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The Fish Kill Mechanism of the Harmful Raphidophyte Chattonella Subsalsa

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THE FISH KILL MECHANISM OF THE HARMFUL RAPHIDOPHYTE *CHATTONELLA SUBSALSA*

By

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ABSTRACT

Harmful algae blooms (HABs) and toxins associated with HAB species are a significant threat to the health of marine species and coastal environments. In the South Carolina coastal zone, HABs have proliferated with intensified anthropogenic eutrophication in coastal waters. The raphidophyte species *Chattonella subsalsa* is a prominent cause of algal blooms resulting in fish kills in South Carolina. Though *C. subsalsa* is a known ichthyotoxic alga, the fish kill mechanism of this species remains unidentified. *C.* subsalsa is likely to elicit fish mortality via one of two mechanisms: (1) gill damage induced by cell contact irritation or (2) the production of a bioactive compound or toxin. We hypothesized physical damage and subsequent mortality was caused by direct contact with *C. subsalsa* cells and that the lethality of *C. subsalsa* is related to the algal growth phase, with the maximum harmful effect occurring during the exponential growth phase. Larvae of *Fundulus heteroclitus* were exposed to cultures of *C. subsalsa* at various phases of growth both directly and indirectly (through a 0.2 μm mesh) for 48 hours. Fish mortality and gill tissue damage were used to measure the effects of *C. subsalsa* exposures. The greatest mortalities and gill damage were observed via indirect exposure to *C. subsalsa* cultures in the exponential growth phase. These results suggest *C. subsalsa* produces a bioactive compound that induces gill damage and subsequent mortalities without the necessity of contact with algal cells and that *C. subsalsa* is most lethal during the exponential growth phase.

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Introduction

Coastal wetlands and marshes act as a buffer zone between the terrestrial and marine environments. One of the most important ecosystem services provided by these habitats is the removal of nonpoint source (NPS) pollutants from stormwater runoff. As these habitats are altered or lost through coastal development, anthropogenic nutrient inputs increase (Lewitus et al. 2008). One of the consequences of eutrophication occurring in coastal wetlands is harmful algal blooms (Lewitus & Holland 2003, Lewitus et al. 2003).

Harmful algal blooms, HABs, are a growing concern in South Carolina's estuary systems. The South Carolina (SC) coastal zone is undergoing some of the most rapid urbanization in the country, including the development of thousands of stormwater detention ponds as catchments for runoff (Allen & Lu 2003, Greenfield et al. 2014a, Kleppel et al. 2006, Smith 2012).

The construction of detention ponds associated with coastal development is a best management practice that can sometimes lead to a worst-case scenario. Water input to these ponds is often from direct runoff or drainage pipes. Increased impervious surfaces, which decrease filtration through vegetation or sediments along with fertilizer use, can lead to highly eutrophic runoff. As this water collects in brackish detention ponds it creates an ideal environment for harmful algae species to accumulate (Drescher et al. 2007, Greenfield et al. 2014b, Kempton et al. 2002, Lewitus et al. 2003, Lewitus et al. 2008). With HABs on the rise, efforts are needed to better understand harmful species and their effects on marine organisms.

Phytoplankton biomass and diversity can be used as an indication of water quality in the marine environment. With the exception of *Pfiesteria* (Burkholder et al. 1995), the only published record of a HAB in South Carolina estuarine or coastal waters was a *Karenia brevis* bloom in 1988 (Tester et al. 1991). A historical lack of HABs in SC may be related to the relatively low impact of anthropogenic nutrient loading in the coastal region (Lewitus et al. 2002, Lewitus & Holland 2003). An initial investigation of HAB species in brackish detention ponds found various harmful genera including four raphidophyte species, *Heterosigma akashiwo*, *Fibrocapsa japonica, Chattonella cf. verruculosa* and *Chattonella subsalsa* (Lewitus & Holland 2003). In Kiawah Island, SC these raphidophyte species were found to be nearly ubiquitous in brackish detention ponds (Lewitus et al. 2003). However, prior to development, (beginning in 1974-75), such taxa had not been identified (Lewitus et al. 2003, Lewitus et al. 2008).

These ponds, often found adjacent to and/or linked to tidal creeks and marsh ecosystem, have the salinity range, stagnant water quality, and high nutrient levels necessary for blooms to occur. Nutrients are a pivotal part of the formation and success of phytoplankton blooms (Burkholder 2006, Evardsen & Imai 2006, Imai et al. 2004). Eutrophication has been linked to the increase and prevalence of harmful algae blooms worldwide and an increase in nutrients in natural systems is often the driving factor influencing raphidophyte blooms (Anderson et al. 2002, Heisler et al. 2008, Imai & Yamaguchi 2012, Lewitus et al. 2008, Zhang et al. 2006).

The presence of harmful raphidophytes in ponds was commonly associated with heightened nutrient concentrations, specifically high levels of phosphate (PO_4^{3-}), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON) (Lewitus et al.

2003). Raphidophyte species have been observed in neighboring tidal creeks when high cell densities occurred in brackish ponds, suggesting transport could take place during tidal exchange (Lewitus et al. 2003, Greenfield et al. 2014b, Greenfield et al. 2014c). Vicinity to wetlands has allowed harmful taxa within these ponds to proliferate into tidal creeks exposing estuary waters to harmful algal species and algal toxins (Drescher et al. 2007, Greenfield et al. 2014a, Lewitus et al. 2003).

In 2003 a bloom of the globally-distributed icthyotoxic raphidophyte *H. akashiwo* marked the second HAB to be recorded in offshore waters in SC. This bloom originated in Bull's Bay and spread 8 km offshore, encompassing an area 200 km^2. Atypical salinity conditions, (21.3 ppt), associated with this bloom were caused by the diversion of freshwater into the marine system. This massive bloom was responsible for a fish kill estimated at 10,000 deaths (Kempton et al. 2008).

A range of environmental and socio-economic consequences can accompany algal blooms. Blooms can cause disruption of ecosystems, alterations of nutrient levels, depletion of dissolved oxygen, production of toxins, and mass mortalities of marine organisms. Socio-economic impacts include closure of commercial fisheries and/or recreation areas and human illnesses. The effects of a bloom can be both immediate and fleeting, and long term and compounding (Anderson et al. 2002, Hallegraeff 1993, Heisler et al. 2008, Landsberg 2002).

Fish kills pose severe threats to the health of marine and estuarine environments. Although the leading cause of fish kills in South Carolina is hypoxia (Greenfield et al. Pers. Commun.), approximately 27% of fish kill events are the result of harmful algal blooms (Greenfield et al. 2015). Fish kills resulting from harmful blooms immediately

effect fish by decimating populations and have the potential to disrupt spawning aggregations as well as reduce larval recruitment (Colman & Ramsdell 2003, Walters et al. 2013). Likewise in blooms of toxin producing algae, toxins may cause immediate death, secondary mortality through health degradation, or transferal of toxins to offspring or predators (Colman & Ramsdell 2003, Kiryu et al. 2002, Samson et al. 2008, Tester et al. 2000).

One of the most common algal species associated with bloom events (defined as cell densities $\geq 10^3$ cells ml^{-1}) in South Carolina is *Chattonella subsalsa* (Lewitus et al. 2008). This estuarine algal species is a naked raphidophyte ranging 25 to 50 μm in size (Imai & Yamaguchi 2012). Blooms of *C. subsalsa* frequently result in fish kills and pose great threats to the health of natural resources and the environment. For example, from 2001 to 2005, a total of 203 harmful blooms, and of the 17 bloom-causing species, *C. subsalsa* was responsible for the greatest number of recorded blooms (Lewitus et al. 2008).

Chattonella is a genus of harmful algae in the class Raphidophyceae of the phylum Heterokontophyta. *Chattonella* species inhabit tropic, sub-tropic, and temperate waters globally. Currently five species of *Chattonella* have been described, *C. antiqua, C. marina, C. ovata, C. minima,* and *C. subsalsa.* This genus is one of many associated with red tides, a pseudonym of harmful algae blooms named for the appearance of discolored water caused by high algal cell densities. Of the five species of *Chattonella* all but *C. minima* have been reported to cause fish killing red tides. Major blooms have been reported in India, Japan, Korea, China, Australia, the southeast USA and California (Imai & Yamaguchi 2012).

Chattonella has the ability to encyst in sediments of coastal waters in cold temperatures or unfavorable conditions. *Chattonella* maintains low density background populations of encysted and vegetative cells that act as a seed population. Warming waters and eutrophication spur germination. Cells become active and dominate the water column with blooms generally occurring in summer months. Encysting is a key part of the *Chattonella* life cycle as cysts can survive in dormancy for several years without germinating. This makes management practices associated with blooms difficult as a bloom does not need to occur in subsequent years for a *Chattonella* population to be maintained (Imai & Yamaguchi 2012).

Irradiance, water temperature, salinity, and nutrients are the most important factors affecting the growth of *Chattonella.* The genus prefers mid brackish conditions but *C.subsalsa* can grow in waters from 6 to 36 ppt with blooms commonly occurring from 11 to 28 ppt (Imai & Yamaguchi 2012). Bloom range temperatures for *C. subsalsa* are from 24 to 31^0C however various strains of the genus have shown adaptability to lower temperatures. Likewise strains have been observed to adapt to various light regimes and may grow successfully outside of their preferred range. *C. subsalsa* does not require intense irradiance and reaches half saturation for light at 69 μmol $m^{-2}s^{-1}$ (Table 1). Such half saturation constants are similar among other species of the genus (Imai & Yamaguchi 2012).

These conditions are common in the estuary system of South Carolina allowing active *C. subsalsa* cells to spread from detention ponds into tidal creeks and open estuary waters. In SC, *C. subsalsa* blooms commonly occur in spring through summer months but are rare in winter (Lewitus et al. 2008).

Harmful effects associated with various HAB species commonly occur at cell densities $\geq 1 \times 10^4$ cells ml^{-1} (Bordelais et al. 2002). However, fish kills caused by the genus *Chattonella* can occur at much lower cell densities. As a genus, *Chattonella* is known to cause fish deaths at cell densities of 4×10^3 cells ml^{-1} (Imai & Yamaguchi 2012). The species *C. antiqua, C. marina,* and *C. ovata* may even kill fish at 1×10^2 cells ml^{-1} (Evardsen & Imai 2006, Imai & Yamaguchi 2012). In a bloom of *C. subsalsa* on Kiawah Island, South Carolina, abundance was estimated at 4.5×10^3 cells ml^{-1} (Keppler et al. 2006). In laboratory studies of *Chattonella marina,* fish mortalities resulted at cell densities from 3.06×10^3 to 1.03×10^4 cells ml^{-1} (Khan et al. 1996). In many *Chattonella* species such low densities can be reached quickly without copious nutrient uptake. As *Chattonella* can kill at such low densities it is regarded an extremely dangerous HAB genus (Imai & Yamaguchi 2012).

Although the genus *Chattonella* is known to cause red tides associated with massive fish kills, the fish kill mechanism remains unclear (Imai & Yamaguchi 2012, Landsberg 2002). There are many hypotheses though it has been suggested that the ultimate cause of death is suffocation (Imai & Yamaguchi 2012). Several bioactive compounds have been found in cultures of raphidophytes including: (i) reactive oxygen species (ROS), (Oda et al. 1997, Woo et al. 2006), (ii) brevetoxin/brevetoxin-like compounds (Bourdelais et al. 2002, Khan et al. 1996, Khan et al. 1997), (iii) free fatty acids (FFAs) (Marshall et al. 2003), and (iv) hemagglutinins and hemolysins (Onoue & Nozawa 1989). These bioactive compounds may be driving forces in fish deaths associated with *C. subsalsa* but they have not been identified as the primary cause of mortality.

Chattonella is likely to impact fish via one of two mechanisms: (1) copious production of mucus on fish gills causing physical blockage or clogging leading to reduced respiration and osmoregulation, or (2) synthesis and exudation of a bioactive compound(s) (Bourdelais et al. 2002, Keppler et al. 2006). The lysis or consumption-related breakage of algal cells may be necessary for such compounds to be released, but it is also possible *C. subsalsa* actively releases a toxic substance(s). In this case two broad possibilities exist; (1) mortality is dependent on physical contact with algal cells or (2) *C. subsalsa* has the ability to produce a water-borne agent(s) capable of inducing fish mortality without the necessity of contact. Investigating these possibilities will aid in determining the mechanism driving *C. subsalsa* fish kills. Identification and understanding of this method is needed to evaluate *C. subsalsa*'s potential effects on the environment and natural resources.

Algal growth follows a predictable pattern both in nature and in vitro. This growth trend has four distinct stages; the lag phase, exponential growth (log) phase, stationary phase, and decline phase. Cultures of harmful algae are thought to be at the highest degree of toxicity when in exponential growth (Khan et al. 1996). This time period represents a point in the life cycle of heightened growth rate and high cell densities.

Exposure and resulting fish kills commonly occur in the exponential or stationary phase during a period of algal maximum (Pettersson & Pozdnyakov 2013). Fish kills occur after acute exposure to toxins or other harmful algal agents. As most deaths occur within short exposure periods, tissue degradation may only be present in primarily infected organs such as the epidermis and gills (Deeds et al. 2006, Marshall et al. 2003, Shen et al. 2011a, Shen et al. 2011b, Skjelbred et al. 2011).

This study was conducted to identify if physical contact with *C. subsalsa* cells is required to induce fish mortality by simulating a bloom scenario in a controlled laboratory setting using a common estuarine fish, *Fundulus heteroclitus*. *F. heteroclitus*, often referred to as mummichog, is part of the family Cyprinodontidae, commonly known as killifishes. *F. heteroclitus* is ubiquitous among the salt marshes of the western Atlantic ranging from Canada to Northeastern Florida (Abraham 1985).

F. heteroclitus is found in great abundance in the western Atlantic, living in temperatures from 6 to 34 ^{0}C and commonly found in brackish tidal creeks. These fish tolerate a wide range of salinities, living in fresh or highly saline waters (Abraham 1985). Adult *F. heteroclitus* are opportunistic feeders with a diet composed primarily of small crustaceans and annelids (Kneib 1984). Adult *F. heteroclitus* range 50 to 100 mm and display sexual dimorphism upon reaching maturity (≥ 40 mm) (Abraham 1985). Its relatively small size and abundance in the salt marsh makes *F. heteroclitus* an ideal food source for a variety of predators. Both an influential predator and prey, this fish is considered important in the transition of energy within the salt marsh ecosystem (Kneib 1984).

Though *F. heteroclitus* is not one of the primary species associated with *C. subsalsa* fish kills, it is known to live in habitats similar to those where *C. subsalsa* blooms have occurred in South Carolina (Abraham 1985, Kneib 1984, Lewitus et al. 2002, Lewitus et al. 2003). Both adult and larval *F. heteroclitus* are likely to interact with *C. subsalsa* during a bloom, however larvae may be more likely to encounter *C. subsalsa* in a natural system as they feed on zooplankton in surface waters where algal cells would be concentrated. The presence of *F. heteroclitus* larvae in the ecosystem also

coincides with bloom range conditions of *C. subsalsa* (Kneib 1984, Imai & Yamaguchi 2012). Adult *F. heteroclitus* spawn in waters from 16.5 to 25 ^{0}C. In South Carolina these temperatures commonly occur in estuary waters from March to October (NEERS, Oyster Landing).

F. heteroclitus has been used extensively in studies concerning toxicology (Bass et al. 2007, Bello et al. 2001, Prince & Cooper 1995, VanDolah et al. 1997). As gill impairment is a common result of exposures to irritants and toxic chemicals, the severity of gill damage incurred by *F. heteroclitus* can be utilized as a measure of *C. subsalsa* lethality (Deeds et al. 2006, Shen et al. 2011b, Skjelbred et al. 2011).

The objectives of this study were to examine the mechanism by which *C. subsalsa* induces fish mortality and determine if a relationship exists between the growth phase of *C. subsalsa* and its effects on *F. heteroclitus*. I examined the fish killing mechanism of *C. subsalsa* by investigating whether mortality was dependent on physical contact with algal cells or independent of physical contact. I hypothesized that (1) *C. subsalsa* cells must be in direct contact with fish to elicit physical damage and subsequent mortality and (2) that the maximum harmful effects occur in the mid to late exponential growth phases of *C. subsalsa*.

Methods

Algal Culturing

A culture of *C. subsalsa* was obtained from Dr. Dianne Greenfield's laboratory located in Charleston, SC (USC/SCDNR). Cultures were raised in a Percival Intellus Environmental Controller (incubator), at 25^{0} C under 12:12 light: dark regime, with an

irradiance value of 85-140 μmol m^{-2} s^{-1} in an f/2 (– Si) nutrient replete medium (Guillard & Ryther 1962). F/2 medium was produced in the lab using a 25 ppt artificial seawater base and an f/2 chemical kit acquired through the Bigelow Laboratory for Ocean Sciences. The seawater base was made in laboratory facilities using Instant Ocean Sea Salt and deionized (DI) water. Artificial seawater and f/2 chemical additions of nitrogen and trace metals were filtered through a 0.22 μm general filtration membrane filter into a clean 1L glass container. Seawater mix was autoclaved on a Liquid Cycle and allowed to cool to room temperature. Upon reaching room temperature the chemical components of vitamins and phosphorus were added and completed f/2 medium was stored in the incubator.

The initial aliquot of *C. subsalsa* was allowed 24-48 hours to adjust to conditions present in the incubator prior to culturing. Culturing was conducted on a clean bench under a laminar flow hood following the standard operating procedure (SOP) used by Dr. Greenfield's lab. A small volume of aliquot culture (2-5 ml) was transferred into f/2 medium in 50 ml glass or polypropylene test tubes. Prior to use all glassware was sterilized via a cycle of DI rinses, acid washing, and autoclaving.

Regular cell counts were conducted on active cultures of *C. subsalsa* to monitor growth. Growth curves were developed for multiple generations of *C.subsalsa* by analyzing cell density counts as the culture progressed through lag, log, stationary, and decline phases. On counting days a sample of approximately 1 ml of culture was removed at 10:00 am from each test tube containing active cultures. Removed culture was preserved using Lugol's iodine solution. The ratio of Lugol's to culture was recorded and applied to determine a dilution factor. Preserved cultures were counted

using a Sedgwick rafter counting chamber. Cell density was calculated using Equation 1(a,b), where *C* is the number of cells counted, *A* is the area of each field within the counting chamber, *D* is the depth of the counting chamber, and *F* is the number of fields counted.

(1)

(a) $$Cell\ density\ mL^{-1} = \frac{(C \times 1000\ mm^3)}{(A \times D \times F)}$$

(b) $Cell\ density\ ml^{-1}_{total} = Cell\ density\ ml^{-1} + (Cell\ density\ ml^{-1}\ x\ dilution\ factor)$

A growth curve was calculated based on cell densities using Equation 2, where μ is growth rate and N_2 and N_1 are number of cells at times t_2 and t_1.

(2) $$\mu = \frac{\ln(N_2/N_1)}{t_2 - t_1}$$

C. subsalsa was cultured regularly to maintain a stock of actively growing cultures. Using the formulated growth curve, the near-end of the log phase was targeted to inoculate new cultures. A volume of 2-5 ml of stock culture in exponential (log) growth phase was used to inoculate new generations of *C. subsalsa* every 10-12 days in accordance with the calculated growth curve (See Appendix I).

To determine if bloom densities are harmful to fish only when achieved by known HAB species, fish were also exposed to bloom levels of the non-harmful species *Isochrysis galbana.* *I. galbana* is a flagellated marine microalgae ranging 5-6 μm in length. *I. galbana* is a member of the class Prymnesiophyceae of the phylum Haptophyta and naturally inhabits the marine waters of the coastal Atlantic. This species is commonly cultivated for use as feed in aquaculture facilities because of its easy maintenance and high fat content (Tomas 1997). As this species has no known negative

effects on marine organisms it was an ideal control species for experimental trials. *I. galbana* treatments also served as control for starvation as fish were not actively fed during experiments.

An initial aliquot of *Isochrysis galbana* (Strain ID: UTEX987) was obtained from Carolina Biological Supply and was cultured and maintained under the same conditions as *C. subsalsa.* Growth rates for *I. galbana* were calculated via regular cell counts with a hemocytometer. Cell densities of *I. galbana* were determined using Equation 3**,** where DF is dilution factor.

(3) $$Cell\ density\ mL^{-1} = \frac{\left(\frac{\sum counted\ cells}{\#\ of\ squares}\right) \times 10^4}{DF}$$

Growth curves were developed from cell densities and used to determine ideal culturing periods. In most cases, *I. galbana* was cultured every 8-10 days (See Appendix I).

Due to the vast difference in *I. galbana* and *C. subsalsa* cell size, cultures were normalized based on carbon content. Carbon content was determined using a loss on ignition method to measure dry weight (DW) and ash free dry weight (AFDW) of *I. galbana* and *C. subsalsa* (See Appendix I). The carbon content ratio of *C. subsalsa* to *I. galbana* was found to be 214.88:1. This ratio was used to determine the cell density of *I. galbana* culture used in *I. galbana* treatment controls.

As morphology and activity are indicative of health, live examinations of active cultures were regularly performed. Cultures were observed to change morphology slightly over the course of growth. In lag and early exponential phases cells were observed to be circular in shape and moderately active. As cultures progressed to exponential growth cell shape was ellipsoid and cells were highly active. The

morphology and activity of live cells was within a healthy range (Marshall & Hallegraeff 1999).

Cell density, growth rate, and divisions per day were calculated for *C. subsalsa* and *I. galbana* (See Appendix I). Growth dynamics may vary considerably in individual clones of the same algae culture even when grown under identical conditions (Turner 2014). Cultures of *C.subsalsa* were variable but overall predictable. High cell densities and division day^{-1} values in the 1.0 and above range were regularly achieved. Overall stock cultures grown throughout the experimental period were healthy.

Fish Husbandry

Though *F. heteroclitus* is not an important human food source or recreational species, it has been the subject of aquaculture. Both large scale and small scale aquarium breeding have been successfully accomplished in past studies (Bosker et al. 2009, Bosker et al. 2013, Hsiao et al. 1996, Janiak & McIntosh 2014, Redway 1980). The small size of adult *F. heteroclitus* removes the necessity for large aquaculture facilities and equipment and enables fairly low-tech breeding (Bosker et al. 2009, Bosker et al. 2013, Hsiao et al. 1996)

Larvae of *F. heteroclitus* bred in Coastal Carolina University's laboratory facilities were utilized for this study. The use of larvae provided several advantages including a constant supply of fish without continued field collection, a captive population potentially free of parasites or outside environmental health consequences, and the ability to work on a small scale and run all experimental trials within an incubator. Conducting all experiments in the incubator allowed for consistence of temperature and lighting conditions throughout all trials.

Adult *F. heteroclitus* were collected from Garden City, SC from an easily accessible marsh location. Sampling dates were set to fall on or around spring tides corresponding with the new and full moon at the fish's natural reproductive peak (Bosker et al. 2009, Bosker et al. 2013, Hsiao et al. 1996, Redway 1980, Shimizu 1997, Taylor et al. 1979).

Fish were captured using killie traps baited with grass shrimp *(Palaemonetes*) or dry dog food. Traps were set at the edge of *Spartina alterniflora* beds during a falling tide when water partially inundated the high marsh. Traps were allowed to soak for a minimum of forty-five minutes before removal. At the time of removal the largest males and females were collected from each trap and the remaining fish were released. Only sexually mature fish, lengths of 40 mm or greater, were retained.

Fish were brought back to laboratory facilities at Coastal Carolina University and acclimated to water conditions in pre-established ten gallon aquaria via a drip system. Prior to acclimation the fish were sorted based on sex. Sex ratios were either even, 1:1, or in female majority. Breeding ratios were female biased to minimize aggressive competition among males (Bosker et al. 2009, Bosker et al. 2013, Hsiao et al. 1996, Redway 1980, Shimizu 1997).

Aquaria were maintained at consistent temperatures and salinities, 31±2 ^{0}C and 34±2 ppt respectively. Routine water changes and tests of water chemistry were performed to maintain water quality. Shell fragments collected at the field site were placed in the aquaria to mimic the natural environment and promote breeding. Adult *F. heteroclitus* were maintained on a diet of frozen grass shrimp (*Palaemonetes*) collected from the field site. Regular feedings, high temperature, and lighting conditions on an

approximate 14:10 light: dark cycle mimicked summer conditions and encouraged fish to spawn in captivity throughout the winter months well beyond the natural spawning season.

Eggs were collected using the Janiak style egg collector (Figure 1) designed for the aquaculture of *F. heteroclitus*. This collector mimics the natural crevices of mussel shells and marsh grasses used by *F. heteroclitus* when spawning in the wild (Janiak & McIntosh 2014). A collector was placed in each of the breeding tanks and left unaltered until egg removal.

Egg collectors were checked regularly by visual examination and eggs were removed approximately once a week. When eggs were harvested the collectors were removed and placed in artificial seawater. Collector disks were loosed and gently shaken to detach eggs. Seawater containing eggs was strained over a nylon mesh screen. The number of isolated eggs was recorded and the eggs were placed in air incubation trays. Incubation trays containing eggs were maintained for a minimum of fourteen days (Coulon et al. 2012).

After incubation fully developed eggs were inundated in 25 ppt artificial seawater to induce hatching. Hatched larvae were held in 4 L plastic containers containing 1.5 L of 25 ppt artificial seawater. Larvae were fed *Artemia salina* nauplii once daily and maintained in aquaria for at least 7 days post hatch (dph) prior to use in experiments. Fish were bred continuously over one year. Measurements of hatching success and larval survival post hatch were determined to ensure the described conditions produced healthy larvae (See Appendix II). All larvae used in experiments were of adequate fitness.

Preliminary Experiments

Prior to beginning formal experiments several preliminary exposures were conducted to determine anticipatory results as well as the exact parameters to be used for formal experiments including, container size and type, mesh separation design, and duration of exposures (See Appendix III).

All preliminary experiments resulted in *C. subsalsa* exposed fish mortalities greater than control fish mortalities (See Appendix III). Preliminary experiments aided in the development of gill analysis protocol and confirmed gill damage was an applicable analysis tool. These experiments provided mortality predictions and supported the results observed in formal experiments.

Exposure Experiments

Due to the variability observed in stock cultures grown under identical conditions over the course of approximately 18 months, it was decided to perform formal experimentation using cultures representing five distinct growth phases. Using cultures of various ages and growth phases allowed room for differences in growth while still minimizing potential bias introduced by variable growth patterns. Cultures were harvested for experiments at lag, early exponential, mid-exponential, late exponential, and stationary growth phases. Culture growth rates were variable, so growth phases were determined by growth rate day^{-1} rather than age in days of culture. A new generation of algae, comprised of 12 individual test tubes, was used for each experiment. The growth phase experiments consisted of six treatments each replicated six times.

Experimental Treatments

1. **EXPD; *Exposure Direct*:** Direct contact of fish and *C.subsalsa* cells; fish held in active algal culture
2. **EXPM; *Exposure Mesh*:** Indirect contact of fish and *C. subsalsa* cells; fish separated from active culture by a 1 micron mesh
3. **IC; *Isochrysis Control*:** Direct contact of fish with control algae *I. galbana* cells; fish held in active algal culture
4. **ASWE; *Artificial Seawater Exposure*:** Artificial seawater experimental control; fish held in artificial seawater and sacrificed as deaths occurred in EXPD
5. **ASWC; *Artificial Seawater Control*:** Artificial seawater control; fish held in artificial seawater
6. **MC; *Mesh Control*:** Artificial seawater and 1 micron mesh; fish held in artificial seawater separated by 1 micron mesh

Based on pilot studies formal experiments were performed in 50 ml test tubes for 48 h. All treatments were conducted in 50 ml polypropylene plastic test tubes in the incubator set to 25 ^{0}C, 12: 12 light: dark cycle, at an irradiance of 85-140 μmol m^{-2} s^{-1}. A total of 30 fish were used for each treatment with each test tube containing 5 fish. A total of 36 test tubes in a 6 x 6 wire rack were utilized in each experiment.

The control treatment (artificial seawater control) was duplicated, consisting of a total of 60 fish divided among 12 test tubes. One of these two controls, ASWC, spanned the entire exposure time uninterrupted minus the removal of any moribund fish. The

other, ASWE, artificial seawater control was used for gill comparison purposes. For each fish mortality in the direct exposure treatment (EXPD), a fish was removed and sacrificed from the corresponding ASWE control treatment. The gill integrity of moribund exposed fish was compared to control fish removed at the same hour of experimental trial. In total five individual experiments were conducted.

Artificial seawater (25ppt) used in control treatments (ASWE, ASWC, and MC) was made in the lab using Instant Ocean Sea Salt and deionized water. Seawater was filtered through a 0.22 μm general filtration membrane filter and stored in the incubator in clean, capped glassware prior to experimental use. Filtration to 0.22 μm removes contaminants including particulate matter and bacteria reducing potential error in controls. ASWC and ASWE treatment tubes were filled with 20 ml of filtered artificial seawater using a graduated pipette.

One generation of algal culture was used for each experiment. Each generation of culture was comprised of twelve 50 ml test tubes of *C. subsalsa*. At 10:00 am algal culture from each tube was pulled, preserved, and counted. A random number generator was used to determine which culture tube (1-12) would be used for each of the experimental exposures, EXPD (1-6) and EXPM (1-6). Each test tube of the treatment EXPD was filled with 20 ml of *C. subsalsa* algae culture.

The final mesh design for treatment EXPM was one utilizing a secondary 15 ml test tube (Figure 2). Six small holes were cut into the middle third of 15 ml test tube and then covered with a layer of 1 μm mesh affixed to the tube with aquarium safe epoxy. The outer 50 ml test tubes of treatment EXPM were filled with 10 ml of f/2 medium. The inner 15 ml test tubes were slowly filled with 15 ml of algal culture until inner and outer

volumes reached equilibrium. The same was done for treatments of MC, however artificial seawater was used for filling both interior and exterior test tubes.

After calculating the cell density of cultures of *C. subsalsa* to be used in EXPD treatments, cell ml^{-1} values were multiplied by the carbon ratio (214.88:1) to determine the cell density of *I. galbana* needed in IC treatments. Cell densities in IC treatments corresponded with the density of EXPD treatments. Stock of various growth phases of *I. galbana* was kept on hand to accommodate for a range of required cell densities. After determining the necessary densities *I. galbana* cultures were diluted accordingly with f/2 medium and added to IC treatment test tubes.

Larvae were maintained for a minimum of 7 dph prior to use in experiments. This time frame was chosen to allow any remaining yolk sac to be absorbed and ensure larvae were actively feeding. Allowing a minimum of 7 dph also confirmed larvae were in good health and acclimated to conditions preceding use in experimental exposures.

Larvae were removed from 4 L aquaria using a 1 ml Pasteur pipette with a cut tip. The cut created a larger opening allowing larvae to be pulled into the bulb. Care was taken to remove larvae without excessive roughing. Five larvae were added to each treatment test tube with as little seawater as possible. Once all tubes had fish the experimental time officially began.

All experimental treatments were conducted simultaneously for a period of 48 h. Fish were monitored at 4 h increments throughout the exposure period and removed upon reaching a moribund state. Fish were monitored at 2 h increments during the mid-exponential growth phase experiment. Sacrificed and moribund fish were anesthetized in

tricaine mesylate (MS-222) and length was measured. Moribund and sacrificed fish were preserved in a 10% formalin buffer.

Gill Surface Area Analysis

After all trials had been completed fish gills were extracted for analysis. Fish were prepared for gill analysis through a series of steps resulting in permanently mounting stained gill tissue on slides. Fish were removed from formalin with clean forceps and submerged in a DI water bath. Fish gills were removed from preserved fish and soaked in a 10% Trichrome stain for 8-10 minutes. Stained gills were dehydrated using 95% ethyl alcohol, cleared with xylene, and rehydrated with glycerin. Processed gills were affixed to slides with Permount mounting medium and cover slip. Completed slides were laid flat for at least 24 hours prior to assessment to ensure tissues had settled and medium was dry.

Images of gill tissue were captured using an Olympus BX60 microscope and Image Pro Plus software. All images were screened and only those meeting standards for analysis were utilized. The standards designated for image analysis were, 1.) Edges of tissue must be clearly defined, 2.) Individual lamellae must have a clear shape and be distinguishable from adjacent lamellae, 3.) The end or attachment point of primary lamellae to the gill arch must be discernable.

Images that met the standards for analysis were measured using *ImageJ* 1.49 software for Mac OS X. For each individual fish at least five individual gills were measured. When possible ten individual gills were measured. As some slides contained multiple individual gill arches, methods were taken to avoid bias in analysis. When more than one

individual gill arch image was available for analysis the number of gill lamellae measured was spread evenly across the separate images.

Two surface area (SA) measurements were taken for each individual gill. The first measurement (*Total* SA) encompassed the perimeter of the total surface area of the gill determined by tracing the outermost points of the secondary lamellae. The second measurement (*Lamellae* SA) traced the exact perimeter of each secondary lamellae **(**Figure 3**)**. The two measurements were compared using Equation 4, where SA is surface area, to determine the percentage of gill surface area.

(4) $$Percent\ Gill\ SA = \frac{Lamellae\ SA}{Total\ SA} \times 100$$

By this design a healthy gill tissue would have a percent gill surface area near 100%. As damaged gills were observed to have disheveled or shriveled secondary lamellae, lower percent gill surface areas were indicative of greater levels of gill degradation. Gill surface areas per treatment were analyzed for individual experiments and as total values per treatment across all experiments. In the analysis of gill surface area the artificial seawater treatments ASWC and ASWE were grouped together and identified as ASW. The mesh control treatment (MC) did not yield enough gill images meeting the standards for analysis and therefore the treatment MC was not included in the statistical analysis of gill surface area coverage.

Statistical Analysis

Five total experiments were conducted targeting the algal growth phases lag, early exponential, mid exponential, late exponential, and stationary. Individual experiments

were identified and referenced by growth phase. The age, average cell density, and average growth rate of *C. subsalsa* cultures used in each experiment can be found in Table 2. A new generation of culture was used for each of the five experiments. As cultures raised under identical conditions may vary in their growth dynamics, growth phase was defined by growth rate rather than day of growth or cell density.

Data from each growth phase experiment were analyzed individually for percent mortality per treatment and percent gill surface area per treatment. There were not enough satisfactory gill images to analyze gill surface area per treatment in the early exponential growth phase experiment. All growth phase experiment data was combined to analyze the total percent mortalities and total percent gill surface areas per treatment.

Statistical analyses were conducted using R-3.2.2 package for Mac OS X 10.9. All percent mortality and percent gill surface area data were tested for normality and homogeneity using Shapiro-Wilk and Fligner-Killeen tests respectively. When analyzing percent mortalities per treatment and percent gill surface area, parametric data were evaluated using an ANOVA followed by the post hoc Tukey's test. Non-parametric data were analyzed using a Kruskal-Wallis rank sum test and the post hoc Dunn's test.

The relationship between percent mortality and *C. subsalsa* growth rate was determined using a general linear model. Percent mortalities observed in mesh exposure and direct exposure treatments were compared to the average growth rate of *C. subsalsa* culture used in each of five growth phase experiments.

Results

Mortality Analysis

The results of Shapiro-Wilk tests for normality and Fligner-Killeen tests for homogeneity for percent mortality and percent gill surface area can be found in Table 3.

With all growth phase experiments combined, percent mortality was found to be significantly different among treatments (Figure 4, Kruskal-Wallis p = 0.007). The mesh exposure to *C. subsalsa,* EXPM, yielded the highest percent mortality over all (26 %) and was significantly greater than the control treatments IC, MC, and ASWE (Dunn's test, p values reported in Table 4). Percent mortality for the direct exposure, EXPD, was significantly higher than the control MC treatment (Table 4, Dunn's test p= 0.05). Percent mortality for most of the control treatments did not significantly differ, except ASWC was greater than MC (Dunn's test p= 0.011).

Although results were not unanimous, a trend was apparent in percent mortalities when broken out by treatment and growth phase (Figure 5). In four out of five growth phase experiments the exposure treatments, (EXPM or EXPD) yielded the highest percent mortalities, and one of the control treatments yielded the lowest percent mortalities.

Direct exposure to *C. subsalsa* (EXPD) yielded lower than anticipated percent mortalities but was found to have significantly higher mortalities than several control treatments (Table 4). The highest percent mortality (30 %) of the direct exposure treatment EXPD was observed in the mid exponential growth phase experiment (Figure 5). The mid exponential growth phase experiment also yielded the highest percent mortality (36.67%) of the mesh exposure treatment EXPM. A 36.67 % mortality was

observed in this treatment in the late exponential growth phase as well. Although total percent mortalities in the mid exponential growth phase were not significantly different, post hoc tests found EXPM to have significantly higher percent mortalities than the control treatments IC (Table 4, Dunn's Test p = 0.05), MC (Table 4, Dunn's Test p= 0.036), and ASWE (Table 4, Dunn's Test p = 0.014). Similarly treatment EXPD was found to be significantly greater than both the control MC (Table 4, Dunn's Test p = 0.049) and ASWE (Table 4, Dunn's Test p = 0.022) in the mid exponential phase experiment.

The mesh exposure, EXPM, regularly yielded the highest treatment mortalities among the various growth phase experiments (Figure 5) and frequently exhibited mortalities significantly higher than controls (Table 4). In the late exponential growth phase experiment the treatment EXPM was found to have significantly higher mortalities than the controls IC (Table 4, Dunn's Test p = 0.014), MC (Table 4, Dunn's Test p= 0.014), and ASWE (Table 4, Dunn's Test p = 0.014). In the stationary growth phase the treatments EXPM and EXPD were found to be significantly greater than the control treatment MC (Table 4, Dunn's Test p = 0.0058 and p = 0.019 respectively).

Experimental mortalities visibly followed the predicted growth phase trend. Algal growth rate was not found to be a statistically significant predictor of percent mortality in treatment EXPD however a significant correlation between growth rate and percent mortality was found in treatment EXPM (R^2=0.7732, p=0.0494) (Figure 6).

Gill Surface Area Analysis

In total, fish from the mesh exposure to *C. subsalsa*, EXPM, had the lowest percent gill surface areas (highest degree of gill damage) (Figure 7). Fish from the direct exposure, EXPD, also had reduced gill surface areas. When combining all growth phase experiments treatments EXPM and EXPD were found to be significantly different than the IC control (Table 5, Dunn's Test, $p = 0.002$ and $p = 0.023$ respectively).

The greatest amount of gill damage in exposure treatments (EXPD and EXPM) was observed in the mid exponential growth phase (Figure 8). Gill damage in exposure treatment in the mid exponential growth phase experiment were not significantly different than controls, however among individual growth phase experiments, exposure treatments were found to be significantly different from controls in the lag and stationary phases. In the lag phase experiments, EXPM was found to be significantly greater than both control treatments ASW (Table 5, Dunn's Test $p = 0.031$) and IC (Table 5, Dunn's Test, $p = 0.005$). In the stationary growth phase experiment, EXPM and EXPD had significantly greater gill damage than the control IC (Table 5, Dunn's Test $p = 0.036$ and $p = 0.046$ respectively).

Discussion

My primary hypothesis stating *C. subsalsa* cells must be in direct contact with fish to elicit physical damage and subsequent mortality, can be rejected. The experimental exposure through 1 micron mesh (EXPM) was anticipated to produce results similar to those of control treatments but yielded both the highest percent mortalities and the greatest gill degradation. These results were unexpected but they were consistent in both the analyses used to determine the effects of *C. subsalsa* on *F.*

heteroclitus. Though it was not hypothesized that EXPM treatments would have high mortalities, other studies have found cell free *C. subsalsa* culture to cause significant fish mortality (Bridgers et al. 2002, Perez-Morales et al. 2014).

Raphidophytes are suspected to be associated with the production and potential exudation of several bioactive compounds (Bourdelais et al. 2002, Dorantes-Aranda et al. 2013, Imai & Yamaguchi 2012, Keppler et al. 2006, Khan et al. 1996, Woo et al. 2006). It is hypothesized that some of these compounds may affect marine organisms when algal cells come in contact with gill filaments (Hiroishi et al. 2005, Shen et al. 2010). Other compounds such as reactive oxygen species, are thought to be exotoxic and actively released by algal cells (de Boer et al. 2012, Marshall et al. 2003). Our results support the theory that a toxicant is produced by *C. subsalsa* and has the ability to cause gill degradation and subsequent fish mortality without the necessity of physical contact or consumption related lysis.

The results suggest contact related lysis is not necessary for *C. subsalsa* to produce an ichthyotoxicant. Though cells may not require lysis to be lethal, cell morphology may be a factor. As *Chattonella* progress through lag, log, stationary, and decline growth phases cell morphology and motility changes. Log phase cells are ellipsoid or spindle shaped with visible flagella. These cells are highly active and spiral as they move. In lag phase as well as late stationary phase cells become spherical in shape and less active. These morphotypes are indicative of algal growth phase and culture health (Khan et al. 1996, Marshall et al. 1999). In our formal experiments morphotype was not documented, however throughout the culturing period these morphological changes were observed and coincided with growth phase.

This study found the percent mortality in EXPM treatments to be correlated to algal growth phase. This correlation matched the secondary hypothesis stating, a correlation exists between the phase of growth and lethality of *C. subsalsa* with maximum harmful effect occurring in the mid to late exponential growth phases. In treatment EXPM percent mortality was found to be equal in both mid and late exponential phases. This percent mortality was also the highest observed in all experiments and treatments. These results were anticipated and have been confirmed by several other studies which link exponential growth to increased lethality in harmful raphidophytes (de Boer et al. 2012, Khan et al. 1996, Marshall et al. 2003, Shen et al. 2010). The results of this and past studies suggest the fish kill mechanism of *Chattonella* is a function of growth phase.

The results of these experiments produced two anomalies. One was the percent of mortalities observed in the artificial seawater ASWC treatment. Mortalities were high in the ASWC treatment in many of the experiments and in some cases significantly greater than the percent mortalities observed in the EXPD treatment. I attribute this to high stress of fish used in this treatment. Care was taken to reduce stress as much as possible when transferring fish to treatment test tubes. Fish were added to treatment test tubes in the same order for each of the five experiments, (EXPD, EXPM, IC, MC, ASWE, ASWC). Fish added to ASWC treatment test tubes were consistently added last. These fish had been the only individuals remaining after several attempts to remove larvae with a 1 ml Pasteur pipette. Fish of treatment ASWC had successfully avoided capture for a period of 30 minutes or more. As these fish had been actively swimming and agitated for a considerable time their fitness may have been significantly lowered resulting in deaths

from exhaustion or shock. Although mortalities in artificial seawater treatments were greater than expected overall percent mortality and gill degradation in artificial seawater control treatments was not significantly greater than either experimental treatment.

As direct contact (EXPD) percent mortalities were found to be significantly greater than deaths in the control MC and gill damage of EXPD treatments was found to be significantly greater than control IC we can rule out EXPD deaths as arbitrary. The significance found in formal experiments as well as the significantly greater gill damages in exposed versus control fish of preliminary experiments, supports deaths of treatment EXPD were the product of exposure to *C.subsalsa*.

The other abnormality in the results is the significance of percent mortality and gill damage in EXPM treatments and lack thereof in EXPD treatments. If *C. subsalsa* is producing water borne exudates that are lethal to fish, the gill damage and subsequent mortalities in both the direct and indirect exposure treatments should have been similar. However the experimental exposure though 1 micron mesh (EXPM) had significantly higher percent mortalities and gill degradation than direct exposure to *C. subsalsa* cells (EXPD). Although the same generation of culture was used for the EXPM and EXPD experimental treatments, treatments of EXPM may have had an environmental advantage.

The design of EXPM treatments incorporated a 15 ml test tube containing a mesh screen, housed inside a 50 ml test tube. The outer tube was filled with 10 ml of f/2 medium preceding the inner 15 ml tube being filled with active culture. This was necessary to have a suitable volume of water for fish, which were confined to the outer test tube. Without the addition of medium, fish in treatment EXPM would be limited to 10 ml where fish in all other single test tube design treatments (EXPD, IC, ASWE,

ASWC) were provided 20 ml. The addition of medium to EXPM treatments created a comparable vertical distribution of water in both exposure treatments. This addition may have also provided a spike of nutrients to the active culture potentially increasing *C. subsalsa*'s activity and successive production of toxicant.

A study of nutrient effects on *C. subsalsa* conducted by Zhang et al. (2006) found increased nitrate and phosphate concentrations to positively affect cell growth rates. The nutrient uptake rates of *C. subsalsa* cultures grown under the experimental conditions was not examined by this study, however f/2 replete medium is known to have a molar concentration of 8.82×10^{-4} M of $NaNO_3$ (Sodium Nitrate) and 3.62×10^{-5} M of $NaH_2PO_4H_2O$ (Sodium Dihydrogen Phosphate)(Guillard & Ryther 1962). As our results and several others point towards increased growth rates (exponential growth phase) increasing algal lethality, the addition of fresh nutrients was likely a contributing factor to deaths in EXPM treatments.

Based on preliminary trials we did not expect the percent of deaths of exposure fish to be outstanding. However percent mortalities were low compared to other studies of *Chattonella* species (Hiroishi et al. 2005, Marshall et al. 2003, Shen et al. 2010, Shen et al. 2011, Khan et al. 1996). Hiroishi et al. (2005) showed that the sensitivity of different fish species may vary considerably when exposed to identical algal cultures. As *F. heteroclitus* is well known as an extremely hardy fish, resulting deaths in exposure treatments may have been more dramatic if a less robust species was used.

Forty-eight hours was a substantial exposure time, however natural blooms may occur over a much longer duration depending on environmental conditions (Anderson et al. 2002, Smayda 1997, Turner 2014). The proliferation and bloom densities of harmful

taxa may succeed one another in nature. In brackish systems in South Carolina harmful raphidophyte species were found to bloom in succession suggesting that as cell densities of one species decline species with similar environmental needs may increase (Lewitus & Holland 2003, Lewitus et al. 2003).

Significant exposure mortalities were observed at cell densities ranging 6,804 cells ml^{-1} to 13,981 cells ml^{-1}. Such densities are not uncommon in natural blooms of *C. subsalsa*. In South Carolina fish killing blooms of *C. subsalsa* have been observed from 4,500 cells ml^{-1} to 4 x 10^4 cells ml^{-1} (Greenfield et al. 2015, Keppler et al. 2006). Fish kills within the genus *Chattonella* have been observed both well below and well above the range employed in this study (Evardsen & Imai 2006, Imai & Yamaguchi 2012, Khan et al. 1996).

Finding significant *C. subsalsa* induced mortalities in *F. heteroclitus* larvae suggests that other resident and seasonally transient estuarine fish species could be severely affected by a natural bloom. Larvae of numerous ecologically and commercially valuable fishes are abundant in salt marsh tidal creeks in the spring to early summer, coinciding with bloom range water temperatures of 24 to 31 ^{0}C (Bozeman & Dean 1980, Imai & Yamaguchi 2012). These include residents such as *Leiostomus xanthurus* (Spot), *Lagodon rhomboids* (Pinfish), *Brevoortia tyrannus* (Menhaden), *Micropogon undulatus* (Croaker), and *Mugil cephalus* (Mullet) (Bozeman & Dean 1980) and transient fish such as *Sciaenops ocellatus* (Red drum) and *Cynoscion nebulosus* (Spotted seatrout) (Reagan 1986, Roumillat & Brouwer 2004). Mass mortalities resulting from a bloom could result in significant population loss, decreased recruitment, and may upset energy flow and nutrient cycling in the marsh.

In contrast to juvenile and adult fish who use their gills to perform a variety of physiological functions including respiratory gas exchange, ion and water balance, excretion of nitrogenous wastes and the maintenance of acid-base balance, developing larvae perform gas exchange through their skin and cutaneous areas (Rombough 2002). Significant damage was observed in gill tissue of larval *F. heteroclitus* but this damage may have been more severe in fully developed adults.

Conclusion

This study supports the hypothesis that growth phase and growth rate are contributing factors in the lethality of *C. subsalsa*. It is likely biochemical alterations occurring in the exponential growth phases result in the production or release of bioactive compounds or ichthyotoxins.

Our results suggest that *C. subsalsa* cells do produce a water borne bioactive compound that causes gill damage and subsequent death in larvae of the highly resilient estuary fish *F. heteroclitus*. This study demonstrated that *C. subsalsa* cells are lethal to fish without the necessity of direct contact opposing the theory that physical irritation via cell contact is the cause of death in *C. subsalsa* fish kills. Cell contact may cause irritation, however this study supports fish mortality extraneous of cell contact. This evidence strongly complements theories suggesting the fish kill mechanism of *C. subsalsa* is a bioactive compound or group of compounds. More research is needed to identify what toxins or toxicants are released by *C. subsalsa* and are instrumental in fish kills.

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TABLES

Table 1
Kinetic constants for growth of *Chattonella subsalsa.* Data from Ichiro & Yamaguchi (2012) μ_{max}, maximum growth rate; K_s , half saturation constant.

Chattonella subsalsa	PO_4^{-3}	NO_3^-	NH_4^+		Irradiance
μ_{max} (divisions day $^{-1}$)	0.81	0.87	0.84	μ_{max} (divisions day $^{-1}$)	1.26
K_s (μM)	0.84	8.98	1.46	K_s (μmol m^{-2} s^{-1})	69

Table 2
Age (days), average density (cells ml^{-1}), and average growth rate day^{-1} of cultures used for individual growth phase experiments.

	Lag	Early Exponential	Mid Exponential	Late Exponential	Stationary
Age (days)	6	4	10	8	26
Density (cells ml^{-1})	3115	730	13,981	6804	13,726
Growth rate day^{-1}	-0.153	0.138	0.477	0.717	0.052

Table 3
Results of Shapiro-Wilk test for normality and Fligner-Killeen test for homogeneity for percent mortality and percent gill surface area data of individual growth phase experiments. Shapiro-Wilk normality defined by p – value ≥ 0.05 * and Fligner-Killeen homogeneity defined by p – value ≤ 0.05 *.

	% Mortality		% Gill Surface Area	
	Shapiro-Wilk	Fligner-Killeen	Shapiro-Wilk	Fligner-Killeen
Lag	$1.97\ e^{-6}$	0.14	$6.84\ e^{-5}$	0.084
Early Exponential	$1.75\ e^{-6}$	0.18	---	---
Mid Exponential	$1.08\ e^{-3}$	0.29	$1.87\ e^{-6}$	0.258
Late Exponential	$1.35\ e^{-8}$	0.011 *		
Stationary	$1.45\ e^{-6}$	0.014 *	$1.09\ e^{-5}$	0.044 *
All Experiments	$9.42\ e^{-16}$	0.10	$9.23\ e^{-14}$	$3.98\ e^{-3}$ *

Table 4
Results of Dunn's Test where experimental treatment percent mortalities were found significantly greater than controls for total experiments and select individual growth phase experiments. Only significant p values are reported, ($p \leq 0.05$ *).

	IC	MC	ASWE	ASWC
All experiments				
EXPD	---	p = 0.05 *	---	---
EXPM	p = 0.0028 *	p = 0.0003 *	p = 0.0028 *	---
Mid Exponential				
EXPD	---	p = 0.049 *	p = 0.022 *	---
EXPM	p = 0.05 *	p = 0.036 *	p = 0.014 *	---
Late Exponential				
EXPD	---	---	---	---
EXPM	p = 0.014 *	p = 0.014 *	p = 0.014 *	---
Stationary				
EXPD	---	p = 0.019 *	---	---
EXPM	---	p = 0.0058 *	---	---

Table 5
Results of Dunn's Test where experimental treatment percent gill surface areas were found to be significantly different than controls for total experiments and select individual growth phase experiments. Only significant p values are reported, ($p \leq 0.05$ *).

	IC	ASW
All Experiments		
EXPD	p = 0.023 *	---
EXPM	p = 0.002 *	---
Lag		
EXPD	---	---
EXPM	p = 0.005 *	p = 0.031 *
Stationary		
EXPD	p = 0.046 *	---
EXPM	p = 0.036 *	---

FIGURES

Figure 1: Janiak style egg collector used for harvesting eggs of *F. heteroclitus*. Composed of a series of large disks 5 inch diameter and 1/8 inch thick separated by small disks 2 inch diameter and 1/16 inch thick threaded on a stainless steel thread closed by wing nuts (Janiak & McIntosh 2014).

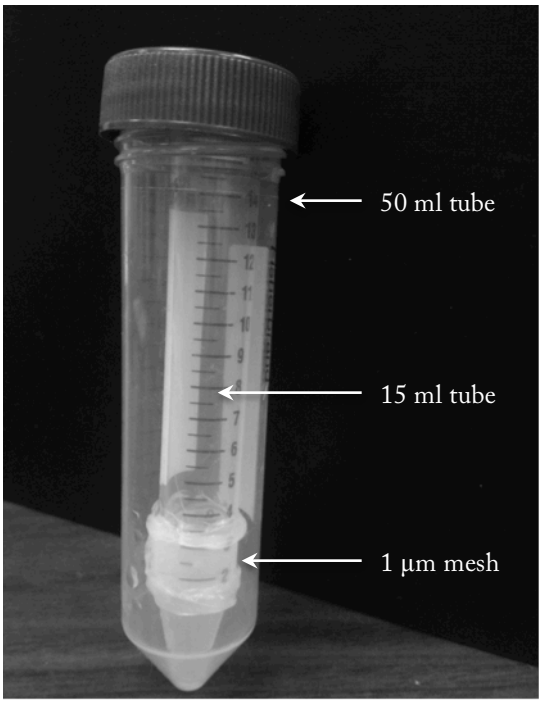

Figure 2: Mesh exposure treatment (EXPM) test tube. 15 ml test tube with 1 micron mesh screen housed within 50 mL test tube.

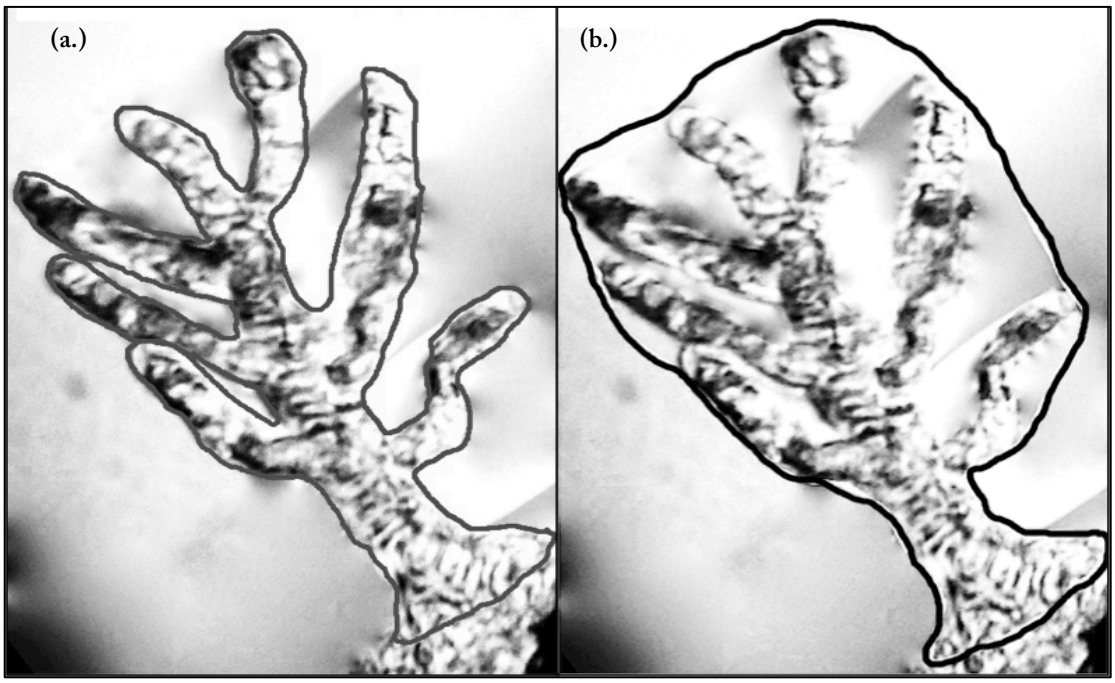

Figure 3: Surface area (SA) measurements, (a.) Lamellae Surface Area and (b.) Total Surface Area used to determine Percent Gill Surface Area in gill analysis.

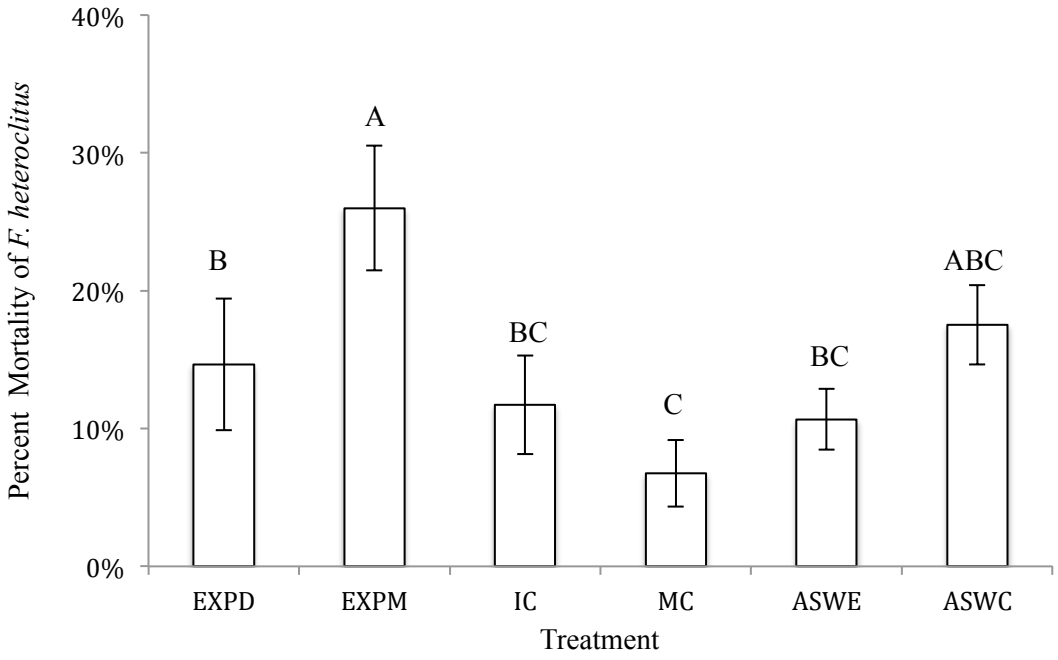

Figure 4: Total percent mortalities of fish larvae per treatment in all growth phase experiments (n = 5) (Kruskal-Wallis rank sum test, $p = 0.007$). Means with different letters are significantly different (Dunn's Test, $p < 0.05$ (Table 4)). Error bars are equal to standard error.

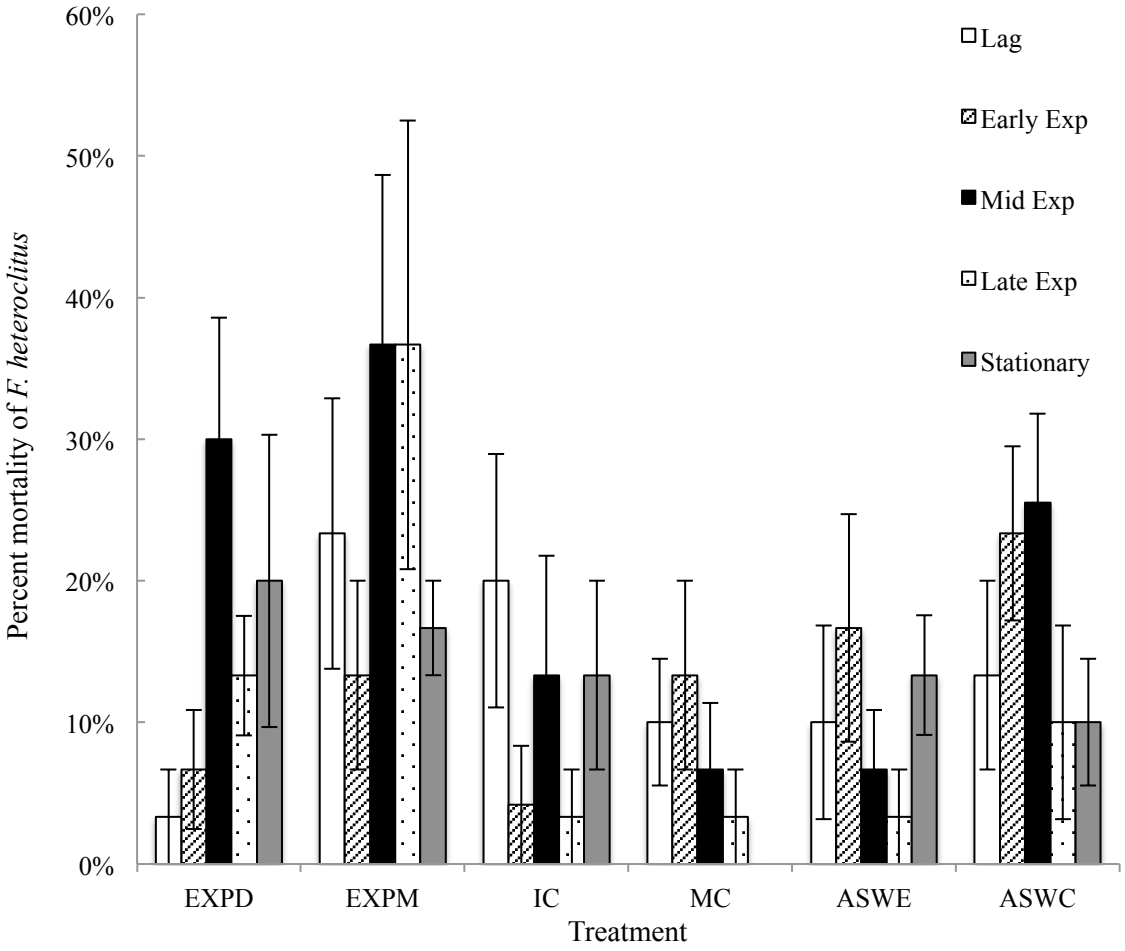

Figure 5: Percent mortalities of larvae fish observed in individual growth phase experiments. Error bars are equal to standard error.

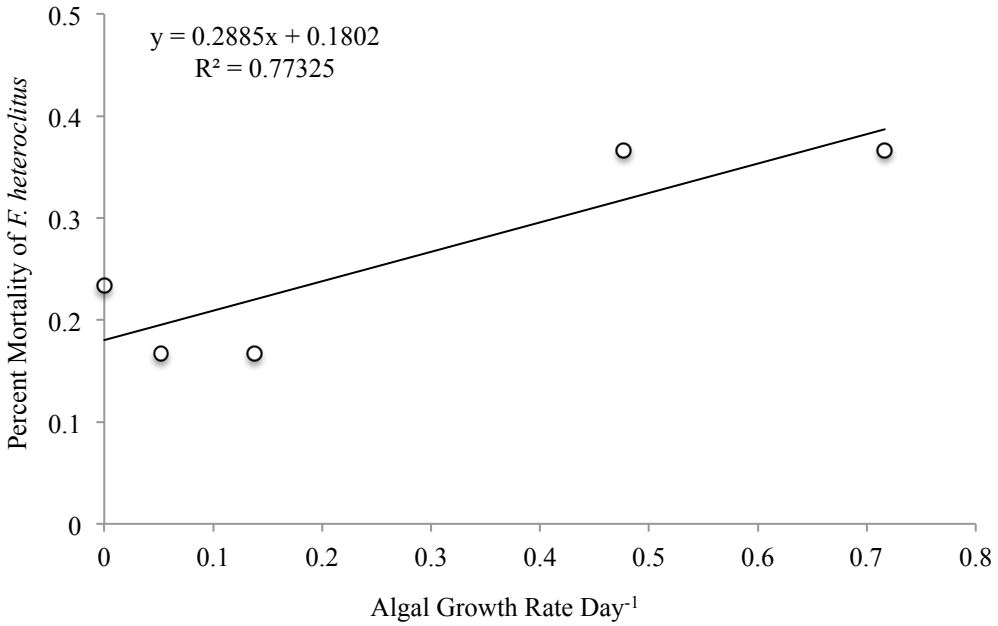

Figure 6: Comparison of average growth rates of *C. subsalsa* cultures per growth phase experiment and percent mortality of larvae fish in treatments EXPM. (General Linear Model, Multiple R^2= 0.7732, Adjusted R^2= 0.6977, p = 0.0494).

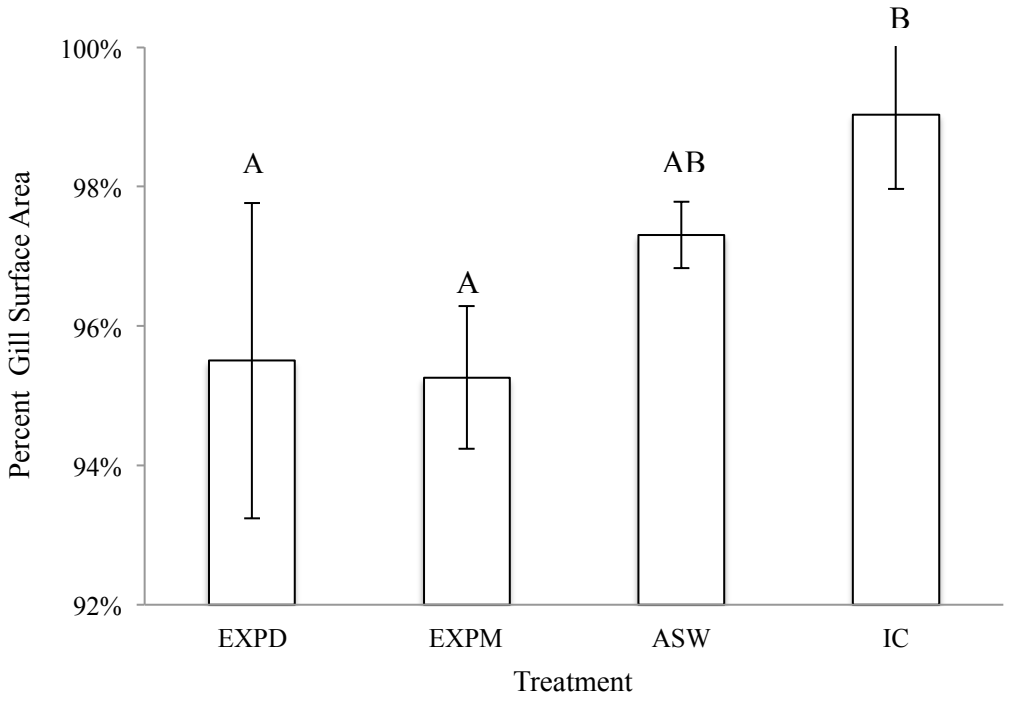

Figure 7: Average percent gill surface areas of all fish per treatment for all experimental growth phases, (Kruskal-Wallis rank sum test, $p = 2.921\ e^{-8}$). Means with different letters are significantly different from one another (Dunn's Test $p < 0.023$) (See Table 5)). Error bars are equal to standard error. (n of EXPD =14, n of EXPM = 31, n of ASW = 58, and n of IC =11).

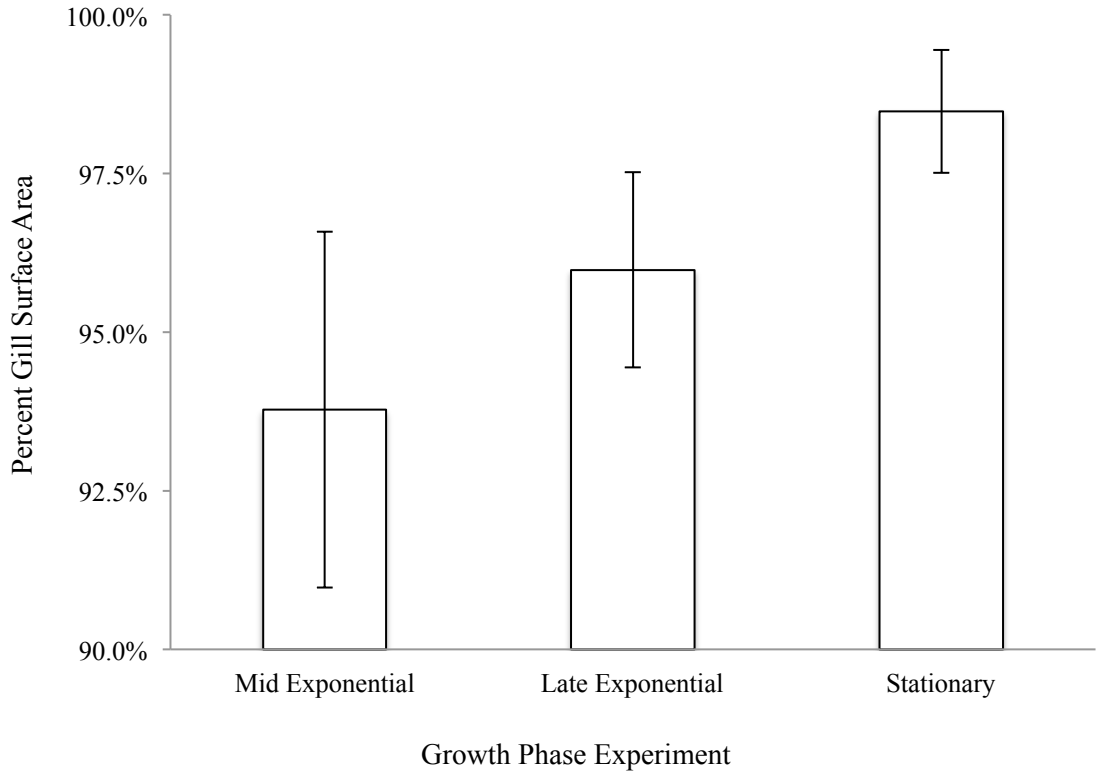

Figure 8: Average percent gill surface areas of exposure treatments EXPD and EXPM observed in Mid Exponential (n = 10), Late Exponential (n = 10), and Stationary (n = 8) growth phase experiments. Error bars are equal to standard error.

APPENDICES

Appendix I: *Algal Culturing*

Abstract

Two algae species were used in this study, the harmful raphidophyte *Chattonella subsalsa* and a benign haptophyte *Isochrysis galbana.* In order to account for starvation and potential physical irritants caused by algal bloom cell densities, a control algae treatment was necessary. The two species were raised under identical conditions and monitored throughout their growth to develop growth curves. After establishing growth dynamics both species were analyzed for carbon content using a loss on ignition method. The carbon content ratio of *I. galbana* to *C. subsalsa* (1: 214.88) was used to calculate comparable cell densities for use in experimental treatments.

Introduction

To properly examine the fish kill mechanism employed by the HAB species *C. subsalsa* the experimental design included a treatment with a control algae species. Physical irritation due to bloom level cell densities, (4×10^3 cells mL^{-1} for the genus Chattonella (Imai & Yamaguchi 2012)), is one of the hypothesized fish kill methods examined by this study. To determine if such densities are harmful to fish only when achieved by known HAB species, fish were also exposed to bloom levels of the non-harmful species *Isochrysis galbana. I. galbana* treatments also served as control for starvation as fish were not actively fed during experiments.

I. galbana is a flagellated marine microalgae ranging 5-6 μm in length. *I. galbana* is a member of the class Prymnesiophyceae of the phylum Haptophyta and naturally inhabits the marine waters of the coastal Atlantic. This species is commonly cultivated

for use as feed in aquaculture facilities because of its ease to maintain and its high fat content (Tomas 1997). As this species has no known negative effects on marine organisms it was an ideal control species for experimental trials.

Methods

A culture of *C. subsalsa* was obtained from Dr. Dianne Greenfield's laboratory located in Charleston, SC (USC/SCDNR). The aliquot was kept in a small styrofoam cooler with an ice pack to prevent over heating of algal culture while being transferred from Charleston to Coastal Carolina University's laboratory facilities.

Cultures were raised in a Percival Intellus Environmental Controller (incubator), at 25^0 C under 12:12 light: dark regime, with an irradiance value of 85-140 μmol/m^2/sec in an f/2 nutrient replete medium (-Si) (Guillard & Rhyther 1962).

F/2 medium was produced in the lab using a 25 ppt artificial seawater base and an f/2 chemical kit acquired through the Bigelow Laboratory for Ocean Sciences. The seawater base was made in laboratory facilities using Instant Ocean Sea Salt and deionized (DI) water. Artificial seawater and f/2 chemical additions of nitrogen and trace metals were filtered through a 0.22 μm general filtration membrane filter into a clean 1L glass container. Seawater mix was autoclaved on a Liquid Cycle and allowed to cool to room temperature. Upon reaching room temperature the final chemical components of phosphorus and vitamins were added and completed f/2 medium was stored in the incubator.

The initial aliquot of *C. subsalsa* was allowed 24-48 hours to adjust to conditions present in the incubator prior to culturing. Culturing was conducted on a clean bench

under a laminar flow hood following the standard operating procedure (SOP) used by Dr. Greenfield's lab. A small volume of aliquot culture (2-5 ml) was transferred into f/2 nutrient replete medium in 50 ml glass or polypropylene test tubes. Prior to use all glassware was sterilized via a cycle of DI rinses, acid washing, and autoclaving.

Regular cell counts were conducted on active cultures of *C. subsalsa* to monitor growth. Growth curves were developed for multiple generations of *C.subsalsa* by analyzing cell density counts as the culture progressed through lag, log, stationary, and decline phases. On counting days a sample of approximately 1 ml of culture was removed at 10:00 am from each test tube containing active cultures. Removed culture was preserved using Lugol's iodine solution. The ratio of Lugol's to culture was recorded and applied to determine a dilution factor. Preserved cultures were counted using a Sedgwick rafter counting chamber. If culture density was approximately 3000 cells ml^{-1}or less, all fields of the counting chamber were counted. If cell densities were above 3000 cells ml^{-1} a set of random numbers was obtained from Random Number Generator (random.org). The list of randomized numbers was used to determine which fields of the chamber to count. Fields were counted until a total of 250 cells had been counted. Cell density was calculated using Equation 1, where *C* is the number of cells counted, *A* is the area of each field within the counting chamber, *D* is the depth of the counting chamber, and *F* is the number of fields counted

(1)

(a) $$Cell\ density\ ml^{-1} = \frac{(C \times 1000\ mm^3)}{(A \times D \times F)}$$

(b) $Cell\ density\ ml^{-1}_{total} = Cell\ density\ ml^{-1} + (Cell\ density\ ml^{-1}\ x\ dilution\ factor)$

A growth curve was calculated based on cell densities using Equation 2, where μ is growth rate and N_2 and N_1 are number of cells at times t_2 and t_1.

(2) $$\mu = \frac{\ln N_2 / N_1}{t_2 - t_1}$$

Cell density and growth rate calculations were used to create growth curves which were averaged to determine the target time frame for culturing and harvesting stock cultures for use in experiments.

The genus *Chattonella* reproduces asexually, doubling cell populations at an average rate of 1 division day^{-1}. Under favorable conditions *C. subsalsa* has been found to grow at a rate of approximately 1.26 divisions day^{-1} (Imai & Yamaguchi 2012). Equation 3 was used to determine the divisions day^{-1} at which a given culture of *C. subsalsa* reproduced.

(3) $$Divisions\ day^{-1} = \frac{\mu}{\ln 2}$$

Cultures of harmful algae are thought to be at the highest degree of toxicity when in exponential growth (Khan et al 1996). Using the formulated growth curve, the near-end of the log phase was targeted to inoculate new cultures. This time period represents a point in the life cycle of active exponential growth and high cell densities allowing continued growth in replicate cultures. *C. subsalsa* was cultured regularly to maintain a stock of actively growing cultures. A volume of 2-5 ml of stock culture in exponential (log) growth phase was used to inoculate new generations of *C. subsalsa* every 10-12 days in accordance with the calculated growth curve (Figure 1).

An initial aliquot of *I. galbana* (Strain ID: UTEX987) was obtained from Carolina Biological Supply, and was cultured and maintained under the same conditions as *C.*

subsalsa. Growth rates for *I. galbana* were calculated via regular cell counts with a hemocytometer. A sample of 100 μm was drawn and preserved in Lugol's iodine. When counting with the hemocytometer cells from each of the four corner squares were counted. Cell counts were applied to Equation 4, where DF is dilution factor to determine cell density.

(4) $$Cell\ density\ mL^{-1} = \frac{\left(\frac{\sum counted\ cells}{\#\ of\ squares}\right) \times 10^4}{DF}$$

Growth curves were developed from cell densities and used to determine ideal culturing periods. In most cases, *I. galbana* was cultured every 8-10 days.

Due to the vast difference in *I. galbana* and *C. subsalsa* cell size, cultures were normalized based on carbon content. Carbon content was determined using a loss on ignition method to measure dry weight (DW) and ash free dry weight (AFDW) of *I. galbana* and *C. subsalsa.*

Three 20 ml samples of each species were filtered through pre-combusted glass fiber filters using vacuum pressure measuring -2.5 inHg or lower. Filters, frits and other glassware were acid washed, autoclaved and pre-combusted prior to use in DW/AFDW analysis. After filtration glass fiber filters were placed in pre-combusted weigh tins in a drying oven at 60 ^{0}C for four hours. After four hours, filters were removed from the drying oven, weighed, and set back in the drying oven until a consistent weight was reached by all samples, approximately 24 hours. Filters were kept in a desiccator while being transferred from oven to analytical balance. After taking final weight measurements, filters were wrapped in pre-combusted aluminum foil envelopes and placed in a muffle furnace at 450 ^{0}C for one hour. Following combustion filters were

weighed a final time to obtain AFDW values. Carbon content per cell of algae was determined using Equation 5, where DW is dry weight and AFDW is ash free dry weight.

(5) $$Carbon\ content/_{cell} = \frac{DW - AFDW}{Total\ cell\ density\ of\ sample}$$

Results

Cell density, growth rate, and divisions per day were calculated for *C. subsalsa* and *I. galbana*. The growth rate of *C. subsalsa* was found to be variable across individual culture generations. When averaging all growth rates a distinct trend was observed. The observed trend had a peak in growth rate in the early days of the culture, usually occurring around day 4-6, followed by a secondary peak, usually occurring between days 10-12 (Figure 2). This trend was apparent among individual generations as well (Table 2).

Average daily growth data only reached a maximum value of 0.845 divisions day^{-1} (Figure 3). However, in nine of the twelve individual culture generations used to calculate data set averages, values of 1.0 division day^{-1} or above were achieved.

As morphology and activity are indicative of health, live examinations of active cultures were regularly performed. Cultures were observed to change morphology slightly over the course of growth. In lag and early exponential phases cells were observed to be circular in shape and moderately active. As cultures progressed to exponential growth. cell shape was ellipsoid and cells were highly active.

I. galbana grew similarly to *C. subsalsa. I. galbana* displayed less variability among individual generations and in most cases grew exponentially for 8 days in culture (Figure 4, Figure 5). In some cases the lag stage of growth was extended in *I. galbana* cultures. This was the case in cultures used for carbon content analysis. Cultures of *I.*

galbana used for carbon content analysis were 19 days old but within late exponential or stationary phase at the time of analysis.

Carbon values per species were averaged and used to determine the ratio of *I. galbana* carbon content to *C. subsalsa* carbon content (*1:214.88) (Table 1). This ratio was used to determine the cell density of *I. galbana* culture to be used in *I. galbana* treatment controls.

Conclusion

Growth dynamics may vary considerably in individual clones of the same algae culture even when grown under identical conditions (Turner 2014). Cultures of *C.subsalsa* were variable but overall predictable. Stock cultures grown throughout the preliminary period were healthy. High cell densities and division day^{-1} values in the 1.0 and above range were regularly achieved.

Morphotype and movement were also used as indicators of algal health. The morphology and activity of live cells was within a healthy range. During exponential life phase live cells were observed to be a spindle shaped and spiraling as they moved (Marshall & Hallegraeff 1999).

Due to the variability observed in stock cultures grown under identical conditions over the course of approximately 18 months, it was decided to perform formal experimentation using cultures representing five distinct life phases. Using cultures of various ages and life phases allowed room for differences in growth while still minimizing potential bias introduced by variable growth patterns. Cultures were harvested for experiments at lag, early exponential, mid-exponential, late exponential, and stationary life phases.

TABLES

Table 1:
Culture information and results from loss on ignition analysis, where DW is dry weight and AFDW is ash free dry weight.

Species	Age (days)	Density cells ml^{-1}	Growth Rate day^{-1}	DW (g)	AFDW (g)	Δ Carbon	Carbon content $cell^{-1}$	Avg. Carbon $cell^{-1}$	I:C Carbon content
C. subsalsa	11	$1.35x10^{4}$	0.29	0.045	0.043	$2.00E^{-3}$	$1.48E^{-7}$		
C. subsalsa	11	$4.55x10^{3}$	0.07	0.043	0.042	$1.30E^{-3}$	$2.86E^{-7}$		
C. subsalsa	11	$1.45x10^{4}$	0.39	0.044	0.042	$1.60E^{-3}$	$1.10E^{-7}$	$1.82E^{-7}$	
I. galbana	19	$1.55x10^{6}$	0.07	0.044	0.042	$1.20E-^{3}$	$7.75E^{-10}$		
I. galbana	19	$1.34x10^{6}$	0.12	0.044	0.043	$1.35E-^{3}$	$1.01E^{-9}$		
I. galbana	19	$2.01x10^{6}$	0.09	0.044	0.042	$1.50E^{-3}$	$7.45E^{-10}$	$8.43E^{-10}$	1:214.88

Table 2:
Comparison of multiple generations of *C. subsalsa,* average growth rates (μ) day^{-1}over the course of sixteen days of growth in culture. Asterisks indicate highest observed growth rate day^{-1} per generation.

Average growth rate day^{-1} (μ)							
Culture ID	**Day 4**	**Day 6**	**Day 8**	**Day 10**	**Day 12**	**Day 14**	**Day 16**
Gen a_1_a	0.373	0.503 *	0.181	0.229	0.291	-0.129	0.172
Gen a_1_b	0.658 *	0.053	-0.269	0.486	0.208	0.489	-0.179
Gen a_2	0.277	0.299 *	0.296	0.106	-0.235	---	---
Gen a_3_1	0.118	0.039	0.270 *	-0.069	-0.079	0.195	-0.049
Gen 12	0.141	0.439	0.439 *	0.003	0.272	0.257	---
Gen 21	0.636 *	-0.214	0.361	0.477	0.410	0.339	-0.026
Gen Y	0.034	0.779 *	-0.473	0.107	-0.165	---	---

FIGURES

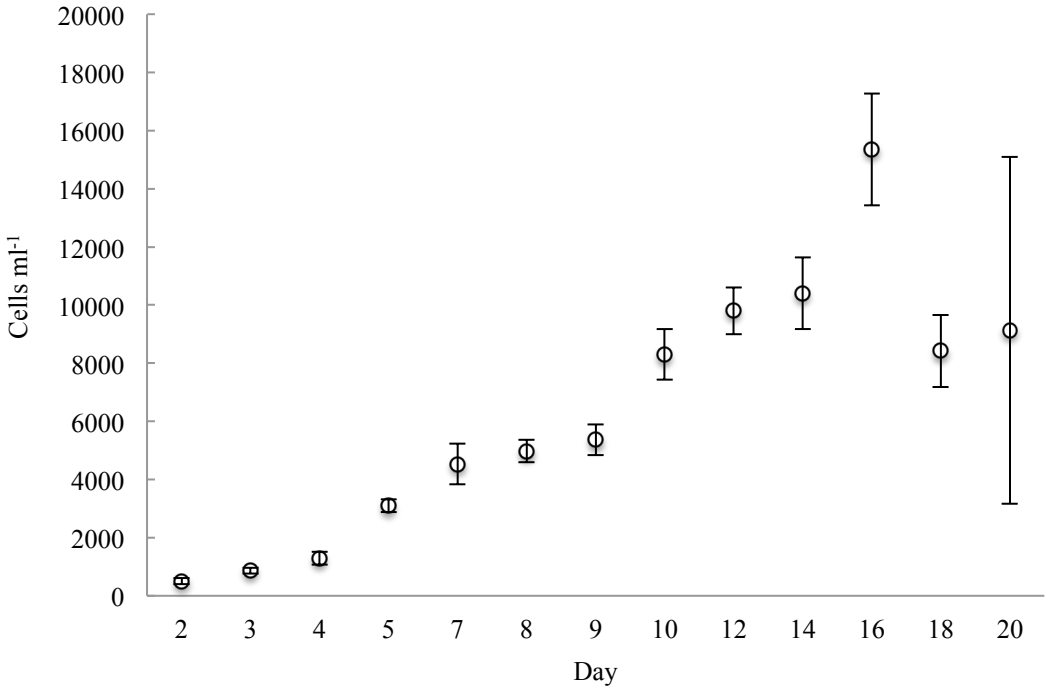

Figure 1: Average cell densities day^{-1} calculated from various generations (n = 260) of *C. subsalsa* over 20 days of growth. Error bars are equal to standard error.

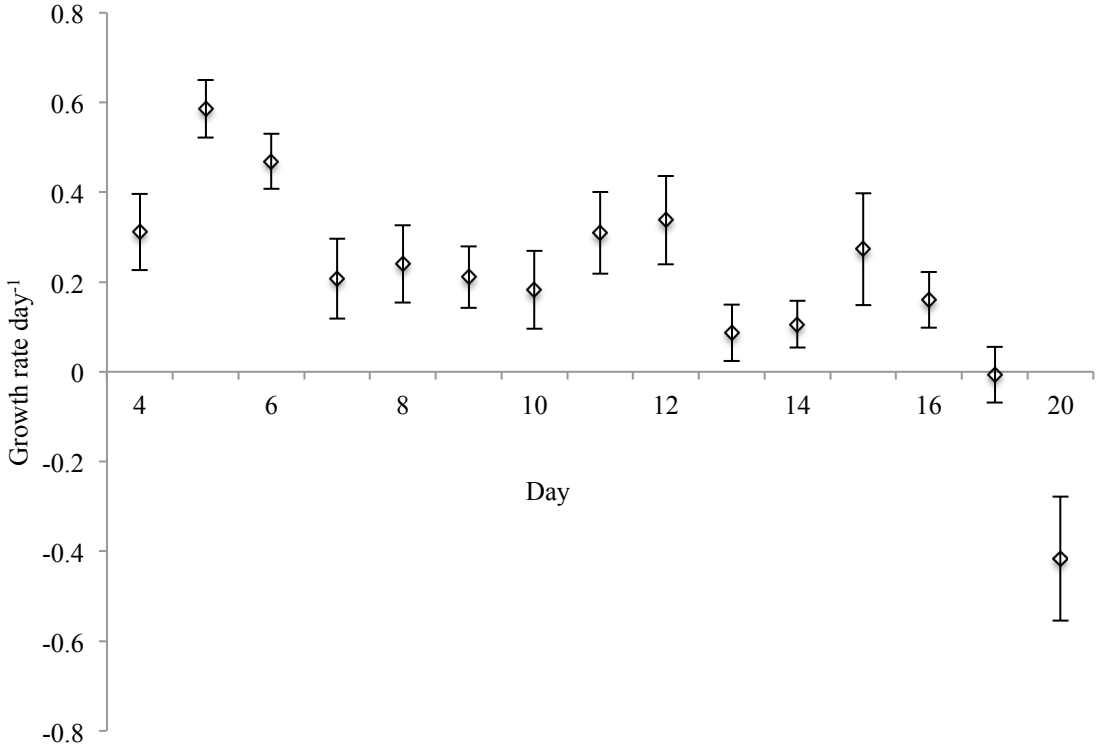

Figure 2: Average growth rates (μ) day^{-1} calculated from various generations (n = 525) of *C. subsalsa* over 20 days of growth. Error bars are equal to standard error.

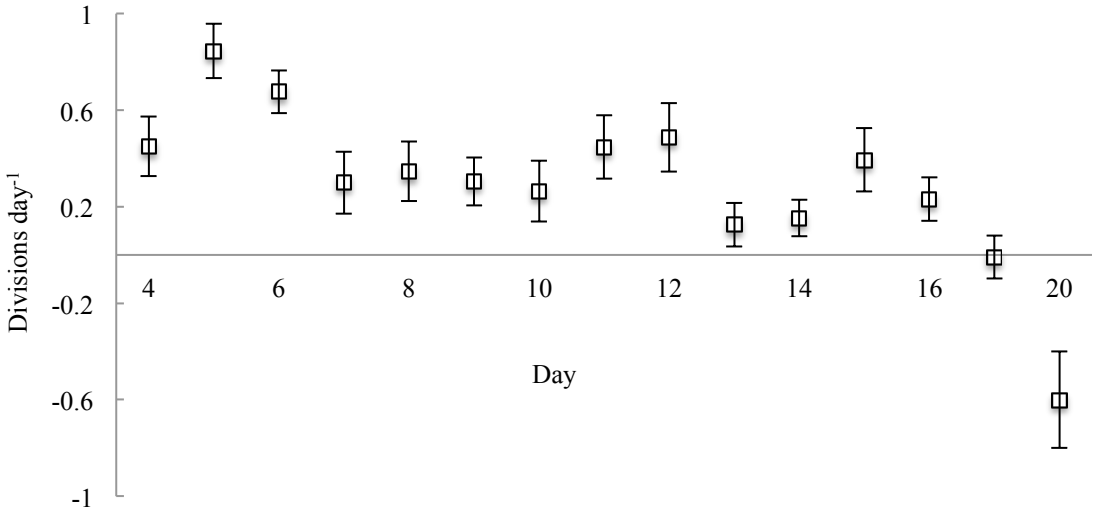

Figure 3: Average divisions day^{-1} calculated from various generations (n = 525) of *C. subsalsa* over 20 days of growth. Error bars are equal to standard error.

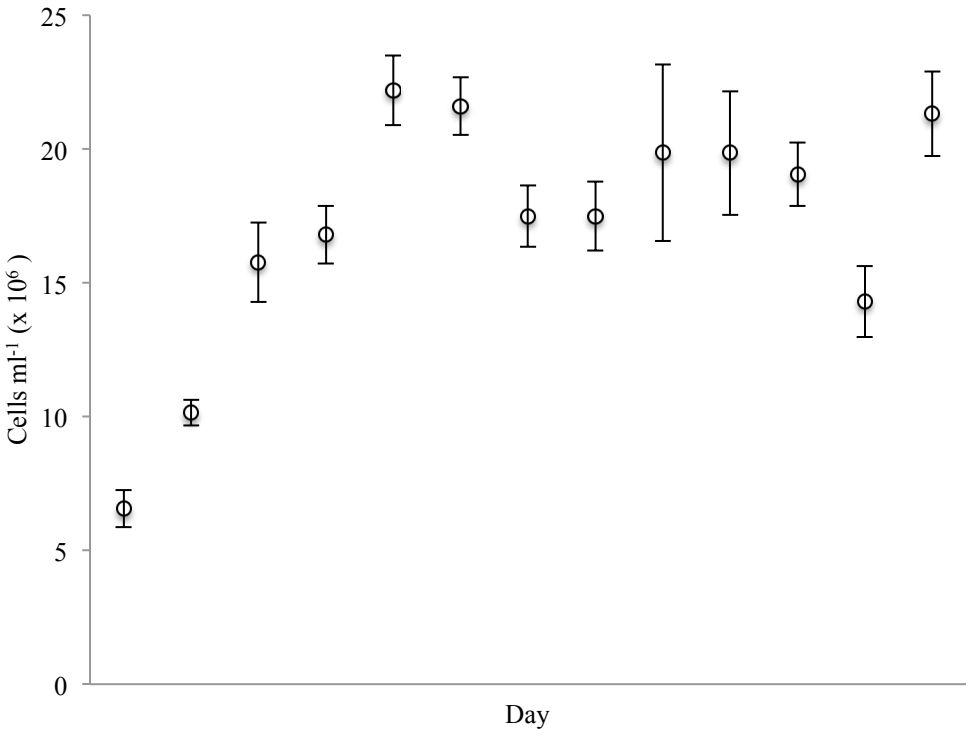

Figure 4: Average cell densities day^{-1} calculated from various generations (n = 156) of *I. galbana* over 20 days of growth. Error bars equal standard error.

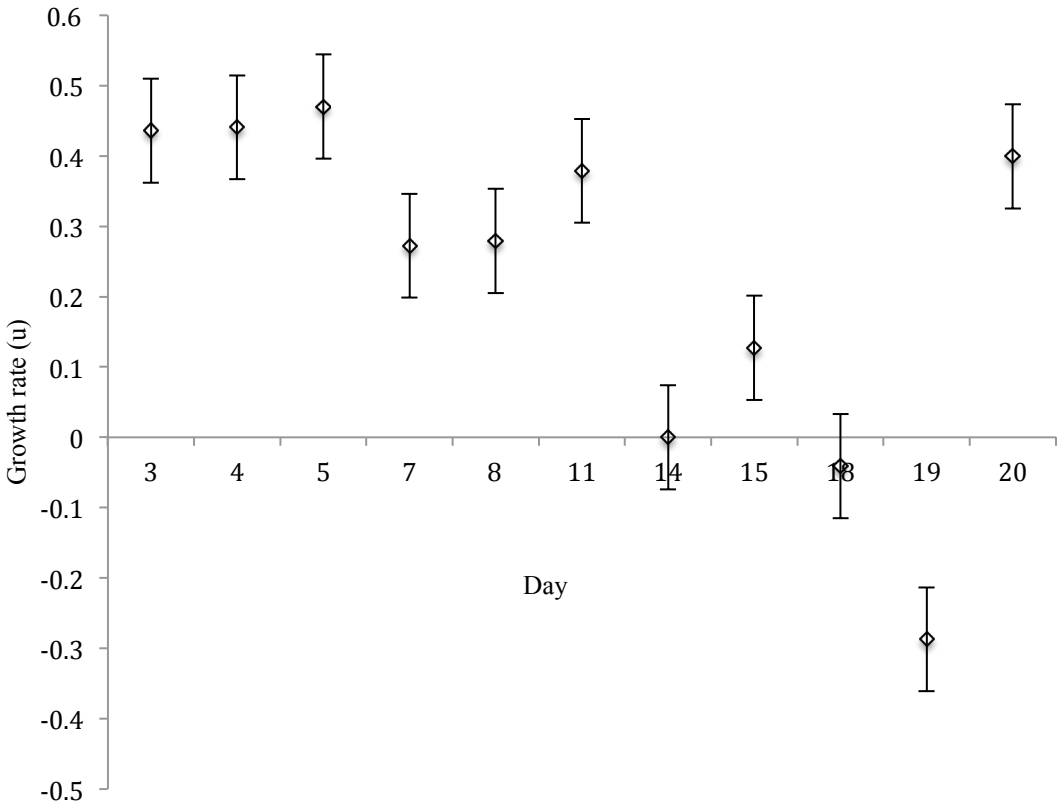

Figure 5: Average growth rates (μ) day^{-1} calculated from various generations (n = 66) of *I. galbana* over 20 days of growth. Error bars equal standard error.

Appendix II: Fish Husbandry

Abstract

This study was conducted using larvae of the common estuarine fish *Fundulus heteroclitus*. Adult *F. heteroclitus* were collected from field sites during the fish's natural reproductive peak. Summer light and water conditions were mimicked in lab facilities to promote breeding activity. This environment proved effective for egg production, yielding an average value of 289.0 eggs per collection. A 92.0% hatch rate was observed in eggs air incubated for 14 days and submerged in 25 ppt and 25 ^{0}C artificial seawater. Larvae were maintained on *Artemia salina* nauplii for a minimum of 7 days post hatch (dph). Conditions and feed were satisfactory yielding a 95.8% survival 7dph.

Introduction

The common mummichog, *Fundulus heteroclitus,* is found in ubiquity within the salt marshes of the Western Atlantic. Though this fish is not one of the primary species associated with *C. subsalsa* fish kills it is known to live in habitats similar to those where *C. subsalsa* blooms have occurred in South Carolina (Lewitus et al 2002, Lewitus et al 2003, Abraham 1985). Adult fish may be less likely to encounter *C. subsalsa* cells directly as they are primarily benthic, commonly known as mud minnows. However, larval *F. heteroclitus* feed on zooplankton in surface waters where algal cells would be concentrated (Kneib 1984, Imai & Yamaguchi 2012). It is very likely that *F. heteroclitus* larvae would directly interact with *C. subsalsa* during a bloom event.

Though *F. heteroclitus* is not an important food or recreational species, it has been the subject of aquaculture. Both large scale and small scale aquarium breeding have been successfully accomplished in past studies (Bosker et al 2009, Bosker et al 2013,

Hsiao et al. 1996, Janiak & McIntosh 2014, Redway 1980). The small size of adult *F. heteroclitus*, max length 100 mm, removes the necessity for large aquaculture facilities and equipment and enables fairly low-tech breeding (Bosker et al 2009, Bosker et al 2013, Hsiao et al. 1996)

Larvae of *F. heteroclitus* bred in Coastal Carolina University's laboratory facilities were utilized for this study. The use of larvae provided several advantages including a constant supply of fish without continued field collection, a captive population potentially free of parasites or outside environmental health consequences, and the ability to work on a small scale and run all experimental trials within an environmental chamber. Conducting all experiments in the environmental chamber allowed for consistence of temperature and lighting conditions throughout all trials.

Methods

Adult *F. heteroclitus* were collected from Garden City, SC from an easily accessible marsh location. Sampling dates were set to fall on or around spring tides corresponding with the new and full moon at the fish's natural reproductive peak (Taylor et al. 1979, Redway 1980, Hsiao et al 1996, Shimizu 1997, Bosker et al. 2009, Bosker et al. 2013).

Fish were captured using killie traps baited with grass shrimp *(Palaemonetes sp.*) or dry dog food. Traps were set at the edge of *Spartina alternaflora* beds during a falling tide when water partially inundated the high marsh. Traps were allowed to soak for a minimum of forty-five minutes before removal. At the time of removal the largest males and females were collected from each trap and the remaining fish were released. Only sexually mature fish, lengths of 40 mm or greater, were collected.

Fish were transferred from the field in five gallon buckets of aerated seawater collected on site. Fish were brought back to laboratory facilities at Coastal Carolina University and acclimated to water conditions in pre-established ten gallon aquaria via a drip system. After drip acclimation for at least one hour fish were removed from acclimation containers with dip nets and placed in ten gallon aquaria.

Prior to acclimation the fish were sorted based on sex. Sex ratios were either even, 1:1, or in female majority (Table 1). Breeding ratios were female biased to minimize aggressive competition among males (Bosker et al. 2009, Bosker et al. 2013, Hsiao et al 1996, Redway 1980, Shimizu 1997).

Aquaria were maintained at consistent temperatures and salinities, 31±2 ^{0}C and 34±2 ppt respectively. Routine water changes and tests of water chemistry were performed to maintain water quality. Shell fragments collected at the field site were placed in the aquaria to mimic the natural environment and promote breeding. Adult *F. heteroclitus* were maintained on a diet of grass shrimp (*Palaemonetes*) collected from the field site. Grass shrimp were collected using a dip net near the end of ebb tide when water was no longer inundating the marsh grasses. Collected shrimp were stored frozen until being used as feed for *F. heteroclitus.* Regular feedings, high temperature, and lighting conditions on an approximate 14:10 light: dark cycle mimicked summer conditions and encouraged fish to spawn in captivity throughout the winter months well beyond the natural spawning season.

Eggs were collected using the Janiak style egg collector (Figure 1) designed for the aquaculture of *F. heteroclitus*. The collector is composed of thin plastic disks tightly spaced on a stainless steel thread closed by wing nuts. This collector mimics the natural

crevices of mussel shells and marsh grasses used by *F. heteroclitus* when spawning in the wild (Janiak & McIntosh 2014). Egg collectors were constructed by cutting disks of 2 and 5 inch diameter from marine grade plastic sheets. The large disks were cut from 1/8 inch plastic and the small disks were cut from 1/16 inch plastic. Once cut disks were sanded smooth and thread onto a steel bolt.

A total of ten large disks separated by nine small disks made up each of six constructed collectors. The threaded disks were tied to plastic floats suspending them in the water column (Figure 2). A collector was placed in each of the breeding tanks and left unaltered until collection.

Egg collectors were checked regularly by visual examination and eggs were removed approximately once a week. When eggs were harvested the collectors were removed and placed in artificial seawater. Collector disks were loosed and gently shaken to detach eggs. Seawater containing eggs was strained over a nylon mesh screen. The number of isolated eggs was estimated and recorded and the eggs were placed in air incubation trays. The incubation trays contained a ½ inch layer of poly foam matting. Eggs were laid on matting and spread out to allow oxygen exchange, which is inhibited by clustering. This was repeated with the collectors from each breeding tank. After all eggs were distributed on the mat the clutch was misted with a spray bottle containing 25 ppt artificial seawater. The incubation tray remained closed while in the incubator and was removed to spray eggs as needed to maintain a damp but not saturated environment (Coulon et al. 2012).

Eggs were air incubated for a minimum of fourteen days using poly foam mats. After incubation fully developed eggs were inundated in 25 ppt artificial seawater to

induce hatching. Hatched larvae were held in 4 L plastic containers containing 1.5 L of 25 ppt artificial seawater. Each container held no more than 150 fish at any given time.

In the lab newly hatched *F. heteroclitus* require 5.5 days at 20 ^{0}C to absorb the yolk sac however yolk may be absorbed prior to hatching if immersion of developed eggs is delayed (Abraham 1985). Eggs were incubated at a temperature of 25 ^{0}C, which may have accelerated development. At the point of hatching all observed larvae had absorbed the yolk sac and were actively feeding.

Larvae were maintained for a minimum of 7 days post hatch (dph) prior to use in experiments. This time frame was chosen to allow any remaining yolk sac to be absorbed and ensure larvae were actively feeding. Allowing a minimum of 7 dph also confirmed larvae were in good health and acclimated to conditions preceding use in experimental exposures.

Larvae were maintained on a diet of *Artemia salina* nauplii, cultivated in lab. *Artemia* are commonly used for feeding small fish in aquaculture or aquarium facilities (Dhont et al. 2013). Dried *Artemia* eggs were acquired from Carolina Biological Supply and *Artemia* hatcheries were constructed from 1L plastic soda bottles (Figure 3). Hatcheries were filled with approximately 500 ml of aerated 25 ppt artificial seawater and housed in the incubator. A desk lamp providing 18 μmol/m^{2}/s^{-1} irradiance was positioned over the hatchery to induce hatching. Hatcheries were started on an every other day basis by adding 2.3 grams of dried eggs to aerated seawater. Eggs hatched after 24 hours of immersion in seawater. At time of harvest aeration was removed and nauplii settled. Nauplii were collected using a 1 ml Pasteur pipette and fed to larvae. Containers of 150 larvae were fed 1 ml of nauplii per day. Partial water changes were conducted 2-3

times a week to maintain water quality and accommodate for evaporation. At this time dead fish and debris were removed.

Results/Conclusions

The egg collection period took place from February 2014 to April 2015. A total of twenty-six documented collections were made during this time. Spawning in brood tanks took place on a continuous basis. Collections were made when eggs were visible on one or more collector. The majority of collection times occurred during new or full moon periods and coincided with the highest overall egg productions. Though collections occurring during lunar transitions (on or around first and third quarter phases) were not as common, all collections at this time fell below 50% of the highest total egg $fish^{-1}$ values (Figure 4). These results were anticipated as a semilunar pattern of egg production has been observed in the southern sub species *F. heteroclitus heteroclitus* (Hsiao et al 1996). A clear trend is visible between moon phase and the number of eggs produced $fish^{-1}$ (Figure 5).

Aquarium containing a total of five fish in a 3:2 female to male ratio, were the most efficient for breeding (Table 2). Of twenty-six total collections aquarium with 3:2 ratio consistently provided a significantly greater number of eggs $fish^{-1}$ (ANOVA $p = 0.0126$) (Figure 6). The average number of total eggs collected $week^{-1}$ was 288.96 with an eggs $fish^{-1}$ average of 26.1. Collected eggs had an average hatch percentage of 92.0% and an average mortality rate of 4.2% seven days post hatch (Table 2).

These results suggest breeding conditions were optimal and breeding fish were healthy. Eggs were properly fertilized and reached full embryonic development over the incubation period under the environmental conditions provided. Artificial seawater

salinity (25 ppt) and temperature (25 ^{0}C) were suitable for hatching success. Water conditions and feed (*Artemia* nauplii) were adequate for larval fishes' survival and growth. The results indicate a healthy stock of larvae could be bred and maintained for use in experimental trials.

TABLES

Table 1: Breeding tank ratios and the number of egg collections made in 2014 and 2015.

	Tank #	Females	Males	Number of Egg Collections
2014	2	2	2	6
	3	3	2	9
	10	3	2	9
	4	3	2	11
2015	4	4	3	2
	5	4	3	4
	1	3	2	7
	2	2	2	7
	3	2	3	7

Table 2: Parameters associated with egg collections, hatching success, and survival rates of larvae, where dph is days post hatch.

	Viable eggs produced	**Eggs female^{-1}**	**Eggs fish^{-1}**	**% Hatched**	**% Survived 7 dph**
Average	288.961	44.910	26.121	92.004	95.834
Standard Deviation	169.144	23.828	13.9748	10.582	6.880

FIGURES

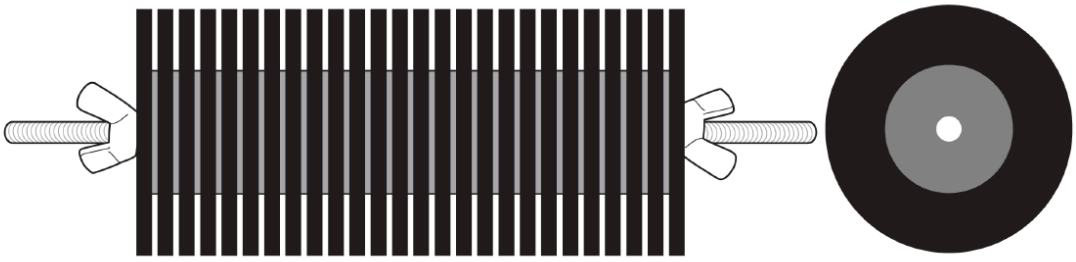

Figure 1: Janiak style egg collector composed of large disks 5 inch diameter and 1/8 inch thick and small disks 2 inch diameter and 1/16 inch thick threaded on a stainless steel thread closed with wing nuts.

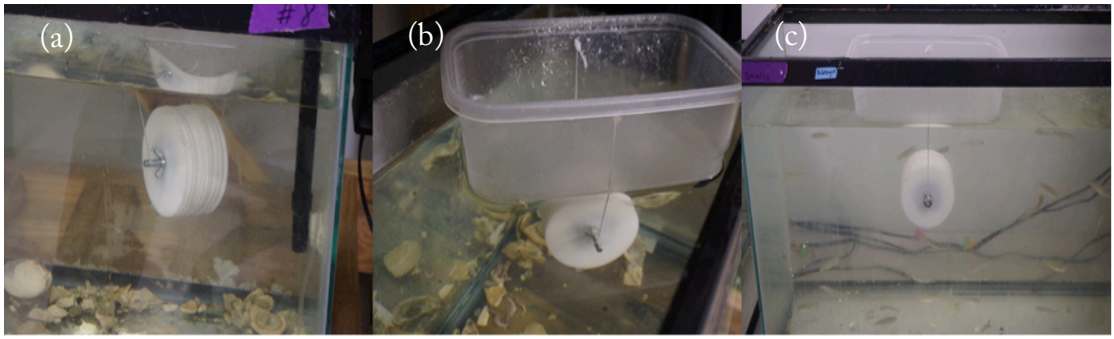

Figure 2: Assembled egg collectors a.) disks of collector, b.) collector float, c.) collector in aquarium.

Figure 3: *Artemia* hatchery constructed from 1L soda bottle, containing rigid tubing and air hose.

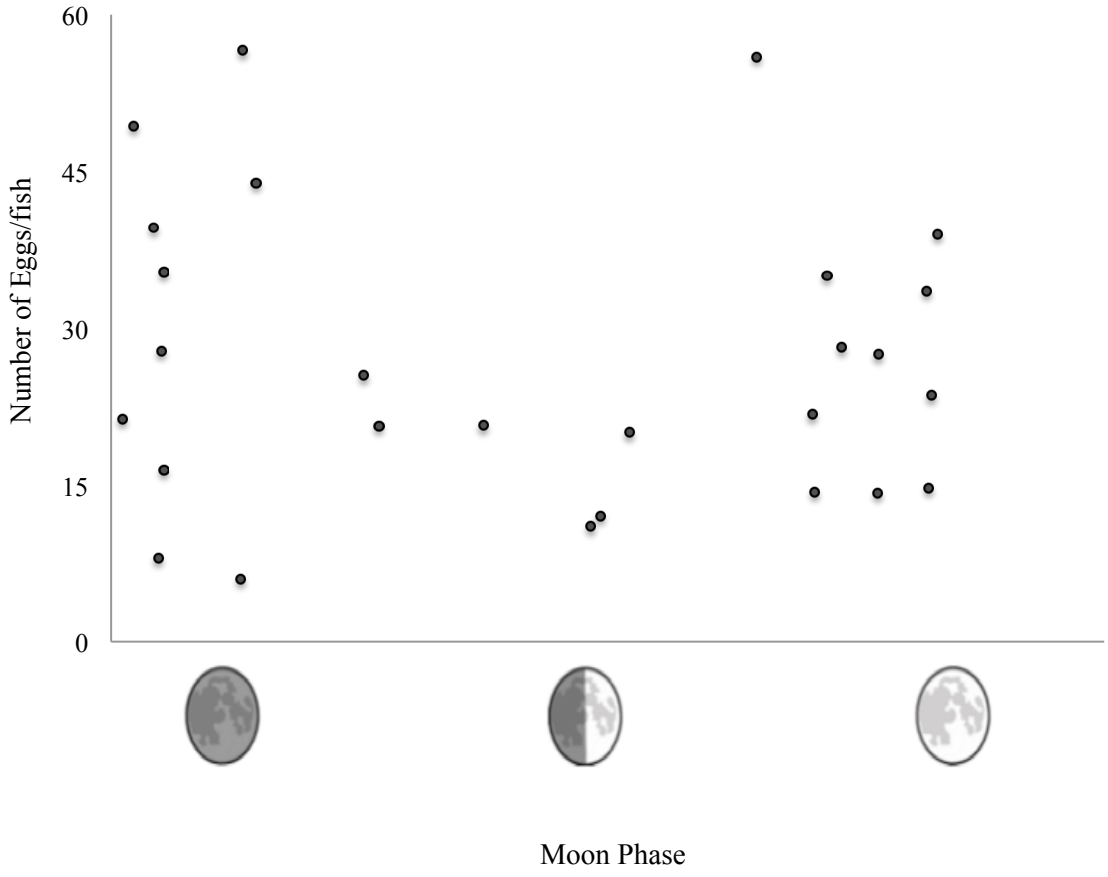

Figure 4: Number of eggs collected per fish at moon phases, new, half, and full (left to right).

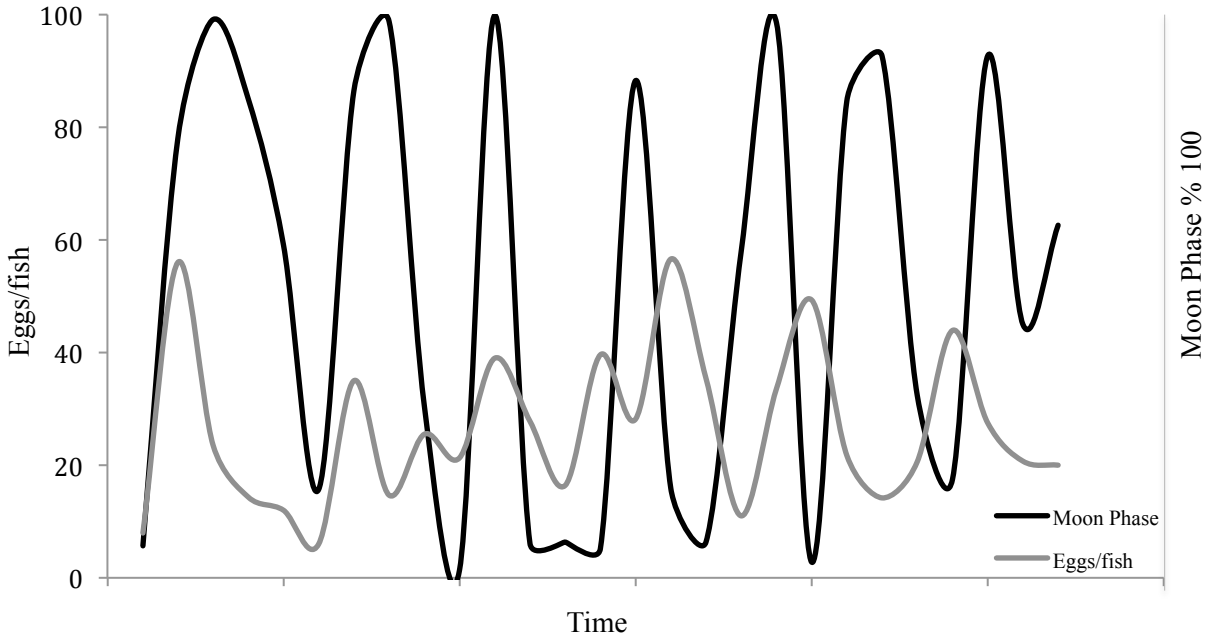

Figure 5: Eggs produced per number of fish and moon phase at time of collection (percent of 100) where 100% is a full moon throughout the egg collection period.

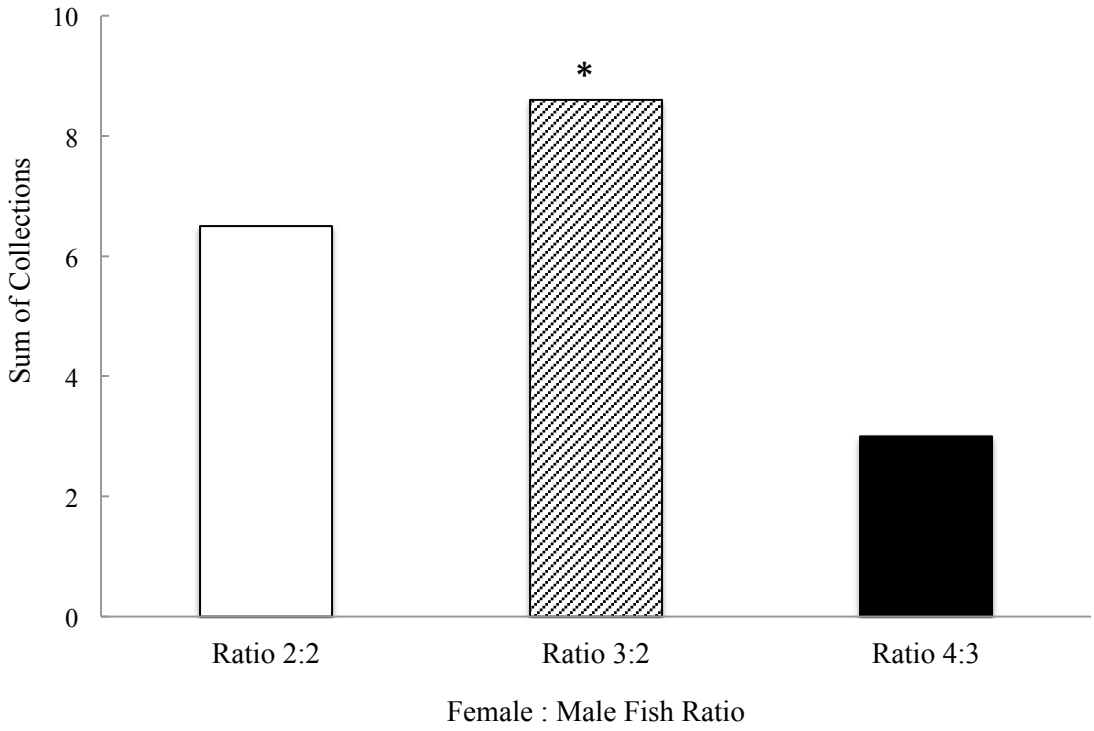

Figure 6: Number of egg collections yielded from various female: male tank ratios, (*p=0.0126).

Appendix III: *Preliminary Experiments*

Introduction

Prior to beginning formal experiments several preliminary exposures were conducted to determine anticipatory results as well as the exact parameters to be used for formal experiments including, container size and type, mesh separation design, and duration of exposures.

Container type and size was determined by experiments with petri dish and 50 ml test tube. Circular containers with large diameters are preferred for maintaining fish in captivity. Such aquaria provide a greater surface area for swimming and schooling as well as better flow dynamics (Oca & Masalo 2013). The petri dish, though suitable to the fish, was not comparable to the vertical volume *C. subsalsa* cells were accustomed to inhabiting. Extensive difficulty was encountered in successfully keeping *C. subsalsa* cultures growing at a healthy rate. To prevent any further stress on algae preliminary exposures were conducted in 50 ml test tubes. Likely due to the fishes' small size (5-7 mm), the diameter constraints of the test tube did not pose any issues.

Another difficulty arose when designing a method to affix mesh to separate test tubes. A vertical design was initially used in which a mesh sheet separated the test tube evenly (Figure 1). This design was not sealed completely on the tubes interior and *C. subsalsa* cells were able to penetrate to either side of the mesh. The final design was one utilizing a secondary 15 ml test tube. Six small holes were cut into the middle third of 15 ml test tube and then covered with a layer of mesh affixed to the tube with aquarium safe epoxy. During experiments the 15 ml test tube was filled with culture and placed inside a 50 ml test tube containing 10 ml of algal medium. This design was cell tight while

allowing the exchange of seawater between the inner and outer tubes. Though a design was perfected prior to formal experiments, no preliminary trials were carried out to test the effects *C. subsalsa* on fish when separated by mesh.

To avoid introducing another experimental variable, *F. heteroclitus* larvae were not fed during experimental trials. This meant that trials could not exceed a certain length of time without starvation becoming a major factor. Little variation in mortality of control fish was present from preliminary trials lasting between 8 to 69 hours, (0 – 26.67%). At 99 hours 100% of control fish had died (Table 1) Based on this information as well as the behavior of natural blooms a 48-hour exposure time was chosen.

Results/Conclusions

Mortality Analysis

The experimental parameters and outcomes of preliminary trials are listed in Table 2 and suggested promising results for formal experiments. Statistical analysis was conducted using R-3.2.2 package for Mac OS X 10.9 and higher.

Percent mortality data was tested for normality and homogeneity. Data was found to be non-normally distributed (Shapiro-Wilk $p=0.0277$) and not homogeneous (Fligner-Killeen $p=0.7636$). As data did not meet the assumptions for an ANOVA, Kruskal-Wallis rank sum test was used to analyze percent mortality of exposed and control fish. No significance was found in percent mortality (Kruskal-Wallis, $p=0.1262$). Test results for *I. galbana* exposure were not included in the statistical analysis. However, in all preliminary trials the percent mortality of fish exposed to *C. subsalsa* was greater than that of the control group.

No visible trend or statistical correlation was found between mortality and cell density or mortality and growth rate. As the time of exposure and environmental conditions were variable across individual preliminary trials such numerical information could not be grouped together for analysis.

Gill Surface Area Analysis

After all trials had been completed fish gills were extracted for analysis. Fish were prepared for gill analysis through a series of steps resulting in permanently mounting stained gill tissue on slides. Fish were removed from formalin with clean forceps and submerged in a DI water bath. Fish gills were removed from preserved fish and soaked in a 10% Trichrome stain for 8-10 minutes. Stained gills were dehydrated using 95% ethyl alcohol, cleared with xylene, and rehydrated with glycerin. Processed gills were affixed to slides with Permount mounting medium and cover slip. Completed slides were laid flat for at least 24 hours prior to assessment to ensure tissues had settled and medium was dry.

Images of gill tissue were captured using an Olympus BX60 microscope and Image Pro Plus software. All images were screened and only those meeting standards for analysis were utilized. The standards designated for image analysis were, 1.) Edges of tissue must be clearly defined, 2.) Individual lamellae must have a clear shape and be distinguishable from adjacent lamellae, 3.) The end or attachment point of primary lamellae to the gill arch must be discernable.

Images that met the standards for analysis were measured using *ImageJ* software. For each individual fish at least five individual gills were measured. When possible ten individual gills were measured. As some slides contained multiple individual gill arches,

methods were taken to avoid bias in analysis. When more than one individual gill arch image was available for analysis the number of gill lamellae measured was spread evenly across the separate images.

Two surface area (SA) measurements were taken for each individual gill. The first measurement (*Total* SA) encompassed the perimeter of the total surface area of the gill determined by tracing the outermost points of the secondary lamellae. The second measurement (*Lamellae* SA) traced the exact perimeter of the secondary lamellae (Figure 2). The two measurements were compared to determine the percentage of gill surface area using Equation 1, where SA is surface area.

(1) $$Percent\ Gill\ SA = \frac{Lamellae\ SA}{Total\ SA} \times 100$$

Gill analysis was conducted to determine if gill damage could be a viable tool for analysis. A visible difference was present in the gill tissue of control (healthy) versus exposed (degraded) fish. Gill surface area measurements were statistically tested for normality and homogeneity. Gill surface area was found to be non-normally distributed (Shapiro-Wilk, $p = 6.43\ e^{-3}$) and homogeneous (Fligner-Killeen, $p = 6.65\ e^{-2}$). As data did not meet assumptions for ANOVA, a Kruskal-Wallis rank sum test was used to determine significance. In preliminary trials the percent gill surface area of fish exposed to *C. subsalsa* was found to be significantly less than the percent gill surface area of control fish (Kruskal-Wallis, $p = 0.02$) (Figure 3).

TABLES

Table 1: Parameters and results of preliminary experiments.

	Start Date	Exp. Time (hrs.)	Age of Culture (days)	Density (cells/mL)	Growth Rate	Life Phase	Age of Fish (days)	Volume	Container Type	Design	Control N (start)	Exposure N (start)	Control N (end)	Exposure N (end)	Mortality Control	Mortality Exposure	% Difference
Chattonella subsalsa	Mar 27 2014	69	49	32467*	-0.008	Stationary	4	10 mL	15 mL petri dish	5 fish/dish	15	15	11	6	26.7%	60.0%	33.3%
	May 1 2014	99	14	28330*	0.549*	Mid Exp	38	10 mL	15 mL petri dish	3 fish/dish	9	9	0	0	100.0%	100.0%	0.0%
	Jun 4 2014	24	10	396*	N/A	Early Exp	45	10 mL	15 mL petri dish	5 fish/dish	20	21	15	14	25.0%	33.3%	8.3%
	Jul 8 2014	54	7	1314*	N/A	Early/Mid Exp	7	12 mL	15 mL petri dish	4 fish/dish	25	24	21	19	16.0%	20.8%	4.8%
	Jul 14 2014	25.5	13	1490	-0.253	Mid Exp	7	7 mL	50 mL test tubes	3 fish/tube	9	9	9	6	0.0%	33.3%	33.3%
	Jul 29 2014	141	15	7054*	0.871*	Late Exp	28	20-20 mL	50 mL test tubes	4 fish/tube	12	12	9	7	25.0%	41.7%	16.7%
I. galbana	Dec 14 2014	40					7	10 mL (*Ig*) 20 mL C	50 mL test tubes	5 fish/tube	15	15	14	15	6.7%	0.0%	-6.7%

FIGURES

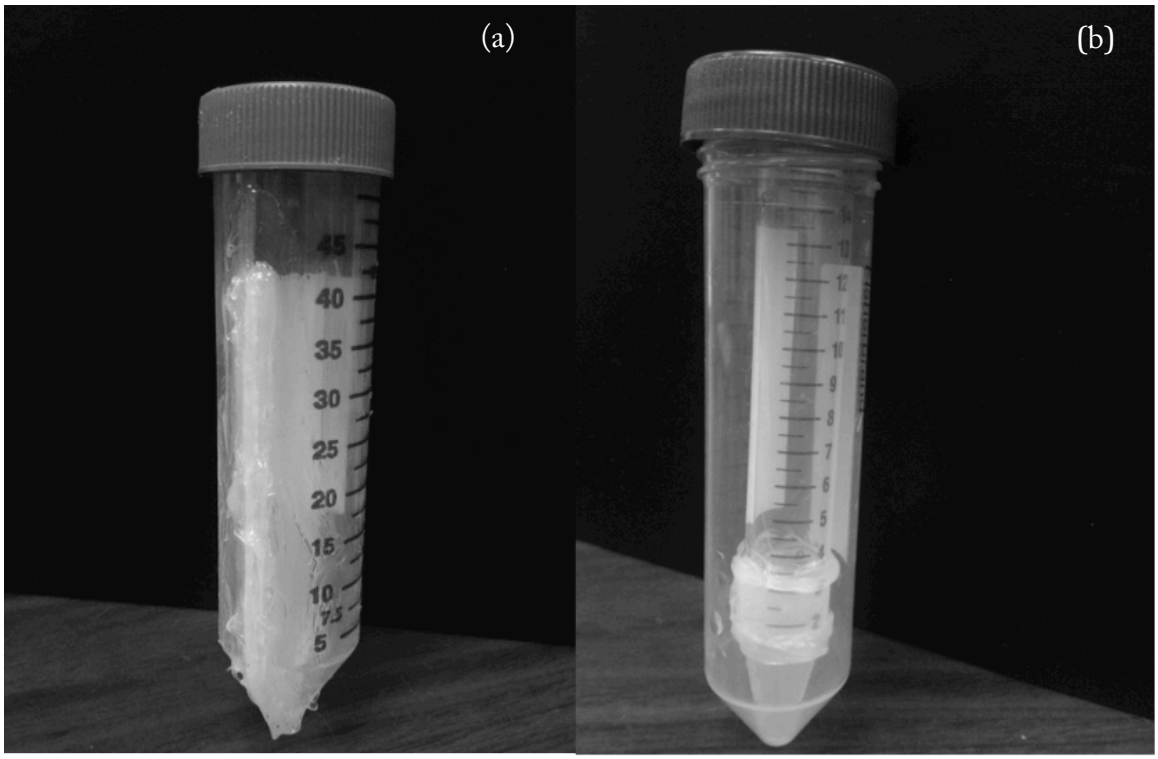

Figure 1: Mesh treatment test tubes, a.) 50 ml test tube separated by 1 micron mesh sheet and b.) 15 ml test tube with 1 micron mesh screen housed within 50 ml test tube.

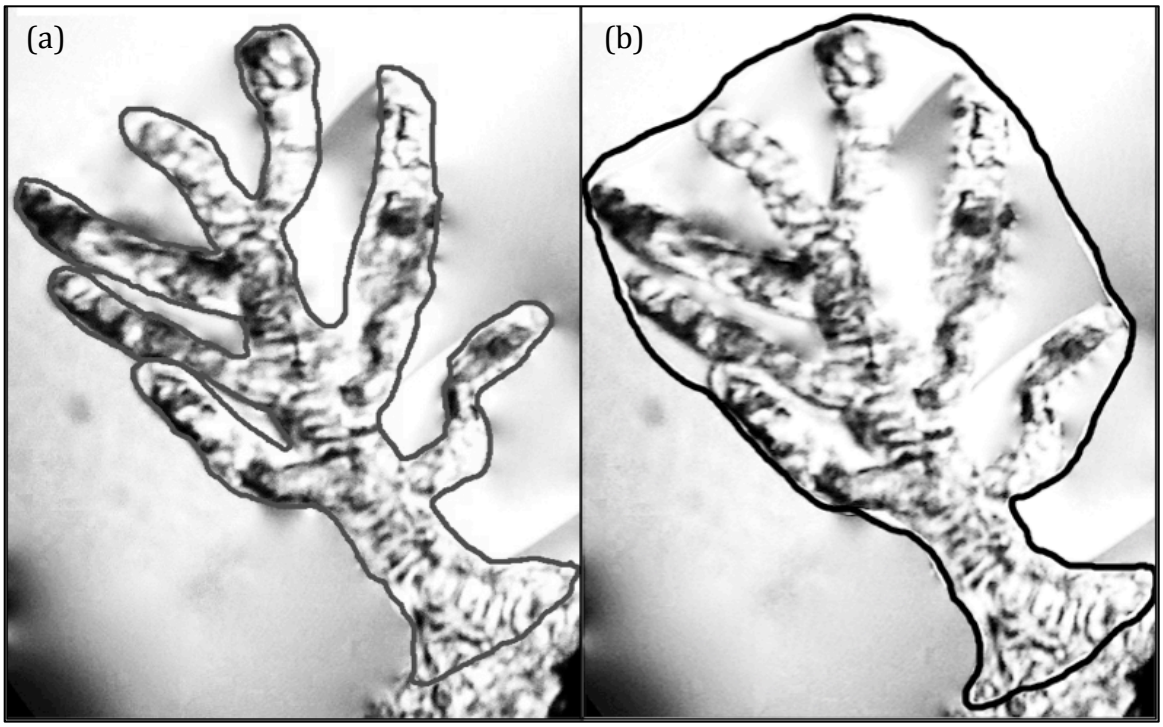

Figure 2: Surface area (SA) measurements, (a.) Lamellae Surface Area and (b.) Total Surface Area used to determine Percent Gill Surface Area in gill analysis.

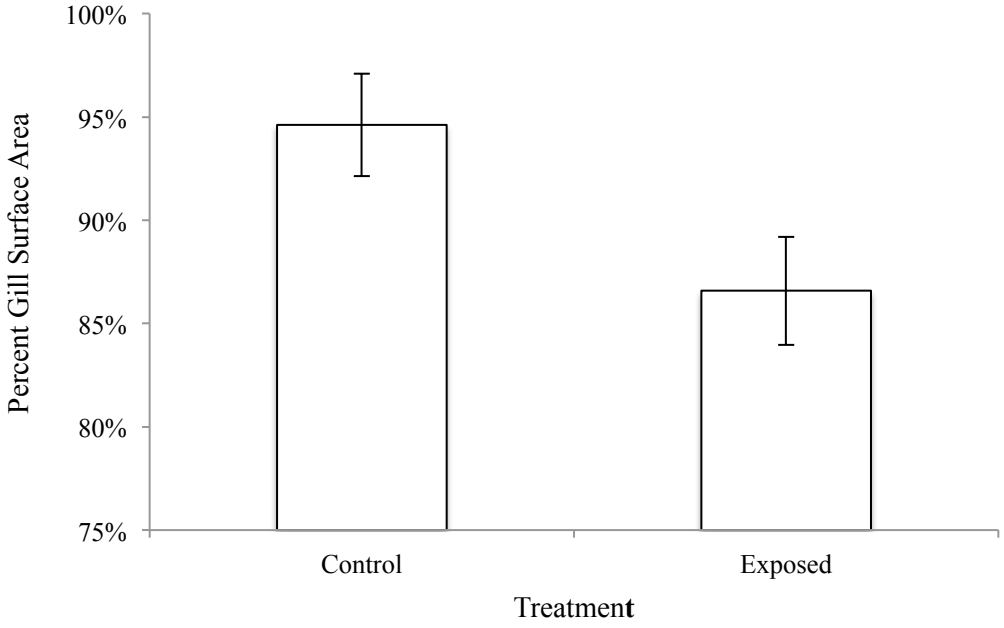

Figure 3: Average percent gill surface area analysis from preliminary trials, control (n = 11) versus exposed gill tissue (n = 13), (p = 0.02). Error bars are equal to standard error.