenaria* to a mixed suspension of *A. anophagefferens* and *T. lutea* and determined a FR between 0.004 and 0.006 mL g wet wt^{-1} min^{-1}. Filtration rate measurements from this thesis were also converted to the units used in Bricelj et al. (2001) for comparison: mean FR of *S. plicata* exposed to *A. anophagefferens* was 0.42 mL g wet wt^{-1} min^{-1}. Although it appears that *S. plicata* might be better at filtering *A. anophagefferens* than *M. mercenaria*, these results are not directly comparable because mixed suspensions were not utilized in this thesis.

Additional comments should be made regarding my calculation and interpretation of filtration rates (FR). Firstly, values for the control of sedimentation of algal cells in beakers containing no tunicates were removed from Coughlan's formula (Coughlan, 1969) for all calculations of FR (with the exception of *T. lutea* controls in the first FR experiment [see Results, 3.1.]). The observed fluctuation of cell density over time in the controls for sedimentation (Figure 2A and Figure 4) does not warrant a correction for sedimentation in Coughlan's formula. Draughon et al. (2010) experienced a similar phenomenon in their study and chose to remove the controls from their FR calculations as well. Controls for sedimentation were utilized in all FR experiments, and were only

removed from the formula *post hoc*. It is not recommended that controls for sedimentation be removed from experimental design in future replicated studies. It is unclear why cells did not settle in the containers as expected, especially in the HAB treatments, because both *Aureoumbra lagunensis* and *Aureococcus anophagefferens* are un-flagellated, non-motile cells (DeYoe et al., 1997). Possible explanation for this lack of sedimentation is the moderate bubbling provided by aeration within the control beakers.

There was a clear disruption of FR when *S. plicata* were fed the pelagophyte HABs, consistent with reductions of FR by bivalves important to the IRL (Bricelj et al., 2001; Gobler et al., 2013). The ecological function of filter-feeding invertebrates is important for maintaining water quality, especially for healthy growing oysters. The latter an important role in reef formation and providing heterogeneous habitat (Boudreaux et al., 2006). Thus, adverse impacts on oysters by HABs can have significant consequences. Buskey et al. (1997) and Gobler et al. (2013) implicate the role of an extra-cellular polysaccharide sheath surrounding cells of *Aureoumbra lagunensis* as a possible cause for the disruption of grazing by filter-feeders. Liu and Buskey (2000) demonstrated that *A. lagunensis* with thick EPS was not suitable food for the protist *Euplotes* sp. and other hypotrichous ciliates; the protist *Aspidisca* sp. had reduced filtration rates and growth rates exposed to thick-EPS *A. lagunensis.* One alternative explanation for the observed reduced filtration rates comes from laboratory studies that showed *A. lagunensis* may be nutritionally inadequate (Buskey and Hyatt, 1995, as cited in Liu and Buskey [2010]). Gainey and Shumway (1991) and Bricelj et al. (2001) discuss a dopamine-mimetic bioactive compound associated with the extra-cellular polysaccharide sheath of *Aureococcus anophagefferens*. This compound reduces the activity of gill lateral cilia in bivalves necessary to generate movement of water through the gills. Cilia are responsible

for the movement of trapped particles in the mucus net of tunicates toward the stomach (MacGinitie, 1939), so perhaps a similar mechanism was operative in my study and may explain the reduced filtration rates when this organism was present.

When filtration efficiency of *S. plicata* exposed to *A. lagunensis* at different initial cell densities over a greater duration (i.e. 24 hours) was examined, the degree to which tunicates were adversely affected by exposure to the HAB, as seen during the preceding study, was not evident. The mean percent decrease of *A. lagunensis* by *S. plicata* were 92, 99, and 77% in treatments with starting cell densities of 5.0×10^3 cells mL^{-1}, 5.0×10^4 cells mL^{-1}, and 5.0×10^5 cells mL^{-1}, respectively (Table 3). Comparatively, mean percent decrease of *T. lutea* by *S. plicata* were 73, 92, and 88% in treatments with starting cell densities of 5.0×10^3 cells mL^{-1}, 5.0×10^4 cells mL^{-1}, and 5.0×10^5 cells mL^{-1}, respectively. The measured reductions in cells between treatments with different initial cell densities and between algal species were non-significant ($p > 0.10$). As in the previous FR experiments, controls for sedimentation were analyzed to determine whether the loss of cells were due to settling or via filtration by tunicates. Four of the six controls had slight to major increases in cell density from Hour 0 to Hour 4 (Table 7). The increase in the *A. lagunensis* control for sedimentation with the starting concentration of 5.0×10^5 cells mL^{-1} by approximately 33% may have been a result of cell replication rather than an artifact of improper mixing at Hour 0. As with the previous FR experiments, there appears to be a consistent lack of sedimentation of cells within these control beakers lacking tunicates. This is likely due to the setup of the containers, the moderate bubbling provided by aeration, and the non-homogenous mixing of cells in suspension.

It cannot be discerned whether the comparable percent decrease in algal cells with either harmful or non-harmful algal treatment can be attributed to acclimation of *S. plicata* following previous extended exposure to HAB cells or to some other effect, including: 1) an artifact of small sample size; 2) the *A. lagunensis*-exposed tunicates maintained a steady yet low FR, which resulted in a decrease in cell density over time; the *T. lutea*-exposed tunicates slowed or stopped feeding after consuming a sufficient food volume at some point during the 24-hour period, as tunicates are able to control feeding based on dietary requirements (MacGinitie, 1939); or a combination of these; and 3) the tunicates responded positively to the extended exposure time to HABs; or a combination of any of the above. An improvement in the methods which may detect one or more of the responses described above would be to collect additional samples throughout the 24-hour period. Collecting samples at Hour 0 and Hour 24 alone is limits interpretation. It is not possible to determine whether the tunicates fed continuously over time, whether feeding occurred for a short period of time before ceasing, or how FR may have fluctuated over time.

It would be advantageous to develop an assay for measuring the activity of styelins or other anti-microbials that may be induced in tunicates following exposure to harmful algal cells. Such studies may elucidate what is occurring at the cellular level, and what impacts such anti-microbials may have on the acclimation of tunicates to harmful cells and hence increase their potential for bioremediation. In *Styela clava*, for instance, the peptide Styelin D is effective against Gram-positive and Gram-negative bacteria, and is hemolytic and cytotoxic to eukaryotic cells (e.g. human cervical epithelial cells, and rabbit red blood cells; Taylor et al., 2000); this activity has also been observed in *S. plicata* (Raftos and Hutchinson, 1995). Plicatamide, an anti-microbial octapeptide

isolated from *S. plicata* blood cells, is effective against wild-type and methicillin-resistant *Staphylococcus aureus* (Tincu et al., 2003). Understandably, these and other studies focus on anti-bacterial applications for medicine; however, because tunicates are able to withstand microbe-laden (i.e. interpreted thus as above normal, background-level cell density) environments (Lehrer et al. 2003), there are promising environmental applications against bacteria and HABs. Lehrer et al. (2003) suggested that *S. clava* be considered for environmental applications as a "green" anti-pollutant and microbicide.

Studies on the immune response to *Escherichia coli* in *S. plicata* have led to recommendations for further study of this species as a model organism for understanding animal response to microbes (Monterio de Barros et al., 2009). It is recommended that similar studies be developed for understanding how animals react to HABs. For example, Hégaret et al. (2011) found that HAB effects on bivalves can be either immunostimulative or immunosuppressive. It is important to understand whether the apparently robust host cell defense in tunicates can be further applied to both bacterial and algal bloom eutrophic conditions. The ubiquity of *S. plicata* in eutrophic coastal systems further supports the need to understand under what conditions this species might serve as an effective bioremediator.

Finally, *S. plicata* fecal deposits collected following exposure to three different cell densities of *A. lagunensis* were cultured in order to ascertain the potential for this tunicate to serve as vector of HAB cells. Of the 27 cultures, 19 had met or exceeded the threshold of 5.0×10^5 cells mL^{-1} during the four-week period; and of these, only two samples established a bloom without their respective controls blooming. The prevalence of false-positive blooms in samples is indicative of *A. lagunensis* cells being present in

exposure water, or by fecal deposits having come into contact with exposure water containing cells.

Observations of fecal deposits using blue-fluorescence microscopy showed that potentially viable cells may be embedded within the fecal deposits (Figure 6). Based on these results alone, it is not clear whether there is a risk of *S. plicata* acting as a vector of *A. lagunensis*. Fecal samples did not form blooms to the order of magnitude determined as the threshold for a re-established bloom. If this study were repeated, it is recommended that fecal cultures be grown in sterilized FSW with nutrient media added in order to account for possible cell stress and nutrient limitation effects.

Hégaret et al. (2008) found that bivalve fecal deposits collected within 48-hours post-HAB exposure were capable of re-establishing growing populations of algae, and recommended that organisms that are transported within aquaculture facilities or for restoration activities be held for at least that long to prevent HAB cell transfer. Similar results were found by Rosa et al. (2013), as HAB cells remained intact in tunicate fecal deposits post-digestion within 48-72-hours post-HAB exposure. *Aureococcus anophagefferens* was not used in this experiment, although both Hégaret et al. (2008) and Rosa et al. (2013) exposed bivalves and tunicates, respectively, to this species and found contradictory results: five of seven species of bivalves in Hégaret et al. (2008) did not produce fecal deposits capable of re-establishing *A. anophagefferens* blooms, although the authors state having had some difficulty confirming the presence of cells in fecal inocula; and Rosa et al. (2013) found that five of six ascidian tunicates tested did have fecal deposits capable of re-establishing growing populations of this species. These studies exemplify the diverse means by which different filter-feeders respond in the presence of HABs.

5 CONCLUSION

A series of experiments studying the potential of the pleated tunicate *Styela plicata* as a bioremediator of the pelagophytes *Aureoumbra lagunensis* and *Aureococcus anophagefferens*, two brown tide harmful algal blooms (HABs) that plague coastal, eutrophic estuarine systems, were conducted.

The filtration rate (FR) of *S. plicata* exposed for five hours to either *Aureoumbra lagunensis* or *Aureococcus anophagefferens* was reduced by approximately an order of magnitude. This suggests that, in the short-term, *S. plicata* cannot effectively remove these HAB cells and its bioremediative function may be limited. Preliminary evidence of *S. plicata* that were exposed to *A. lagunensis* for a longer exposure period (i.e., 24 hours) demonstrated that this tunicate might be capable of acclimating to HAB conditions. Prior acclimation may thus result in their ability to remove HAB cells from suspension, as the percent removal was similar to that of the control algae.

Data on *S. plicata* acting as a vector of pelagophyte cells is not supported here, but this may be a consequence of lack of feeding on *A. lagunensis* or stress on potentially viable cells to replicate following survival through the tunicate gut. Previous research has demonstrated some varied results regarding the potential for bivalves and ascidian tunicates to serve as vectors of HABs, with the latter having greater vector potential. Tunicates are able to feed upon a variety of particle sizes and at large volumes, but this often results in less effective digestion (Hughes et al., 2005). This coupled with their bio-fouling characteristics and invasive potential (Bullard and Carman, 2009) increases

concerns regarding their transport of HABs. Further research is recommended, especially in light of the occurrence of HABs in coastal ecosystems.

Future directions for research should include studies on the mechanisms which account for reduced grazing of pelagophyte algae by tunicates and other grazers. This understanding will provide vital information regarding which taxa are more susceptible to brown tides and whether adaptability in these eutrophic systems is possible. In general, eutrophic ecosystems will be best served by changes in anthropogenic activities related to development, reduction in nutrient pollution, and other water management best-practices. However, environmental restoration, habitat quality improvement projects, and bioremediation remain promising tools to combat eutrophication and the suite of its negative effects.

APPENDICES

Tables

Table 1. Summary of *Styela plicata* specimens used in the determination of FR (*Aureoumbra lagunensis*) experiment, including whole animal wet weight (WAWW; grams), hourly FR (HFR), and average FR (AFR).

***A. lagunensis* treatment**				***A. lagunensis* treatment con't**				***T. lutea* treatment**				***T. lutea* treatment con't**			
ID	WAWW (g)	HFR	AFR	ID	WAWW (g)	HFR	AFR	ID	WAWW (g)	HFR	AFR	ID	WAWW (g)	HFR	AFR
1	33.0	-739	-138	6	42.2	449	158	11	26.2	422	615	16	51.2	2600	2094
		-87				272				591				1902	
		-158				84				744				1941	
		205				-37				669				1875	
		90				23				648				2149	
2	41.3	-226	199	7	33.0	1016	542	12	23.9	323	611	17	34.1	3630	2462
		595				740				462				2709	
		402				349				734				2305	
		96				333				909				1930	
		129				272				627				1737	
3	20.6	37	90	8	22.1	-1244	-282	13	16.8	3165	2975	18	22.1	1584	1487
		314				-102				2856				2477	
		212				171				3529				1510	
		-60				-90				2995				1050	
		-54				-144				2328				816	
4	30.3	502	271	9	45.4	-9	52	14	21.6	1788	1722	19	37.0	2474	2650
		437				207				1414				2834	
		77				-29				2657				2546	
		214				69				1221				2776	
		123				23				1529				2619	
5	21.1	560	248	10	25.4	365	136	15	38.8	2425	2151	20	30.6	2334	2408
		185				159				2619				2272	
		200				47				2258				2567	
		125				79				1819				2680	
		166				28				1631				2187	

Table 2. Summary of *Styela plicata* specimens used in the determination of FR (*Aureococcus anophagefferens*) experiment, including whole animal wet weight (WAWW; grams), hourly FR (HFR), and average (AFR).

***A. anophagefferens* treatment**				***A. anophagefferens* tmnt. con't**				***T. lutea* treatment**				***T. lutea* treatment**			
ID	WAWW (g)	HFR	AFR	ID	WAWW (g)	HFR	AFR	ID	WAWW (g)	HFR	AFR	ID	WAWW (g)	HFR	AFR
21	18.6	975	577	26	24.9	337	446	31	15.6	745	967	36	14	2554	1990
		1057				524				646				1870	
		370				598				1003				1917	
		318				367				1204				2051	
		165				404				1238				1558	
22	10.8	1121	489	27	24.6	427	475	32	20.1	2746	2133	37	17	1132	2482
		604				727				2028				3363	
		198				577				1492				2635	
		288				340				2319				2702	
		234				305				2080				2580	
23	15.4	455	364	28	22.4	958	574	33	14.7	303	600	38	21	1734	1405
		390				866				1051				1531	
		265				248				1089				786	
		357				393				-376				1187	
		353				405				935				1788	
24	28.7	808	410	29	14.5	587	343	34	16.8	2750	2051	39	18	2962	2216
		346				289				1569				1692	
		228				406				1760				2406	
		361				194				2071				1890	
		305				241				2104				2129	
25	16.3	1317	578	30	15.0	1134	593	35	13.1	926	1489	40	16	821	1168
		426				754				1457				1429	
		435				538				1635				925	
		361				339				1753				1084	
		354				203				1673				1582	

Table 3. Mean percent decrease in cell density of *Aureoumbra lagunensis* (AL) and *Tisochrysis lutea* (TL) by *S. plicata* at different initial starting densities (5.0×10^3 cells mL^{-1}, 5.0×10^4 cells mL^{-1}, and 5.0×10^5 cells mL^{-1}) over 24 hours.

MEAN HOUR 0	MEAN HOUR 24	Decrease (%)	MEAN HOUR 0	MEAN HOUR 24	Decrease (%)
AL 10^3 treatment			TL 10^3 treatment		
1.0E+04	8.6E+02	92	4.6E+03	1.2E+03	73
AL 10^4 treatment			TL 10^4 treatment		
4.8E+04	4.9E+02	99	6.5E+04	5.2E+03	92
AL 10^5 treatment			TL 10^5 treatment		
5.5E+05	1.3E+05	77	4.7E+05	5.6E+04	88

Table 4. Frequency (%) of potentially viable *Aureoumbra lagunensis* cells in *Styela plicata* fecal samples collected 24, 48, and 72 hours after exposure to different initial cell densities (5.0×10^3 cells mL^{-1}, 5.0×10^4 cells mL^{-1}, and 5.0×10^5 cells mL^{-1}; n=3 per density), observed microscopically.

Hours after exposure	Initial Cell Density (cells mL^{-1})		
	5.0×10^3	5.0×10^4	5.0×10^5
24 hours	66	33	100
48 hours	100	33	0
72 hours	66	33	0

Table 5. *Aureoumbra lagunensis* bloom occurrence in *Styela plicata* fecal sample cultures and their respective exposure water controls by initial cell density and collection time. A "+" indicates a bloom developed (at 5.0 x 10^5 cells mL^{-1}) during the four-week culture period.

Rep	Treatment (cells mL^{-1})	24 HOURS		42 HOURS		72 HOURS	
		Fecal Culture	Control	Fecal Culture	Control	Fecal Culture	Control
1	5.0 x 10^3	-	+	-	-	-	-
2	5.0 x 10^3	-	-	-	-	-	-
3	5.0 x 10^3	-	-	-	-	-	-
1	5.0 x 10^4	+	+	-	-	-	-
2	5.0 x 10^4	+	+	+	-	+	-
3	5.0 x 10^4	+	+	-	-	-	-
1	5.0 x 10^5	+	+	-	+	-	-
2	5.0 x 10^5	+	+	-	+	-	-
3	5.0 x 10^5	+	+	-	+	-	+

Table 6. Filtration rates (L g dry wt^{-1} hr^{-1}) of the hard clam *Mercenaria mercenaria* and eastern oyster *Crassostrea virginica* (Gobler et al., 2013), and the pleated tunicate *Styela plicata* (this thesis) provided diets of 5.0 x 10^5 cells mL^{-1} Tisochrysis lutea (TL) and 4.0 x 10^5 cells mL^{-1} or 5.0 x 10^5 cells mL^{-1}* *Aureoumbra lagunensis* (AL).

Filter-Feeder	**Diet**	
	TL	**AL**
M. mercenaria	0.50 ± 0.13	0.04 ± 0.09
C. virginica	1.27 ± 0.46	0.18 ± 0.07
S. plicata	0.87 ± 0.37	0.05 ± 0.09*

Table 7. Mean difference (%) in cell density of *Aureoumbra lagunensis* (AL) and *Tisochrysis lutea* (TL) in controls for sedimentation at different initial starting densities (5.0×10^3 cells mL^{-1}, 5.0×10^4 cells mL^{-1}, and 5.0×10^5 cells mL^{-1}) over 24 hours.

MEAN HOUR 0	MEAN HOUR 24	DIFF (%)	MEAN HOUR 0	MEAN HOUR 24	DIFF (%)
AL 10^3 control			TL 10^3 control		
5.9E+03	6.3E+03	+6.25%	7.3E+03	8.7E+03	+19%
AL 10^4 control			TL 10^4 control		
5.3E+04	4.8E+04	-9%	5.6E+04	5.7E+04	+2%
AL 10^5 control			TL 10^5 control		
4.2E+05	5.6E+05	+33%	4.8E+05	4.7E+05	-2.5%

Figures

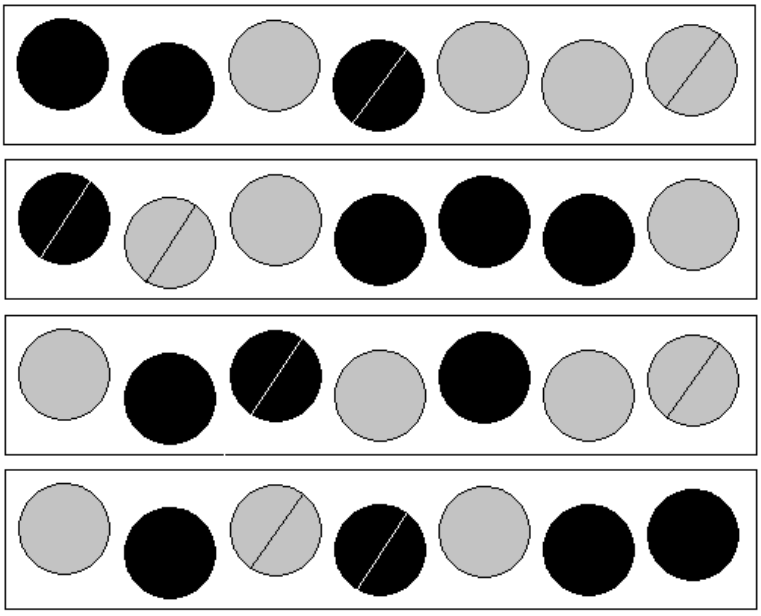

Figure 1. Example unbalanced, randomized block design for FR experiments. Rectangles illustrate shallow 3.05 m-long, 45 cm-deep tanks (i.e. individual "blocks" [not to scale]). Black circles are HAB replicates, gray circles are Tisochrysis lutea replicates; circles with a diagonal line through are control beakers for sedimentation.

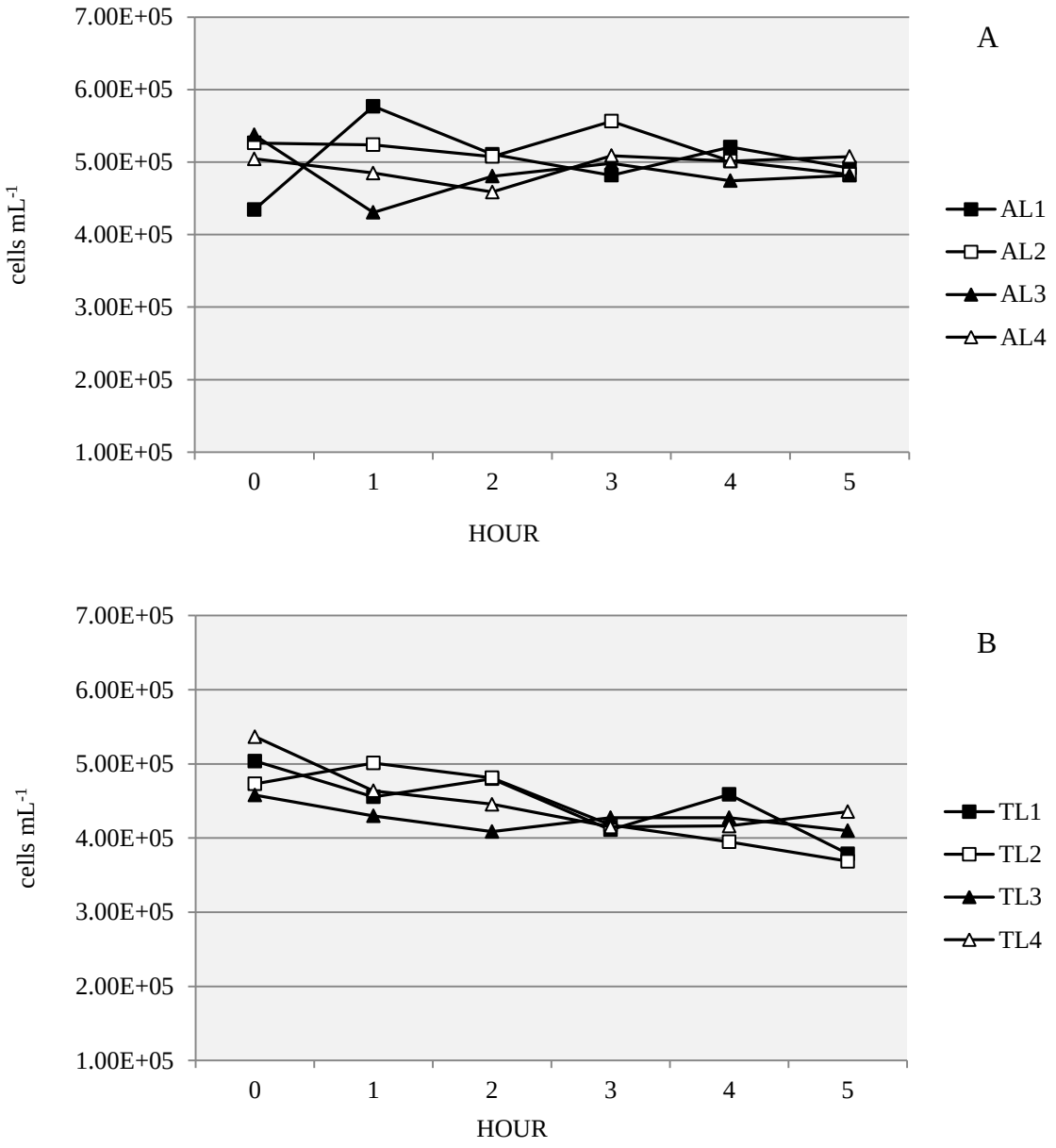

Figure 2. Cell densities of *Aureoumbra lagunensis* (AL; A) and *Tisochrysis lutea* (TL; B) in replicate (n=4 for each species) control beakers for sedimentation (tunicate excluded). AL densities fluctuated around a mean of 4.9 x 10^5 cells mL^{-1}, whereas TL densities declined from a mean of 4.9 x 10^5 cells mL^{-1} at Hour 0 to 4.0 x 10^5 cells mL^{-1} at Hour 5.

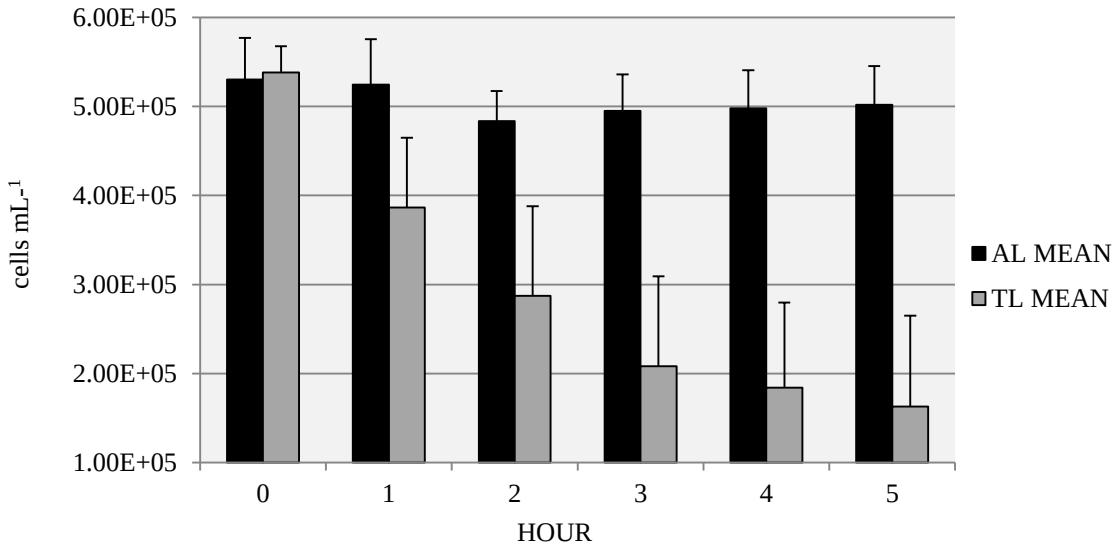

Figure 3. Mean algal cell densities (± s.d., n=10 for each algae species) over time. AL) *Styela plicata* exposed to *Aureoumbra lagunensis* (starting ~5.2 x 10^5 cells mL^{-1}); TL) *Styela plicata* exposed to *Tisochrysis lutea* (starting ~5.3 x 10^5 cells mL^{-1}).

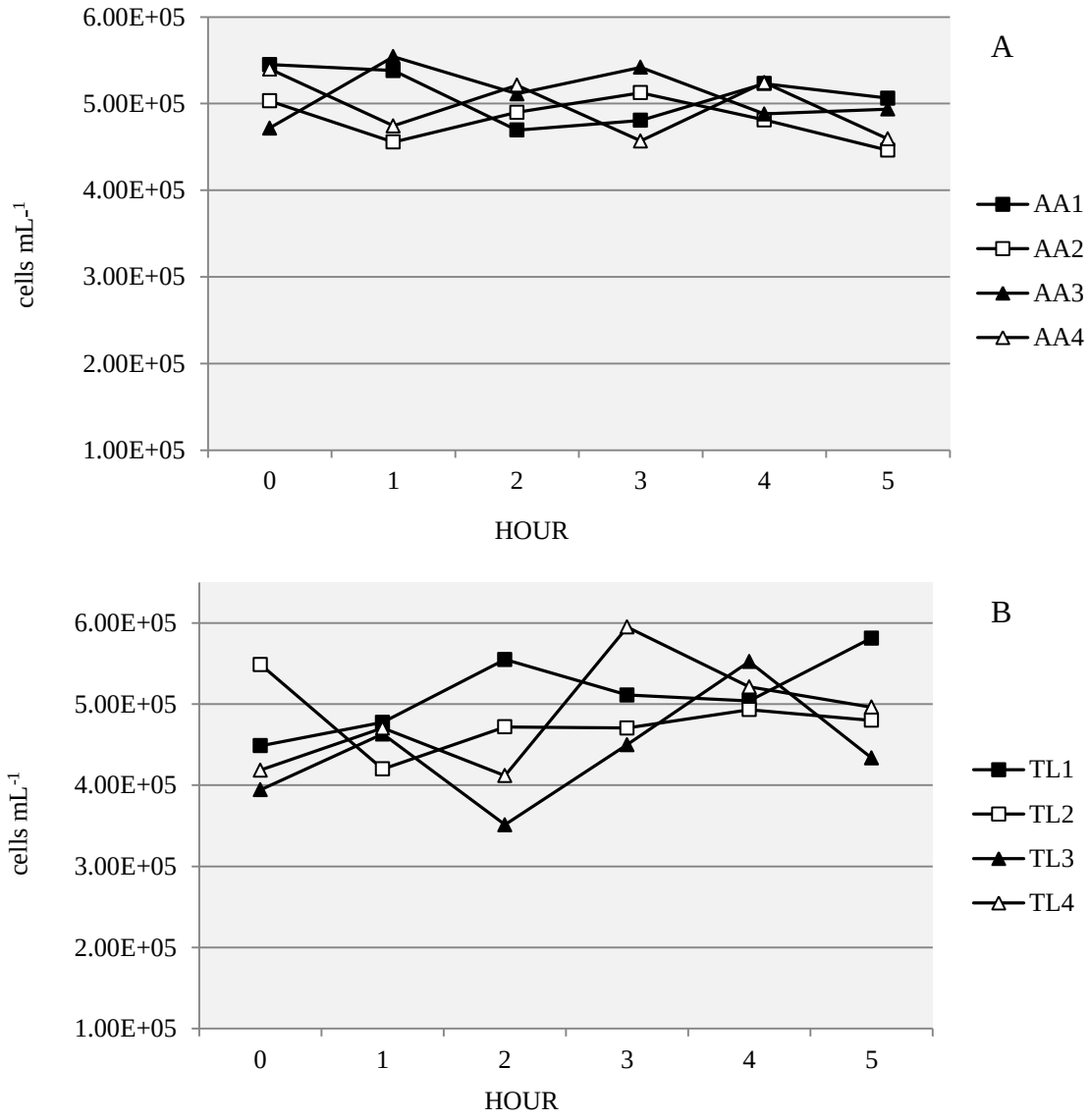

Figure 4. Cell densities of *Aureococcus anophagefferens* (AA; A) and *Tisochrysis lutea* (TL; B) in replicate (n=4 for each species) control beakers for sedimentation (tunicate excluded). AA densities fluctuated around a mean of 5.0 x 10^5 cells mL^{-1}, whereas TL densities fluctuated around a mean of 4.8 x 10^5 cells mL^{-1}.

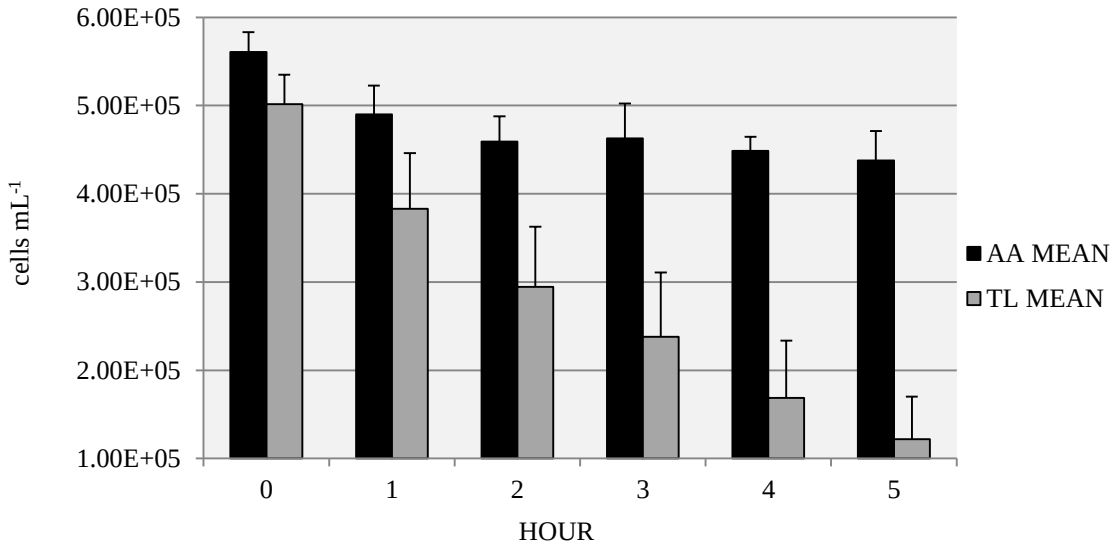

Figure 5. Mean algal cell densities (± s.d., n=10 for each algae species) over time. AA) *Styela plicata* exposed to *Aureococcus anophagefferens* (starting ~5.6 x 10^5 cells mL^{-1}); TL) Styela plicata exposed to *Tisochrysis lutea* (starting ~5.0 x 10^5 cells mL^{-1}).

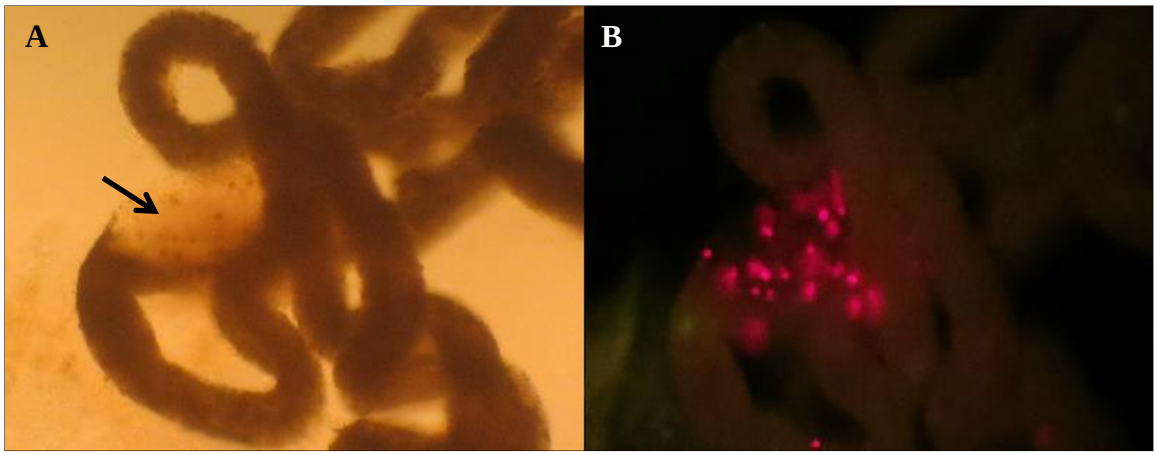

Figure 6. Microscopic image (200X magnification) of A) *Styela plicata* fecal deposit with visible light microscopy and B) Same sample under fluorescence (excitation filter 490 nm; emission filter 515 nm). Active chlorophyll fluoresces red (B) and was used to identify potentially viable algae cells. A cluster of cells is visible, and encapsulated in either an air bubble or mucous pocket embedded within the fecal deposit, as indicated by the arrow.

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