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Cultivation of a Commercially Important Macroalga (Ulva sp) for Polyculture Research in Coastal Georgia

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CULTIVATION OF A COMMERCIALLY IMPORTANT MACROALGA (*ULVA* SP) FOR POLYCULTURE RESEARCH IN COASTAL GEORGIA

by

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(Under the Direction of John Carroll)

ABSTRACT

The Georgia coast has shown great potential for large-scale oyster aquaculture, and the rate at which the sector has grown demands implementation of novel techniques to ensure sustainability and success in the face of climate change. One practice – integrated multi-trophic aquaculture (IMTA) or polyculture – is known to have positive effects on the health and quality of farmed organisms and the surrounding environment through low pH/alkalinity amelioration, while also being a unique and viable way for a farming operation to diversify its products. To date, there has been no such attempt at investigating or demonstrating macroalgae culture in Georgia. One species, sea lettuce (*Ulva* sp), is widely cultivated around the world for its many useful applications. Before any large-scale polyculture can occur in Georgia, there is a need to investigate the feasibility of farming sea lettuce and the potential interactions with oysters. *Ulva* specimens were collected from coastal Georgia for use in spore settlement and ocean grow-out trials. Seeded ropes were placed at two experimental small-scale plots on Skidaway Island, GA and grown in the field for ~3 months. 890 g of *Ulva* was cultivated in total (~15 $g \cdot cm^{-1}$ of rope) at a maximum daily growth rate of 1.1 $g \cdot day^{-1}$. Additionally, a 1-month lab experiment and several 50-min incubations were conducted in which Eastern oysters (*Crassostrea virginica*) and *Ulva* were cultivated together and separately to determine any influences on water quality or organismal growth. Under daytime laboratory conditions, total alkalinity, pH, and dissolved

oxygen were higher in *Ulva* and polyculture treatments than controls and oyster-only treatments, illustrating the buffering influence of a photosynthesizing organism on water chemistry. The results of this study can inform future research in shellfish-seaweed polyculture in the Southeast US.

INDEX WORDS: Bioremediation, Climate change mitigation, CO_2 removal, *Crassostrea virginica*, Georgia, IMTA, Integrated multi-trophic aquaculture, Oyster farming, Polyculture, Sea lettuce, Seaweed farming, Sustainable aquaculture, *Ulva* sp

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by

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DEDICATION

This thesis is dedicated to my parents Brian and Phyllis Mundy, my grandparents Jerie Chromchik and Larry and Claudia Mundy for their unconditional love and support, the single temperature logger tragically lost at sea that taught me ecology is often messy, and the piece of corrugated aluminum that almost took my eye but gave me a great scar.

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CHAPTER 1

PROJECT BACKGROUND

1. Introduction

1.1 A Growing Aquaculture Industry

Driven by demands for a sustainable protein source, growth of the aquaculture sector has risen significantly over the past 30 years and shows no signs of slowing down. Most recent estimates have shown that production of extractive species like bivalves and seaweeds has increased to almost 60 million metric tons, roughly on par with fed species like finfish and crustaceans (Verdegem et al. 2023). Global fisheries and aquaculture production exceeded 122 million metric tons in 2020 and represents 57% of total world seafood production, surpassing traditional capture fisheries (Chopin & Tacon 2020), with the aquaculture of seaweeds representing approximately 35 million metric tons (FAO 2022). Global production is dominated by Asian aquaculture, which accounts for over 90% of production. Conversely, the United States is one of the largest importers of seafood, with imports representing up to 90% of consumption (Botta et al. 2020).

Despite representing only a small percentage of the aquaculture sector, Eastern oyster (*Crassostrea virginica*) aquaculture in the US is a rapidly growing industry and there is a strong push to increase oyster culture capacity domestically, particularly in the southeastern US (Revell 2021). Eastern oyster production exceeded $134.4 million in sales (USDA 2019). Although traditional aquaculture faces criticisms for its potential negative impacts to water quality, shellfish aquaculture is generally considered sustainable and environmentally beneficial (Buschmann et al. 1994, Botta et al. 2020, Smaal et al. 2019). The main threats to oyster aquaculture include climate change, mostly in the form

of ocean acidification, sea level rise, and global temperature increase (Clements and Chopin 2016, Ahmed et al. 2019). In order to fulfill increasing demand for marine "blue food" options and safeguard against an uncertain climate future, new developments and unique strategies are needed in marine aquaculture production.

One proposed strategy is the integrated aquaculture of shellfish and seaweed, referred to by many as Integrated Multi-Trophic Aquaculture (IMTA) or simply polyculture (Chopin et al. 2001). The majority of industrial agriculture and aquaculture in the United States focuses on optimization of a single crop species for maximum yield, often to the detriment of the environment. In contrast, polyculture practices focus on cultivating multiple crop species, often of different trophic levels in ecological equilibrium. Wastes or uneaten food produced by one trophic level become the inputs needed to support adjacent levels. Polyculture has existed for millenia, with some of the earliest examples found in South and Southeast Asia in the farming of carp, mulberry trees, and silkworms (Ruddle et al. 1983, Fig. 1.1). Polyculture practices such as this can also be found elsewhere. For example, in the southern US crayfish were historically co-cultured with rice in large fields (Ackefors 2000). Rice is planted in the spring, and when the plants reach optimum heights in June, the fields are flooded and (if necessary) stocked with crayfish. The crayfish emerge from their burrows in the fields to breed and feed on rice stubble, the material that is left over after harvesting rice. Rice and crayfish are then harvested in alternating seasons.

Recently, increasing attention has been given to polyculture in fully aquatic and marine systems. These practices can be categorized along an intensive-extensive scale (Fong et al. 2023). On the extreme end of the intensive scale, there are recirculating

aquaculture systems (RAS). These systems are high yield, high intensity operations that cultivate multiple organisms in land-based systems. The most complicated of these systems features a main crop (fish or shrimp), a deposit/filter feeder (sea cucumbers or shellfish), and a seaweed (Al-Hafedh et al. 2015). Uneaten food and particulate waste produced by the main crop species are absorbed by deposit or suspension feeders, and dissolved waste like ammonia and phosphate are taken up by seaweed. RAS are capable of significant yields, but require equally high energy, labor, and capital to maintain operation.

On the opposite end, there are relatively low-intensity methods that feature ocean-based extensive operations. The best examples can be found in Sanggou Bay in China, where large floating kelp farms are cultivated adjacent to shellfish cages/ropes and finfish cages (Li et al. 2021). In these scenarios, seaweed cultivation can benefit shellfish by absorbing CO_2 through photosynthesis, alleviating environmental stress caused by low pH (Tang et al. 2011, Xiao et al. 2021, Young et al. 2022). Within these dense kelp farms, seawater pH was significantly higher than the surrounding area (Li et al. 2021), providing evidence that can alter water chemistry significantly. Extensive polyculture of macroalgae and shellfish has the potential to diversify product availability, mitigate water quality, and benefit the health of farmed organisms and the surrounding environment (FAO 2009). Additionally, the lower costs and energy associated with this type of polyculture may make it easier to integrate into existing shellfish farming in the US.

1.2 A History of Seaweed Farming

Macroalgae farming and harvest has an equally long history of practice. For example, *limu* (seaweed) is a component of traditional Hawai'ian cuisine (Abbott 1984),

and seaweeds have been harvested as a cultural crop for centuries in Ireland (Monagail & Morrison 2020). The bulk of seaweed farming globally is composed of only 4 taxa: *Saccharina japonica* (kombu), *Eucheuma* spp, *Gracilaria* spp, and *Porphyra* spp (nori). Kombu alone represented over a third of all global seaweed production in 2018 (FAO 2020). The United States may lack any major historical or cultural identity associated with seaweed, but demand has been steadily increasing in recent years due to consumer interest in alternative protein sources, enhanced food security, and sustainability (Kim et al. 2017).

Seaweed farming has also been proposed as a method of short-term carbon sequestration and an important component of blue carbon, which can be used by farmers to move their operations towards carbon neutrality (Gao et al. 2018a, Raven 2018). Natural macroalgal habitats together with salt marshes, seagrass beds, and mangrove forests represent Blue Carbon Ecosystems (BCEs). Although they cover less than 10% of the ocean, BCEs have the potential to store massive amounts of carbon due to photosynthesis (Bertram et al. 2021). The majority of this carbon storage is found in below-ground biomass, sediments, and in the case of macroalgae, exported to the deep sea through currents. In order to enhance this natural ability to sequester carbon, scientists and other agencies have begun to explore farming and sinking macroalgae into deep ocean layers as a mechanism for carbon removal (Krause-Jensen et al. 2018). However, there is much research to be done into the long- and short-term implications of these practices.

Other ecosystem services of macroalgae include removal of nitrogen and other excess nutrients (i.e. bioremediation), providing habitat for other organisms, and limiting harmful algal blooms (HABs) through allelopathy (Tang & Gobler 2011, Lv et al. 2021, Pezzolesi et al. 2021); extracts from *Ulva* and *Gracilaria* were found to inhibit the growth

of both *Aureococcus anophagefferens* and *Heterosigma akashiwo* in a laboratory environment (Sylvers & Gobler 2021, Nan et al. 2008). These findings have major implications for shellfish aquaculture and fisheries specifically, as HAB presence is a common reason for the closures of farming seasons.

Sea lettuce (*Ulva* spp.) is a cosmopolitan genus of green algae that has a high nutrient uptake rate and is commercially viable in the edible seaweed market, pharmaceutical, and biofuel industries (Macchiavello & Bulboa 2014, Korzen & Israel 2015). Green algae as a whole represent less than 1% of the global seaweed market, but their diverse applications show potential. In many land-based IMTA and RAS operations, *Ulva* is used to absorb effluent from the main crop species, usually finfish and shrimp, and research suggests that high nutrient loads can improve the nutrient profile of *Ulva*, increasing its value (Gao et al. 2022, Stedt et al. 2022). The polysaccharide ulvan, a major component of the *Ulva* cell wall, has shown promise in medical and agricultural applications (Coiai et al. 2021, Li et al. 2023). *Ulva* is commercially farmed in the North Atlantic in ocean-based operations, and in land-based operations in Europe and South America (Olsson et al. 2020, Garcia-Poza et al. 2022). The US-based seaweed aquaculture sector is incredibly small, however according to a recent GIS-based suitability assessment of the continental United States, much of the Southeast and Gulf Coast regions could support expansion of seaweed farming (Geddie & Hall 2020).

However, some research suggests that *Ulva* might not be a great candidate for polyculture. For example, extracts taken from *Ulva compressa* may decrease oyster larval survival, especially in combination with additional nutrient loading (Green-Gavrielidis et al. 2018). Additionally, the larval Pacific oyster (*Crassostrea gigas*) showed impaired

development when exposed to extracts taken from two other bloom-forming ulvoids (*Ulva lactuca* and *Ulvaria obscura*; Nelson & Gregg 2013). *Ulva* species are notoriously difficult to identify without the use of molecular techniques, though some cell morphologies are unique to certain species (e.g. number of pyrenoids, cell shape/size, plastid location within cell; Guidone et al. 2013). This difficulty in identification coupled with the potential for species-specific effects on co-occurring organisms means that identifying *Ulva* species is paramount when proposing polyculture of *Ulva* and shellfish. The examples of *Ulva* farming above pale in comparison to the farming of red and brown seaweeds, which together make up over 99% of global seaweed mariculture (Moreira et al. 2021).

1.3 Roadblocks to Advancements in Aquaculture

The majority of seaweed farming is concentrated in Asian countries (FAO 2020), with the US held back by various legislative, financial, and cultural barriers (Kinney 2017). Seaweed is currently classified as a raw agricultural commodity (RAC) by the FDA (Sec. 201 (r) of the Federal Food, Drug, and Cosmetic Act 2023). Although this group also includes foods such as fruits, vegetables, and meats, the Act does not clearly identify seaweed as either produce or seafood at the present time. This distinction means that US-based seaweed aquaculture exists in a legal gray area difficult to traverse for farmers interested in expanding their crops to include macroalgae.

IMTA itself is fraught with confusing and ill-defined terminology. Kinney (2017) found that some respondents of their survey disliked the term, due to the use of the word “aquaculture” and its associated bad reputation in the public eye. Some respondents were also unsure as to how close organisms had to be to each other to qualify the operation as

IMTA, and few practiced what is typically considered to be full IMTA - that is, an integrated system containing 3 trophic levels: finfish, shellfish, and seaweed. Still, that definition is arbitrary, and to date there is no universally accepted definition for IMTA. Financially speaking, diminishing returns and high startup costs were cited as the primary reason practitioners abandoned or did not consider IMTA or polyculture as a long-term option. For the practice to become more adaptable, the policies outlining the practice must be streamlined, terminology needs to be well-defined or expanded, and initial costs need to be curtailed, although these objectives lie well outside the scope of my thesis research.

2. Project Objectives

US shellfish aquaculture has much to gain by implementing new and sustainable techniques, and it stands to reason that polyculture of oysters and seaweed would have numerous economic and ecological benefits. My research consisted of two main sections: a series of lab-based polyculture experiments & a field-based farming experiment. The objective of the polyculture experiments was to examine the effects of *Ulva* on water quality, shellfish and seaweed growth, and survival in a fully factorial long-term (1 month) coculture experiment and a series of short-term (50 min) incubations with living oysters (*Crassostrea virginica*).

There were four objectives of the field experiment. Seaweed was first collected from jetties, floating docks, and any rocky outcroppings on North Tybee and Skidaway Islands, GA. The second objective was to establish a long-term culture of *Ulva* to increase biomass for use in farming trials and laboratory-based polyculture experiments. The third objective was to induce spore formation and release of *Ulva* and settle spores onto synthetic rope in accordance with existing commercial farming methods. Finally, the fourth objective was to investigate the viability of macroalgae farming in coastal GA by transitioning the seaweed-embedded ropes to two simulated farm sites on Skidaway Island, GA, where they were grown for a period of ~3 months.

3. Figures

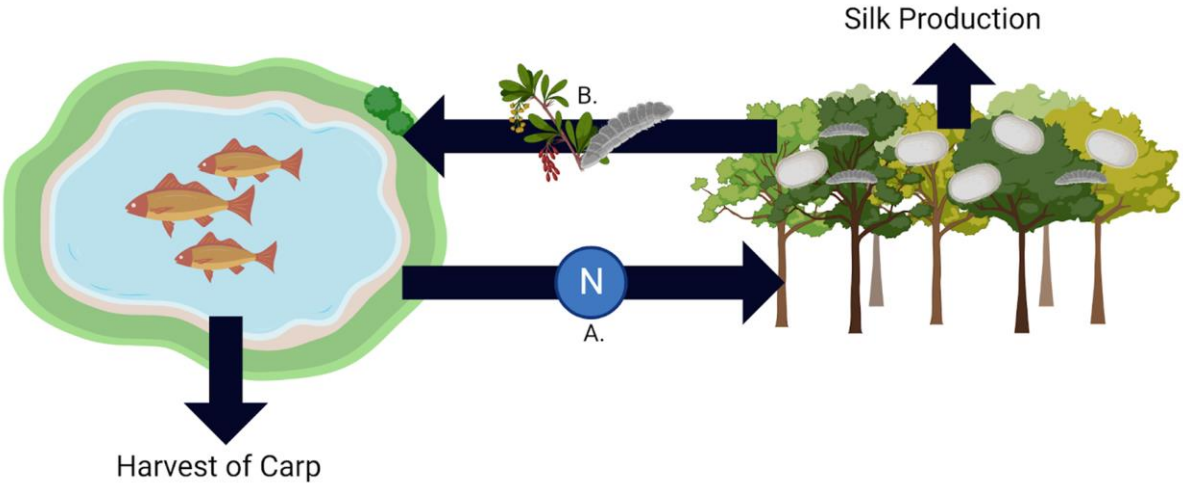


Figure 1.1 Carp were cultured in ponds, and the waste produced by the carp was taken up by mulberry trees. Mulberry trees are the preferred host plant of the domesticated silk moth, the cocoons of which are used in the production of silk. The trees absorb nutrients from ponds (A) and mulberry fruits and a portion of the silkworms not needed for sericulture (B) could then be fed to the carp, thus completing the cycle. Created in BioRender.

CHAPTER 2

FEASIBILITY OF SEA LETTUCE (*ULVA*) MARICULTURE AND EFFECTS OF OYSTER-*ULVA* POLYCULTURE

1. Introduction

Although global aquaculture has been on the rise since the 1950s, the bulk of this increase is due to China dominating the sector; other countries have failed to replicate this phenomenon (FAO 2020). As it stands, the US is a net importer of premium half-shell oysters, the majority of which come from Canada, Mexico, and South Korea (Botta et al. 2020). Increasing domestic shellfish aquaculture production remains a high priority in order to fill the gap created by the decline in wild oyster populations. Historically, advancement of shellfish aquaculture has been curtailed by lack of new technologies that are cost effective and functional at scale. Additionally, the threat of ocean acidification (OA) and its impact on the future of shellfish aquaculture remains on the minds of growers and scientists (Clements & Chopin 2016). Incorporating seaweed aquaculture into shellfish farming may function as a way to address both of these concerns.

Although the bulk of seaweed diversity in North America occurs in temperate regions and along the west coast, research suggests that seaweed farming may be feasible in areas less dominated by macroalgae, such as the southeast US (Geddie & Hall 2020). *Ulva*, commonly referred to as sea lettuce, may not be the most cultivated genus of macroalgae, but it is considered an ideal candidate for aquaculture due to its high growth rate, edibility, and numerous industrial applications (Bird et al. 2011, Craigie 2011, Morais et al. 2020). Cultivation of algae alongside shellfish also provides important ecosystem services and economic benefits, such as improved water quality, reduction of

OA, and diversification of crop yield for farmers. Shellfish and seaweed polyculture has been shown to increase the CO_2 removal capacity of coastal ecosystems (Tang et al 2011). Central to this study is the growing body of research suggesting that macroalgae and other submerged aquatic vegetation (SAV) may positively influence calcification rates and overall health of bivalves by alleviating OA stress through CO_2 removal (Chung et al. 2017, Wahl et al. 2017, Groner et al. 2018, Ricart et al. 2021, Hengjie et al. 2023). Bivalve and seaweed aquaculture has also been associated with higher abundance and species richness of fish and invertebrates, suggesting that the benefits of these operations may extend to habitat-related interactions (Theuerkauf et al. 2020).

Such shellfish-seaweed polyculture systems exist in many parts of the world and have been shown to be manageable even at relatively large scales. The practice has a long history in Chinese aquaculture, particularly in Sanggou Bay in which polyculture operations have been maintained for decades (Shi et al 2013). In other parts of the world, polyculture is extensively utilized in land-based aquaculture as a method to remediate water prior to discharge (Cahill et al. 2010, Chopin and Tacon 2020, Gao et al. 2022). Recent adoption of floating oyster cage culture by many shellfish growers has made it more feasible to incorporate seaweed farming into these types of oyster farms in the form of parallel placement of shellfish cages and seaweed lines (Kim et al. 2017).

In coastal Georgia, there is tremendous interest in growing local aquaculture capacity. Recently, the state adjusted its management strategies regarding oyster aquaculture in an attempt to promote industry growth. It is crucial for new farms to explore polyculture of oysters and seaweed as a Best Management Practice for maximizing and diversifying product yield, building a sustainable local seafood market,

and safeguarding the industry and environment from the threats posed by climate change and ocean acidification. The proposed experiments were intended to explore polyculture between oysters and *Ulva* in Georgia. Specifically, I sought to 1) characterize the influence of oysters and algae on water chemistry using a 1 month lab experiment and several short-term (50 min) chamber incubations, and 2) explore the potential for ocean-based native *Ulva* farming in coastal Georgia by way of a proof-of-concept study in the field. The results of these lab experiments and field study will be critical information for Georgia growers interested in pursuing seaweed as a second crop. Furthermore, investigating changes in water chemistry due to algae presence can help scientists and stakeholders understand how macroalgae can affect the environment at different scales; an important step in quantifying the carbon removal capacity of these organisms.

2. Methods

2.1 Long-Term Polyculture Experiment

In order to determine the potential impacts of the macroalga *Ulva* on oysters in co-culture, I conducted a fully-factorial tank experiment consisting of 4 treatments: *Ulva* and oysters together in polyculture (P), oysters only (O), *Ulva* only (A), and a control containing saltwater (C). The experiment lasted for 33 days from October 9th to November 11th of 2023. Oysters were obtained from the UGA MAREX and Georgia Sea Grant Shellfish Hatchery on Skidaway Island, GA (from this point on referred to as UGA hatchery). The tank experiment consisted of 10 L aquaria (n = 6 per treatment) filled with artificial seawater (20 ppt, 20-22 ℃). While there was not direct temperature control within tanks, the experiment was conducted in a climate-controlled room. However, to capture any variation in room temperature, data loggers (n = 7, HOBO 64k Pendant Temp) were randomly assigned to treatment tanks. All tanks received a daily addition of 0.25 mL commercial shellfish feed diluted 50% with filtered saltwater (Reed Mariculture Shellfish Diet 1800, ~2 billion cells/mL undiluted), following Reed Mariculture volume recommendations. This amount increased slightly to 0.3 mL halfway through the experiment to account for any increases in oyster size. Light was provided via LED grow lights (Barrina LED 42W grow lights, 80-100 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}, 12:12 photoperiod). Light requirements were determined from existing *Ulva* aquaculture research (Steinhagen et al. 2022), and while experimental light level was much lower than environmental levels typically experienced by *Ulva* (1160.60 ± 466.94 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} at UGA Hatchery Jan-Apr), 80-100 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} more accurately represents light levels achievable by growers.

Treatments with oysters received 10 oysters (length = 1.08 cm ± 0.17) affixed to ceramic tiles. Sets of 5 oysters were glued to tiles for pre- and post-experiment imagery to allow for individual oyster measurements (Fig. 2.1). Two tiles were placed into each of the P and O aquaria. On average, each oyster tile weighed 32.18 g (± 0.57 g). I subtracted individual tile mass from the total mass of the tile + oysters to determine oyster mass per tile. This measure was then divided by 5 to calculate an average single oyster mass per tile. This mass was used both to determine the amount of algae to use in P treatments and to estimate the oyster biomass change at the end of the experiment. The mass of a single oyster was only 0.18 g (+/- 0.03 g) at the start of the experiment, a very small measurement that necessitated the use of a bulk mass of 5 oysters per tile. To prevent overstocking of treatment tanks, oysters and algae were added at a ratio of ~1.2:1, with an average total oyster mass of 1.77 g (+/- 0.15 g) per tank and an average algal mass of 1.53 g (+/- 0.04 g) in P treatments. Though prior research has found ideal stocking ratios of oysters and seaweed to be 4:1, this was too extreme of a ratio given the small size of my experimental units (Han et al. 2017). A treatments received similar algal biomass to the P treatments. Algae and oysters were physically separated from each other in P treatments using a mesh barrier. *Ulva* for the experiment was purchased from a macroalgae supplier (AlgaeBarn, LLC).

Oyster growth was measured at the beginning and end of the period (see Appendix A for full protocol). Following termination of the experiment, oysters were grouped by tile, photographed, placed in labeled plastic bags and frozen for at least 24 hours.

Shell area was measured via ImageJ using initial and final photos of each oyster tile. The instantaneous growth coefficient for shell area (*k*) was calculated using the following formula:

$$k = (ln x_2 - ln x_2)/(t_2 - t_1) \times 100$$

Where *k* is the instantaneous growth rate and x_2 and x_1 represent oyster shell area (in cm^2) at times t_2 and t_1, respectively (Krebs 1972). Oysters were then thawed, removed from their tiles, weighed, and dried in an oven for ~36 hours at 60℃, after which dry weight was recorded. Mass and soft tissue content measurements could not be measured individually due to the small size and fragility of the oysters and represent aggregated measurements in which all 10 oysters per tank were averaged. All oysters were then combusted in a muffle furnace for ~4 hours at 450℃ to remove any organic matter. Approximate soft tissue content was determined by subtracting the combusted weight from the dry weight.

To measure *Ulva* growth, algae were spun in a salad spinner to remove excess water and weighed every other day for the first 2 weeks, followed by weekly measurements (see Appendix B & C for full protocols). Following termination of the experiment, algae were placed in labeled plastic bags and frozen for at least 24 hours. Samples were then thawed and placed in a drying oven at 60℃ for 24 hours to prepare them for C:N ratio analysis. All samples were pulverized in a shaker ball mill until a fine and uniform consistency was achieved, after which 2.59 ± 0.28 mg of each replicate were loaded into tin capsules and stored in 96 well plates for future elemental composition analysis.

Water quality (pH, alkalinity, NH_3, and $PO4^{3-}$) was measured bi-weekly over the course of the 6-week experiment using auto-titration (Metrohm Titrando) and a HACH DR3900 spectrophotometer (see Appendix D for full protocol). Water samples (~350 mL glass bottles) destined for alkalinity and pH testing were fixed with 400 µL $HgCl_2$ prior to testing (NOAA 2010). Samples taken for nutrient analysis (two 30 mL centrifuge tubes per tank) were frozen prior to analysis to preserve integrity in accordance with prior research (Fellman et al. 2008). In order to better visualize treatment effects on water chemistry, all values were also subtracted from mean of the control at each time point; these are referred to as delta (Δ) values. In order to examine the relationship between the change in total alkalinity (ΔTA) and dissolved inorganic carbon (ΔDIC) and how it relates to calcification, both measurements were separated by treatment and plotted against each other. Relative calcification was determined through regression analysis of ΔTA and ΔDIC (Cyronak et al. 2018). Salinity was monitored 2-3 times per week using a refractometer. Total alkalinity, pH, temperature, and salinity were used to calculate pCO_2, total CO_2 (i.e. DIC), and saturation state of calcite (Ω_{cal}) with the program CO2SYS. To ensure survival of the oysters, 20% water changes were performed 3 times a week following collection of water samples.

2.2 Polyculture Incubation Experiment

In order to isolate the effects of the oysters and *Ulva* on water chemistry from any background influences in the previous long-term experiment (e.g tank aeration, food, water changes), I ran short-term incubation experiments (see Appendix E for full protocol). Approximately 65 live oysters (mass 0.67 ± 0.2 g each) were obtained from the UGA Shellfish Hatchery. Wild *Ulva* was collected from the dock at the same location and

held in laboratory conditions prior to the experiment. Experiments were conducted in 350 mL glass BOD bottles (n = 4 per treatment) and incubated for 50 minutes using artificial seawater (22 ppt, 17-19 ℃) using the same treatment designations as the long-term experiment.

A total of 4 incubation experiments were conducted; two were light incubations under LED grow lights (80-100 μmol · m^{-2} · s^{-1}), and two were dark incubations in total darkness. One dark incubation served to explore water quality changes during algal respiration, the other dark incubation consisted entirely of control bottles (i.e. only saltwater) to correct for background oxygen production during the light runs, which varied by ~0.47 mg/L DO and ~0.05 pH units. For reference, probe accuracy for DO and pH were ± 0.1-0.2 mg/L and ± 0.02 units respectively. During each run, all bottles were agitated using a shaker table. Algae and polyculture treatments received 1.50 ± 0.17 g of *Ulva*. Oyster and polyculture treatments received 1.50 ± 0.16 g of oysters. All bottles were filled with artificial seawater until overflowing, ensuring no air bubbles were present. Prior to capping the bottles, initial dissolved oxygen, pH, temperature, and conductivity were measured using a Hach HQ2100 series portable meter. Bottles were then incubated under their respective conditions for 50 minutes, after which final water chemistry measurements were taken. Temperature remained relatively constant during each run.

2.3 Native Ulva *Collection, Culture & Field Growth*

Ulva stock was collected from North Tybee Island, GA May-Jul 2023 and Dec 2024, and from the dock at the UGA Shellfish Hatchery in Dec 2023 and Jan 2024. A web map of the project and collection sites can be found at https://arcg.is/1avjr01. Mature *Ulva* thalli were hand-collected from exposed rock, mudflats, docks, and marsh grass stems at

the various collection sites. Any free-floating macroalgae present within sample sites were also collected. Collected algae were rinsed in seawater to remove any large fouling organisms or debris and placed in partially sealed plastic bags and in a cooler to maintain temperature (unsealed storage of algae allows off-gassing). Once transported to campus, samples were further rinsed in sterile filtered saltwater to remove debris, placed in holding tanks containing seawater analogous to site-specific temperature and salinity conditions, then drip acclimated to lab conditions (20-22 ppt artificial seawater, 20-22 ℃) over the course of 1 hour.

Ulva specimens were identified using a combination of dichotomous keys, light microscopy, and cross-sectioning (Fig. 2.2) (Schneider & Searles 1991). However, due to the cryptic nature of the taxa and current state of taxonomic discourse around the *Ulva* genus, species identification was largely impossible without the use of DNA barcoding techniques (Guidone et al. 2013, Melton & Lopez-Bautista 2021). Instead, *Ulva* specimens were kept in tanks labeled by site name and date. Microscopy and cross-sectioning yielded no distinct differences between *Ulva* specimens, apart from currently accepted variation in cellular morphology taken from identification guides (e.g. cells with rounded edges, 1-2 pyrenoids per cell, flattened blade with no discernible tubular morphology). Identification without the use of DNA techniques leads me to determine that collected specimens most closely resemble *Ulva compressa* (Melton & Lopez-Bautista, Schneider & Searles 1991). Laboratory culture and spore-settlement trials of *Ulva* began in June 2023 according to existing protocols (Kaladharan & Gireesh 2003, Redmond et al. 2014, Steinhagen et al 2021). Collected *Ulva* was acclimated to aerated culture units (20-22 ppt artificial seawater, 20-22 ℃) and inoculated with fertilizer medium (45 g Osmocote slow-release

fertilizer) under a 16:8 photoperiod (Barrina LED 42W grow lights, 80-100 PAR). Additionally, a solution of GeO_2 (0.5 mL L^{-1}) was added to each culture tank to prevent the growth of diatoms (Shea & Chopin 2007; see Appendix F for full protocol). *Ulva* was then allowed to propagate asexually through growth and fragmentation, punctuated by weekly water changes.

Synthetic polypropylene rope was used as a settlement substrate for field grow-out, as described by Geng et al (2015). Ropes were rinsed in DI water and immersed in boiling water to remove any residue left over from manufacturing, followed by another DI water rinse. The ropes were cut to the experimental length (~60 cm), strung onto a PVC frame (40 cm x 20 cm), and placed in the bottom of a 20 L glass aquarium. To induce swarmer (i.e. spore and gamete) release for settlement trials, I used the fragmentation method described by Steinhagen et al. (2021). Propagule formation was confirmed through microscopy (Fig. 2.3). Water containing swarmers was then added to a small tank (10 L) filled with sterile saltwater and the settlement substrate. Following a small period of light exposure (~5 minutes) to allow any fertilization of gametes to occur, the tanks were kept in the dark for 24 hours to facilitate settlement, after which settlement success was again confirmed through microscopy (see Appendix G for full protocol).

These ropes were then placed in lightly aerated culture tanks held at previously described conditions until algae were large enough to be transitioned to a field setting. A solution of GeO_2 (0.5 mL L^{-1}) was added to prevent the growth of diatoms. Approximately ~4 weeks post-settlement when the *Ulva* germlings were <1 cm in length (Fig. 2.4A), *Ulva* was deployed into the field. Seaweed ropes were transitioned to 2 sites on either side of Skidaway Island, GA (leeward and windward, Fig. 2.4B) so their growth could be

evaluated in a simulated farm setting. Nine ropes were placed parallel to each other within a buoyant PVC frame (60 cm x 180 cm, n = 1 per site) and tied to the dock at each site. They were deployed on January 17th, 2024 and were kept in the field until April 3rd, during which their growth was measured bi-weekly. Ropes were removed from the array, spun in a salad spinner and weighed on a balance. Water temperature, salinity, turbidity, and photosynthetic photon flux density (PPFD) were measured at these time points at both locations. Towards the end of the experiment, I measured pH within and away from the seaweed rope array at the UGA hatchery using HOBO *in situ* pH loggers. At the end of the experiment, all *Ulva* was removed from ropes for each site, spun to remove excess water, separated from any fouling organisms, weighed, and frozen. Additionally, cross-sections were taken and visually compared to parent *Ulva* to confirm similarities. I then subsampled the *Ulva* from each site for elemental analysis, the methods of which can be found in section 2.1.

2.4 Statistical Analyses

Normality and homogeneity of variance were checked via Shapiro-Wilk and Levene's tests, and all data fulfilled sphericity. In the long-term experiment, oyster weight change, soft tissue, and instantaneous growth rate of shell area (k) were analyzed using a nested ANOVA with tile (A or B) nested into treatment (O or P) with an added random effect. *Ulva* growth by treatment was analyzed using repeated measures ANOVA. Due to numerous dependent variables and known interaction/correlation among water chemistry measurements, principal components analysis (PCA) was used to identify major trends in the data. Variables used in PCA were: pH, TA, DIC, pCO_2, Ω_{cal}, TA:DIC ratio, NH_3, and PO_4.

In order to further examine whether oysters and algae affected water quality, I used a repeated measures ANOVA with individual water quality parameters (e.g. pH, alkalinity) as the response variable and aquaculture treatment (A, C, O, or P) as categorical factors. Additionally, to better visualize treatment effects on water chemistry compared to controls, all values were also subtracted from the mean of the control at each time point, represented as delta (Δ) values. When assumptions were met, Tukey HSD post-hoc tests were done on each measurement and date to determine which treatments differed from each other. In situations where assumptions were not met, nonparametric Steel-Dwass tests were used. TA:DIC ratio as a proxy for relative calcification was analyzed using linear regression. In the incubation experiment, pH differences in light runs were analyzed using randomized block ANOVA and Tukey HSD post-hoc tests with experiment date as the blocking factor. Since the experiment run on 4/20/24 consisted entirely of control runs intended only to correct for background oxygen production, this was also omitted from analysis to preserve a uniform sample size.

pH differences in the dark run were analyzed using an ANOVA and Tukey HSD post-hoc tests. Light and dark DO differences did not satisfy assumptions and were analyzed separately using Friedman's and Kruskal-Wallis tests respectively. One outlier in the oyster treatment on 4/10/24 was omitted from analysis due to an unusually high DO value. The experiment run on 4/20/24 consisted entirely of control runs and was also omitted from analysis to preserve a uniform sample size. Field environmental measurements (PAR at 0.5 m depth, salinity, and turbidity) and final *Ulva* rope weight differences between sites were analyzed using t-tests. All tests were performed at an alpha of 0.05 in JMP Pro 16.0. ArcGIS Online was used to create a field site web map.

3. Results

3.1 Long-Term Polyculture Experiment

All oysters survived the experiment, regardless of treatment and in spite of increasing biofouling in the tanks throughout the experiment. Mean weight, soft tissue content, and instantaneous growth rate of shell area did not differ significantly between polycultured (P) and monocultured (O) oysters ($DF_{weight} = 3$, $F_{weight} = 1.34$, $p_{weight} = 0.29$; $DF_{soft\ tissue} = 3$, $F_{soft\ tissue} = 0.92$, $p_{soft\ tissue} = 0.43$; $DF_k = 3$, $F_{kt} = 0.29$, $p_k = 0.84$; Fig. 2.5). On average, oysters increased by 135.30 ± 47.76mg in the O treatment and 120.67 ± 47.46 mg in the P treatment. Instantaneous growth rate (k) was almost 20% higher in P treatments ($k_P = 0.49 \pm 0.48$ cm^2) compared to O treatments ($k_O = 0.41 \pm 0.50$ cm^2), although due to high variability, these were not statistically significant differences. Loss of shell area was also observed in both treatments. Prior to analysis, any negative soft tissue values were removed. Mean soft tissue content was slightly higher in O treatments(34.00 mg ± 12.70 in O vs. 27.08 mg ± 8.39 in P).

Algal biomass increased significantly over time, however these increases did not differ significantly by treatment ($DF_{time} = 4, 40$, $UUE_{time} = 1$, $p_{time} < 0.0001$; $DF_{time*treatment} = 4, 40$, $UUE_{time*treatment} = 1$, $p_{time*treatment} = 0.33$; Fig. 2.6A). *Ulva* biomass in the polyculture (P) treatment tripled in weight by the end of the experiment, and *Ulva* in the algae-only (A) treatment nearly quadrupled in weight. Across each of the 6 experimental tanks, a total of 35.74 and 27.27 g of *Ulva* was grown in A and P treatments, respectively. Variation in weight increased towards the end of the experiment, and was particularly high (± 2.47 g) in the A treatment on the last day. Overall algal growth was lower and less variable in the P treatment. Additionally, though we did not measure aspects of *Ulva*

morphology other than mass, experimental *Ulva* darkened significantly over time and became much more compacted, compared to what all pieces looked like before the experiment (Fig. 2.6B).

Principal components analysis of water chemistry revealed interesting trends in the data (Fig. 2.7-2.9). Clear clustering can be seen by treatment, particularly at day 21 (Fig. 2.8). Overall, the bulk of variation was captured by the first 2 principal components (day 7 = 89.82%; day 21 = 86.74%; day 33 = 80.52%). At day 21, PC1 was primarily made up of pH, Ωcal, and the TA:DIC ratio and PC2 was made up of the remaining measurements. At this time point, TA:DIC ratio, pH, and Ωcal were positively associated with A treatments and negatively associated with O treatments. NH_3, PO_4, pCO_2, TA, and DIC were positively associated with C (control) treatments, and P treatments did not show strong association with any parameters. This suggests a strong influence of treatment on the water chemistry parameters in the experiment, and was further examined using RM ANOVAs.

Of the measured carbonate chemistry parameters, all except pCO_2 were higher at the end of the experiment than at the beginning. However, the behavior of these parameters varied by treatment and collection date (Fig. 2.10, 2.11). Across all parameters, treatment and time were significant (Table 2.1). Treatment*time was significant in all except ΔTA ($DF_{time*treatment}$ = 4, 30, $UUE_{time*treatment}$ = 1, p = 0.0617). The highest total alkalinity values were observed in the algae (A) and control (C) treatments and lowest in the O treatment, with P often falling between these two extremes (Fig. 2.10A, 2.11A). pH behaved similar to alkalinity, but there was a dramatic decrease on 10/30/23 in the O treatment, which then rebounded by the end of the experiment (Fig.

2.10B, 2.11B). Dissolved inorganic carbon (DIC) follows a similar trend to total alkalinity (Fig. 2.10C, 2.11C). On the final day, the distribution of pH values resembled that of total alkalinity. This observation is evidently inverted in the pCO_2 response, sufficiently highlighting the relationship between pH and pCO_2, apart from the low pCO_2 at day 7 (Fig. 2.10D, 2.11D). Ω_{cal} behaved similarly to pH, with the highest values observed in C and A treatments and lowest in the O treatment (Fig. 2.10E, 2.11E). TA:DIC ratio mirrors pH and higher ratios result in increased buffering capacity (Fig. 2.10F, 2.11F). The A treatments showed the least amount of calcification (Fig. 2.12). Calcification did not differ significantly between A and P treatments, though O treatments did differ from both (DF = 2, $X^2 = 1.24$, $p = 0.006$; Fig. 2.12).

Ammonia ($[NH_3]$) and phosphate ($[PO_4^{3-}]$) concentrations differed significantly among treatments and across time (Table 2.1). P, A, and O treatments were able to keep ammonia levels below 0.5 ppm by day 7 and below 0.25 ppm by day 21 of the experiment. The control treatment showed the highest ammonia levels, and it took an additional 12 days for $[NH_3]$ to drop below 0.25 ppm as compared to the three other treatments (Fig. 2.13A). Phosphate levels showed a less dramatic trend, increasing over the course of the experiment to >1 ppm by day 7, after which levels remained similar in all treatments except A, which dropped to ~0.5 ppm (Fig. 2.13B). Of all treatments, A showed the greatest dampening effect on both nutrient levels.

3.2 Polyculture Incubation Experiment

Results from the short-term incubation trials resemble those from the 1 month experiment. In the dark incubations, the change in pH (ΔpH) over 50 minutes was significantly greater in the polyculture treatment than control treatments (DF = 3, F = 3.75,

p = 0.0413), but the ΔpH of oyster and algae treatments was not significantly different from either control or polyculture (Fig. 2.14). In general, pH was lower in all treatments at the end of the dark runs. Contrary to this, we saw drastic and significant increases in pH in the light runs for most treatments and more variation in the ΔpH response to treatment (DF = 3, F = 8.59, p = 0.0004). The greatest increases were seen in the algae treatment, the lowest were in oyster treatments, and polyculture treatments showed a wide range of responses. The decrease in DO (ΔDO) during dark experiments was the greatest in the polyculture treatments, intermediate in algae treatments, and lowest in oyster and control treatments (Fig. 2.15). During light runs, algae and polyculture showed significantly higher DO than oyster and control treatments (DF = 3, F = 28.52, $p < 0.0001$). DO did not change significantly in control and oyster treatments and were similar to dark runs (DF = 3, $X^2 = 12.00$, p = 0.0074). Blocking factor (i.e. date) in Friedman's and randomized block ANOVA tests was not significant ($DF_{DO\ Light\ On} = 1$, $F_{DO\ Light\ On} = 0.76$, $p_{DO\ Light\ On} = 0.39$; $DF_{pH\ Light\ On} = 1$, $F_{pH\ Light\ On} = 3.93$, $p_{pH\ Light\ On} = 0.0582$).

3.3 Ulva *Field Growth*

From 1/17/24 to 4/3/24, I successfully grew 890 g of *Ulva* using seeded rope culture. This was all new growth, as starting biomass on the ropes was negligible and no major differences were seen in cell structure between parent algae and field-grown algae. More biomass was grown at Priest Landing (491.50 g) than at the UGA hatchery site in the Wilmington River (398.50 g), though not a significant amount (DF = 13.82, t = -0.97, p = 0.3505). Seaweed growth at both sites remained near zero until around 2/7/2024, after which it increased at both sites (Fig. 2.16). Between 2/7/2024 and 4/3/2024, daily mean growth rates at Priest's Landing and UGA hatchery dock were ~1.1 g and ~0.89 g per 60

cm rope, respectively. Both sites performed similarly, though biomass grown at the UGA hatchery site was more foliose in its morphology, and it reached this stage faster than the algae at Priest's Landing (Fig. 2.17). UGA algae showed slightly higher mass at all subsequent dates except for the final day, with this loss being attributed to adverse weather conditions on 4/2/2024. Ropes were dominated by *Ulva*, although other fouling organisms were present. The most commonly observed fouling organism was the sea grape *Molgula manhattensis*, followed by *Balanus* sp. barnacles. Fouling organisms were mostly found on the PVC and foam components of the array and not on *Ulva* ropes. Growth rate was highest during February and March, after which bleaching and loss of biomass was observed on ropes. These observations coincided with water temperatures >20 ℃ at the UGA site (Fig. 2.18).

Unfortunately, the temperature probe was lost at Priest Landing, so no temperature data is available for the duration of the study for that site. Salinity, PPFD at 0.5 m depth, and turbidity did not differ significantly between sites ($DF_{salinity}$ = 13.38, $t_{salinity}$ = -1.28, $p_{salinity}$ = 0.2213; DF_{PPFD} = 4.12, t_{PPFD} = -0.14, p_{PPFD} = 0.8949; $DF_{turbidity}$ = 10.56, $t_{turbidity}$ = -0.13, $p_{turbidity}$ = 0.8970; Fig. 19). In an attempt to detect any elevating influence of *Ulva* presence on environmental pH, I deployed several pH loggers at the UGA hatchery dock on 2 occasions. 3 loggers were placed within the seaweed array, and 2 more were placed >3 m away to serve as controls. The first deployment (4/3/24 - 4/4/24) was done with *Ulva* present, and the second (4/17/24 - 4/18/24) was done with no *Ulva* present in the array. During both observations, pH values within the seaweed array were consistently lower by 0.10 ± 0.03 units (Fig. 2.20).

4. Discussion

The presence of algae and oysters within both long-term and incubation experiments significantly impacted water chemistry. These observations were in line with what is known about primary productivity, nutrient absorption, respiration, and calcification in marine environments (Jiang et al 2014, Cyronak et al 2018, Han et al. 2020, Liberti et al. 2022). Photosynthetic *Ulva* drove pH and alkalinity up during the day by removing CO_2. By contrast, oysters - a calcifying organism - drove pH and alkalinity down through the processes of calcification and respiration. The most drastic changes were seen on day 21 of the experiment when the highest pH and TA values were found in the A treatments, the lowest values in the O treatments, but importantly, the P treatment often fell between the algae and oyster extremes. Overall, the water quality results suggest that macroalgae may help alleviate and buffer against stressful water quality conditions for shellfish (Anthony et al. 2013, Fernández et al. 2019, Wahl et al. 2018, Xiao et al. 2021, Young et al. 2022). This buffering capacity is further highlighted by examining calcification via the change in TA versus the change in DIC. According to Cyronak et al. 2018, relative calcification can be determined through regression analysis of TA and DIC. For every mole of $CaCO_3$ precipitated or dissolved, the TA:DIC ratio changes at a rate of 2:1. Therefore, net community calcification (NCC) of a system increases as the slope of the line approaches 2. Calcification was highest in O treatments, and differed significantly between P and A treatments. Calcification was lowest in A and P treatments, as these systems contained higher net community production (NCP) in the form of photosynthesis. The reason slopes in the A treatments were not closer to 0 is likely due to the addition of alkalinity to the system in the form of water changes.

Algal presence was expected to decrease both ammonia and phosphate concentrations, and this expectation was supported by the outcome of the experiment. This is in line with existing research, as both ammonia and phosphate are essential in regulating metabolic activities in macroalgae and are reduced by algal absorption. Macroalgae cultivation has historically been used in enclosed bays and IMTA systems as a method of bioremediation (Tsagkamilis & Danielidis 2010, Huo et al. 2011, Al-Hafedh et al. 2015, Gao et al. 2018b). Nitrogen species (NH_3, NO_3^-, etc.) are of concern in many land-based aquaculture systems because they can become toxic in moderate/high levels, and this research has shown that macroalgae can be utilized as a biofilter, in some cases surpassing traditional bacterial biofiltration in its efficiency (Cahill et al. 2010). O, A, and P treatments were able to significantly lower ammonia to near 0 ppm by day 21, while the control took an additional 12 days to reach the same level. Overall, A treatments were the most effective at reducing both ammonia and phosphate levels.

While the decrease in nutrient levels in algae-containing treatments can be attributed to macroalgal absorption, the ammonia response in oyster treatments followed a similar but unexpected trajectory. Shellfish aquaculture has been seen as a potential nutrient sink through assimilation of particulate organic matter (Clements & Comeau 2019), however oysters are also a source of nitrogen and ammonia concentration was expected to increase in these tanks. The nutrient reduction in the oyster tanks may have resulted from a combination of this nutrient assimilation (i.e. ingestion of food) and absorption of excreted ammonia by phytoplankton and associated periphyton, similar to Pietros and Rice (2003), as all oysters and the majority of tanks had abundant microalgal

presence. The most abundantly seen microalgal species was *Tetraselmis marina*, assumed to have originated from shellfish diet as this species is the primary component (Fig. 2.21).

Despite the overall impacts on carbonate chemistry, I found no statistically significant differences in oyster responses such as soft tissue content or surface area. Previous research with *Ulva* and oysters suggests potential benefits to oysters, especially regarding shell growth (Young and Gobler 2018), which I also expected to observe. Shell growth was almost 20% higher in polyculture treatments relative to oysters alone in this experiment, and while this was not statistically significant, it may be biologically relevant. Prior studies have demonstrated significant differences in oyster shell growth between algae and control treatments, specifically in experiments with larger juvenile oysters (Young & Gobler 2018). Increasing shell size as spat may be particularly important for oysters to reduce the risk of predation, so any difference in shell growth could have important biological and ecological implications (Carroll & Finelli 2015). It is likely that the lack of statistical effects regarding shell growth and soft tissue could be due to variability and the nested sampling design, as well as the relatively short experimental time period (33 days). Other studies on similar timescales have focused on the effects of polyculture on water quality and algal growth, but not shellfish growth (Mao et al. 2009, Nardelli et al. 2019). Likely, much longer timescales are needed in order for differences in shell growth to manifest, as observed in a months-long study of pearl oyster *Pinctada martensi* growth in proximity to the red alga *Kappaphycus alvarezii* (Qian et al. 1996).

Though there were no differences in *Ulva* growth between treatments, the changes in *Ulva* pigmentation before and after the experiment are due most likely to the low light conditions. Irradiance, flow rate, nutrient concentration, and microbial community are all

known to significantly alter morphology of seaweed. The *Ulva* genus is well-adapted to low light levels. For example, in *Ulva lactuca*, the light compensation point for growth is ~2.5 μmol·m^{-2}·s^{-1}, significantly lower than experimental light levels (Sand-Jensen 1988). *Ulva* increases its photosynthetic light harvesting efficiency at low light levels by increasing the number of photosynthetic units, corresponding with an increase in chlorophyll concentration that darkens pigmentation (Riccardi and Solidaro 1996, Gao et al. 2016). Similarly, Bermejo et al. 2020 found that pigment concentration of the red alga *Gracilariopsis longissima* can be altered through manipulation of light intensity and nutrient levels. Although the light intensity used in this experiment falls within existing research, increasing light intensity would have likely had significant effects on *Ulva* productivity and pigmentation. It is commonly accepted that high SA:V ratios of algae grown in low-flow environments enhances the flux of nutrients and ions across the diffusion boundary layer; this would explain the increase in rugosity of *Ulva* in the long-term experiment (Hurd et al. 2000). In land-based aquaculture operations that utilize algae, the rate of flow and concentration of nutrients can be manipulated to increase biofiltering capacity or biomass production, depending on the priorities of the operation (Buschmann et al. 2001). In the field, *Ulva* morphology has been found to not only be dependent on environmental parameters but also associated bacterial communities (Toth et al. 2020, Mantri et al. 2020).

One caveat about the initial polyculture experiment is that water quality parameters were only measured during daylight hours in order to capture the effect of photosynthesis, as this is the primary mechanism contributing to the macroalgae-mediated seawater buffering described in existing research (Mongin et al. 2016, Groves & Rachootin 2019,

Li et al. 2021, Hengjie et al. 2023). It is well established diurnal fluctuation in pH occurs due to nightly respiration (Baumann & Smith 2017, Enochs et al. 2018, Cyronak et al. 2020), and this implied oscillation between high and low pH was likely more extreme in the polyculture treatment due to the compounding effect of both oyster and algal respiration in a closed system, and this may also have contributed to the lack of difference between polycultured and monocultured oysters. This was the motivation behind the short-term light and dark incubation trials, with the dark incubation demonstrating this compounding effect of polyculture on pH. The presence of *Ulva* had drastic effects on both DO and pH in these incubations, much more than the presence of oysters.

The minimal response of pH and DO in oyster incubations could have resulted from a number of factors, namely the presence of periphyton on the surface of the oyster shells or the possibility that 50 minutes was not long enough for oysters to acclimate to the bottles and reopen their valves. In these situations, oxygen production resulting from microalgae on the surface of the oysters could have slightly skewed measurements. The presence of air bubbles on oysters at the conclusion of the light incubations further suggests this was the case, even though oysters were cleaned prior to experiments (Fig. 2.22). Similar studies suggest incubation times on the order of several hours, though with the small volume of the BOD bottles used in this experiment (350 mL), the effect of *Ulva* on water chemistry would have likely greatly overshadowed any effect of the oysters if they were incubated for longer than 45-50 minutes at a stocking ratio of 1:1 (Han et al. 2107, Han et al. 2020). Ensuring that oysters are free of periphyton and altering the stocking ratio of *Ulva* and oysters is essential for future studies.

Results of *Ulva* rope culture verify that this type of farming is possible in coastal Georgia on a small timescale (~ 3 months), an area not known for seaweed diversity or seaweed farming, although there was a considerable delay in growth following deployment and a noted disconnect between the oyster and algae growing seasons. Although the oyster harvest season in Georgia is open from September to June, co-culture of *Ulva* would likely only be possible between December/January and April. Growers deploy oyster spat in grow-out cages beginning in March and April at the tail end of the *Ulva* growing season, suggesting that polyculture of these two species is not possible year-round (Bliss & Manley 2017). Growth rates in this experiment were lower than those in large-scale commercial harvest, which often approach 2 g *Ulva* per meter of rope per day (Steinhagen et al. 2022). However, to my knowledge, this is the first attempt to employ *Ulva* rope culture in Georgia; most research is conducted in the North Sea where temperatures rarely exceed 17°C. The time lag may have been due to acclimation, as temperatures were fluctuating at this time (Fig. 2.18). Additionally, research into the presence of an *Ulva* microbiome suggests that the assemblage of the holobiont is thought to play an important role in thallus morphology (Califano et al. 2020, Mantri et al. 2020). *Ulva* species grown in axenic culture do not develop the archetypal *Ulva* foliose morphology; this may also explain the delay in growth as lab-grown *Ulva* not only acclimated to field temperature, but also to a natural microbiome (Wichard 2015). Cultivating *Ulva* in contact with a natural microbiome instead of in axenic culture would be essential to producing an ideal and marketable blade morphology.

Although I was able to achieve seaweed growth in the field, the potential impacts of *Ulva* on water chemistry in the open field setting were less clear. Prior studies have found significant impacts of macroalgal presence on water column carbonate chemistry (Li et al. 2021, Hendriks et al. 2014). My field pH measurements within the seaweed array suggest that, while the buffering effect of macroalgae can be measured in a closed system, the observation of this phenomenon is entirely dependent on prevailing field conditions. Any pH increases mediated by *Ulva* in the array were overshadowed by a significant lowering effect of the environment, though the exact cause can only be speculated on. First, following deployment of the seaweed array, large amounts of vegetation and seafoam would become caught between ropes and required removal on a regular basis (Fig. 2.23). Second, the PVC and foam frame may have lowered water flow within the array. The presence of decaying organic matter inside the array, as well as the sheltering effect of the PVC frame itself, may have contributed to low pH measurements in comparison to the outside area. These observations highlight the importance of appropriate sampling and the considerable environmental variability that can be found in estuarine ecosystems, even in a relatively small area.

It is clear that *Ulva* and oysters have measurable opposing influences on water quality in closed-system experiments, and the co-culture of these two organisms resulted in water quality values that fell between the means of both solo treatments (i.e. *Ulva* (A) and oysters (O) in isolation). Moreover, these effects were strong enough to overcome the influence of regular aquarium maintenance such as water changes, feedings, and ample aeration. These findings clearly illustrate the “push and pull” relationship between these two organisms, suggesting that *Ulva* may be able to counter the pH-lowering effects of

calcification and respiration. This relationship can potentially be leveraged in multiple ways. Previous studies have revealed similar synergistic relationships between shellfish and seaweed, with seaweeds able to mediate biogenic acidification and nutrient concentrations in aquaculture operations (Macchiavello & Bulboa 2014, Han et al. 2020). For example, a land-based polyculture operation cultivating shellfish and seaweed could utilize *Ulva* not only as a nutrient absorber but as a way to increase pH, contributing to the health and survival of shellfish stock and allowing for the increase in water residence time.

With the field-based sea lettuce farming component of this study, we have demonstrated that *Ulva* mariculture is possible in coastal Georgia, however the limited window for growth and low amount of biomass produced suggest that large-scale polyculture of the two species is not feasible or profitable year-round and would not benefit existing oyster aquaculture. As it stands, the US lags behind other countries in macroalgae production, and there is considerable room for growth of seaweed aquaculture. Sustainable and locally produced seaweed represent a potential new product that may appeal to consumers. Research suggests local residents are interested in a source for fresh, locally sourced seafood and willing to pay premium for these products (Tookes et al. 2018, Shamshak et al. 2020). By continuing to refine these protocols and expand to other candidate macroalgae such as *Gracilaria* or *Agardhiella*, seaweed farming can become a viable additional practice for aquaculture leaseholders to utilize in order to diversify their crops and meet existing demands for locally produced seafoods. Unfortunately, the high summer temperatures and turbidity of coastal Georgia would not be ideal for an *Ulva* farming operation.

Lab and field findings from this research ultimately suggest that any commercial scale *Ulva* cultivation in Georgia would have to exist in a land-based operation. However, oyster growers may be able to harvest wild *Ulva* from their own floating cages, as it is known to recruit to aquaculture gear. In this case, significant work needs to be done to establish and clarify legislation regarding seaweed as a food product. Integrating seaweed farming into existing shellfish aquaculture should still be explored in other regions in order to answer questions about the economics of the practice (i.e. costs, benefits, marketability) as well as further illuminate the complex biogeochemical interactions between shellfish and seaweed.

5. Tables & Figures

Table 2.1 RM ANOVA results from long-term polyculture experiment. Data fulfilled assumption of sphericity. All tests performed at an alpha of 0.05.

	Factor			Time			Factor*Time		
Parameter	DF	F ratio	*p*	DF	Univar unadj E	*p*	DF	Univar unadj E	*p*
TA ($\mu mol \cdot kg^{-1}$)	3, 20	0.53	0.0003	2, 40	1	<0.0001	6, 40	1	0.0002
pH	3, 20	1.60	0.0002	2, 40	1	<0.0001	6, 40	1	0.0051
DIC ($\mu mol \cdot kg^{-1}$)	3, 20	1.27	0.0008	2, 40	1	<0.0001	6, 40	1	0.0128
pCO_2 (µatm)	3, 20	0.66	0.0152	2, 40	1	0.0002	6, 40	1	0.0128
Ω_{cal}	3, 20	2.50	<0.0001	2, 40	1	<0.0001	6, 40	1	<0.0001
TA:DIC ratio	3, 20	1.32	0.0006	2, 40	1	<0.0001	6, 40	1	<0.0001
ΔTA ($\mu mol \cdot kg^{-1}$)	2, 15	1.02	0.005	2, 30	1	<0.0001	4, 30	1	0.0617
ΔpH	2, 15	2.31	0.0001	2, 30	1	<0.0001	4, 30	1	<0.0001
ΔDIC ($\mu mol \cdot kg^{-1}$)	2, 15	1.50	0.0001	2, 30	1	<0.0001	4, 30	1	<0.0001
ΔpCO_2 (µatm)	2, 15	0.53	0.0417	2, 30	1	<0.0001	4, 30	1	<0.0001
$\Delta\Omega_{cal}$	2, 15	3.37	<0.0001	2, 30	1	<0.0001	4, 30	1	<0.0001
ΔTA:DIC ratio	2, 15	2.35	0.0001	2, 30	1	<0.0001	4, 30	1	<0.0001
$[NH_3]$ (ppm)	3, 20	4.59	<0.0001	2, 40	1	<0.0001	6, 40	1	<0.0001
$[PO_4]$ (ppm)	3, 20	1.22	0.001	2, 40	1	0.0002	6, 40	1	<0.0001

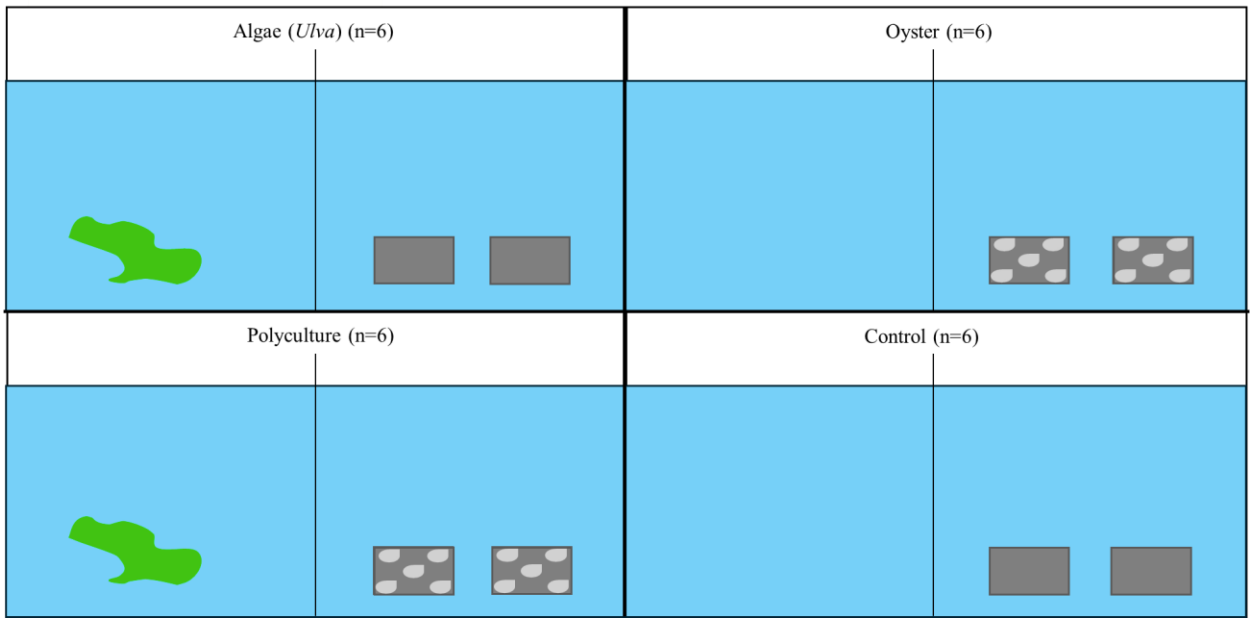


Figure 2.1 Diagram depicting long-term experimental design. Gray squares represent granite tiles used as oyster substrate. Blank tiles were used in algae and control treatments. Light gray dots represent oysters affixed to tiles in oyster and polyculture treatments. All tanks were under constant aeration.

Figure 2.2 Collection of *Ulva* photos, clockwise from left: *Ulva* specimen collected from Tybee Island; natural stand of *Ulva* attached to marsh grass and exposed at low tide; cross section of *Ulva* specimen at 1000x magnification. The distromatic cell arrangement in cross-section confirms identification.

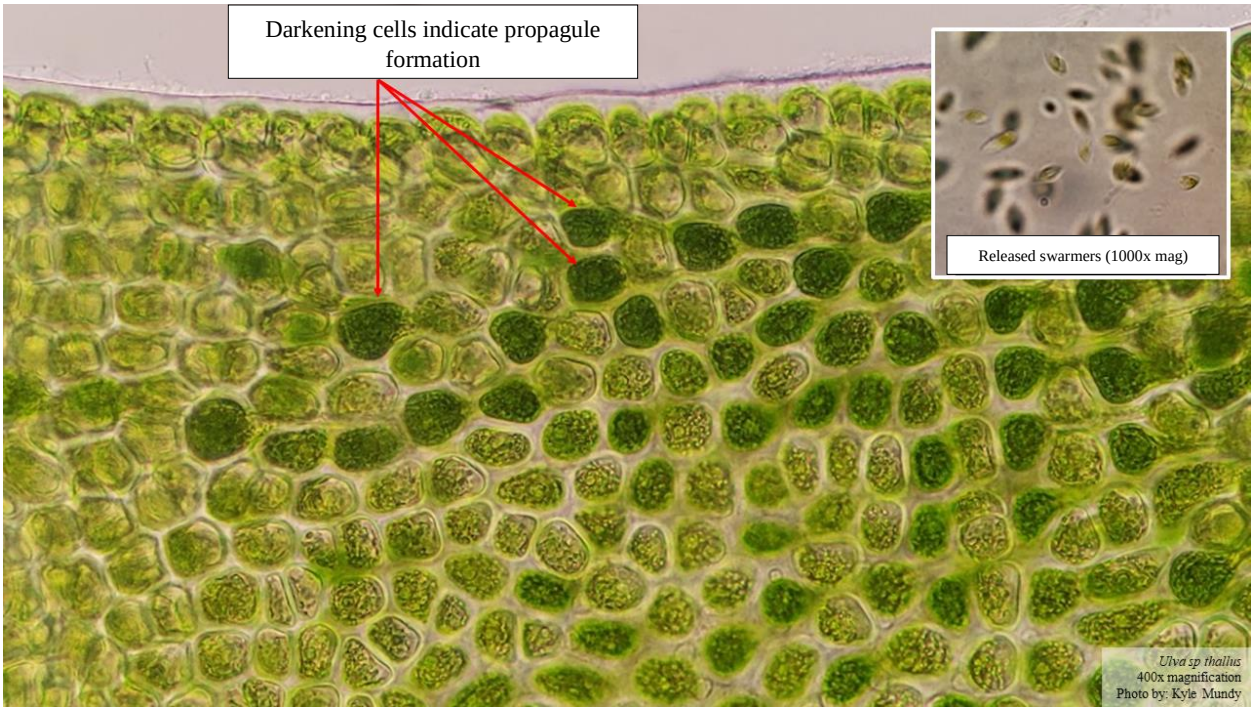


Figure 2.3 Propagule formation and release of swarmers in *Ulva* spp can be observed via microscopy.

Figure 2.4 (A) Closeup of a seaweed rope prior to deployment at the UGA hatchery dock, showing early stages of sea lettuce blade development. (B) Satellite imagery of the two Skidaway Island field locations with overlaid images of each site's seaweed array. Sites were chosen based on prevailing conditions and distance from the ocean, UGA being more sheltered in comparison to PL. Arrays were loaded with 9 *Ulva* ropes on Jan 17 2024 and attached to the dock at each location, after which *Ulva* was harvested at the 3 month mark (April 3 2024).

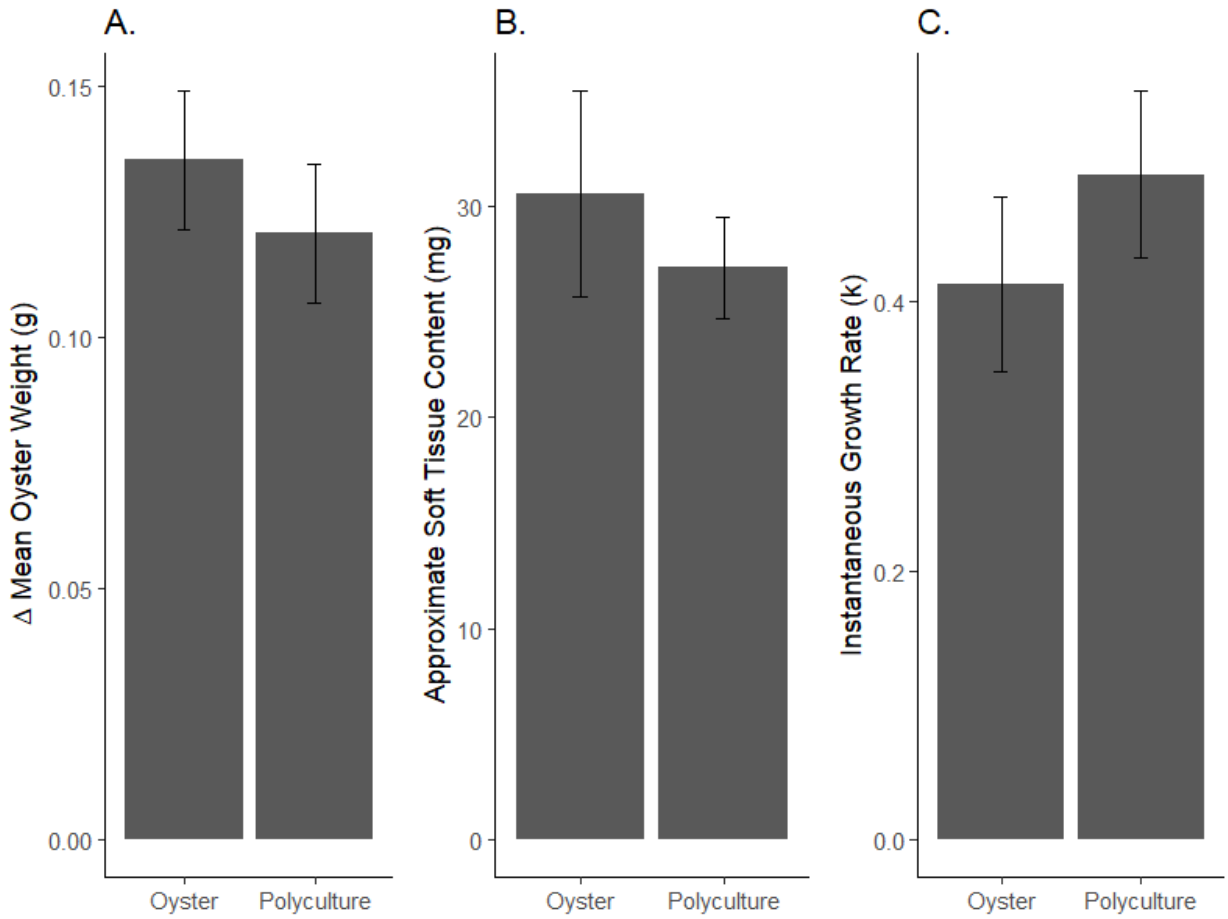


Figure 2.5 Mean measurements of oysters grown in monoculture and *Ulva* polyculture for 33 days. (A) Mean change in oyster weight (final - initial). Oyster tiles were dried with a kimwipe prior to weighing. Actual oyster weights were determined by subtracting total mass from tile weights. Values represent mean total oyster mass per tank (10 oysters per tank, n = 6 per treatment). (B) Approximate total tissue content per tank at the end of the experiment, determined through desiccation and combustion of oysters to calculate weight difference. (C) Instantaneous growth rate (k) of individual oyster shell area (cm^2) on each tile within each treatment. Error bars are standard errors. Oysters were measured using ImagJ.

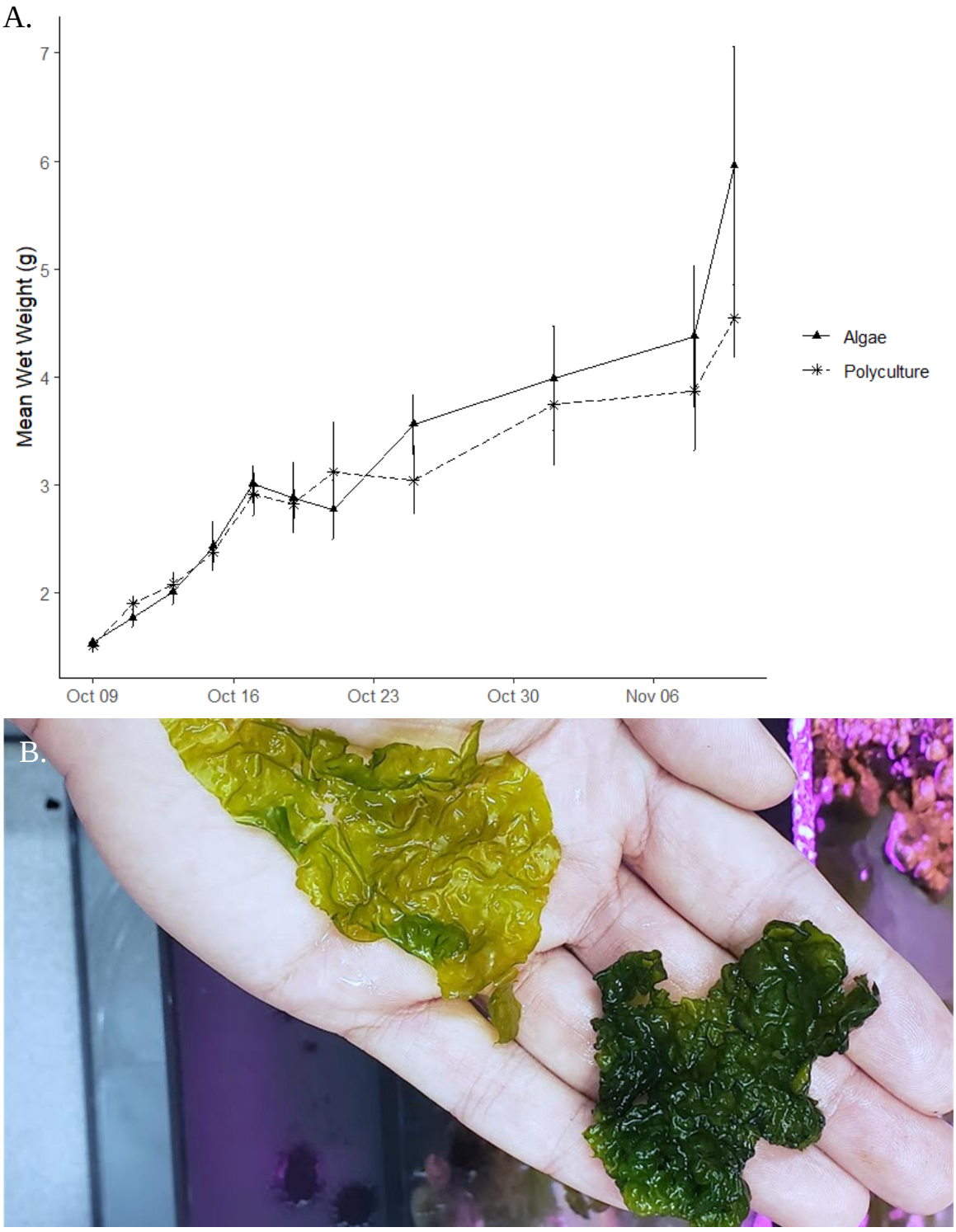
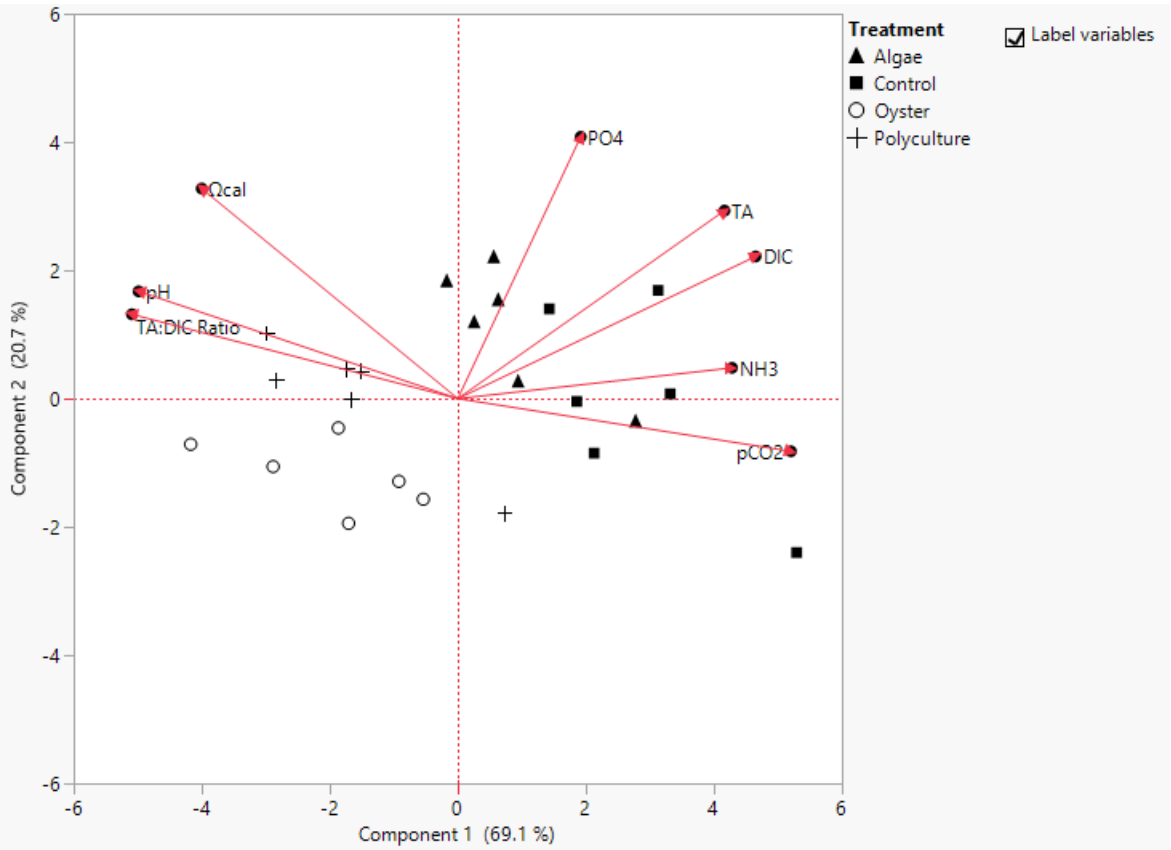


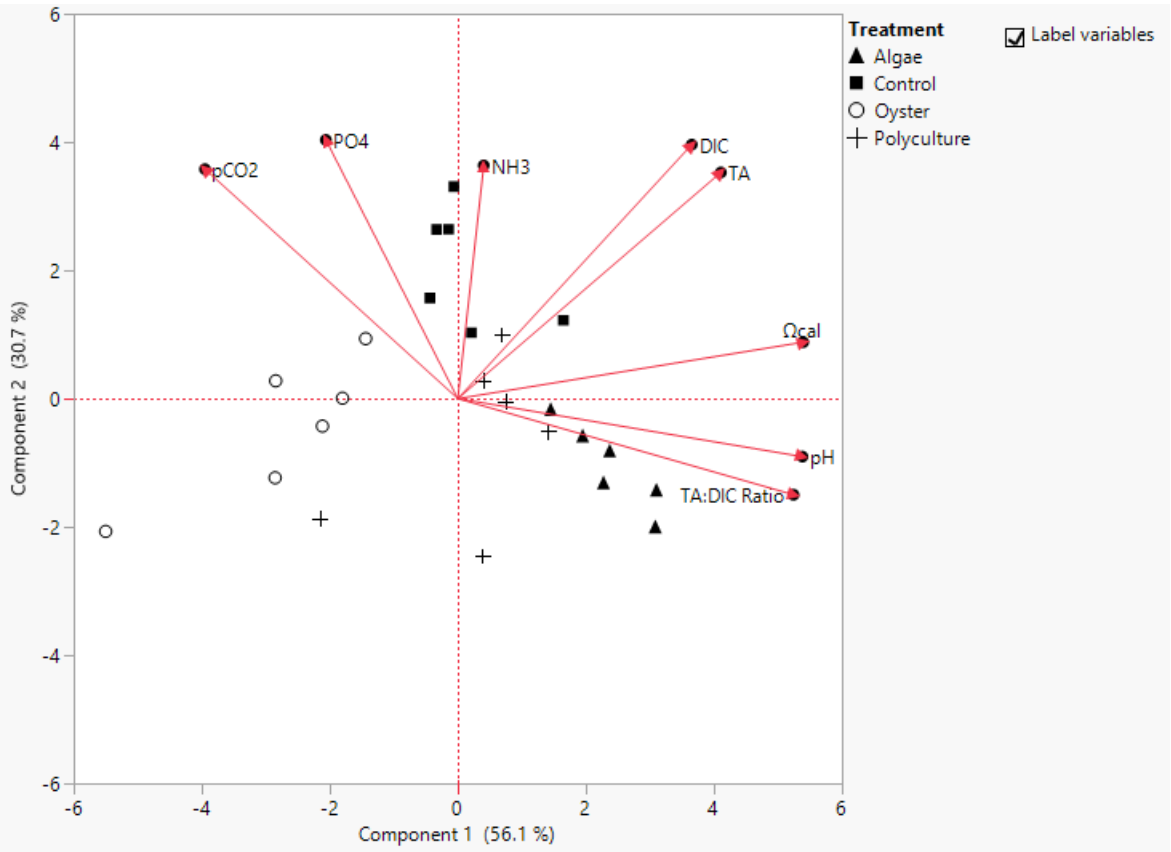
Figure 2.6 (A) Mean wet weight of experimental *Ulva* throughout the long-term polyculture experiment (n = 6). Thalli were spun in a salad spinner for 30 seconds before weighing to remove excess water. Error bars depict standard error. (B) Comparison of *Ulva* characteristics between non-experiment (left) and post-experiment (right) *Ulva* thalli.



Eigenvectors Day 7 (2023-10-16)

	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7	Prin8
TA	0.33471	0.43075	0.23163	-0.38184	-0.13295	0.16626	-0.10938	-0.66760
DIC	0.37373	0.32566	0.17013	-0.34183	-0.14695	0.21004	-0.01765	0.73651
pH	-0.40075	0.24579	0.15105	0.04473	-0.09595	0.38037	0.77426	-0.02853
pCO2	0.41802	-0.12054	-0.06112	-0.11262	0.81604	0.03731	0.35452	-0.03630
Ωcal	-0.32151	0.48118	0.28844	-0.11396	0.27255	-0.69811	0.01100	0.08460
NH3	0.34408	0.07062	0.57567	0.73812	-0.02013	-0.00034	-0.00161	-0.00018
PO4	0.15417	0.59968	-0.67636	0.39866	0.01447	0.00340	-0.00154	-0.00025
TA:DIC Ratio	-0.40918	0.19318	0.13709	0.07700	0.45903	0.54296	-0.51229	0.05076

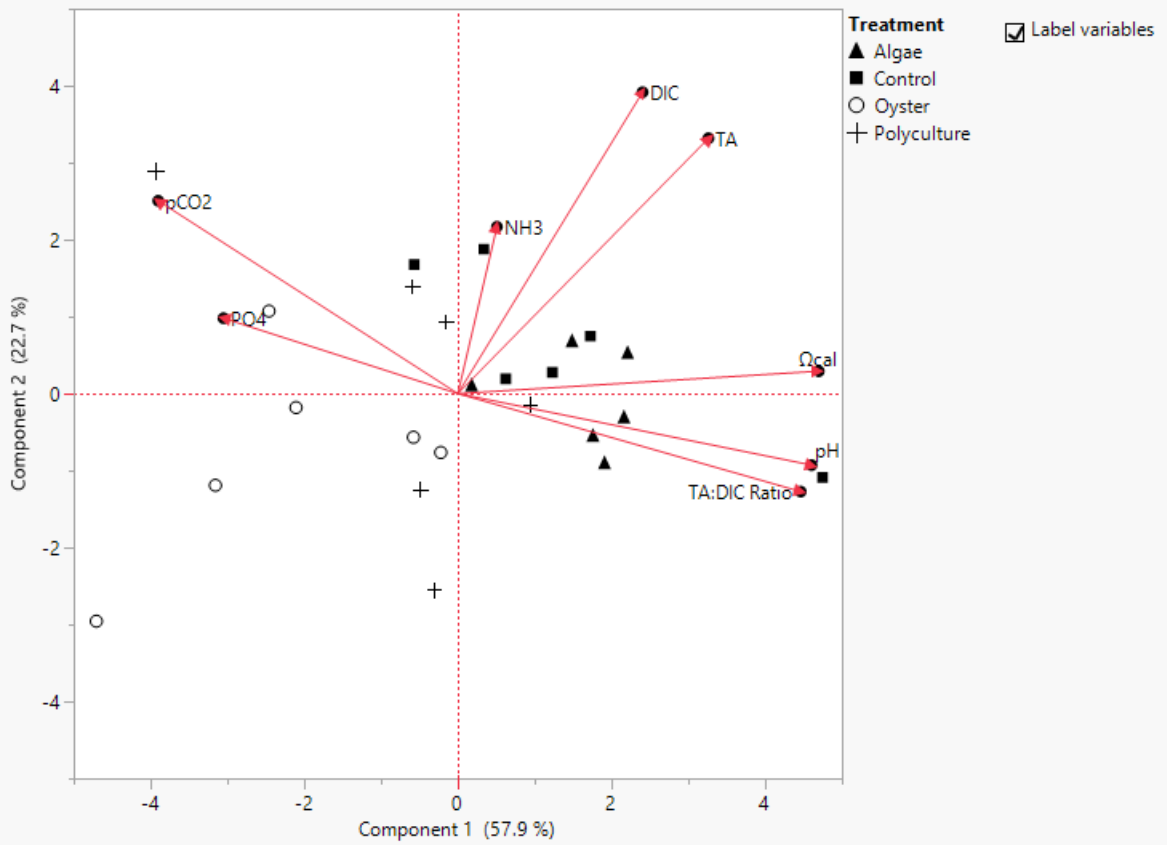
Figure 2.7 PCA biplot Day 7 (2023-10-16) with eigenvector table.



Eigenvectors Day 21 (2023-10-30)

	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7	Prin8
TA	0.35253	0.40928	0.12761	-0.24652	-0.18526	-0.08331	-0.15843	-0.75153
DIC	0.31353	0.45967	0.13586	-0.28332	-0.30640	-0.03212	-0.27260	0.64997
pH	0.46179	-0.10489	0.02714	0.19063	-0.39106	0.47692	0.59827	0.01899
pCO2	-0.33905	0.41530	0.06638	-0.41351	0.38770	0.56031	0.27004	-0.00057
Ωcal	0.46290	0.10214	0.06398	-0.04645	0.60122	-0.45990	0.42858	0.11129
NH3	0.03462	0.42185	-0.85912	0.28691	0.01964	0.00735	0.00178	-0.00004
PO4	-0.17696	0.46809	0.46670	0.72738	0.05020	-0.01060	0.00698	-0.00010
TA:DIC Ratio	0.44993	-0.17383	0.01134	0.19512	0.45187	0.48883	-0.53480	0.00146

Figure 2.8 PCA biplot Day 21 (2023-10-30) and eigenvector table.



Eigenvectors Day 33 (2021-11-10)

	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7	Prin8
TA	0.31934	0.51911	-0.17578	0.11604	-0.11074	-0.01226	-0.07416	-0.75251
DIC	0.23531	0.61276	-0.23202	0.09659	-0.22039	0.14832	-0.16772	0.63820
pH	0.45055	-0.14517	0.11177	0.13245	-0.03551	0.57343	0.64459	0.01773
pCO2	-0.38180	0.39202	-0.15414	-0.08850	0.74839	0.22153	0.24443	-0.00707
Ωcal	0.45976	0.04564	0.06592	0.13254	0.35464	-0.72049	0.30632	0.16098
NH3	0.04979	0.33924	0.78970	-0.50608	-0.05144	0.00697	-0.00037	0.00014
PO4	-0.29864	0.15330	0.47239	0.81412	-0.03645	-0.00669	-0.00198	-0.00011
TA:DIC Ratio	0.43686	-0.19908	0.16703	0.12392	0.49807	0.28417	-0.63031	0.01234

Figure 2.9 PCA biplot Day 33 (2023-11-10) and eigenvector table.

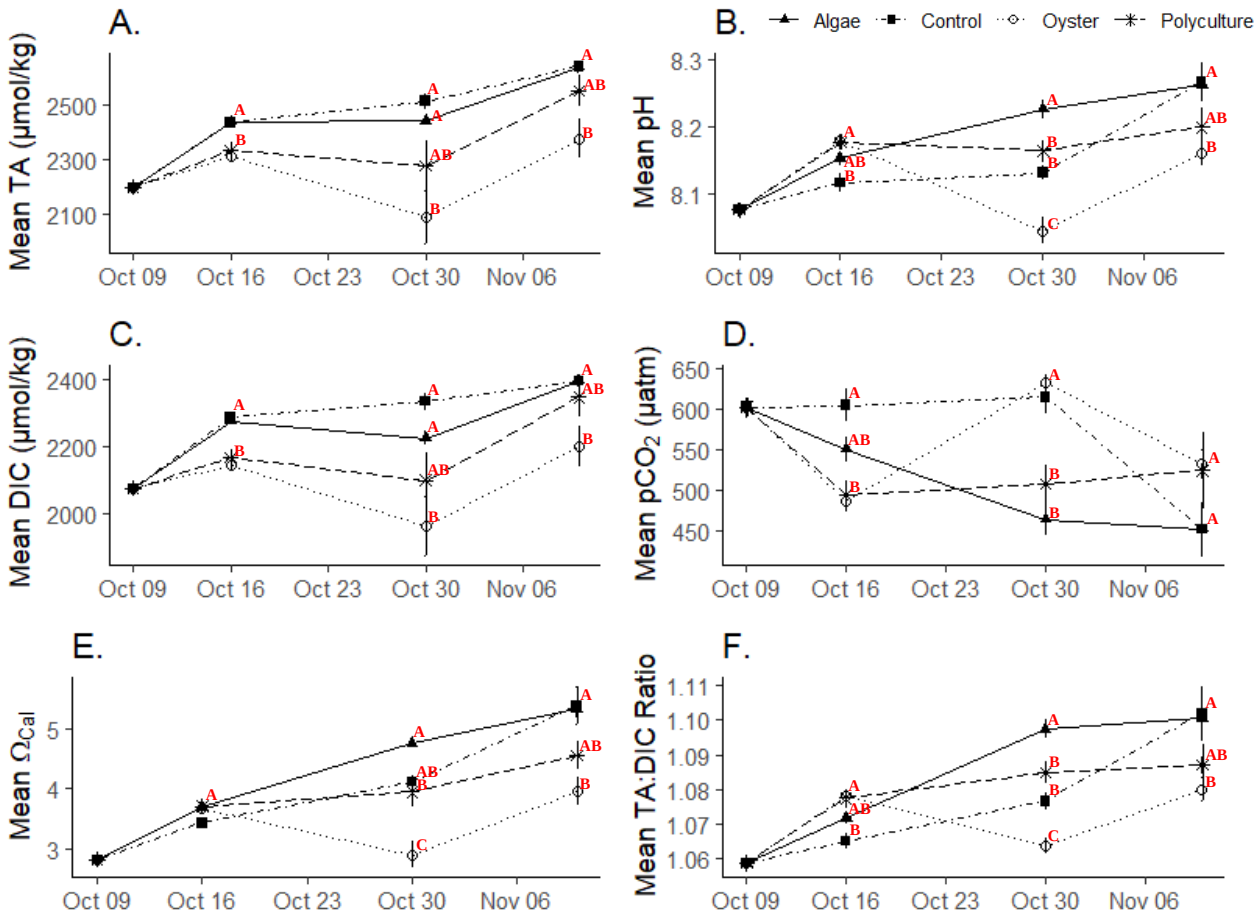


Figure 2.10 Bi-weekly carbonate chemistry measurements: (A) total alkalinity, (B) pH, (C) DIC, (D) pCO_2, (E) calcite saturation state, (F) TA:DIC ratio. within each treatment. TA and pH were measured directly via titration. All other values were calculated using CO2SYS. N=6 per treatment per time point. Levels not connected by the same letter are significantly different. Measurements at the initial time point (Oct 09) are the mean values of source saltwater (n = 5). Error bars are standard error.

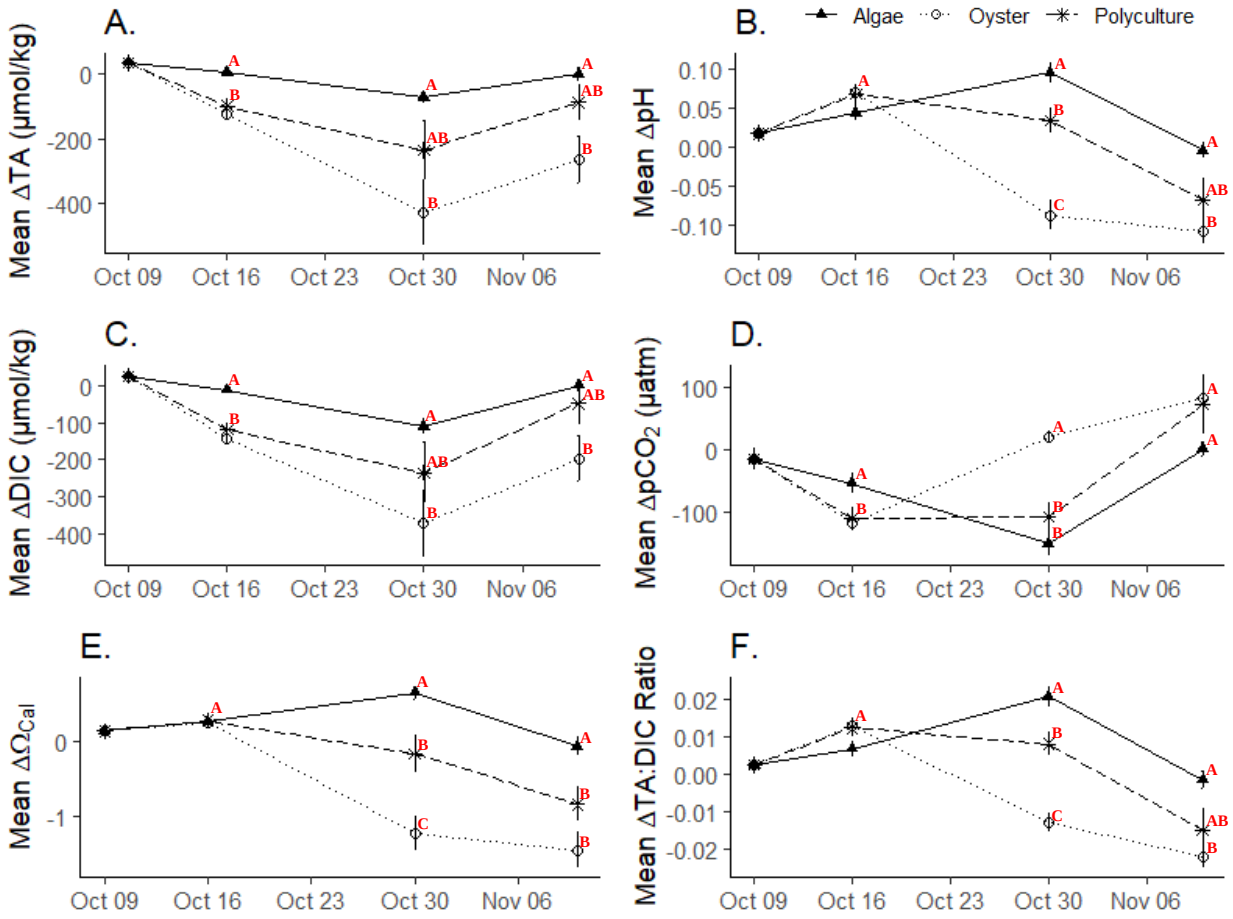


Figure 2.11 Bi-weekly carbonate chemistry measurements corrected to the mean of the control at each time point: (A) Δtotal alkalinity, (B) ΔpH, (C) ΔDIC, (D) ΔpCO_2, (E) Δcalcite saturation state, (F) ΔTA:DIC ratio. within each treatment. TA and pH were measured directly via titration. All other values were calculated using CO2SYS. N=6 per treatment per time point. Measurements at the initial time point (Oct 09) are the mean values of source saltwater (n = 5). Error bars are standard error.

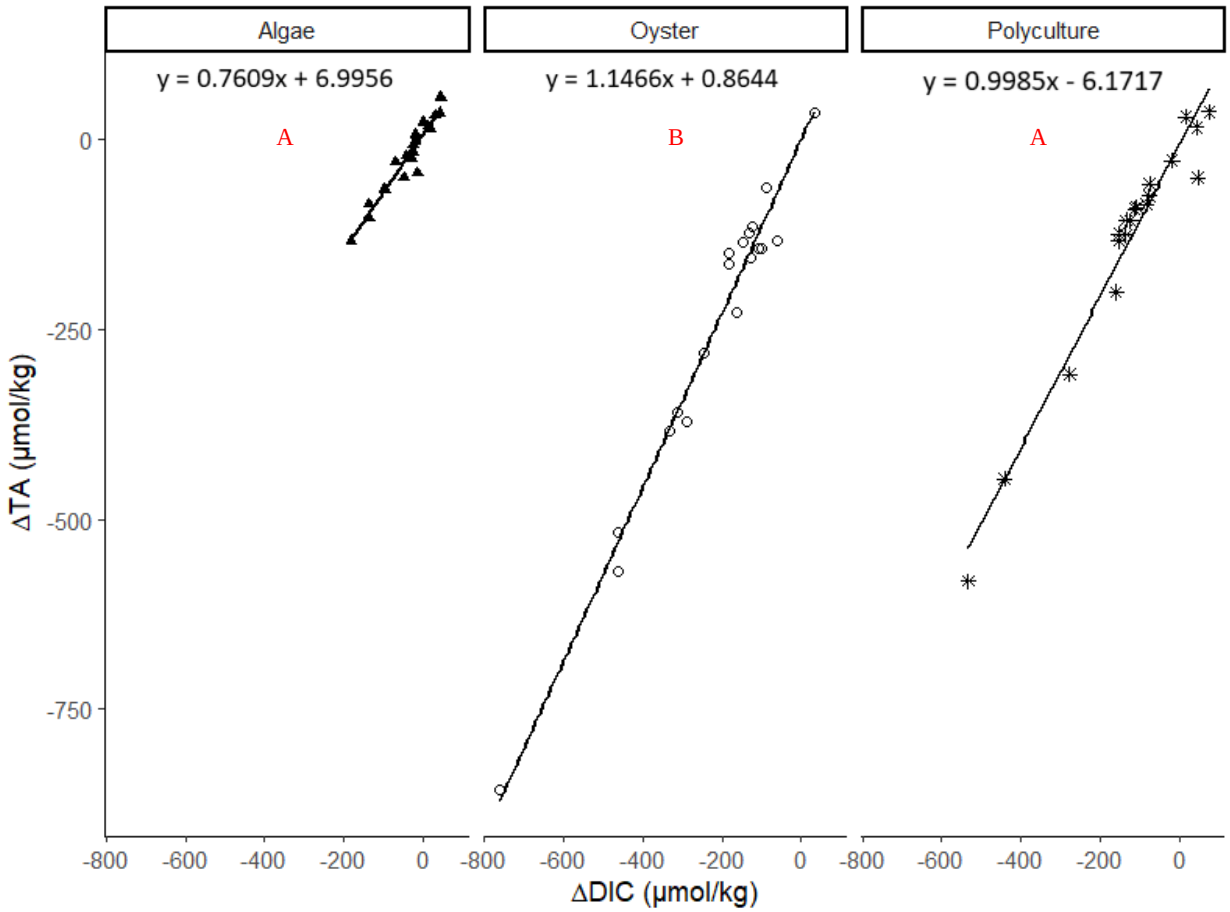


Figure 2.12 ΔTA vs. ΔDIC by treatment. Slope of the line indicates the level of calcification occurring in the system. Slopes closer to 2 indicate higher calcification (Cyronak et al. 2018). Levels not connected by the same letter are significantly different from each other.

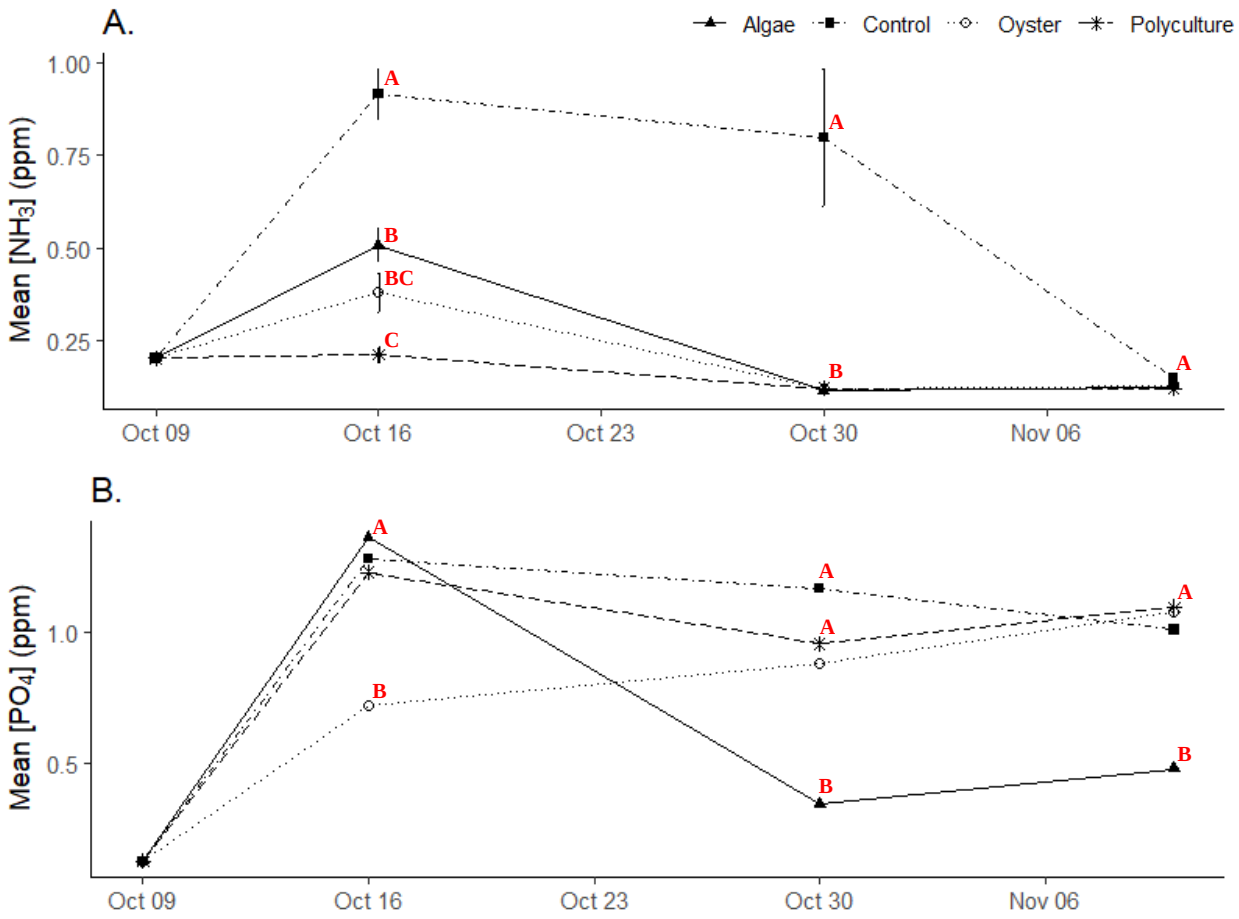


Figure 2.13 Bi-weekly concentrations of (A) phosphate (PO_4) and (B) ammonia (NH_3) within each treatment. Measurements at the initial time point (Oct 09) are the mean [NH_3] and [PO_4] of source saltwater (n = 5). Concentrations were measured via spectrophotometry (Hach DR3900). Samples were diluted 50% with DI water prior to analysis to minimize the influence of pH on test results. Error bars are standard error.

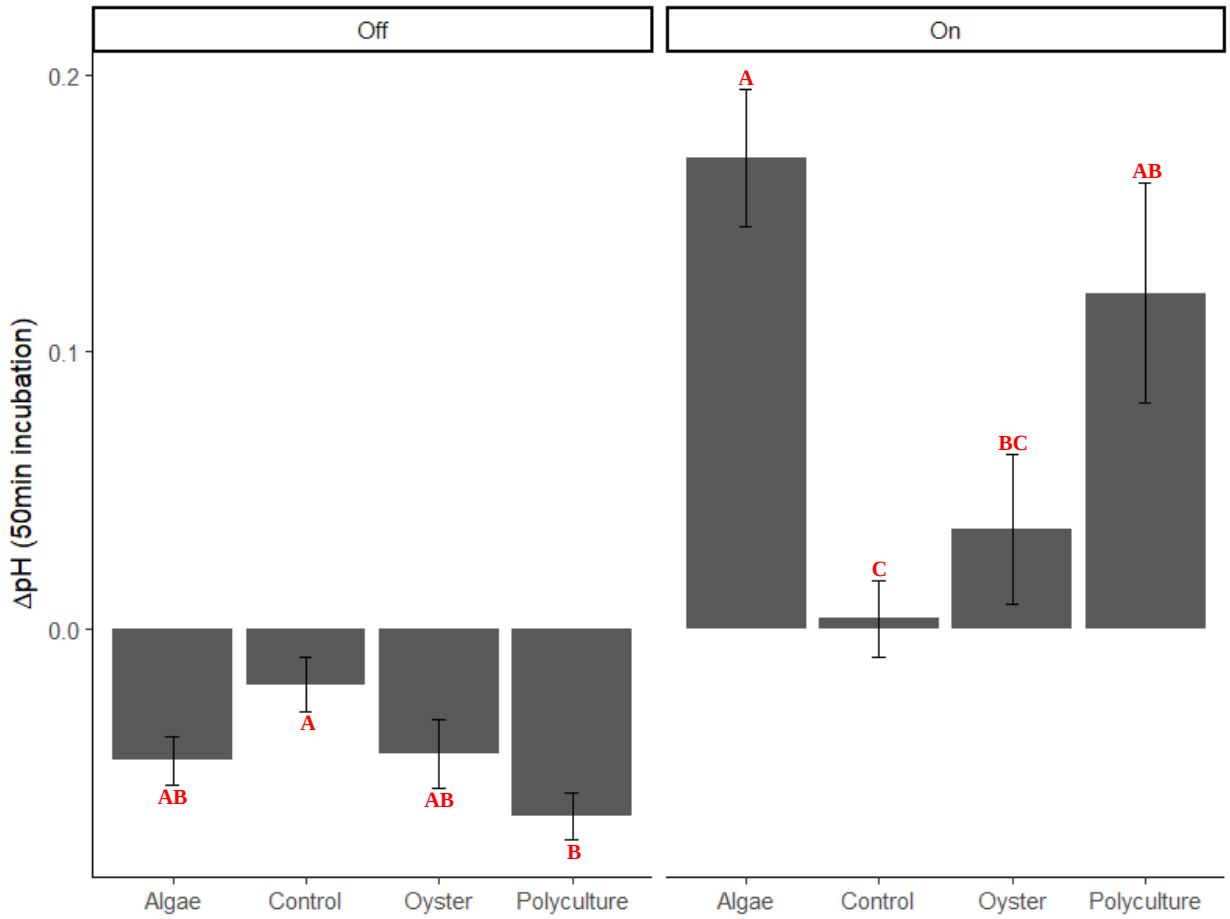


Figure 2.14 Change in experiment pH over 50 minute incubations. Error bars are standard errors. Levels not connected by the same letter are significantly different (Tukey Post-Hoc). Tests were performed separately by light level. "Off" was analyzed using an ANOVA, "On" was analyzed using a randomized block ANOVA. Blocking factor (date) in the randomized block ANOVA was not significant.

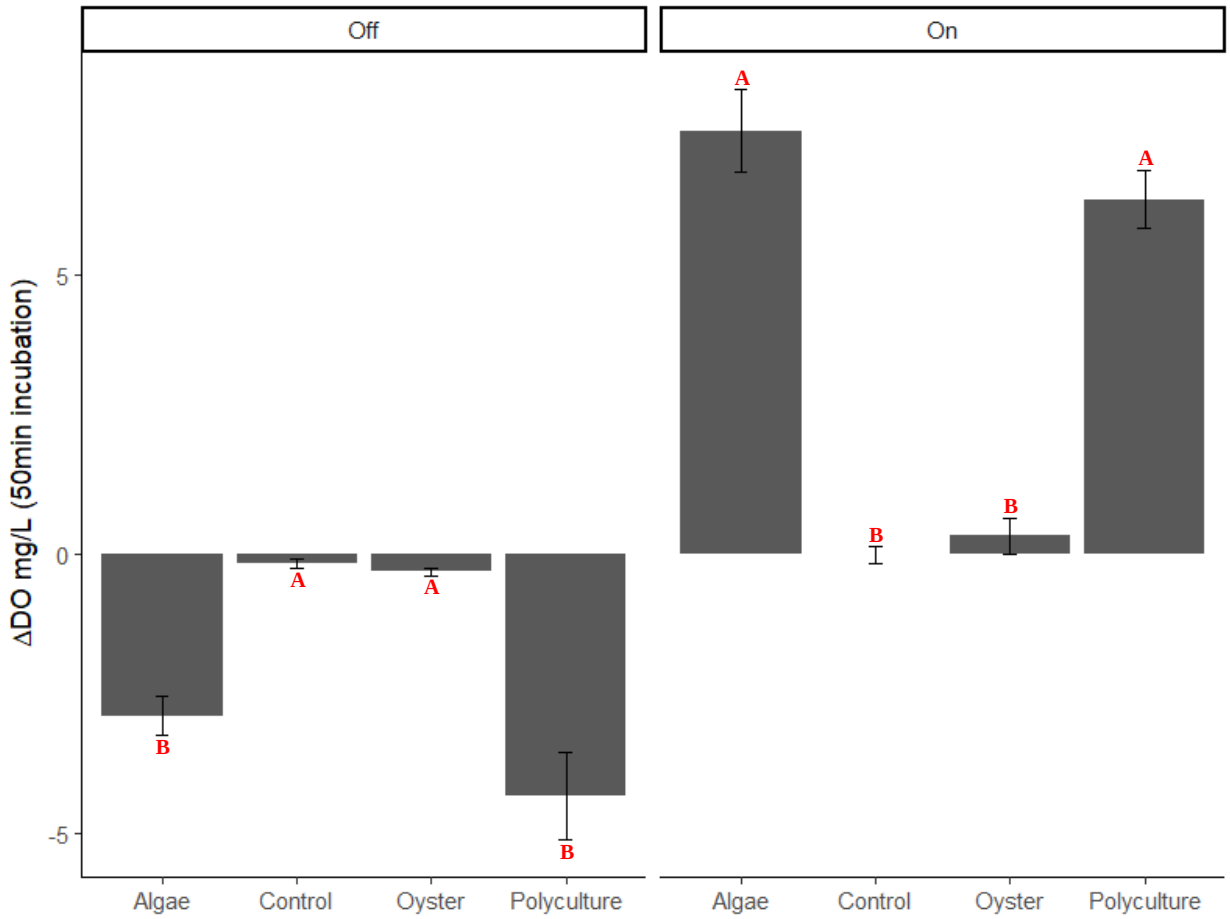


Figure 2.15 Change in dissolved oxygen (DO) over 50 minute incubations. Error bars are standard errors. Levels not connected by the same letter are significantly different (Steel-Dwass or Tukey Post-Hoc). Tests were performed by light level. "Off" was analyzed using a Kruskal-Wallis, "On" was analyzed using a Friedman's Test. Blocking factor (date) in the Friedman's Test was not significant.

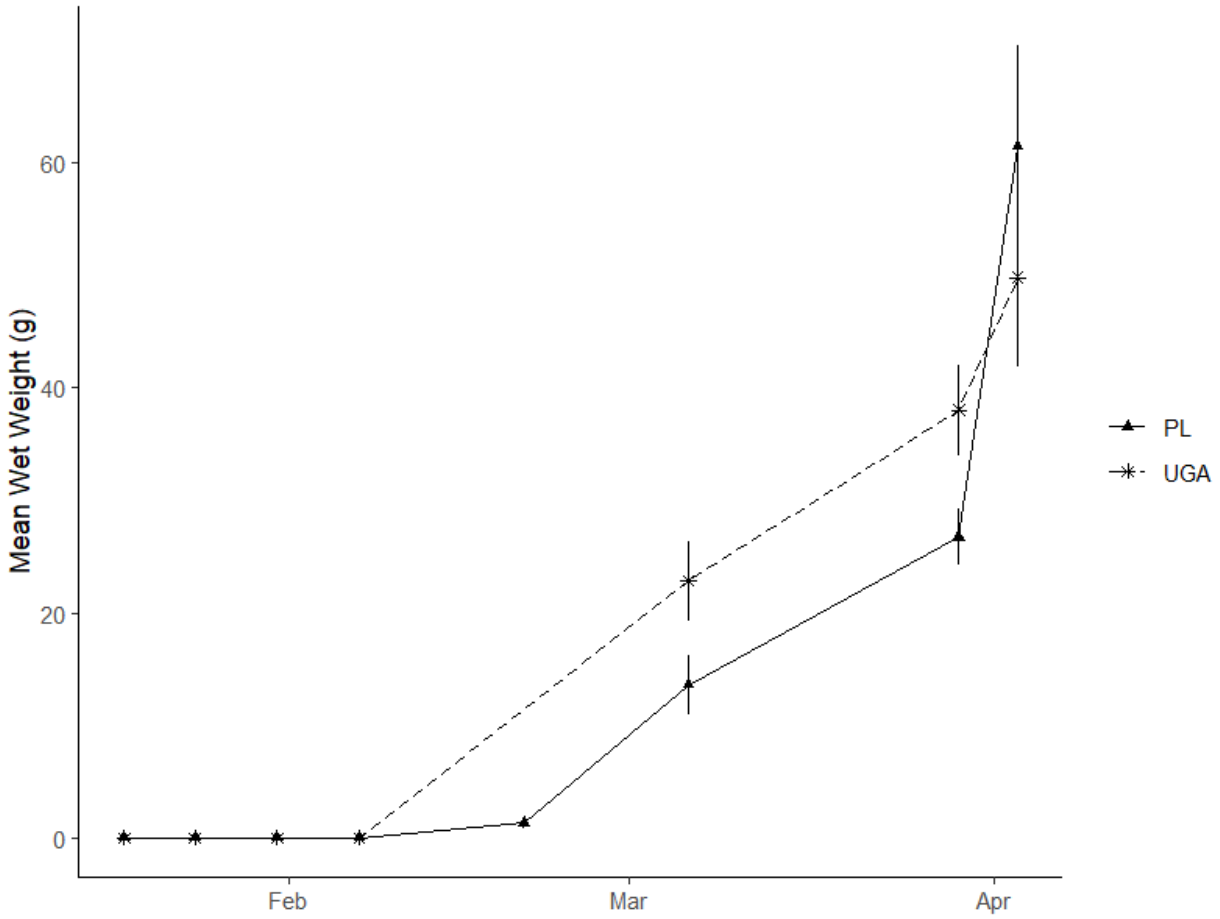


Figure 2.16 Bi-weekly weight measurements of *Ulva* cultivated via rope culture (n = 9) at two field locations, Priest's Landing (PL) and UGA MAREX Hatchery Dock (UGA). Weight of the ropes was subtracted from total weight to determine *Ulva* mass. Ropes were spun in a salad spinner for ~30 seconds prior to weighing. Error bars are standard error.

Figure 2.17 Visual comparison of *Ulva* grown at two different field locations (A) Priest's Landing and (B) UGA Hatchery Dock from Jan-April 2024, with the latter developing a more foliose and robust morphology than the former.

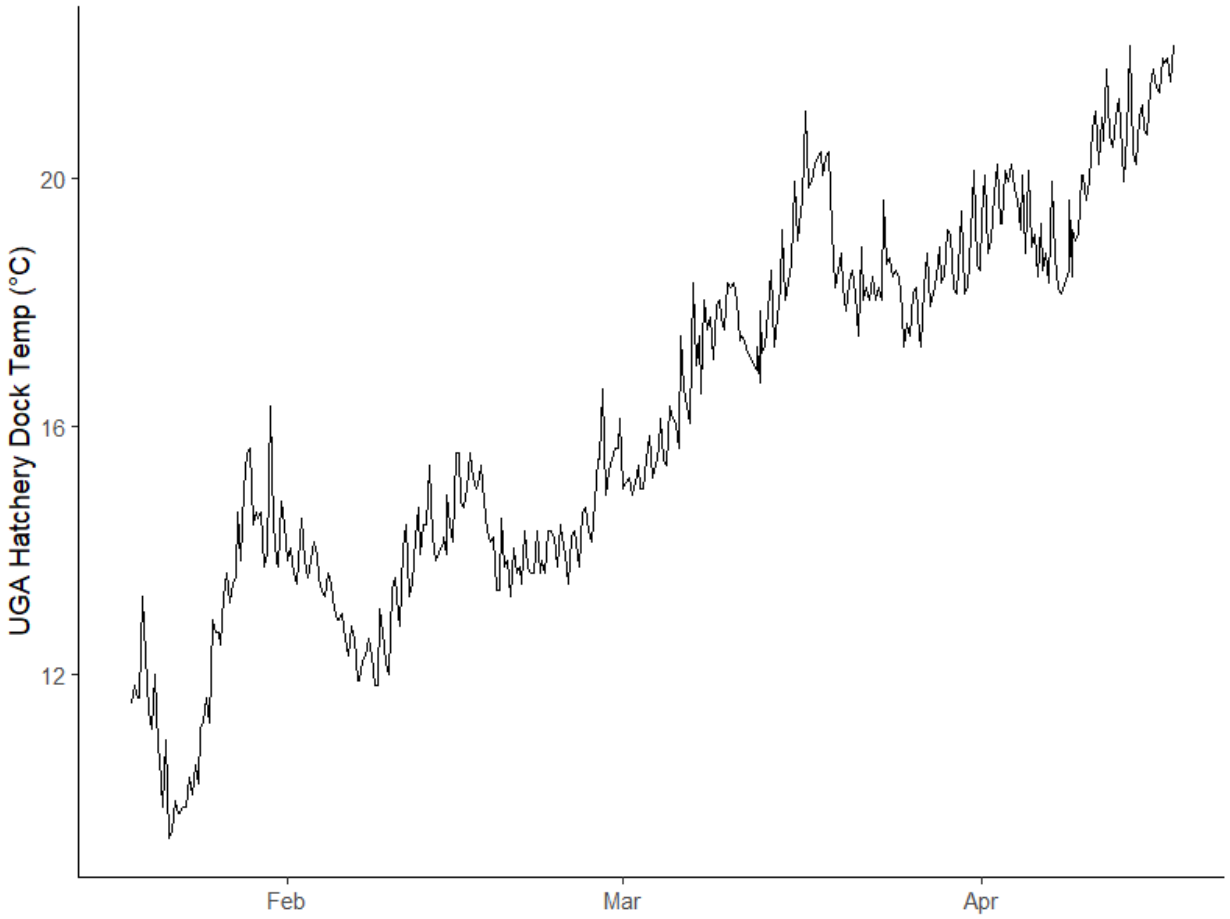


Figure 2.18 Temperature variability at the UGA Hatchery Dock throughout the study period (Jan 17 to April 3, 2024. Measurements were recorded every 6 hours.

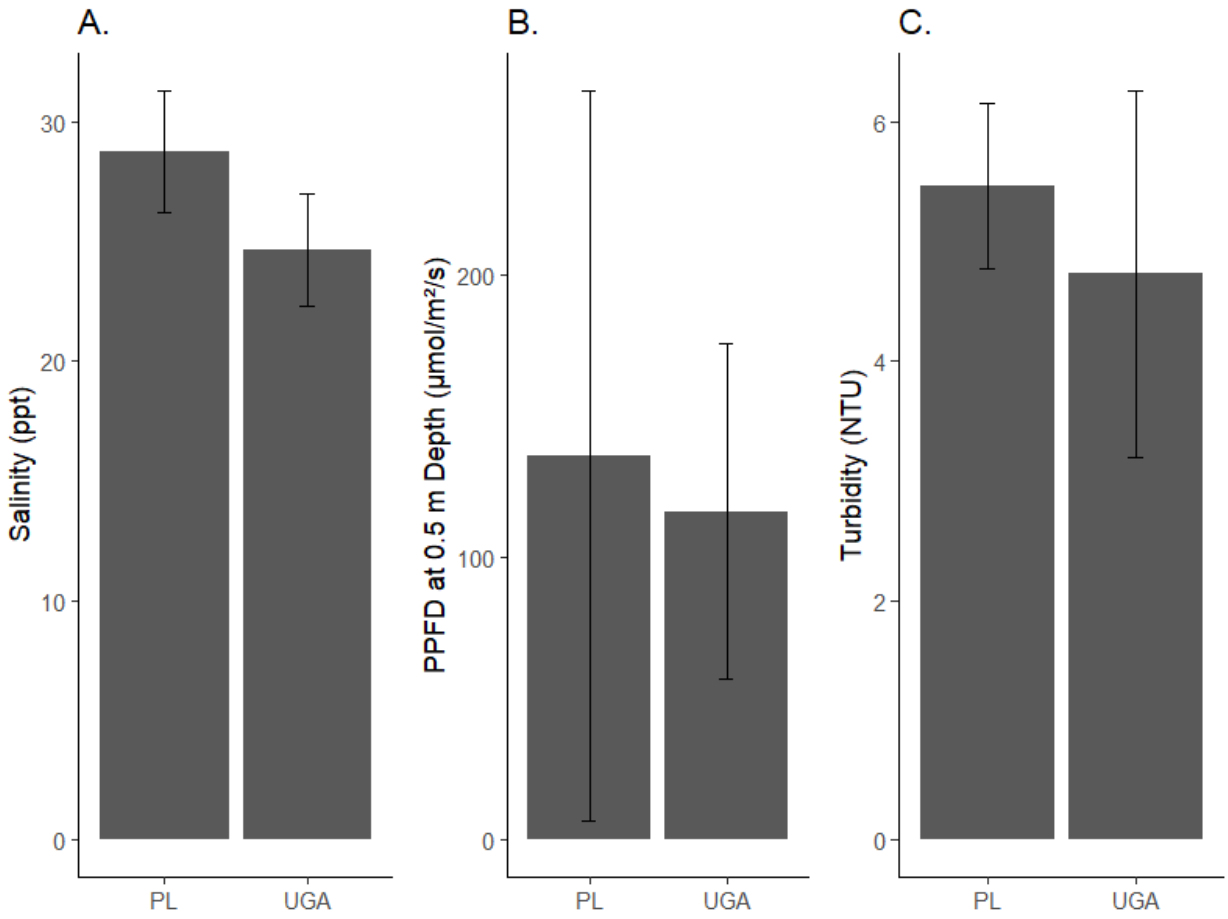


Figure 2.19 Comparison of mean environmental parameters (A)salinity, (B) photosynthetic photon flux density, and (C) turbidity at the two field sites Priest Landing (PL) and UGA MAREX Hatchery Dock (UGA). Error bars are standard errors. Field measurements were taken between January and April of 2024.

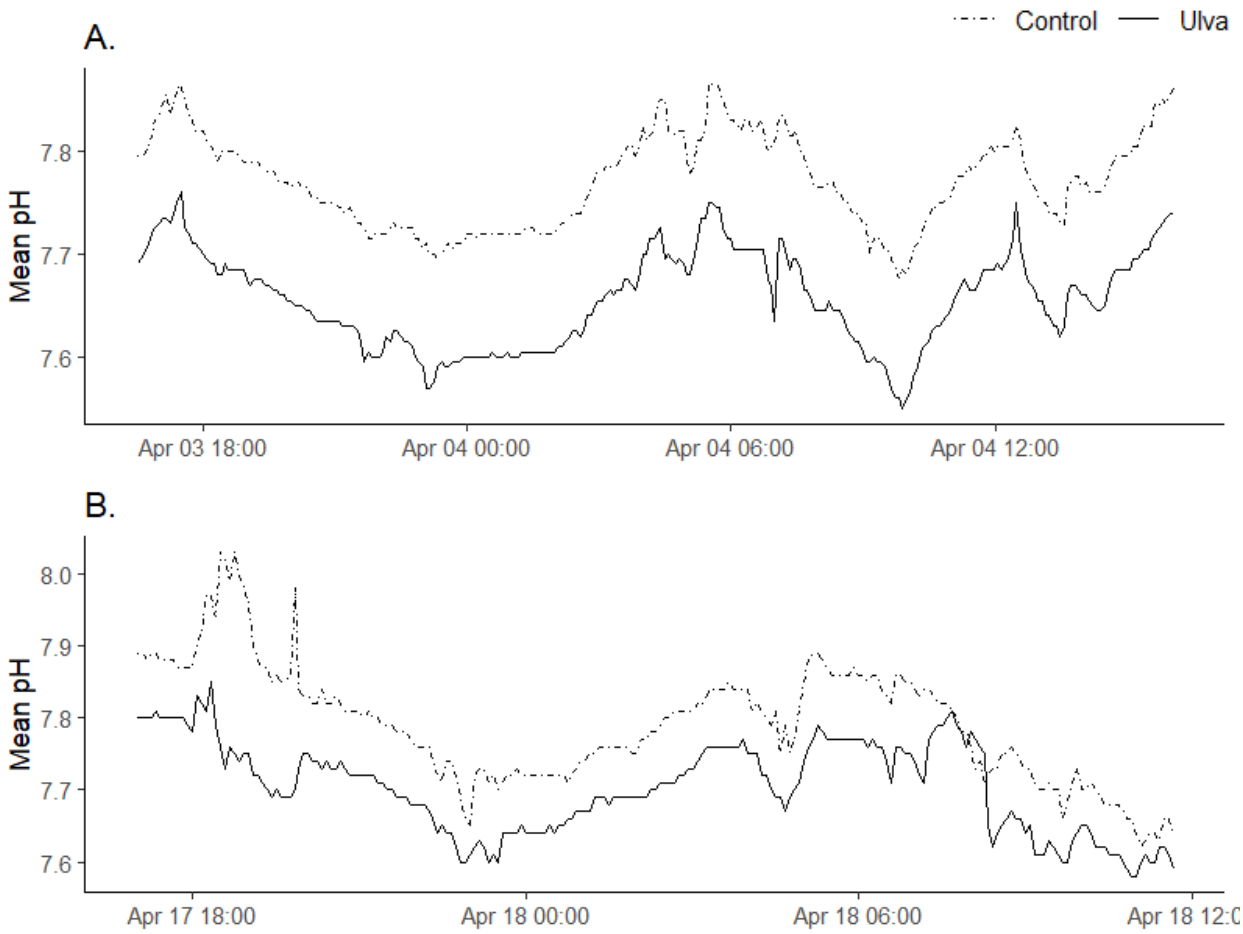


Figure 2.20 Comparison of UGA MAREX Hatchery Dock field pH from within (solid line, n = 3) and away from (dot-dashed line, n = 2) the seaweed array at 2 time periods: (A) seaweed present, and (B) seaweed absent.

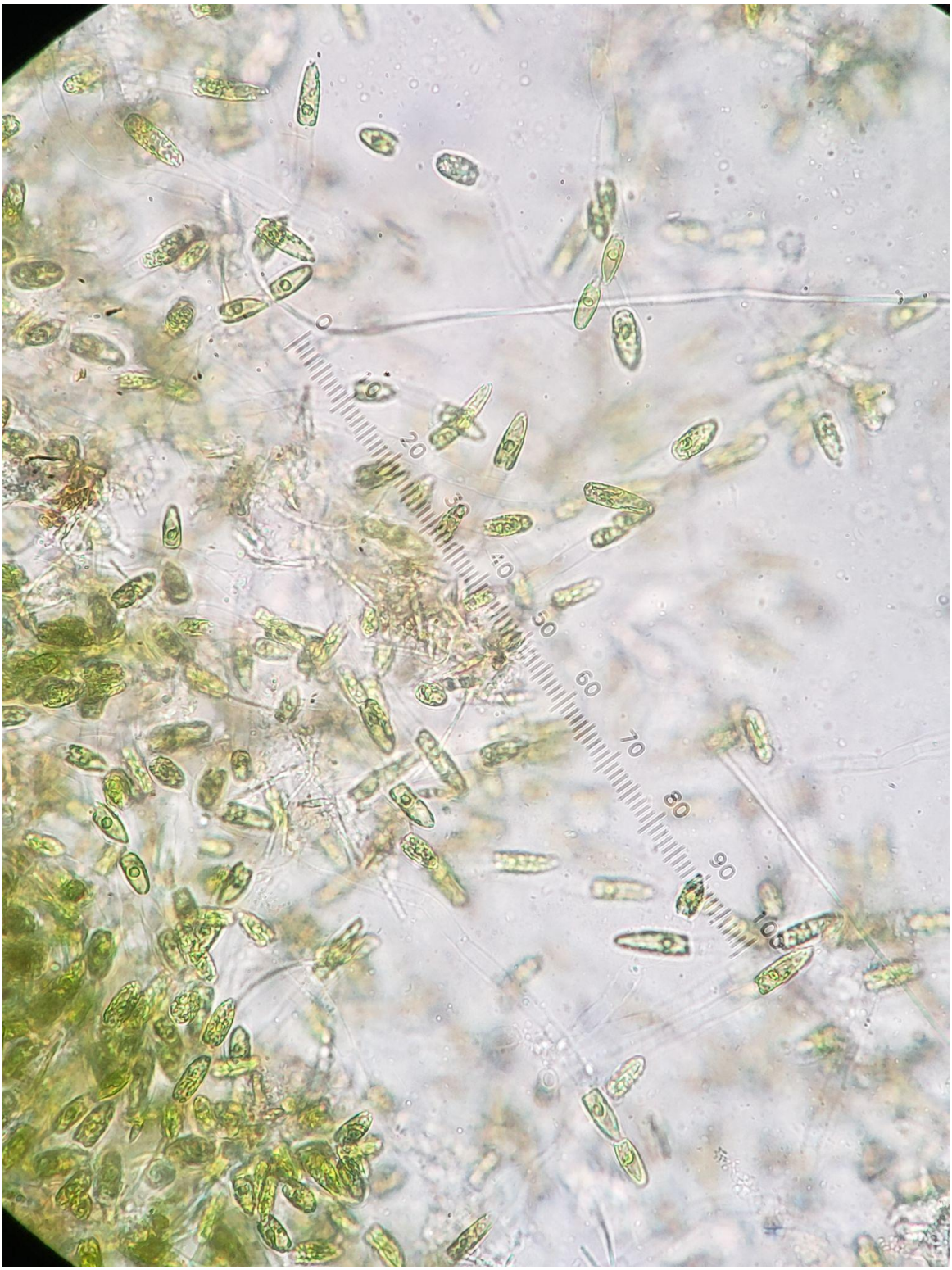


Figure 2.21 *Tetraselmis marina* collected from the wall of one the experimental tanks (400x magnification); similar abundance was found in the majority of other tanks towards the conclusion of the experiment.

Figure 2.22 The obvious presence of a microalgal biofilm and air bubbles on oyster shells in oyster-only experiments suggests this contributed to the lack of any significant differences in dissolved oxygen between oyster and control incubations.

Figure 2.23 Accumulation of decaying vegetation, seafoam, and other debris within the seaweed array at Priest's Landing. This debris had to be removed on a regular basis and may have both hampered growth of the *Ulva* and affected pH measurements taken inside the array.

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APPENDIX A

PROTOCOL: OYSTER PROCESSING

I. Purpose: To instruct the researcher on processing oysters for soft tissue and shell growth analysis following completion of polyculture trials.

II. Materials

- Labeled plastic bags (x12)
- Analytical balance
- Ruler or other scale bar
- Camera for photographing oysters
- Freezer
- Drying oven
- Foil trays (for drying)
- Muffle furnace

III. Procedure

1. Remove oysters from treatment tanks, group by replicate
    a. Be careful to keep them on the tiles and don't mix up the tiles. If the permanent marker has come off the tiles, the tiles on the left are A and the tiles on the right are B.
2. Lightly dry the oysters and tiles with a kimwipe, making sure to wipe off any debris or fouling algae **but don't knock the oysters off the tiles**
3. Relabel each tile if needed, or write the sample ID on a small piece of paper so you don't lose track of the tile's ID
4. **Weigh** each tile on the balance and record the weight to 3 decimal places
5. Place the tile on a counter next to a ruler. **Make sure the sample ID and ruler are visible in the frame and take a picture of the tile.**
6. Repeat with every tile, upload the photos to the shared drive for future ImageJ analysis
7. Place each tile in a ziploc bag for further processing, label with:
    a. Sample ID (treatment and replicate)
    b. Tile ID
    c. Species
    d. Date frozen
8. Thaw oysters overnight and remove from tiles, group by treatment and tile
9. Weigh and record thawed oysters
10. Place oysters in foil trays (labeled by treatment and tile) and dry at 60℃ until desiccated
11. Weigh and record dried oysters

12. Place dried oysters in a muffle furnace and combust them at 450°C for at least 4 hours
13. Remove, weigh, and record combusted oysters
14. Subtract combusted weight and dried weight to determine approximate soft tissue weight of oysters

APPENDIX B

PROTOCOL: WEIGHING *ULVA*

I. Purpose: To instruct the researcher on weighing and recording of *Ulva* biomass.

II. Materials

- Nitrile gloves
- Pen/pencil and journal, or computer, for recording data
- Salad spinner
- Analytical balance
- Weigh boat or beaker

III. Procedure

1. To weigh *Ulva*, remove all pieces from a treatment tank carefully using a net
    a. **Do not** place ungloved hands in the tanks at any time to avoid influencing water chemistry
    b. Rinse the net in DI water between each tank
2. Place the algae in the salad spinner and spin for 30 seconds to remove excess water
3. Remove *Ulva* and measure mass, record
    a. Wipe out the salad spinner in between uses
4. Place *Ulva* back into its respective tank
5. Repeat steps 1-4 for all 12 tanks

APPENDIX C

PROTOCOL: *ULVA* PROCESSING FOR ELEMENTAL ANALYSIS

I. Purpose: To instruct the researcher on processing *Ulva* samples for elemental analysis following completion of polyculture trials.

II. Materials

- Labeled plastic bags (x12)
- Freezer
- Salad spinner
- Drying oven
- Foil trays (for drying)
- Ball grinder mill w/ assorted stainless steel ball bearings
- Labeled glass scintillation vials
- 96-well plate(s)
- 8mm x 5mm tin capsules
- Metal chemical spoon
    1. For transferring samples into tin capsules
- Curved tip forceps (2)
    1. For folding tin capsules
- Analytical microbalance
- Lab notebook for recording sample data

1. Remove *Ulva* from treatment tanks, group by replicate
2. Place seaweed in labeled plastic bags and freeze for at least 24 hours
3. Thaw seaweed, remove excess water through gently squeezing
    a. Salad spinner may also be used, but this may fragment the seaweed into small pieces which will be harder to manage
4. Dry seaweed samples in a drying oven at 60°C for 24-36 hours
5. Once dry, pulverize seaweed samples into a uniform fine powder using a food processor or ball grinder
6. Place powder in scintillation vials, label each vial with:
    a. Sample ID (treatment and replicate)
    b. Species
    c. Date frozen
    d. Date processed (aka pulverized)
7. Subsample ~2 mg of algae powder using the microbalance, load into a tin capsule using metal spoon

8. Carefully fold the tin capsule into a ball using the 2 forceps and place into one of the wells in the 96-well plate
    a. Practice with a few blanks first. This is a skill that takes some time to develop.
9. Repeat this process for as many samples and/or replicates you want
10. Record sample ID and well location in your lab notebook
11. EA samples can be run in house with the combustion analyzer in the Moore Lab or sent to the Whitney Lab in St. Augustine

APPENDIX D

PROTOCOL: COLLECTING & FIXING WATER SAMPLES

I. Purpose: To instruct the researcher on collecting and fixing water samples for carbonate chemistry and nutrient analysis. Adapted from Cyronak et al. 2018.

HANDLING OF $HgCl_2$ MUST BE DONE IN A FUME HOOD

II. Materials

A. Nitrile gloves
B. Lab coat
C. 350 mL glass BOD bottles for carbonate chemistry analysis
D. 50 mL centrifuge tubes for nutrient analysis
E. Plastic syringe (for starting siphons)
F. Siphon hose w/ ball valve (for collecting samples)
G. Bucket (for wastewater)
H. Labeling tape & sharpie
I. 100 mL of saturated $HgCl_2$ **in fume hood**
J. 200 µL pipettor & pipette tips

III. Procedure

1. Label all bottles with tank IDs, collection date, and corresponding analysis
    a. e.g. Tank 1 of the control group collected 9/7 for carbonate chemistry would be labeled as "C1 9/7/23"
        i. An ammonia sample for the same tank would be labeled "C1 9/7/23 A"
2. To collect **Carbonate Chemistry** samples:
        i. Note: Samples should be collected and fixed within a standardized time frame (i.e. the time it takes to collect and fix one sample should set the pace)
    b. Remove the glass stopper from the corresponding sample bottle
    c. Place the bottle in a 1L beaker so the water can overflow
    d. Place the bucket on the floor in front of the tank
    e. Use a syringe to start the siphon, then immediately close the valve to maintain pressure
    f. Fill the sample bottle with a small amount of water. **This is not your sample**. **Rinse and discard**.

        i. Make sure there are **no bubbles** on the inside walls of the bottle after rinsing
    g. Place the tube at the bottom of the bottle and fill the sample bottle again **to overflow**. **This is your sample**.
    h. Cap the sample with the glass stopper and place bottle in cardboard box
    i. Repeat with **all 24 replicates.** Flush the siphon hose with DI water between every tank sample.
        i. It may be useful to carry around a bucket of fresh water for flushing
    j. Once done, take all samples upstairs so they can be fixed with $HgCl_2$
        i. Steps k-o must be done in a fume hood
    k. Uncap the sample and pour a small amount of water out
        i. You want about ¼ inch of space between the neck of the bottle and the sample water level
    l. Carefully add 400 µL of $HgCl_2$ to the bottle (twice with the 200 µL pipette)
        i. This needs to be done **as quickly as possible** and time should be consistent between samples. Work with another person if possible.
            1. All samples should be fixed within **30 minutes** of collection
        ii. Do not submerge the pipette tip in the sample
    m. Add 3 **small** dabs of bottle grease to the glass stopper
    n. Place the stopper in and twist it around to spread the grease
    o. Gently invert several **3 times** to mix the $HgCl_2$ thoroughly
    p. Repeat with **all 24 samples**
    q. Store all samples in a cardboard box/out of direct sunlight until they can be processed

3. To collect **Nutrient** samples:
    a. Label all 50mL centrifuge tubes with tank ID, collection date, and corresponding analysis (A for ammonia and P for phosphate)
        i. Each tank needs 2 sample tubes, one for ammonia and one for phosphate. Both will be filled to 50 mL.
    b. Fill with a small amount of sample water. **This is not your sample. Rinse and discard.**
    c. Fill the tube with sample water up to the **50 mL** line
    d. Screw the cap back on tight.
    e. Do this twice for each tank.
    f. Immediately (or as soon as possible) freeze **nutrient** samples for future analysis.
4. Once sampling is complete, continue to drain water out of all tanks until you reach the line. This constitutes a water change of ~ 10% volume (~1 L).
5. Rinse all equipment thoroughly with DI water and set to dry
6. Discard $HgCl_2$ waste in the hazardous materials bin

7. Refill all treatment tanks with **20 ppt filtered saltwater.**
    a. **Note:** there are **two valves** on the hoses for the storage tanks. When you are done, make sure both valves are **closed**
8. Feed tanks with **0.5** mL Reed Mariculture Shellfish Diet 1800 daily

APPENDIX E

PROTOCOL: CHAMBER POLYCULTURE EXPERIMENT

I. Purpose: To instruct the researcher on performing a chambered experiment using glass BOD bottles in order to investigate the carbonate chemistry dynamics of oysters and seaweed in isolation and co-culture.

II. Materials

- Glass BOD bottles(n = 16)
  - Labeled with sample ID
- 20 mL scintillation vials (n = 64)
  - Labeled with sample ID (half for initial measurements, half for final measurements, pull 2 samples per replicate so we have backups)
- Shaker table (can fit 16 bottles at a time)
- Oysters
  - Need 2-3 per bottle, 4 bottles per treatment
  - Total oyster weight per bottle: **1.4 +/- 0.1g**
- Algae (*Ulva* and/or *Gracilaria*)
  - Total algae weight per bottle: **1.4 +/- 0.1g**
- 20-22 ppt filtered saltwater
- LED growlight (n = 2)
- Timer
- Refractometer
- Handheld DO/pH probe
- $HgCl_2$ & micropipette
  - Dose = 1uL/mL
  - **Must be done in fume hood, wear lab coat & gloves**

III. Procedure

1. The day before, make sure source water is the correct salinity
2. Label experimental bottles & collection vials with appropriate sample IDs if not already done
3. Fill 16 20mL scintillation vials with initial saltwater
   a. Measure and record DO/pH of source water **at least 16 times** (this can be done while prepping for the rest of the experiment)
4. Fix all samples with 20uL $HgCl_2$ and store in labeled plastic container for WQ analysis
   a. Must be done in fume hood, wear lab coat & gloves
5. Oysters and algae will be stocked at a ratio of **1:1**

6. Record the weight of the oysters in each bottle (should be ~1.4g)
7. Weigh out & record an appropriate amount of algae
    a. The algae can be cut to achieve the desired mass
8. Place algae and/or oysters in the correct bottles
9. Fill with source seawater & cap with the glass stopper so that there are no air gaps
10. Randomly arrange the 16 bottles on the shaker table
11. Make sure grow lights & shaker table are on, **incubate for 50 minutes**
    a. Use this time to begin WQ analysis on the initial samples
12. After the 50 minutes is up, immediately measure DO and pH of each sample by immersing the probes directly into the BOD bottle (see photo)
    a. 

13. Fill the remaining 16 labeled scintillation vials with 20mL of each sample's water
14. Fix all samples with 20uL $HgCl_2$ and store in labeled plastic container for later WQ analysis
    a. Must be done in fume hood, wear lab coat & gloves

APPENDIX F

PROTOCOL: GERMANIUM DIOXIDE SATURATED SOLUTION

I. Purpose: To instruct the researcher on preparing a saturated solution of GeO_2 for dosing aquaculture systems to inhibit the growth of fouling diatom species.

MUST BE DONE IN A FUME HOOD

II. Materials

- Nitrile gloves
- Safety goggles
- Germanium Dioxide powder
- Analytical balance
- Measuring spoon
- Weigh boat
- 200 mL volumetric flask w/ glass stopper
- DI water
- Stir plate & magnetic stir bar

III. Procedure

1. Measure out **0.894 g** of GeO_2 onto a weigh boat, set aside
2. Measure out exactly **200 mL** of DI water using a volumetric flask
3. Carefully place the stir bar in the volumetric flask
4. Carefully scrape the GeO_2 into the flask using a measuring spoon
5. Place the glass stopper in the flask and invert once to mix in any GeO_2 stuck to the sides
6. Place the flask on the plate & turn on, allow the solution to mix for **24 hours**
7. Once complete, transfer the solution to a labeled stock bottle
8. Dose culture tanks at a rate of **0.5 mL/L** upon startup and following every water change

APPENDIX G

PROTOCOL: *ULVA* SWARMER RELEASE & SETTLEMENT

I. Purpose: To instruct the researcher on inducing spore formation & release of *Ulva* spp followed by settlement on substrate for use in sea lettuce aquaculture studies. Adapted from Steinhangen et al. 2021 & 2022.

II. Materials

- Glass or plastic aquaria for holding & cultivating *Ulva* fragments
- Synthetic rope for spore settlement
- Natural or artificial seawater
  - Water should be UV sterilized and filtered to 0.2 um
  - Other forms of sterilization:
    - Bleaching followed by sterilization with sodium thiosulfate
    - Pasteurization
    - Autoclave
  - Whatever method of sterilization is used, the goal is to keep *Ulva* cultures axenic until settled germlings are at least 1 mm in blade length
- Natural or artificial light
  - This can be done in a temperature-controlled greenhouse under natural light or through the use of LEDs/fluorescent tubes
  - PAR levels should be at least 100 umol m^{-2} s^{-1} for adult thalli and 80 umol m^{-2} s^{-1} for germlings
- GeO_2 saturated solution
  - Useful for diatom exclusion
  - Dose at 1 mg L^{-1} at every water change (once or twice a week)
  - See GeO_2 protocol for more information
- Nutrient media
  - Too many varieties to name all (Guillard's, PES, F/2, etc.), but whatever formulation you choose to go with it should be heavy on nitrogen and contain less phosphorus
  - Fertilizer should also not contain silica in order to further excluded diatoms
- Air supply, silicone and rigid airline tubing
  - Air supply to tanks with *Ulva* is useful to prevent self-shading and keep cultures suspended
  - CO_2 addition to algae cultures is a common practice in microalgae propagation, although I have come across few instances of its use in macroalgae cultures
- Access to a temperature-controlled room for algae cultivation (at least 15 C)

III. Procedure

1. In the field, remove large pieces of debris and fouling from collected *Ulva* thalli & rinse with seawater
2. Before adding to lab culture, briefly rinse *Ulva* in DI water to further remove any debris or fouling
3. Acclimate collected thalli to laboratory holding tank(s)
    a. These conditions should closely match the conditions under which the *Ulva* was originally collected (i.e. 14-16 C, 100 PAR, 20-22 ppt)
4. Spore Formation & Settlement Methods:
    a. Steinhagen et al. 2022
        i. Cut *Ulva* into discs measuring ~4 cm in diameter and transfer to a small aquarium filled with sterile, filtered seawater
        ii. Keep under constant aeration
        iii. Observe discs for darkening coloration, indicating spore formation
            1. May take up to 4-5 days but may be faster or slower depending on individual variation
        iv. Once darkening occurs, wash discs with sterile filtered seawater and transfer to a beaker filled with 80-100 mL of seawater, after which they should immediately begin to release spores
        v. Life stage of spores (i.e. "swarmers") can be determined by counting the number of flagella under a microscope
            1. 2 flagella = gametes
            2. 4 flagella = spores OR fused gametes
                a. Both may be present in cultures
        vi. Swarmer solutions may then be quantified with a cytometer or stock solutions may be created
        vii. Add swarmers to another tank filled with sterile filtered seawater and settlement substrate of your choice
            1. 10 m coiled nylon cord is what was used in this study
            2. Rope substrate should be boiled to remove factory residue
        viii. Supply the grow tank with 80-100 PAR under a 12:12 photoperiod
        ix. Perform water changes weekly, followed by addition of fertilizer of choice and GeO_2 (1 mg L^{-1})
        x. Cultivate for 6 weeks, then acclimate *Ulva* to prevailing field conditions over the course of 1 week
    b. Modification of the Steinhagen Method
        i. Begin by stringing synthetic rope onto a PVC frame to fit in the bottom of the culture tanks (see photo)

ii. Place rope array in the bottom of the culture tank and cover with filtered sterile seawater
iii. Add clean collected *Ulva* thalli to the culture tank and aerate constantly
    1. GA *Ulva* has proved to be much more volatile than its North Atlantic counterpart, and will sometimes spore prematurely
    2. As a safety precaution, I've found it to be most effective to place settlement substrate in with the collected thalli, in case sporulation occurs without preparation
iv. Follow Steinhagen protocol as previously stated up to step vii.
v. After adding swarmers, cover the culture tank and keep in the dark for 24 hours under low/no aeration to facilitate attachment to the substrate
    1. Optionally, this can be repeated by flipping the rope array upside down and adding additional swarmers to ensure complete coverage on the substrate
vi. After the attachment period, resume normal culture conditions at step viii.

5. Troubleshooting
    a. Cyanobacteria are a nuisance, particularly in the later stages of germling culture. To my knowledge, they cannot be chemically controlled like diatoms, so the only solution I've found is attempted exclusion.