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Exposing Artemia Salina to Chattonella Subsalsa: A General Toxicity Test

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Abstract

The raphidophyte Chattonella subsalsa has been reported to cause harmful algal blooms in every major ocean. In South Carolina, C. subsalsa blooms have been observed in brackish stormwater detention ponds as well as estuarine waters neighboring urbanized areas. Blooms frequently cause fish kills although the fish kill mechanism of C. subsalsa is currently unknown. In many harmful species, the lethality of algal cells is thought to correspond with algal growth phase. Algal growth is known to progress through five distinct phases; lag, early exponential, late exponential, stationary, and decline. In nature, harmful algal blooms commonly occur in the late exponential or stationary growth phases; however, in vitro studies of Chattonella have identified the early exponential phase as most lethal. The strain of C. subsalsa used for this study was found to progress through the five growth phases in a period of twenty days. To examine the lethality of C. subsalsa at various growth phases, the zooplankton species Artemia salina was exposed to C. subsalsa culture at two-day intervals for twenty days. Deaths fluctuated among the growth phases of C. subsalsa with the highest mortalities observed in the late exponential and stationary growth phases. The late exponential and stationary growth phases were found to have significantly greater percent mortalities than the early exponential, lag phase, and control groups (Kruskal-Wallis rank sum test, p=0.05).

Introduction

The raphidophyte algae genus *Chattonella* has been responsible for several notable fish kills across the globe. This phytoplankton can cause serious damage to economically important fish populations and marine ecosystems during and after a bloom event (Imai and Yamaguchi 2012). One of the five known species of *Chattonella, Chattonella subsalsa* is found in temperate and subtropical estuarine waters. In South Carolina, this species as well as several others, has been known to form harmful algal blooms (HABs) in brackish stormwater ponds as well as adjacent tidal creeks (Lewitus et al. 2003; Lewitus and Holland, 2003). These blooms have the potential to disrupt tidal ecosystems, causing large-scale mortality in economically important fish species and other marine organisms.

C. subsalsa has been found in estuarine environments with salinities ranging from 6-36 ppt and temperatures ranging from 17-33 °C (Imai and Yamaguchi 2012). The wide range of conditions allows *C. subsalsa* to thrive in multiple ecosystems globally. Anthropogenic activity such as ballast water transportation (Hallegraeff 1998) has

allowed this species to reach numerous areas and *C. subsalsa*'s adaptability has allowed it to flourish. Optimal growth occurs in waters 24-31°C and 11-28 ppt, ranges common in the coastal waters of South Carolina. Though *C. subsalsa* has been responsible for many fish kill HABs, the fish kill mechanism is currently unknown.

Some HABs, like those of the dinoflagellate *Karenia brevis*, are associated with toxin production that can be harmful to humans. The toxins of this dinoflagellate, known as brevetoxins, can cause illness through acute or chronic exposure via foodborne toxins or aerosolized pathways (Van Dolah 2000). Similar toxins are thought to be associated with raphidophyte species.

Two broad theories exist on how fish are impacted by *C. subsalsa* during blooms: 1.) copious production of mucous on fish gills causing physical blockage or clogging, 2.) production of a brevetoxin-like compound (Keppler et al. 2006; Bourdelais et al. 2002). In either case, the eventual cause of death is suffocation. It is unknown whether consumption related lysis of the individual cells is necessary for potential phycotoxins to cause mortality or what role such toxins may play in fish kill events. As multiple theories exist, the mechanism of *C. subsalsa* fish kills remains enigmatic.

Algal lethality and growth rate are thought to correspond among various HAB species with maximum harmful effects occurring in exponential or stationary phases (Marshall and Hallegraeff 1999). Algal growth generally follows a predictable pattern (Marshall and Hallegraeff 1999). Cell division begins in the lag phase, which is followed by a period of exponential growth. Cell numbers reach a peak in the stationary phase and finally decline. This cycle of growth can vary temporally with species and growth conditions (Turner 2014). Within the *C. subsalsa* strain examined, the four phases of growth were generally observed in a period of 20 days.

Zooplankton are a critical part of the aquatic food web. As primary consumers that graze upon phytoplankton, they provide an important link between primary producers and higher trophic levels. There are several different zooplankton species considered viable for toxicity testing, including larvae of mysids, copepods, decapods, and echinoids but none have been more widely used than *Artemia* (Sorgeloos et al. 1978; Persoone and Wells 1987; Caldwell et al. 2003; Nunes et al., 2006; Mohamed and Al-Shehri 2012; Libralato 2014). *Artemia* have an extreme tolerance for salinity and have been successfully cultured in water with salinities of 5 to 150 ppt (Persoone and Wells 1987) and temperatures of 6-40 °C (Libralato 2014; Browne and Wanigasekera 2000).

Artemia have been used in several toxicology tests involving other HAB species such as the raphidophyte *Heterosigma akashiwo* (Mohamed and Al-Shehri 2012) as well

as the toxic dinoflagellate *Alexandrium* spp. (Zhenxing et al. 2006). In each incidence, 24-hour post hatch (hph) naupliar stage *Artemia* were exposed to the respective HAB species. As a model species, *Artemia* can be utilized to study the effects of toxins. Toxin intake by *Artemia* can be limited by age and life stage. Artemia undergo several molts during their life cycle. The initial molt absorbs a yolk sac from which the newly hatched *Artemia* obtain nutrients. If the yolk sac is still attached to the *Artemia* nauplius when exposed to a harmful algae or toxicant and death occurs, this may indicate consumption is unnecessary for death. Focusing on the naupliar life stage also allows for easily replicated and controlled conditions for experimentation (Sorgeloos et al. 1978; Vanhaecke et al. 1981; Caldwell et al. 2003). *Artemia* are found in a wide geographic range, resist manipulation, have a short life cycle, and produce a large number of offspring making them ideal for laboratory ecotoxicity testing (Nunes et al. 2006).

This study was conducted to determine how the lethality of *C. subsalsa* changes throughout its growth in culture, using the zooplankton species, *Artemia salina* as a model organism. Investigating a potential correspondence between growth phase and lethality provides insight into the fish killing mechanism of *C. subsalsa* and the possibility of toxin production. Assuming a correlation exists, we hypothesized the highest percent mortalities of *A. salina* to occur in the late exponential and early stationary growth phases.

Methods

Husbandry of Organisms

A sample of C. subsalsa was obtained from the Hollings Marine Laboratory in Charleston, South Carolina. Cultures were maintained in 25 ppt f/2 nutrient replete medium (-Si) (Guillard and Ryther 1963) in 50 ml borosilicate glass test tubes in a Percival incubator set at 25°C on a 12:12 light dark cycle at 80-110 umol/m²/sec irradiance value. Every other day after initial culturing, the algae were counted using a Sedgewick-rafter counting chamber to create a growth curve. The growth curve indicated all life phases were accomplished after a 20-day period.

For the purposes of this study, synchronization was vital to keeping physiological conditions consistent between *Chattonella* and *Artemia* cultures. *Artemia* were reared in 25 ppt artificial seawater in a hatching cone with light aeration. The hatching cone was housed within the incubator and supplemented by a constant light source (5-18 umol/m²/sec). After 24 hours, ten individuals were taken from the hatchery and examined under a dissecting microscope to confirm life stage.

Experiment

A total of six 2-centimeter deep petri dishes, three containing *C. subsalsa* culture and three containing 25 ppt artificial seawater, were used for each experiment. Each dish held 5 ml of culture or artificial seawater. Artificial seawater was filtered through a 0.22 um glass fiber filter prior to experimental use and maintained at a consistent temperature of 25°C. Ten *Artemia* nauplii were added to each of the treatment dishes (N = 60). Experiments were conducted for two hours during which time the number of deaths and time of death of *A. salina* were recorded. Death was defined as a lack of movement in all appendages for 10 seconds (Vanhaecke et al. 1981). This process was repeated at two day increments for a period of 20 days completing an experiment. Three full experiments were conducted.

Statistical Analysis

The phases of algal growth could be defined and grouped for the purposes of this experiment into lag, early exponential, late exponential, stationary, and decline. Phases were defined as: lag (days 2 and 4), early exponential (days 6 and 8), late exponential (days 10 and 12), stationary (days 14 and 16), and decline (days 18 and 20). Mortality was analyzed for both day and growth phase for each experiment and total experiments. Tests for normality (Shapiro-Wilk test) and homogeneity (Fligner-Killeen test) were performed prior to further analysis. Percent mortalities per growth phase were analyzed using an ANOVA in normal data sets or Kruskal-Wallis rank sum test in non-normally distributed data. Algal growth rates were then compared to percent mortalities using a general linear model.

Results

Only one death was observed in control treatments throughout all experiments (N = 900). Zooplankton death fluctuated following the various life phases of *C. subsalsa*. The greatest number of deaths (n= 32) was observed during the late exponential phase of growth on day 12 (Figure 1). A significant polynomial trend between number of deaths and day of algal growth was found (R^2=0.6082; Figure 2). This trend fits the growth rates observed over approximately 1.5 years of continuous growth of this strain of *C. subsalsa* (Grogan, 2015). However, when examining the experimental growth rates and number of deaths, a significant linear trend was not found. The absence of a significant R^2 value when running a general linear model could be credited to the small sample size of growth rates calculated during this study. ANOVA and Kruskal-Wallis rank sum tests showed significant statistical difference ($p<0.05$) within the five life stages of *C. subsalsa* most notably between the lag and late exponential

phases (Table 1). The late exponential phase showed the highest percent mortality in two out of three experiments. In the third experiment, the highest percent mortality was observed in the stationary phase.

Figure 1: Total number of *A. salina* deaths of various *C. subsalsa* growth phases in all experiments.

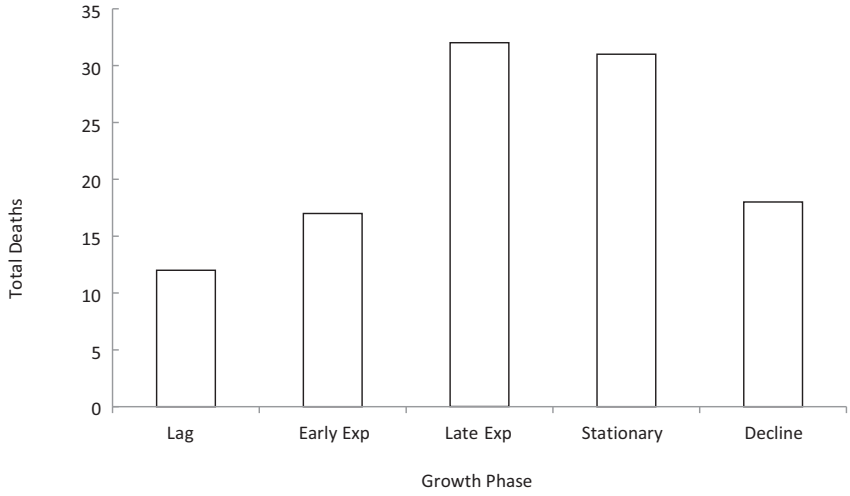

Figure 2: Total experimental A. salina deaths by day of C. subsalsa growth, polynomial trend line (R^2=0.6082).

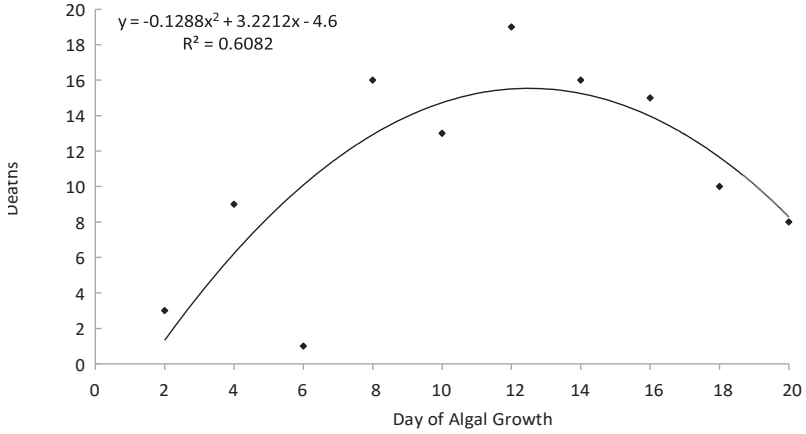

Table 1. Significant p-values* (<0.05), late exponential and stationary phases were found to have significantly higher percent mortalities than both lag and early exponential phases.

	Decline	**Early Exponential**	**Lag**	**Late Exponential**
Early Exponential	0.4473			
Lag	0.2383	0.2812		
Late Exponential	0.0639	**0.0489***	**0.0127***	
Stationary	0.0599	**0.0457***	**0.0117***	0.4868

Discussion

The maximum number of deaths occurred in the late exponential growth phase suggesting that *C. subsalsa* reaches its most potent lethality during this stage. It may also indicate that although different harmful algal species have different ranges of toxicity throughout their growth, *Chattonella* species have similar lethality fluctuations. *Chattonella* antiqua is thought to fluctuate lethality over the course of algal growth (Khan et al. 1996). The most toxic stage in *C. antiqua* was found to be in the early- to mid-exponential growth phase and decreased drastically once the cells reached stationary phase (Khan et al. 1996). *Chattonella* marina was also found to have lethality highly correlated with its growth rate in a study by Shen et al. (2010). The results of these studies as well as the current study further confirm the theory that *Chattonella* species have similar lethality fluctuation throughout their growth.

Shen et al. (2010) also showed that there was a high correspondence between growth rate and lethality but not with cell density (cells ml-1) and lethality in *C. marina*. This study supports this trend, as cell density was not found to significantly effect mortality. Our results support those observed in natural HAB-related fish kills, which commonly occur in the exponential or stationary phases (Pettersson and Pozdnyakov 2013).

In the third experimental trial, the highest percent mortality was observed in the stationary phase, contradicting the other two experiments. This development could perhaps be attributed to *C. subsalsa* having an extended lag phase and not reaching the exponential phase of growth until day 10 in culture. Often, algal growth can vary among the same algal strain in culture even if environmental standards are kept consistent (Turner 2014).

It is unknown whether or not direct consumption of *C. subsalsa* is required to produce mortality in zooplankton species. This study showed that mortality occurs in *Artemia* when exposed, but it may not indicate that consumption is necessary. As *A. salina* were exposed after 24-hour immersion of cysts, all nauplii should still have had attached yolk sacs. However yolk sacs can be absorbed after 12 hours and suspension feeding can begin (Sanders 2008). If the yolk sac was still attached to the nauplii at time of death, this would indicate that consumption is not necessary for *C. subsalsa* to cause mortality, as *Artemia* would not actively feed. Consumption was shown to be unnecessary for lethality in a recent study by Grogan (2015) when exposing *Fundulus heteroclitus* larvae to *C. subsalsa*. The effects of the algae on this much larger consumer coincide with the effects observed on the much smaller zooplankton of the present study. Assuming all nauplii used in this study maintained yolk sacs, our results suggest that *C. subsalsa* releases a waterborne toxin.

Although the effects of *C. subsalsa* on feeding and motion were not measured, observations were recorded as the motion of the *Artemia* ceased or became exasperated after harmful algae exposure. In nearly all experimental exposures, exasperated motion was observed. The time until exhausted motion varied depending on the concentration and growth rate of *C. subsalsa* but overall observations of exposed *Artemia* found substantially slower movement than in all control trials. Control groups appeared to be unaffected during the entire two-hour experiment in artificial seawater.

One issue in using a uni-algal species in exposure is that nearly all HABs in the natural environment occur with multiple species, harmful and benign, present and potentially blooming (Turner and Tester 1997). Various phytoplankton may be present in a single bloom event including diatoms, dinoflagellates, and cyanobacteria. Multiple algal species allow for zooplankton to potentially avoid harmful species and become selective grazers. The replication of a natural bloom would be nearly impossible to perform in a controlled setting, so single species are most commonly targeted (Khan et al. 1996; Shen et al. 2010; Imai and Yamaguchi 2012). The use of a single species in toxicity testing provided the zooplankton with one potential food source. If *A. salina* chose not to feed upon *C. subsalsa*, starvation would likely be the cause of death over a long-term study. In the case of this study, the time without food was not long enough to cause death by starvation, as there was only one observed control group fatality. Using 24 hph nauplii also suggests active feeding did not occur.

Conclusion

Our study has shown that *C. subsalsa* is most lethal to *A. salina* during the late exponential to stationary growth phases. Only one death was recorded in all of the

control groups, and exasperated or slowed movement in the experimental *Artemia* was prevalent. The aforementioned observations would indicate toxin production by *C. subsalsa* is likely and toxins are most lethal during exponential growth.

Our results suggest a lethal waterborne bioactive compound is produced by *C. subsalsa* and that this compound is most harmful in the late exponential growth phase. As this growth phase is commonly associated with blooms these results have serious implications for the environmental impact of *C. subsalsa.* Further studies on the fish kill mechanisms of *C. subsalsa*, potential toxin production, and trophic transfer of toxins are needed to further understand the effects of this species and similar raphidophyte algae.

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