Measuring the Effects of Recycled Water on the Growth of Three Algal Species: Tisochrysis lutea, Chaetoceros calcitrans, and C. muelleri in a Commercial-Scale Oyster Hatchery

Lisa Marie Bourassa

Louisiana State University and Agricultural and Mechanical College, lbourassa474@gmail.com

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_theses

Part of the Environmental Sciences Commons

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_theses/4602

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master's Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
MEASURING THE EFFECTS OF RECYCLED WATER ON THE GROWTH OF THREE ALGAL SPECIES: *Tisochrysis lutea, Chaetoceros calcitrans, AND C. muelleri* IN A COMMERCIAL-SCALE OYSTER HATCHERY

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in The School of Renewable Natural Resources

By
Lisa M Bourassa
B.A., Roger Williams University, 2010
May 2017
For my dear friend, Todd Massari, who’s never ending encouragement, friendship and shared passion for marine conservation has supported me the past ten years. May your inspiration and guidance never end.
Acknowledgements

I would like to thank the Louisiana Sea Grant College Program for the opportunity to work on this research and my master’s degree while working for the Michael C. Voisin hatchery. I appreciate the guidance and support from my committee advisors: Drs. John Supan, C. Greg Lutz, and Ralph Portier.

This work would not be possible without the understanding and support of my coworkers and supervisor. Thank you to the hatchery team: Megan Gima, Erin Leonhardt, Brian Callam, Sarah Woolley, Stephanie Grodeska, and Jenessa Kay for your assistance over the past 3.5 years while I’ve worked on this degree, Josh Chavers for his assistance taking water quality measurements, and Dr. Blouin and Joseph Madere from the LSU Experimental Statistics department for their help with SAS and statistics.

Special thank you to my parents, Barbara and Jim, my sisters, Jen and Lauren, my brother Chris, and my many friends who have supported me throughout the years. Your encouragement, enthusiasm and support has been instrumental.
# TABLE OF CONTENTS

Acknowledgements ........................................................................................................ iii

Abstract .......................................................................................................................... vi

Introduction ..................................................................................................................... 1
- Louisiana Oyster Production ......................................................................................... 1
- Michael C. Voisin Oyster Hatchery ............................................................................. 4
  - Flow through seawater maintenance ........................................................................ 5
  - Recycling seawater filtration ................................................................................... 7
- Recycling Aquaculture Systems .................................................................................. 8
- Nitrogen Cycle ............................................................................................................. 11
- Phosphorus Cycle ....................................................................................................... 12
- Description of Algal Production ................................................................................ 14
  - Algal Growth .......................................................................................................... 16
  - *Tisochrysis lutea* ................................................................................................ 18
  - *Chaetoceros* spp. .................................................................................................. 19
- Objectives and Hypothesis .......................................................................................... 19

Methods .......................................................................................................................... 20
- Water Collection and Preparation ............................................................................. 20
- Media Preparation ...................................................................................................... 21
- Inoculations ................................................................................................................ 21
- Culture Conditions ..................................................................................................... 22
- Data Collection .......................................................................................................... 22
  - Biomass and Productivity ....................................................................................... 22
  - Water Chemistry .................................................................................................... 23
- Statistical Analysis ..................................................................................................... 24
Results .................................................................................................................................................................24
  Water Chemistry ......................................................................................................................................................24
  Biomass and Productivity ........................................................................................................................................26
    Dry Weight ............................................................................................................................................................26
    Growth Rate ..........................................................................................................................................................27
    Cell Concentrations ..............................................................................................................................................28
Discussion .................................................................................................................................................................30
  Applications to Daily Hatchery Operations ..............................................................................................................34
Conclusions and Future Research ..................................................................................................................................36

References .....................................................................................................................................................................37

Appendix ....................................................................................................................................................................41
  A. Nutrient Formulas................................................................................................................................................41
  B. Algae Protocols at the Michael C. Voisin Oyster Hatchery ..................................................................................42

Vita .............................................................................................................................................................................49
Abstract

Algal production is often the limiting factor in large-scale oyster hatcheries, and constant, reliable production is required to grow enough algae to support all oyster larvae and broodstock grown and conditioned in a hatchery. The algal rooms in the Michael C. Voisin Oyster Hatchery at Grand Isle, LA are temperature-controlled to maintain consistent temperature, but this hatchery is also unique in its ability to recycle natural seawater pumped from the northern Gulf of Mexico. Effects of recycling seawater on algal production in an oyster hatchery, however, are undocumented.

In this study, *Tisochrysis lutea*, *Chaetoceros calcitrans* and *C. muelleri* were grown in different water sources to determine if productivity would be affected by water source. Algae were grown in ambient filtered seawater, recycled filtered seawater, and artificial seawater for a period of 10 days. To evaluate algal production, cell concentrations were measured every other day and dry biomass and growth rate were calculated. Water chemistry (nitrate, phosphate, and silicate concentrations) was measured initially and at ten days.

Dry weights and growth were significantly different between species (p=0.0475, p<0.0001), but not water sources. *C. muelleri* grew the greatest in biomass and the slowest growth rate, followed by *C. calcitrans*, and *T. lutea*. Nitrate and phosphates had no significant effects on growth between species, although silicate content was significantly higher in *T. lutea*.

Overall there were no statistically significant effects for the interaction of the three water sources on the growth of these algal species over time (p=0.2882). Although there were
no significant differences, algae grown in ambient bay water grew denser and greater biomass than those in recycled bay water or artificial seawater.
Introduction

The Eastern oyster (*Crassostrea virginica*) is an environmentally, economically, and commercially important animal in Louisiana. Due to overharvesting, coastal erosion, sedimentation, freshwater diversions, storms and other environmental disasters, however, natural oyster populations are in decline. As a response to low natural populations, aquacultured oysters are being grown at the Michael C. Voisin Oyster Hatchery at Grand Isle, Louisiana. The hatchery is operated by the Louisiana Sea Grant College Program (LSG) and the Louisiana Department of Wildlife and Fisheries (LDWF). Hatchery-reared oyster larvae are being raised to rehabilitate wild stocks on public oyster grounds, to be sold to local oystermen to grow and sell on their leases, and for research purposes to provide for the future of the oyster industry in Louisiana.

Louisiana Oyster Production

Louisiana leads the nation in oyster production, providing 34% of the total volume of oysters landed in the United States, and 60% of the Gulf of Mexico region (LDWF 2015). In the Gulf of Mexico, Eastern oysters are very important economically, with an annual value of $67 million, and provide many ecosystem services including: a hard substratum and three-dimensional habitat for at least 33 different species of fish and invertebrates (LaPeyre, Schwarting and Miller 2013), coastal protection by reducing wave action and storm surge (Piazza et al. 2005) and cleansing water by filtering out particulates and sediment (Maxwell 2008).
The Louisiana oyster industry relies on the health and productivity of public oyster grounds, which are managed by the Louisiana Department of Wildlife and Fisheries (LDWF). These public oyster grounds are spread throughout coastal Louisiana, and cover a total of 667,731 ha, 24,000 of which are hard bottom, in addition to 155,802 ha of privately leased bottom (Soniat et al. 2014). For the last 150 years, many commercial oystermen in Louisiana have had private leases on which they grow and harvest oysters, but they also rely on the public grounds to seed their own beds (Supan, 2002; LDWF 2015; Leonhardt et al. 2017). Typically, oystermen will collect seed oysters from the public grounds and relocate them to stock their private leases, allowing them to grow to market-size, and then harvest them. However, relocating oysters also removes shell from the environment, limiting the amount of hard substratum for future oyster larval recruitment (Soniat et al. 2014). The most recent Oyster Stock Assessment (LDWF 2015) reports decreasing oyster populations on public grounds: 3.29 million barrels below the long term mean, and a 40% overall decrease in oyster populations across Louisiana when compared to 2014.

Oyster populations in Louisiana have historically been cyclical: a year or two of good harvest and recruitment, followed by one or two poor years, but always comparatively stable (LDWF 2014). Despite this cyclical ability for stocks to recover, following Hurricanes Katrina and Rita, the Deepwater Horizon Oil spill, the 100-year flood, and decades of overharvesting and shell loss, oyster populations on public grounds have not rebounded as expected, and the seed and market sized oysters was 40.7% below the long term mean in 2015, following ten years of consistently fewer oysters available (Fig 1) (LDWF 2015). This lack of recovery and unreliability
has encouraged oystermen to turn to alternative, more reliable methods for their seed supply: hatchery grown seed.

Fig 1: Historical estimated oyster stock size in the public areas in Louisiana. LTA denotes long-term average.
Source: LDWF 2015.

As a response to low oyster populations, there are now efforts underway to rehabilitate the public grounds and provide alternatives to Louisiana growers. Oyster larvae grown at the Michael C. Voisin Oyster Hatchery will be remotely set onto whole oyster shell and other substrate set at the LDWF Buras Remote Setting facility for relocation to select sites on the public grounds after metamorphosis. Oystermen may also purchase oyster larvae or seed from the hatchery through the Louisiana Oyster Growers and Dealer’s Association to produce spat-on-cultch via remote setting or to grow single oysters using Alternative Oyster Culture (AOC) in off-bottom cages. An additional benefit of AOC is that oysters that grow quicker, are more
resistant to disease, such as *Perkinsus marinus*, or that have been adapted to different salinity regimes can be selectively bred in the hatchery, resulting in oysters that are better suited to particular environments (Leonhardt et al. 2017). Triploid oyster seed (reproductively sterile oysters that do not lose meat yield or quality to spawning in the summer and grow faster than normal diploids) is also available to growers to be sold at a higher price due to enhanced quality (Degremont et al. 2012).

Michael C. Voisin Oyster Hatchery

Rehabilitation efforts and the shift to AOC would not be possible without an oyster hatchery. The Michael C. Voisin hatchery requires many factors to be successful including reproductively competent broodstock, good water quality and consistent algal production. Algal production is often the limiting factor in large-scale oyster hatcheries. Moderate and consistent temperatures, sufficient lighting and good water quality are required to grow enough algae to support larvae and broodstock grown and conditioned in the hatchery. Due to its location close to the mouth of the Mississippi River in Southeastern Louisiana, the Michael C. Voisin Oyster Hatchery (hereinafter referred to as “the hatchery”) is subjected to hot summer temperatures (>30°C) and there are often periods of poor water quality from runoff and riverine inputs. Future river diversions, coupled with the hatchery’s close proximity to the river, will likely cause low salinity events (less than 10 ‰) that may limit oyster and algal production in the hatchery. To help combat these problems, the algal production room in the hatchery is temperature-controlled and utilizes natural sunlight and LED lighting to maintain high growth. Additionally, the hatchery can recycle water when it is suspected that ambient water quality will degrade, to ensure consistent algal and larval production.
Recycling water will be beneficial during periods of low salinity or poor water quality events in Caminada Bay, as well as during tropical storms or hurricanes. There are many finfish hatcheries and several aquariums that are supported by optionally recycling and/or recirculating water, with much research dedicated to studying optimal water quality levels. To our knowledge, however, there are no large-scale recycling bivalve aquaculture facilities other than the Michael C. Voisin Oyster Hatchery. Due to this unique status of being the first large-scale recycling bivalve hatchery, there are many unknowns about how recycling water may affect algal production and oyster larval culture success.

Flow through hatchery seawater maintenance

Typically, the hatchery uses natural seawater pumped directly from Caminada Bay stored in 30,000-liter (8000 gallon) tanks after being filtered rapidly using an Arkal disk filter system (Amiad Water Systems, Mooorseville, North Carolina) (Fig 2). Disk filtration allows the filtration of thousands of gallons of water to 20-microns (nominal) in a short time period. This 20-micron water is pumped directly to one of the three 30,000-liter storage tanks and subsequently filtered with a 50 GPM propeller bead filter (Aquaculture Systems Technologies, New Orleans, Louisiana) for 24 hours to remove additional solids and zooplankton to 10-microns. The bead-filtered water returns to its storage tank and is treated with sodium carbonate (soda ash) to buffer pH to 8.25 as needed (Fig 3). Prior to being pumped to the hatchery, the seawater is further filtered or “polished” to 1-micron with cartridge filters and then distributed to larval tanks or the algal production system in the Algal Production Room (APR).
The algal production system has additional filtration to remove contaminants and supply pure, natural seawater to algal cultures (Fig 4). Seawater is continuously-filtered through a 1-micron cartridge filter in a return loop to its storage tank to remove additional particulates.
Next, seawater is pumped through a tangential flow filter (Maxcell CFP-1-E-45, GE Healthcare Life Sciences, Pittsburg, Pennsylvania) to remove particles larger than 0.1 microns. This filtered water is collected in a reservoir for the algal system, and water is pumped through a pasteurizer at 80°C and a heat exchanger before being distributed to the algal system.

![Image](image)

**Fig 4: Algal Production Room water filtration; A) 1-micron filter with hatchery seawater supply line, B) seawater storage tank, C) 1-micron filter to polish seawater, D) tangential flow filter, E) water reservoir for system, F) pasteurizer, G) heat exchanger.**

**Recycling seawater filtration**

The hatchery enters recycling mode when ambient water quality is expected to degrade, prior to anticipated low salinity events, or during tropical storms so the staff can safely evacuate. The three 30,000-liter storage tanks are filled with seawater and valves on the tanks allow for 90,000-liters of seawater to be held for the duration of recycling period. A valve that typically drains the hatchery to the bay is closed, and the water is returned to the system to be recycled (Fig 3). Tanks are normally filled using the disk filter to 20 microns and subsequently filtered using the bead- and cartridge filters, however the bead filter also acts as a biofilter for
nitrifying bacteria during this time (during typical hatchery flow-through mode, the bead filter only provides solids removal, not nitrification). Additionally, a protein skimmer or foam fractionator is used to remove excess proteins in seawater stored in the tanks. Seawater is treated with soda ash to adjust pH to 8.25, and cartridge-filtered prior to being pumped into the hatchery.

Recycling Aquaculture Systems

Recycling or recirculating aquaculture systems (RAS) are dependent on the maintenance of water chemistry and maintaining stable bacterial communities. A typical RAS system pumps water from tanks to mechanical filtration, biofiltration and denitrification, aeration or oxygen enrichment, UV sterilization or ozone disinfection, pH regulation, heating, etc. to remove solids and maintain water chemistry within the optimal range (Bregnballe 2015). Optimal salinity and temperature may vary by species and location, but general conditions are listed in Table 1 (Buchanan et al. 1998).

Table 1: Target Water Quality Values in a conditioning RAS for C. virginica adults in Louisiana

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity</td>
<td>15 ‰</td>
</tr>
<tr>
<td>Temperature</td>
<td>15°C to 25°C</td>
</tr>
<tr>
<td>pH</td>
<td>8.0 - 8.5</td>
</tr>
<tr>
<td>Dissolved Oxygen</td>
<td>&gt; 3.6 mg/L</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.0 - 5.5 mg/L</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.0 – 460.0 mg/L</td>
</tr>
</tbody>
</table>

Source: Buchanan et al. 1998
Two of the biggest sources of failure in recirculating aquaculture systems are poor water quality and high heterotrophic bacterial loads (Badiola et al. 2012). Bivalves are generally much more tolerant of marginal water quality levels that may affect finfish, although stable water quality is desired for nitrification. Water quality can become degraded when solids are not adequately removed and high bacterial loads interfere with the nitrification cycle. A disrupted nitrification cycle can lead to high concentrations of ammonia that may become toxic or detrimental, and higher carbon dioxide levels that may affect pH (Kuhn et al. 2010). Generally, in RAS nitrite levels do not accumulate as they are quickly oxidized into nitrates, which are comparatively non-toxic to marine organisms, (Epifanio et al. 1975) and are readily assimilated by algae for growth (Guillard 1975).

Although large-scale recycling bivalve aquaculture hatcheries for oyster larvae have not been studied previously, there has been some limited research completed on smaller scales with juvenile and adult oysters and a few species of algae grown in these systems. Epifanio and Mootz (1976) successfully grew post-set *Crassostrea virginica* oysters in a RAS, with a growth rate comparable to oysters grown in nature. Four species of live algae were cultured to feed oysters in the Epifanio and Mootz study, but not using RAS water. Algae were grown in non-recirculated, ambient seawater from Delaware Bay, supplemented with Guillard’s f/2 in a semi-continuous system that grew stable cultures for up to three or four weeks.

Thiekler et al. (1982) reported an intensive bivalve recirculating system used to culture seed and adult oysters for over a year, and algal production (*T. lutea* and *Thalassiosira pseudonana* (“3H”)) cultured using pretreated natural recirculated seawater in Delaware.
Cultures were supplemented with Guillard’s f/2 in Kal-Wall tubes (Thiekler et al. 1982) semi-continuously, and grew well for part of the year, but became light-limited in the winter. Densities ranged from 2.5x10^6 cells/mL to 10x10^6 cells/mL, and an average of 5.0 x10^6 cells/mL was desired for culture longevity. Algal production was satisfactory, and water quality remained within target ranges, but the oysters grew poorly, possibly due to mishandling, poor diet, or poor genetics (some oysters grew better than others despite similar conditions).

Although not a recycling system, wastewater supplemented with varying amounts of seawater was used in a flow-through polyculture system by Ryther et al. (1975) at Woods Hole Oceanographic Institute. This system successfully used wastewater to grow local, natural microalgae in ponds (mostly diatoms including Phaeodactylum tricornutum and Chaetoceros sp.), seed oysters (C. virginica) and clams (Mercenaria mercenaria), polychaetes, artemia, winter flounder in tanks, and two species of macroalgae (Ulva lactuca and Chondrus crispus) with varying levels of total nitrogen (5-10 mg/L), also in ponds (Ryther et al. 1975). Algal biomass directly increased with increasing amounts of total nitrogen, until nitrogen concentrations reached 3-4 mg/L. High levels of nitrogen did not appear to negatively affect algal growth, but there are limits as to how much nitrogen can be assimilated by algae.

Throughout the course of their study, Ryther et al. (1975) used both natural and artificial seawater for algal culture, but did not indicate if one yielded better results than the other.

More recently, Kuhn et al. (2013) discussed the feasibility of culturing oysters in a recirculating system using Crystal Sea synthetic sea salt. Two common species of microalgae used in oyster aquaculture, Chaetoceros spp. and Isochrysis spp. were cultured using
wastewater from oyster tanks, and cultures were supplemented with Kent A and B Pro Algae Culture nutrient solutions (Kent Marine, Franklin, Wisconsin). Using oyster wastewater and supplemental nutrients, high cell counts (4.5 million cells/mL) were achieved within 3 to 5 days. Water quality was less than 1% of the lethal range for oysters, and due to the high calcium demand of adult oysters, supplementation with calcium in the form of quicklime to concentrations of 225 mg/L was all that was required. Overall, very high survival rates of adult oysters (greater than 99.0%) were attained, with comparable growth rates to adult oysters in the field, and success with algal culture in wastewater from an oyster RAS using artificial sea salt.

Nitrogen Cycle

Nitrogen is an essential element as an important component in nucleic and amino acids, and in biogeochemical cycles in the environment. It is most commonly present in five forms: organic nitrogen (N$_2$), nitrate (NO$_3^-$), which is the form most used in biological processes, nitrite (NO$_2^-$), particulate nitrogen, and dissolved organic nitrogen (DON) (Fig 5). Nitrogen can be introduced into the marine ecosystem via riverine, atmospheric, and anthropogenic inputs (Brandes et al. 2007).

In RAS or other forms of aquaculture, ammonia is a byproduct of animal waste and decomposing matter. It can be toxic to adult oysters at concentrations of 8.2 mg/L, and juveniles at 2.9 mg/L (Epifanio and Srna 1975). Nitrifying bacteria (ammonia-oxidizing) (Nitrosomonas sp.) convert ammonia and ammonium (NH$_3$ and NH$_4^+$) by reacting with oxygen to form nitrite (NO$_2^-$) (lethal at 470 mg/L for oyster adults, 570 mg/L for juveniles). Nitrite is
converted to nitrate (NO$_3^-$) by denitrifying bacteria (nitrite-oxidizing) (*Nitrospira* sp. and *Nitrobacter* sp.) that grow naturally in a RAS biofilter, and are not toxic to marine organisms (Brandes et al. 2007; Epifanio and Snra 1976; Kuhn et al. 2010; Timmons and Ebeling 2013).

Fig 5: The nitrogen cycle (from Brandes et al. 2007)

Phosphorus Cycle

Phosphorus is essential to living organisms, functioning as the structural backbone for DNA, RNA, and ATP, which is used for intracellular energy transfer and cell division during photosynthesis. It also contributes to the phospholipid structure in cell membranes (Paytan and McLaughlin 2007). Phosphorus is commonly found in the form of orthophosphate (PO$_4^{3-}$), also called reactive phosphorus. It can be used in biological uptake and is commonly limiting in marine ecosystems.

Phosphorus is introduced into the marine ecosystem via continental weathering, or through riverine influx with anthropogenic inputs (Payton and McLaughlin 2007) (Fig 6). In the
ecosystem, reactive phosphorus is taken up by primary producers via photosynthesis, and then consumed by zooplankters, etc. then eventually transferred up the trophic web. Ultimately, these organisms die and decompose, and the phosphorus is released and sinks to the ocean floor and continental shelf where it is essentially lost unless returned to the water column from upwelling (Payton and McLaughlin 2007).

In a RAS, phosphorus is introduced into the water through detritus, dead oyster larvae, broodstock or algae. Much of extra phosphorus is released, but some of the phosphorus is removed from the system through enhanced biological phosphorus removal (EBPR) from sludge recyclers through aerobic and anaerobic processes. De-nitrifiers can also store phosphorus if it is in excess (Barak et al. 2003).

Fig 6: The Oceanic Phosphorus Cycle
Source: Payton and McLaughlin 2007
Description of Algal Production

Aquacultured organisms that feed on algae throughout different phases of their lifecycles rely on diets that provides different nutritional benefits (Phatarpekar et al. 2000). Enright et al. (1986) reported that providing a mixed algal diet to oysters resulted in organisms with faster growth and higher meat yield. Furthermore, it is important in aquaculture to have temperature tolerant, fast growing algae that are within the correct size range, are non-toxic, provide good nutritional profiles and can be digested by the intended consumer organisms (Enright et al. 1986; Brown et al. 2002). Five species of algae are grown at the hatchery to provide a full nutritional spectrum for oysters in all life stages: eggs, D-stage (or straight-hinge) veligers, swimming late veligers, pediveligers, spat, seed and adults (Fig 7).

![Oyster Life Cycle Diagram](image)

Fig 7: The life cycle of an oyster.
Source: South Carolina Department of Natural Resources

*Tisochrysis lutea, Isochrysis galbana, Pavlova lutheri (Mono strain), Chaetoceros calcitrans,* and *C. muelleri* are grown mono-specifically in the Algal Stock Room. The algal stock
room is a small, climate controlled room isolated from the rest of the hatchery, where all five algae strains are grown in small-batch culture. Cultures are started in 500mL non-aerated flasks using starter cultures obtained from the NOAA algal culture collection and library, Milford, Connecticut. Cultures are transferred to aerated 1 L Erlenmeyer flasks, then to 2.8 L Fernbach flasks that are used to support the algal production system. Algae are grown in media comprised of natural seawater filtered to 0.1 microns with Guillard’s F/2, sodium meta-silicate, and Tris buffer solutions. Flasks with media are autoclaved at 121°C, with a sterilization time of 15 minutes, and allowed to cool overnight prior to use.

Aliquots of stock culture are transferred aseptically to new culture flasks under a positive pressure hood, which has HEPA filtration and is disinfected by UV light between uses to reduce chance of contamination (Combination PCR Workstation 600, Airclean Systems, Creedmoor, NC). Cultures are illuminated on mirrored shelves to reflect light for optimum efficiency with red (660 nm), blue (420-460 nm) and white LED strip lights with a total intensity of approximately 1200 LUX.

When cultures reach log phase, flasks from the Algal Stock Room are transferred to the continuous system in the Algal Production Room. Cultures are kept monospecific, aerated and illuminated via LED light strips and natural sunlight (3000-4000 LUX) in a temperature controlled room (25-29 °C), with supplemental nutrients solution. Natural, filtered and pasteurized bay water is continuously pumped to the cultures and continuously harvested upon reaching the desired volume and density. Algae are combined after harvest to produce 1700 L of algae daily with an average concentration of 5.5 million cells/mL. These algae are held in an
aerated storage tank until used in the hatchery to feed oyster larvae or adults; diluted with filtered seawater if necessary.

**Algal Growth**

Marine algae grown in this study (*Tisochrysis lutea*, *Chaetoceros calcitrans* and *Chaetoceros muelleri*) are unicellular autotrophs: requiring sunlight, carbon dioxide, and enriched seawater with the major nutrients of nitrate, phosphate, silicate (for diatoms), iron, trace metals, and vitamins to thrive (Guillard 1975). These algae typically grow via binary fission, also known as asexual reproduction, wherein they produce daughter cells from themselves to replicate (Bold and Wynne p.9; Borowizka, Beardall and Raven, pp. 1-7).

An algal culture undergoes five different phases of growth: lag phase, exponential (or log) phase, stationary phase, and senescent (or death) phase (Fig 8) (Creswell 2010). Batch cultures grow until mid to late exponential phase, when an aliquot is transferred to a new stock or aerated flask, or transferred into a semi-continuous or continuous system. Semi-continuous cultures are large-volume vessels that are grown to a dense concentration in late log phase. The majority of the culture is harvested, then refilled with new media (seawater and nutrients), and allowed to grow before harvesting again. Continuous systems maintain cultures in log phase, and maintain the culture in “steady-state” by maintaining a constant supply of fresh media (seawater and nutrients), that produces a constant volume of algae (Droop 1974).
Algal growth and quality varies between species and growth conditions, such as light intensity and photoperiod, temperature and nutrient concentrations (Fidalgo et al. 1998; Tantanasarit, Englande, and Babel 2013). Algae assimilate their required nutrients directly from the environment in which they are grown, maintaining an elemental composition of $106\text{C} : 16\text{N} : 1\text{P}$, known as the Redfield ratio in the open oceans (Leonardos and Geider 2004). In a laboratory, however, the concentrations of nitrogen and phosphorus may fluctuate and become limiting, hampering algal growth. The Michaelis-Menton model is commonly used to determine the maximum nutrient uptake by an algal culture (Droop 1974; Tantanasarit, Englande and Babel 2013), and the uptake rate can be different for each strain or isolate of algae, even within the same species (i.e. a culture can adapt to certain conditions) (Carpenter and Guillard 1971). Varying concentrations of these nutrients may affect culture growth and biochemical properties (carbohydrates, proteins and lipid fractions).

A large body of research has been focused on nutrient-limited algae and its effect on production and biochemical properties. Generally, under nutrient-limiting conditions the
growth rate slows or stops (nitrogen-limitation is most efficient). The algae instead put that energy into neutral lipids (triacylglycerides (TAG)) that act as an energy reserve for metabolism (Fidalgo et al. 1998; Lopez-Elias et al. 2014), instead of phospholipids that are used for cell structure and photosynthesis (i.e. growth and replication) (Fidalgo et al. 1998). If growth conditions change and nutrients become available, a culture can typically recover and switch back to biomass production (d’Ippolito et al. 2015).

Production and biomass can be measured several different ways. Among the most common methods (the ones used in this research) are cell concentration or density, dry weight and growth rate (Guillard 1973). Dry weight and cell concentration are often used to determine feeding volume or rate to the intended consumer (oysters). Dry weights are indicative of the amount of algae produced because the size of cells vary among the same species (Ketchum and Redfield 1949). Young or dilute cultures grow quicker and are much more variable in size compared to older, denser cultures that become more homogenous in size. There can also be a discrepancy in the amount of solids present in the algae by 20-30% (Ketchum and Redfield 1949). Therefore, it can be more representative to use dry weight measurements to compare growth and production.

Tisochrysis lutea

*T. lutea* are a species of Prymnesiophyceae (Haptophyte) algae from the order Isochrysidales (Sanchez et al. 2000). They are photosynthetic, unicellar, non-calcifying, mobile flagellates that are commonly used in aquaculture. *T. lutea* was formerly identified as *Isochrysis galbana*, (clone T-Iso) and was isolated from Tahiti. Previously believed as the same species, but
isolated from the Caribbean, *I. galbana* (clone C-Iso) are morphologically similar, but have slightly different chemical properties and some differences in taxonomy (Bendif and Pruder 2013). These algae are predominantly motile cells, in spherical to ovate or oblong shape of 3.5-6 microns and have 2 flagellae measuring up to 7 microns long, with mean cell volumes of 40-50µm$^3$ (Helm et al. 2004).

Chaetoceros spp.

*Chaetoceros calcitrans* and *C. muelleri* are both diatoms of the class Diatomophyceae (Bacillariophyceae). They are both non-mobile, can form chains with their spines, and are surrounded by crystalline coverings made of silica, called frustrules (Olenina et al. 2006). *C. calcitrans* is a smaller alga with an oval shape, with a size range of 3-9 microns (Olenina et al. 2006), and a mean cell volume of 35µm$^3$ (Helm et al. 2004). *C. muelleri* is larger at 7-9 microns (Olenina et al. 2006), and mean cell volume and range is 80 µm$^3$ (Helm et al. 2004).

Objectives and Hypothesis:

Degraded oyster populations have led to a need for aquacultured oysters, requiring hatchery grown larvae. Hatchery success relies on high algal production, which may be affected by the use of recycled seawater. This study focuses on the affects recycled water may have on algal production in the hatchery. To test for effects from recycling water for algal culture, several different types of water were compared. These included: 1) natural, ambient seawater from Caminada Bay filtered via bead, mechanical, and tangential flow filtration (TFF) to 0.1 micron; 2) recycled seawater discharged from oyster hatchery operations (i.e., algal culture, flow through or static oyster larval culture, broodstock conditioning tanks, etc.) treated with
soda ash to adjust pH, passed through a protein skimmer, and filtered to 0.1 micron via tangential flow filtration; and, 3) Instant Ocean® artificial seawater prepared with tap water after reverse osmosis treatment.

The null hypotheses for this study was that algal production will be equal between each water source: \( H_{01}: \mu_{Bw} = \mu_{Rw} = \mu_{Aw} \) (where \( Bw \)= bay water, \( Rw \) = recycled water and \( Aw \) = artificial water), and will be equal between species: \( H_{02}: \mu_{Tl} = \mu_{Cc} = \mu_{Cm} \) (where \( Ti \) = *Tisochrysis lutea*, \( Cc \) = *Chaetoceros calcitrans*, and \( Cm \) = *C. muelleri*). Alternatives to the null hypothesis include that algal production will vary by water sources and species of algae: \( H_{A1}: \mu_{Bw} \neq \mu_{Rw} \neq \mu_{Aw} \) and \( H_{A2}: \mu_{Tl} \neq \mu_{Cc} \neq \mu_{Cm} \).

**Methods**

**Water Collection and Preparation**

Bay and recycled water were collected from the hatchery’s algal production system. Filtered seawater was collected after the tangential flow filter (natural seawater filtered to 0.1 microns), stored in a five-gallon bucket, and frozen in a chest freezer until the trial or block could be conducted. Two days before the start of the block, the previously collected bay and recycled seawater were thawed and artificial seawater was made with Instant Ocean to match the natural seawater salinity.

Three blocks were conducted from three different recycled water events. The first event began September 30, 2015 when bay water was collected, and water was recycled in the hatchery until October 6, 2015 (1 week), when the recycled water was collected. The second
event was during a low salinity event in the bay, from May 30, 2016 to June 13, 2016 (two weeks), and the third block was from September 13, 2016 to September 20, 2016 (one week).

Media Preparation

Media was prepared using the appropriate water source (bay water, recycled water, or artificial seawater) with the recommended levels of Guillard’s F/2, sodium meta-silicate, and Tris buffer solutions (Appendix A).

After collecting, freezing, thawing and preparing all water sources as necessary, media was prepared by adding Guillard’s F/2, sodium meta-silicate, and Tris buffer at concentrations of 2.5 mL/L of water for Guillard’s F/2 and sodium meta-silicate, and 10 ml/L of water for the Tris buffer. Acid-washed Erlenmeyer flasks each received 300 mL of media, with foam closure covered with aluminum foil, and autoclaved (Hirayama Model HV-110, by Amerex Instruments, Concord, California) in the hatchery for 45 minutes at 20 psi and 121°C. For each block, ten flasks of each water source were prepared for the three species, with three replicates for each and an extra flask that was used to measure initial water quality prior to algae inoculations. Flasks were allowed to cool at room temperature overnight prior to inoculations.

Inoculations

Cultures of *Tisochrysis lutea*, *Chaetoceros calcitrans* and *C. muelleri* were acclimated to nutrient composition, lighting and temperature conditions at the hatchery. Cultures were maintained as monocultures, but it is unknown if they were axenic.

Parent cultures were prepped for four days prior to the beginning of each trial. Each parent culture was a 1L, non-aerated Erlenmeyer flask, with 600 mL of inoculum, containing f/2
and silica solutions, and tris buffer, and was used for all transfers of that species (i.e. all research cultures of the same species originated from the same parent culture). Each treatment water source was kept in triplicate and inoculated with each species of algae. On the first day of the block, 50 mL aliquots were transferred from the mature parent flask in log phase. Transfers were completed under the algal stock room hood. Each flask was flame-sterilized prior to transfer, using a propane torch. A bulb pipette was used to transfer algae to maintain accuracy between replicates. Immediately upon inoculation, each flask was sampled for initial counts and labeled with the species name, date of the transfer, and water source.

Culture Conditions

Research cultures were grown in non-aerated 500 mL Erlenmeyer flasks with 350 mL volume, with a 12:12 hour light-dark cycle under red (660 nm), white, and blue (420-460 nm) LED strips with a total illumination of ~ 1200 LUX. All research cultures were maintained in the oyster hatchery algal stock room, randomly distributed on one shelf. Temperature of the stock room was kept constant between 23-25°C, and flasks were hand-swirled twice daily for aeration throughout the duration of 10 days, as per hatchery protocol (Appendix B).

Data Collection: Biomass and Productivity

One milliliter samples of algae were collected from each flask on day 0 (immediately after inoculation), and days 2, 4, 6, 8, and 10 at approximately the same time to measure algal growth. Samples were stored in micro-centrifuge tubes with 50µL of Lugol’s solution in a refrigerator (5°C) to halt algae growth. On day 10, water quality parameters were tested again
from each flask, and samples of algae were collected for cell density and dry weight for biomass analysis.

To measure growth and productivity, cell densities were estimated on designated days (0, 2, 4, 6, 8, 10). Cell counts were completed in triplicate using a haemocytometer and compound microscope at 10x magnification with Lugol’s solution to ensure accurate counts. The growth rate was estimated as: \( \mu = \frac{\ln B_n - \ln B_0}{t_n - t_0} \), where \( \mu \) = growth rate, \( B_0 \) = initial cell density (cells/mL), \( B_n \) = cell density at time \( n \), and \( (t_n - t_0) \) period of culture time since inoculation (as described in Guillard 1973 and Martinez-Cordova et al. 2012).

Dry biomass was completed by collecting 25 mL algae on pre-weighed 0.2 µm glass fiber filters, and dried in an oven at 70°C for two hours or until constant weight. Upon drying, filters were weighed again, and the dry biomass was measured by calculating the difference, and dividing by 25 to obtain the result as mg/mL (simplified to g/L) (Martinez-Cordova et al. 2012).

Water Chemistry

Salinity, temperature, pH, nitrate, reactive phosphorus, and silicate were measured in the spare flask at the start of the experiment. Salinity, temperature, and pH were measured using a pH probe (Hach HQ11D, Hach Company, Loveland, Colorado). Other parameters were measured using a spectrophotometer (Hach DR/890, Hach Company, Loveland, Colorado), following manufacturer protocols. Nitrate was tested using the Hach Cadmium Reduction method (Method 8192), reactive phosphorus was tested using the Hach PhosVer3 Method, Test N Tube Procedure (Method 8048), and silicate was measured using the Silicomolybdate Method (Method 8185). All parameters were measured in each replicate after 10 days of growth using the water filtered from the dry weight samples, as completed by Tantanasarit et al. (2013).
Statistical Analysis

Production of algae across different water treatments and species was analyzed with generalized linear mixed models (Proc GLIMMIX), using the Statistical Analysis System 9.4 (SAS) (Gibbs 2008). At the completion of this study, the total sample size was 81. Three species of algae were grown in three different water sources over three individual blocks, for a total of 27 replicates per species and per water source. A level of \( \alpha=0.05 \) was considered significant.

Results

Water Chemistry

Nitrate, phosphate and silicate concentrations were measured in each replicate at the beginning and end of the block, and compared against water source and their effects between species. Nitrates were not different between the three species of algae (\( p=0.1739 \)) or between the different water sources (\( p=0.3904 \)) (Fig 9). Phosphorus and silicate were significantly different between the water sources (\( p<0.001 \)), but only silicate concentrations were different between species; silicate concentrations were much higher in \( T. lutea \) replicates (\( p<0.001 \)) (Figs 9 and 10).

Differences were also found between initial and final concentrations of all three water chemistry parameters (nitrate: \( P=0.0357 \), phosphate: \( p<0.0001 \), and silicate: \( p<0.0001 \)) (Fig 10). These differences, however, were not found in the overall interaction of species and water source and time (nitrate: \( p=0.3193 \), phosphate: \( p=0.9878 \), and silicate: \( p=0.6445 \)).
Initial water quality by each water source, block, and water chemistry: nitrate, phosphate, or silicate, prior to the start of the study is presented in Figure 11.

Fig 9: Mean water chemistry (nitrates, phosphates, and silicates) ±SE by species and water source. T-Iso = *Tisochrysis lutea*, CC = *Chaetoceros calcitrans*, CHGRA= *C. muelleri*. Bay= ambient bay water, Rec= recycled bay water, and Art= artificial seawater. α=0.05 Error bars indicate ± standard error.

Fig 10: Comparisons of initial and final water chemistry. Error bars indicate ± standard error.
Dry weights were measured in each of the replicates at the conclusion of each block and compared by species and water source (Fig 13). Dry weights were significantly different between species (p=0.0475). *T. lutea* was the lowest (0.4180 g/L), followed by *C. calcitrans* (0.4521 g/L) and *C. muelleri* was the highest with a mean weight of 0.4696 g/L. Water source had no effect on the dry weight of the algae tested (p=0.8679).
The overall growth rate was determined using calculations from initial cell concentrations and final concentrations on day 10 using the equation: \( \mu = \frac{\ln B_n - \ln B_0}{(t_n-t_0)} \).

Growth rate was significantly different between species (P<0.001). \textit{T. lutea} grew fastest (\( \mu=1.46327 \text{ d}^{-1} \pm \text{SE} \)), followed by \textit{C. calcitrans} (\( \mu=1.45197 \text{ d}^{-1} \pm \text{SE} \)), and \textit{C. muelleri} (\( \mu=0.1.44268 \text{ d}^{-1} \pm \text{SE} \)) grew the slowest (Fig 13).

Growth rate was not significantly affected by water source (p=0.5004), nor by the interaction of species and water source (p=0.1242). While not statistically different, algae grown in bay water grew the fastest (\( \mu=1.45672 \text{ d}^{-1} \pm \text{SE} \)), followed by recycled and artificial water (\( \mu=1.445507 \text{ d}^{-1} \pm \text{SE} \) and \( \mu=1.44613 \text{ d}^{-1} \pm \text{SE} \)).
Fig 13: Mean growth rate (doublings per day) (±SE) of 81 different cultures of algae, grouped by species and water source. T-Iso = *Tisochrysis lutea*, CC = *Chaetoceros calcitrans*, CHGRA = *C. muelleri*, Bay = ambient bay water, Rec = recycled bay water, and Art = artificial seawater. α=0.05. Error bars indicate ± standard error.

Cell concentrations

Each replicate was sampled for the cell concentration every other day for 10 days and compared between species and water source (Fig 14). Log transformed cell counts of the replicates showed that there were significant differences between species (p=0.0001). The species that yielded the highest cell concentrations at the completion of ten days was *T. lutea*, (2.448 x10⁶ cells/mL), followed by *C. calcitrans* (2.267 x10⁶ cells/mL), with *C. muelleri* growing the least (2.053 x10⁶ cells/mL). Water source did not significantly affect algal growth (p=0.1463) (Fig 8). Despite lack of significance, bay water produced the highest cell concentrations (2.333 x10⁶ cells/mL), followed by recycled water (2.297 x10⁶ cells/mL), and artificial water produced the least (2.137 x10⁶ cells/mL). Overall, the interaction between species and water source over the period of this study was not significant (p=0.2882) (Fig 15).
Fig 14: Mean final algal concentration, (cells/mL) (±SE) of 81 different cultures of algae, grouped by species and water source. T-Iso = Tisochrysis lutea, CC = Chaetoceros calcitrans, CHGRA= C. muelleri. Bay= ambient bay water, Rec= recycled bay water, and Art= artificial seawater. α=0.05. Error bars indicate ± standard error.

Fig 15: Mean log algal concentration (cells/mL) of algae by water source, over a period of 10 days. T-Iso = Tisochrysis lutea, CC = Chaetoceros calcitrans, CHGRA= C. muelleri. Bay= ambient bay water, Rec= recycled bay water, and Art= artificial seawater. α=0.05.
Fig 16. Mean algal concentration (cells/mL) of algae by water source, over a period of 10 days. 
T-Iso = *Tisochrysis lutea*, CC = *Chaetoceros calcitrans*, CHGRA = *C. muelleri*. Bay = ambient bay water, Rec = recycled bay water, and Art = artificial seawater. α=0.05.

**Discussion**

Algae is a critical component of any bivalve hatchery, and is often the limiting factor for production of larvae, requiring testing to be completed to determine if algal production may be jeopardized. Algae is routinely grown in wastewater systems, but extensive algal culture has not been studied using recycled seawater from an oyster hatchery rearing larvae. Three species of algae common in aquaculture for bivalves were grown in ambient seawater, recycled seawater, and artificial seawater, and their growth rate and biomass were compared.

The growth rates of the algae in this study were not affected by water source, but the overall growth rate was different between species. Literature is inconsistent on whether diatoms or flagellates grow faster, but in our study, both *Chaetoceros* spp. of diatoms grew
slower than the flagellate, *T. lutea*. Cultures were grown statically under 12:12 light at 1200 lux in 23-25°C with f/2, and had the overall growth rates of *T. lutea*: $\mu = 1.463 \text{ d}^{-1}$, *C. calcitrans*; $\mu = 1.452 \text{ d}^{-1}$ and *C. muelleri*; $\mu = 1.443 \text{ d}^{-1}$. Thompson et al. (1992) compared these same species using enriched media, but under very different conditions: continuous light at 220 $\mu$mol m$^{-2}$s$^{-1}$, a stirrer bar and bubbling aeration. In this environment, *C. calcitrans* grew the fastest (25°C, $\mu = 3.39 \text{ d}^{-1}$), followed by *C. muelleri* (20°C, $\mu = 1.67 \text{ d}^{-1}$), and *T. lutea* (25°C, $\mu = 1.38 \text{ d}^{-1}$) grew the slowest. Despite a much higher light intensity (220 $\mu$mol m$^{-2}$s$^{-1}$= approximately 16280 lux), and being aerated, *T. lutea* grown in the hatchery grew at a rate comparable to those in Thompson’s research.

Renaud et al. (2002) compared the growth rate between several different species in aerated cultures with temperatures ranging from 25 to 35°C under 12:12, 80 $\mu$mol m$^{-2}$s$^{-1}$ (5900 lux). *T. lutea* had the highest growth rate at 27°C ($\mu = 0.97 \text{ d}^{-1}$), compared to 30 °C ($\mu = 0.87 \text{ d}^{-1}$) for *Chaetoceros* sp. CS256. McGinnis et al. (1997) also support a temperature of 30°C for *C. muelleri* with a growth rate of nearly 3.5 d$^{-1}$. Growth rate of *C. calcitrans* was also measured in aerated cultures in 27-30°C, and found to be 0.94 d$^{-1}$ (Tantanasarit et al. 2013). Despite the differences in temperature, growth rate of *C. calcitrans* was higher in the hatchery, but it is likely that the growth rate of the *Chaetoceros* spp. in the hatchery is optimal at 30°C, instead of the 23-25° range in which they were grown.

Variation in growth rate between studies is most likely due to various growth conditions: aerated vs non-aerated, light intensity and photoperiod, and temperature are the main factors influencing growth rate when nutrients are not limiting (Guillard 1975). Aeration provides constant mixing and agitation to cultures that prevents over-shading, keeps the cells in
suspension, break up chains of diatoms, and introduces additional carbon dioxide into solution, providing extra carbon for photosynthesis (Guillard 1975). The algae grown in the hatchery were static, but were swirled twice daily to mix in carbon dioxide and agitate cells. The limited amount of swirling/agitation may have been a contributing factor to slower cell growth in the diatoms. Although lower growth rates compared to the literature were likely caused from the lower than recommended level of silicate present in the initial water sources for the diatoms, production and growth were comparable for the flagellate.

Hatchery algae were grown under 2:2:1 of blue (420-460 nm): red (660 nm): white light strips. It is possible that *T. lutea* grew faster than the two *Chaetoceros* spp. due to a photosynthetic preference for a different light profile (different red: blue ratios). A review by Schulze et al. (2014) explained that algae with different structures, levels of chlorophyll α, β or c, or other accessory pigments, react to different wavelengths of light and intensities differently and may photosynthesize under wavelengths differently. For example, algae with more chlorophyll α, are more photosynthetically efficient under the LED wavelengths of 420 – 470 nm (blue) and/or 660-680 nm (red) wavelengths. It would be advantageous to culture them under red and blue lights for highest growth. In the way that oyster larvae perform better when fed a variety diets, algae grow best under a mixture of wavelengths so they are not limited.

Dry weights and final cell concentration were also compared between the three different species and water sources. Water source had no effect on either result, but production was different between species. Despite growing the fastest and the densest, *T. lutea* was the lightest by dry weight, followed by *C. calcitrans* and lastly *C. muelleri*. Contrary to this result, a study by Volkman et al. (1989) analyzed ten common species of algae grown for
aquaculture under 12:12 light (70-80 \mu mol m^{-2}s^{-1} or 5100-5900 lux), in Guillard’s f/2 and found that *C. calcitrans* grew the densest, followed by *T. lutea*, and lastly *C. muelleri*. Data provided by Volkman et al. (1989) indicate that *C. calcitrans* is the smallest of the three algae, followed by *T. lutea*, and lastly *C. muelleri*.

Previous work and theories suggest that smaller species of algae may grow faster because a greater surface area to volume ratio allows for faster uptake of nutrients (Fogg and Thake 1965; Phatarpekar et al. 2000). Research has indicated that phosphate concentrations do not limit growth, but silicate can become limiting in diatom cultures and nitrogen limitation will alter the biochemical composition of algae (Lomas and Glibert 2000; Lopez-Elias et al. 2014; Thomas and Dodson 1968). To prevent cultures from becoming nitrogen limited too soon after inoculation, Guillard (1975) recommends an initial concentration of 75 mg/L of nitrate, 5 mg/L of phosphate, and 30 mg/L of silicate in diatom cultures.

In this study, nitrates were lower than the recommended levels in the first block across all three water sources, and were higher in the second and third blocks except for block 3 artificial seawater. Phosphates exceeded the recommended level by over four times in all treatments, except for block 3 bay water, when it was slightly lower. The recommended level of 30 mg/L of silicate was not met in any treatment, and may be limiting in the diatoms. Despite the water source and initial concentration of nitrate prior to the addition of Guillard’s F/2, nitrates were not significantly different between water sources, though phosphate and silicate were.
A significant difference was found between silicate concentrations between water sources and species. It was much higher in *T. lutea* vs. the diatoms since flagellates do not require silicate. At the completion of the study, there was still silicate present, so the algae were not completely silicate-limited in the duration of the 10-day study.

The RAS of the hatchery was operated differently during the first block versus the second and third. The first block was in October of 2015 for 1 week at the end of a short hatchery season in a new facility. During this block, the water storage tanks were cycled with a UV sterilizer after the bead filter, and water was pumped through an activated carbon filter prior to distribution to the rest of the hatchery. The second and third block did not undergo UV sterilization, or activated carbon, due to concerns that UV-generated ozone can be harmful to oyster larvae with the absence of activated carbon (Supan, personal comm). Absence of UV sterilization possibly led higher levels of ammonia and nitrite in blocks 2 and 3, and *Nitrosomas* sp. and *Nitrobacter* sp., converted them to nitrate, yielding higher concentrations than the first block.

Applications to Daily Hatchery Operations

While the majority of the algae produced at the hatchery are grown in the continuous system under many different conditions, media for all cultures in the stock room are made using water collected from the algae system. Considering that all hatchery operations succeed or fail on the success of algal cultures in the stock room, it was imperative to study possible effects on algal culture, such as water source. Since this study concludes that the production of static cultures grown in the stock room do not differ by water source, we now know that no
special care must be used when preparing media during recycling periods vs flow through periods, and it is not necessary to use artificial sea salt.

Mass production of algae grown with recycled seawater may be different from the results found in this study due to the dynamic culture conditions that mass production can endure. This study used controlled, static, batch culture to grow the algae where the mass production of algae grown in a continuous system (like the hanging-bag algal system at the Michael C. Voisin Oyster Hatchery) with many more variables and inconsistencies. Algae grown in non-aerated, batch cultures begin with a finite amount of nutrients, without supplemental carbon dioxide, and are intentionally grown slowly in a small, windowless, temperature-controlled room with artificial illumination; i.e. a consistent/steady environment (Helm et al. 2004, pp. 34-58). Growth is slow and relatively steady, but exponential over time.

Continuous culture, however, has a much more dynamic environment. Continuous culture typically starts with an inflated, empty polyethylene bag that is inoculated with a dense flask grown in intermediate-scale batch culture. Pasteurized sea water and nutrients are delivered slowly to allow the algae to grow as the volume of the culture grows. When the bag is full, it is harvested at an equal rate of water input. The goal is to keep algal density high and steady, so the culture can provide high algal biomass with less work (large-scale batch culture production is very time and labor intensive). Bags are kept monospecific and they are aerated with carbon dioxide supplemented air, illuminated by both artificial and natural sunlight. Temperature is less controlled, and water and nutrient delivery is more variable. Nutrients are delivered via a peristaltic pump into the seawater line every hour, instead of a high concentration of nutrients initially (batch culture), that can be assimilated as the algae grow.
Nutrient concentrations in the system can vary, from nitrate or phosphate limited to replete, depending on volume and density of the algal culture. This may result in inconsistent biochemical composition of the algae, despite the overall goal to maintain constant growth and concentration.

Conclusions and Future Research

Although there were no statistically different effects of water source on algal growth for these three species, ambient bay water appears to be more robust than the other water sources. More research will be needed to see if water chemistry changes in recycled water for periods of longer than two weeks, and to determine how long the hatchery can operate in recycling mode until there is a difference in water chemistry. Previous research was completed in Delaware and Virginia on adult and juvenile oysters in RAS, but not in Louisiana or with any species of bivalve larvae. It would be beneficial if that research is also completed.

Additionally, research is needed to conclude if the biochemical compositions (proteins, lipids and carbohydrates) of the three species of algae differ when grown in the different water sources, and if this will affect the growth of algae in the continuous system. Furthermore, according to many visitors to the hatchery over the past two seasons, we are the only hatchery utilizing LED lights to grow our algae. It would be helpful to measure algal biomass and growth rates under varying LED light levels, red: blue ratios, etc., to further maximize production, since the diatoms experienced slower growth rates than described in the literature. Despite the slower growth, however, the diatoms currently grow sufficiently in the hatchery stock room.
References


Louisiana Oyster Dealers and Growers Association.  


Appendix

A. Nutrient Formulations for solutions used at the Hatchery

Nutrient Solution (modified Guillard’s F/2)

- Vitamin Solution to the 2.5” mark (stored in the refrigerator)
- Add Iron Solution to the 6” line
- Add R.O. water to the 9” line
- Add 25 ml of trace metal solution (stored in the refrigerator)

Vitamin Solution

- 10 L RO water
- 450 g sodium phosphate
- 30 g thiamine
- 150 mL biotin solution (0.25 g/L – stored in refrigerator)
- Vitamin B12 (just enough to turn it pink, add very slowly)

Iron Solution

- 10 L RO water
- 1,500 mL (dry measure) sodium nitrate
- 250 mL (dry measure) chelated iron

Trace Metal Solution

- 1 L RO water
- 1.2 g Copper sulfate
- 0.76 Sodium molybdate
- 2.64 g sodium molybdate
- 2.64 Zinc Sulfate
- 1.2 g Cobalt chloride
- 2.16 Manganese chloride

Silica Solution

- 20 L HOT water
- 600 g sodium metasilicate monohydrate
- Add 630 mL muriatic acid (31.4%) very slowly and stir constantly

Tris Buffer Solution

- 950 mL RO water
- 100 g Trisma Base (Sigma T1503)
- 75 mL muriatic acid
Media Preparation:
For 1 L of seawater, add 2.5 mL of Nutrient solution, 2.5 mL Silicate solution and 10 mL of Tris buffer.

B. Algae Protocols at the Michael C. Voisin Oyster Hatchery

(Adapted and modified from Guillard 1975 and the FAO Hatchery Culture of Bivalves)

When performing algal work always have clean hands and wear gloves. Use isopropanol OR rubbing alcohol liberally (without getting it into algae cultures). Always do any algal work at the beginning of the day and always before touching raw seawater or larvae.

Glassware and Flask Prep

All glassware is washed in 5% muriatic solution. Wear long acid gloves and safety goggles to help protect yourself. If the acid solution splashes on you, neutralize it with a sodium bicarbonate (baking soda) or alconox solution and rinse well with water. If it splashes into your eye, flush with eyewash and seek medical attention.

5% Muriatic Solution:

10 L RO and add ~250 mL of 31.4% muriatic acid.

Cleaning Glassware

1. To wash flasks, first rinse with tap water. If there is any build up on the sides of the flask, soak for a bit in water then scrub with a brush.
2. Wearing gloves, dunk flask in 5% muriatic acid solution bucket. Dump (back into bucket) and repeat several times to ensure the entire surface of the flask has been cleaned.
3. Rinse with R.O. water at least 3 times (discard first rinse into waste bucket and any subsequent rinses can be dumped down the drain).
4. Erase any pencil markings before moving to the rack to dry.
5. To clean air sticks, put sticks in acid bucket, and use a beaker to pour acid through the air sticks.

   If there is any salt build up or algae caked onto the air stick, use a brush or pipette to scrape it out. Dump more acid through the stick to rinse the salt residue out from both top and bottom and make sure the stick is clean.
6. Put in a 2L beaker filled with R.O. water to rinse, and flip the sticks so each side gets rinsed with R.O. water.
7. Dump out water and set aside to dry.
8. To clean foam stoppers:
a. put them in a beaker with RO water and squeeze them so they suck in the water.
b. Dump and squeeze out water two times, then allow to dry.
c. Once dry, autoclave, then put on lab bench by the silica bottle so they can be used.

Flask Prep

1. All flasks should be cleaned according to previous glassware cleaning procedure.
   a. Clean flasks will be on shelf in algal stock room, or on drying rack. Any flasks to the right of the sink with pencil/sharpie marks are dirty
2. Choose the appropriate size flasks that need to be prepped by looking at what is currently growing in the stock room, and transfers that will need to be made in the next few days.
3. Fill flask with media to the correct volume:
   a. 500 mL Erlenmeyer stock culture: Fill to ~300-325 mL
   b. 1000 mL Erlenmeyer aerated culture: Fill to ~600 mL
   c. 2800 mL Fernbach aerated culture: Fill to 1 cm below “No. 2240” label
4. Put correct air stick in the flask and make sure it’s not too long or too short. (You may need to switch them around). Put OPEN clamps on tubing (White pinch clamp on long tubing, beige screw clamp on short tubing, if aerated. 500 mL cultures use the foam stoppers. Put a stopper on (make sure it’s not too loose so it doesn’t fall in the culture) and cover it with aluminum foil.

5. Ready to Autoclave

Autoclave Procedures

For the Hirayama HICLAVE HV110 Toploading Autoclave

1) Drain the chamber by opening the valve underneath the water basin
2) Fill water basin so the water level is between the “Low” and “High” markers
3) Close valve and put water basin back into its slot
4) Pour 5L of R.O. water into chamber
5) Put flasks filled with media into baskets (with appropriate salinity tags) and put into autoclave. Make sure the airlines are not pinched or obstructed
   a. All aerated flasks should have an open beige screw clamp on the short tubing and an open white pinch clamp on the long tubing. Stock cultures should have a foam stopper wrapped with “pinchable” aluminum foil caps
6) Close and lock lid
7) Select mode, time and exhaust level
1. To change the mode, press the “Mode button” until it is flashing on the desired setting. Use MODE 2 for autoclaving anything with liquid in it (i.e. flasks with media, stoppers being processed in water, etc.). To change the mode, autoclave should be ON and press the MODE button until it flashes with the one you want. Always mode 2 for filled flasks, mode 3 for glassware ONLY.
   a. To change time or exhaust level, press “Set/Ent” then the “Next” button until the desired variable is flashing. Then use arrow buttons to change the numbers. When done, press “Set/Ent” again to exit.

8) Press start

9) When the cycle completes (about 2 hours later) carefully open the door and remove the baskets.

10) Put flasks on the beige cart, immediately closing beige screw clamp, and spraying an air filter with isopropanol and attaching to the long tubing

11) When cool, mark with salinity and move into stock room on appropriate shelf

Drain chamber and empty water basin between loads

Glassware - Use mode 3 for 20 minutes at 121 ℉
- Flasks with algae media – Use mode 2 at 121 ℉, exhaust level 10%
  o 500 mL Stock cultures (filled to 300 mL) - 37 minutes
  o 1 L aerated flasks (filled to 600 mL) – 48 minutes
  o 2.8 L Fernbach flasks (filled to ~1cm below the NO. 4290 mark) – 60 minutes
  o Carboys – 250 minutes
- Always select time for the largest flask present

Algae Inoculation Procedures

Always inoculate algae cultures in the beginning of the day with clean hands, arms, and gloves and use the “Pinch, spray, flick” method

Flasks

1. Swirl flasks as normal during “morning chores”
2. Turn the UV light to sterilize the hood for 5 minutes prior to transfers.
3. Select the new flasks to be transferred and spray the exterior surfaces with Isopropanol. Put in hood. (These flasks should have been acid washed, filled with new media (filtered seawater, nutrients, silica, and tris buffer), autoclaved, allowed to cool overnight and the salinity written. Make sure the new salinity is appropriate (not too big of a change: keep within 4-5 ‰ for larger cultures, should be same or only 1-2 ‰ different for 500 mL stocks).
4. Sterilize the hood for 20 minutes with the UV light. (while the hood is sterilizing, complete Erlenmeyer to fernbach transfers and
5. Allow any flocculants in starter flask to settle on the bottom.
6. Select stock culture flask to be transferred (choose older, darker flasks– it’s in late log/stationary phase and has poor growth and would not be beneficial in the APR. Make sure it’s good quality algae- not milky/cloudy, clumpy, etc”. Spray with Isopropanol and put in hood.
   a. We typically do all of the same strain at the same time (ie. All the α or β at once, then a few days later transfer the γ and Δ, each one weekly. Erlenmeyers to Fernbach transfers as needed, typically. 4-5 days after inoculation.) Always recreate the same stock in to a new 500 mL flask, then 1-3 erlenmeyer flasks OR 1 Fernbach flask.

In the hood
7. Pinch aluminum foil/foam stopper on the parent flask to remove it and put to the side, being careful not to allow the stopper to touch the hood.
8. Pinch aluminum foil/foam
9. Flame tops of flasks quickly to sterilize (without raising temperature of the media). If inoculating an aerated flask, raise the stopper to just above the flask, but do not pull it out.
10. Flame sterilize and hold the stopper to just above the flask. Slowly and carefully pour algae from its parent stock into its new container (25-50mL for stock cultures, ~100 mL for 1L aerated flasks, and as much of the remaining stock into a Fernbach). Pour algae straight through the top of the opening; try not to spill or allow the algae to touch the side of the flask. Always inoculate stock cultures first.
   a. Avoid pouring flocculent into new cultures, if necessary; use a UV sterilized pipette to suck up the best quality algae. Always select the best culture. If the flask is too sticky or discolored, discard the culture and select another.
11. Quickly flame sterilize the top of the flask and replace stopper into its original position.
12. Wipe surface of flask with a KimWipe and Isopropanol, if necessary.
13. Label flask with species name, date of transfer, previous transfer date, and salinity.
14. Connect aerated flask culture to air filters. Sterilize tubing with Isopropanol (be careful not to drip Isopropanol into culture) and air filter (“Pinch, spray, flick”!).
15. Turn down air, connect to airline, and remove clamps. Adjust air flow to proper aeration.
   a. Slow aeration for new/light cultures and increase velocity of bubbles as the culture grows darker.

1L Erlenmeyers to Fernbachs
1. Pinch off short tubing and disconnect air from 1 L Erlenmeyer that is to be transferred.
2. Select inoculation tubing from inside the hood that has been cleaned with Isopropanol, UV sterilized and dried.
3. Connect inoculation tubing short tubing on the erlenmeyer and fernbach (remember to spray with Isopropanol to clean)
4. Clean the long tubing on the outflow of the Erlenmeyer with Isopropanol.
   a. Pinch it close to the glass stick, spray with Isopropanol to make sure it has coated the surface, let it drain, then connect a clean air filter to the long tubing you just cleaned. Algae should move from the old, parent flask to the new Fernbach.
   b. Adjust airflow if necessary to make the algae move.
5. When transferring algae, make sure flocculants don’t transfer to the new flask. Once complete, disconnect the air filter then tubing, and remove inoculation tubing. Clean the short tubing piece on the Fernbach and reconnect the air filter. Adjust aeration as necessary.
6. Label the new Fernbach with species name, date, date of parent flask and salinity.
7. Clean inoculation tubing with R.O., use syringe in the Tupperware to push Isopropanol through it, drain, then put under the UV light.
8. Clean flasks as per the glassware cleaning protocols.

Algae Bags

Setting up a bag

1. Unroll a sealed and skewered bag from the bin. Make sure there are no holes, tears, stretches, etc. and hang on outrigger with the skewer facing you.
2. Get an air filter that has been UV sterilized, spray with Isopropanol and connect to airline with the “IN” side facing up toward the rack. Be careful not to spray up into filter.
3. Clean airline pipette tip with tweezers, and using the syringe, push Isopropanol into the airline through the pipette end, pinch clamp and hold for 15 sec. (It’s helpful to close white clamp prior to putting syringe in, open the clamp when it’s in place, then open the clamp to push isopropanol up.) Allow the Isopropanol to be pushed out of the tubing and collect it in the Tupperware to be reused.
4. Once the Isopropanol has been pushed out, spray the top of the bag beneath the seal, poke a hole through, and clean then connect the airline to inflate the bag.
5. Fill the bag with air, but make sure it doesn’t pop into a corner or pop like a balloon. If it does, discard the bag and get a new one.
6. When the bag is full of air, disconnect air line from the top of the bag.
7. Immediately after, using the clean poker, poke a *single* hole at the bottom of the bag and put the air line in through the hole. Be careful not to poke through the bag. If you do, either seal it with a plug or discard bag and get a new one.

**Inoculating a bag**

1. Spray in the spot where you’re going to put the belly and immediately after poke a hole (spray the poker first) for the belly button, facing the drain about 3 feet off the floor and insert cleaned belly button.
2. Clamp the airline, disconnect at the coupling, and gently lower the tubing connected to the bag so it doesn’t get pulled out (or use beige clamp to hang it on the rack) and use the airline to push algae from the flask into the bag.
3. Clean the inoculation tubing by shooting Isopropanol through it, and use the airline to push out excess Isopropanol. Connect it to the short piece of tubing and the other side to the belly button.
4. Connect the airline to the long tubing. Open belly button clamp, open beige clamp, then open airline clamp on the flask.
5. Algae will pump from the flask to the bag. (you can make 1-3 bags from 1 Fernbach and 1 bag from 1 Erlenmeyer, depending on need).
6. Careful not to move sticky algae/flocculants to the bag, and stop transfer prior to air being pushed through the bag/ after all of the algae has been pumped to new bag.
7. Close clamps, and disconnect all of the tubing.
8. Spray the belly button with isoporanol and pinch the clamp on the belly button shut (don’t leave algae in the belly button) and reconnect the airline tubing to the other half of the airline tubing in the bag,
9. Label the bag with bag #, species, date, date of parent flask, salinity of the algae system, and its culture origin and salinity. (ex. Bag A3, T-Iso β on 7/28 (7/20) ½ FB @ 18‰ -> 20 ‰) i.e. system is at 20 ‰ and you used 1/2 Fernbach from 7/20 that was at 18‰,

**Side transfers**

Side transfers are to be done only when there is a great algae demand but nothing is quite ready in the stock room to be transferred.

1. Select a bag that is in really good condition and relatively new (nice color, no stickiness, etc).
2. Set up a new bag close to the bag you will be side transferring over, just as normal SOP.
3. Insert the belly button in new bag.
4. Sterilize inoculation tubing (you may need to connect several depending on how far apart the bags are) and sterilize belly button on parent bag.
5. Connect inoculation tubing to both belly buttons.

(You may need to turn the air way down (but not off) on new bag because of the pressure/gravity differences, and may need to put a clean plug in the outflow hole on parent bag to increase pressure/algae flow rate to new bag.

6. Once enough algae have moved over to start the new bag, clean and clamp both belly buttons, and adjust air as necessary. After transferring algae from flasks, always bring empty, dirty flasks to sink, and rinse/soak with water and clean promptly.
Vita

Lisa Bourassa was born and raised in Shrewsbury, Massachusetts. She graduated from Shrewsbury High School in 2006, and attended Roger Williams University from 2006 to 2010. At Roger Williams University, she graduated with a dual Bachelor of Arts degree in marine biology and French language, with a minor in environmental science. During this time, she worked as a research assistant in the Marine Ornamental Aquaculture WetLab where she helped raise several ornamental species including seahorses, clownfish, dottybacks, fire shrimp, and queen triggerfish. To raise these fishes, she cultured algae and copepods for a live feed, and completed a research project on Parvocalanus sp. copepods at different temperatures. She also worked as an intern for the Giant Ocean Tank at the New England Aquarium in Boston, Massachusetts. While at Roger Williams University, Lisa studied abroad in Bermuda at the Bermuda Institute of Ocean Sciences (BIOS) and completed a research project on the association of color and coral bleaching.

Upon graduating from Roger Williams, Lisa was recruited in June 2011 to work with Dr. John Supan at the Sea Grant Oyster Research Laboratory (now the Michael C. Voisin Oyster Hatchery) as a research assistant 2, and became the senior phycologist of the hatchery in March 2015. In the Fall of 2013, Lisa began taking classes for her graduate degree at Louisiana State University in the School of Renewable Natural Resources for her master’s degree in Aquaculture and Fisheries while continuing to work full time at the hatchery. She will graduate in May 2017.