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ECOLOGICAL AND EVOLUTIONARY INTERACTIONS BETWEEN THE COPEPOD *ACARTIA TONSA* AND THE DINOFLAGELLATE *COCHLODINIUM POLYKRIKOIDES*

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Abstract of the Dissertation

Ecological and evolutionary interactions between the copepod *Acartia tonsa* and the dinoflagellate *Cochlodinium polykrikoides*

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The dinoflagellate *Cochlodinium polykrikoides* Margalef has formed dense blooms and caused severe fish kills on a global scale in recent decades. The effects of *C. polykrikoides* on survival, feeding, and fecundity of the copepod *Acartia tonsa* Dana were examined to determine if it is harmful to grazers. *C. polykrikoides* significantly reduced copepod survival, feeding, and fecundity within the range of bloom densities. Two bioassay experiments suggested that copepod mortality was due to multiple harmful compounds produced by *C. polykrikoides*.

Although *Cochlodinium polykrikoides* is harmful to *Acartia tonsa* at high cell densities, the mixed-diet experiments indicated that *C. polykrikoides* was nutritious to *A. tonsa* at low densities. The density-dependent nutritional value of *C. polykrikoides* to *A. tonsa* was also demonstrated by the survival experiments. These results suggest a putatively 'harmful' alga is not always deleterious to grazers and its ecological effects may be distinctly different during bloom and non-bloom periods.

The beneficial effects of *Cochlodinium polykrikoides* at low cell densities put forth a new question as to how *C. polykrikoides* cells avoid being completely decimated by grazers before they gain a window of opportunity for bloom formation. Field populations of *C. polykrikoides* displayed a significantly larger variation in chain length compared to laboratory cultures without grazers. Chain length of *C. polykrikoides* was significantly increased when exposed to *Acartia tonsa* adults for 48 h or to fresh (<24 h post-isolation) exudates of *A. tonsa*. Chain length of *C. polykrikoides* was correlated with *A. tonsa* abundance in the field. These results suggest that dissolved chemical cues released by *A. tonsa* induce chain formation in *C. polykrikoides*. Ingestion rates of *A. tonsa* on the 4-cell

chains of *C. polykrikoides* were lower than on single cells, suggesting chain formation may be an effective anti-grazing defense.

To quantitatively assess the population dynamics of zooplankton during harmful algal blooms, high-frequency (sampling interval 2 d) time series of *Acartia tonsa* abundance, egg production rate, and egg hatching success were documented during a *Cochlodinium polykrikoides* bloom. Embryonic mortality (combined egg-through-the-second-naupliar-stage, egg-N2) was higher than birth rate on most sampling days, resulting in the consistent decline of *A. tonsa* abundance. Embryonic mortality was correlated with cell density of *C. polykrikoides* with a 4-d delay. Birth rate was negatively dependent on adult abundance of *A. tonsa*, but not on cell density of *C. polykrikoides*. Therefore, the population dynamics of *A. tonsa* was regulated by algal-density-dependent mortality and copepod-density-dependent birth rate.

The potential for evolutionary responses of the copepod *Acartia tonsa* to the harmful dinoflagellate *Cochlodinium polykrikoides* was investigated using a common garden experiment and an artificial selection experiment. Copepod resistance to *C. polykrikoides* was evaluated by egg production rates when feeding on *C. polykrikoides* relative to the non-toxic flagellate *Rhodomonas lens*. After six years from the first occurrence of *C. polykrikoides* in eastern bays of Long Island, *A. tonsa* populations from bloom areas were significantly more resistant to *C. polykrikoides* than conspecifics from nearby non-bloom areas. In the laboratory, *A. tonsa* taken from a non-bloom area gradually increased the resistance when exposed to *C. polykrikoides*. Copepod resistance in the selection line became three times greater than the control line after four generations. Following a two-generation relaxation of selection, the elevated resistance in *A. tonsa* was completely lost. The annual occurrence of *C. polykrikoides* blooms has resulted in the divergence of *A. tonsa* populations in eastern bays of Long Island from their conspecifics in nearby non-bloom waters. The rates of gain and loss of resistance in *A. tonsa* to *C. polykrikoides* were comparable, suggesting these evolutionary changes could occupy the same time scale. The rapid gain and loss of resistance to harmful algae highlight the need to consider the evolutionary responses of grazers in understanding and management of harmful algal blooms.

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Chapter 1 Dissertation introduction

Ecological and evolutionary processes are traditionally thought to occur on different time-scales. Recent studies, however, have shown that many organisms develop adaptations to selective forces over just a few generations (Thompson 1998, Carroll et al. 2007). Such rapid or contemporary evolution often occurs between prey and predators. While prey develop numerous defenses to avoid predation, predators evolve means to breach these defenses resulting in an evolutionary "arms race" of adaptation and counteradaptation (Vermeij 1994, Brodie and Brodie 1999, Agrawal 2001). Selection on prey is often stronger than on predators due to the "life-dinner principle" which argues that it is worse to lose life than to miss a dinner (Brodie and Brodie 1999). Reciprocal adaptations between species regulate population dynamics, shape community structure, affect biogeochemical cycles, and drive genetic diversification (Agrawal 2001, Pohnert et al. 2007, Hay 2009).

In aquatic environments, phytoplankton have developed morphological and chemical defenses against predation. Many phytoplankton have protective external structures, such as siliceous or calciferous shells, spines, and horns (Litchman and Klausmeier 2008). Thickened cell walls, increased extracellular mucilage, or fast gut passage of some phytoplankton can lead to incomplete digestion and subsequent imbalances in lipids or unknown compounds important for the reproductive success of herbivores (Dutz et al. 2008). A wide variety of harmful compounds are produced by more than 200 algal species from 20 genera to deter grazing or directly kill grazers (Landsberg 2002).

Harmful algae may therefore exert selective pressure on their grazers. Grazer populations that have experienced recurrent HABs can evolve local adaptations to toxic algae (Colin and Dam 2004). By hatching long-dormant eggs of *Daphnia galeata* found in lake sediments, Hairston et al. (1999) showed *D. galeata* evolved to become more resistant to dietary cyanobacteria associated with the occurrence of eutrophication. An artificial selection experiment even showed that copepods can evolve resistance to toxic *Alexandrium* only over 2-5 generations (Colin and Dam 2004). These results have important implications for management and control of spreading HABs. The rapid evolution of resistance may be an important feedback mechanism potentially leading to bloom control. On the other hand, this adaptation may lead to more toxins being accumulated in zooplankton and transported to higher trophic levels.

The unarmored, chain-forming dinoflagellate *Cochlodinium polykrikoides* has formed dense blooms and caused severe economic damage in Southeast Asia during the past two decades (Lee 2008). Recently, the emergence of *C. polykrikoides* blooms has been

documented in many coastal waters ranging throughout temperate, sub-tropical and tropical latitudes (Curtiss et al. 2008, Gobler et al. 2008, Tomas and Smayda 2008, Fig. 1.1). *C. polykrikoides* blooms have occurred annually in late summer and last for 1 to 2 months in the eastern bays of Long Island since 2004 (Gobler et al. 2008, Fig. 1.2). Typical cell densities of *C. polykrikoides* range from 10^3 to 10^4 cells mL^{-1} (Fig. 1.2), sometimes even exceeding 10^5 cells mL^{-1}, during blooms (Gobler et al. 2008). However, the interactions between *Cochlodinium* and zooplankton have been poorly studied.

Fig. 1.1. *Cochlodinium* blooms reported in peer-reviewed journals.

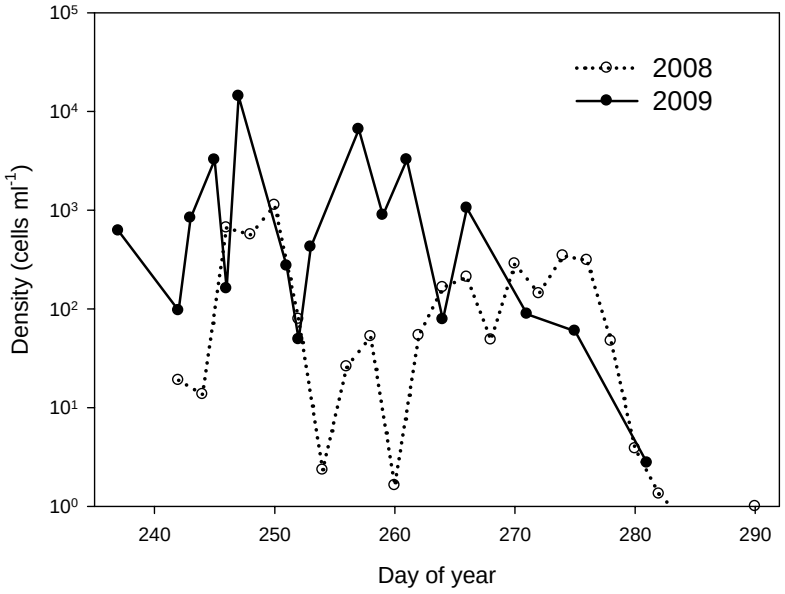

Fig. 1.2. Cell density of *Cochlodinium polykrikoides* in Old Fort Pond, Shinnecock Bay, NY.

The copepod *Acartia tonsa* Dana is an abundant species in many neritic and estuarine environments, where *Cochlodinium polykrikoides* blooms occur, and is capable of consuming *C. polykrikoides*. The effects of *C. polykrikoides* on copepod survival, feeding, and fecundity were examined to determine if it is harmful to grazers (Chapter 2). *C. polykrikoides* significantly reduced copepod survival, feeding, and fecundity within the range of bloom densities of this alga. Two bioassay experiments suggested that copepod mortality was due to multiple harmful compounds produced by *C. polykrikoides*.

Although *Cochlodinium polykrikoides* was harmful to *Acartia tonsa* at high cell densities, I used the mixed-diet approach to determine whether *C. polykrikoides* was always harmful to *A. tonsa* over a range of ecologically-relevant cell densities (Chapter 3). Based on egg production rate, egg hatching success, and naupliar recruitment rate of *A. tonsa*, mixed-diet experiments showed that the nutritional value of *C. polykrikoides* changed from beneficial to deleterious with increasing cell density. The survival experiments also supported this conclusion.

The density-dependent nutritional value of *Cochlodinium polykrikoides* inspired a new question regarding how cells at low densities avoid being completely decimated by grazers before they gain a window of opportunity for bloom formation. I tested the hypothesis that the presence of zooplankton grazers would induce chain formation in *C. polykrikoides*, which may be an effective anti-grazing defense (Chapter 4). I compared chain structures in a field population (presence of grazers) and a cultured population (absence of grazers), investigated chain length following grazer addition, and explored the relationship between chain length and grazer abundance. Given the variation of chain length in cultured cells through its natural growth cycle even without grazers, I further tested the hypothesis that some nutrients would influence chain formation in *C. polykrikoides* using culture-based nutrition amendment experiments.

I also quantitatively assessed the population dynamics of *Acartia tonsa* during a *Cochlodinium polykrikoides* bloom (Chapter 5). A high-frequency (sampling interval 2 d) time series for abundance, egg production rate, egg hatching success, and gut fluorescence of the copepod *A. tonsa* was documented during a bloom of the dinoflagellate *C. polykrikoides* in Old Fort Pond, Shinnecock Bay, NY, from August 29 to October 18, 2008. The results showed that *C. polykrikoides* was the main causative agent for embryonic mortality of *A. tonsa* and copepod-density-dependent birth rate was the secondary force for the population regulation.

From the perspective of an evolutionary arms race, copepods are hypothesized to evolve means to breach the chemical and morphological defenses of *Cochlodinium polykrikoides*. I investigated the potential for evolutionary response in copepods to harmful *C. polykrikoides* (Chapter 6). Evidence for the evolution of resistance was tested in two ways. Resistance of *A. tonsa* populations from bloom and non-bloom areas was compared using a common garden experiment. In addition, copepods from a non-bloom area were subjected to the artificial selection of resistance to *C. polykrikoides* for four generations. Both avenues of research demonstrated rapid adaptation of copepods to this

alga. To test whether evolved resistance is reversible, the exposure to *C. polykrikoides* was halted and the resultant evolutionary trajectory was tracked for another four generations. The results provide the empirical evidence of the intrinsic capacity for reversal of grazer resistance to harmful algae. Rapid adaptation to harmful algae and loss of resistance in copepods highlight the need to consider the evolutionary consequences of grazers in understanding and management of harmful algal blooms.

Chapter 2 Deleterious consequences of *Cochlodinium polykrikoides* for *Acartia tonsa**

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Introduction

Harmful algal blooms (HABs) have increased in frequency, duration, and distribution in recent decades. Fish kills and accumulation of phycotoxins in shellfish with subsequent poisoning of humans have been well documented. However, studies of the interactions between harmful algae and their zooplankton grazers have been less common, and results are often controversial (Turner and Tester 1997, Turner 2006). These complex and inconsistent interactions are partly due to the wide variety of phycotoxins associated with more than 200 algal species from 20 genera (Landsberg 2002), substantial changes in toxicity levels of a single algal clone with culture age and nutrients (Granéli and Flynn 2006), and variations of grazers in terms of feeding patterns, binding sites of toxins, and structures of nervous systems (Turner and Tester 1997). Furthermore, phenotypic plasticity and rapid evolution of resistance to harmful algae can significantly shape the interactions between algae and herbivores (Hairston et al. 1999, Colin and Dam 2004).

Despite this complexity, zooplankton grazers are often considered as adversely affected by harmful algae. Effects include impaired feeding, avoidance behavior, physiological dysfunction, depressed growth and reproduction, and reduced population fitness (Turner and Tester 1997, Landsberg 2002, Prince et al. 2006, Barreiro et al. 2007, Cohen et al. 2007, Flynn and Irigoien 2009). Reduced feeding rates of zooplankton may be due to behavioral rejection of harmful algae prior to ingestion or physiological incapacitation (Ives 1987). Inability to continue feeding may result in low growth and reproduction, eventually causing a decline in population abundance. Zooplankton grazing may impact the development and termination of HABs. However, many studies suggest that the top-down controls are limited due to poisoning of grazers by phycotoxins and/or their relatively low growth rate (Turner and Tester 1997). Beyond directly feeding on harmful algae, zooplankton grazing may transport toxins along the food web and they may serve as vectors for higher trophic levels (Jester et al. 2009).

The unarmored, chain-forming, gyrodinioid dinoflagellate *Cochlodinium polykrikoides* Margalef has formed dense blooms and caused severe economic damage in Southeast Asia during the past two decades (Lee 2008). Recently, *C. polykrikoides* blooms have been documented in many coastal waters ranging throughout temperate, sub-tropical, and tropical latitudes in both Asia and North America (Anton et al. 2008, Gobler et al. 2008, Curtiss et al. 2008, Park et al. 2009). Cell densities during blooms usually range from 10^3 cells mL^{-1} to 10^4 cells mL^{-1} (Anton et al. 2008, Gobler et al. 2008, Curtiss et al. 2008, Park et al. 2009). Bloom patches can achieve cell densities exceeding 10^5 cells mL^{-1} (Gobbler et al., 2008). Some studies have shown that *C. polykrikoides* isolated in East Asia can be mixotrophic, feeding on small phytoplankton species (<11μm) by engulfing the prey through the sulcus (Jeong et al. 2004). Strong diel vertical migration has been observed in field populations of *C. polykrikoides* (Park et al. 2001). *C. polykrikoides* has been reported to be resistant to attacks by six algicidal bacteria (Imai and Kimura 2008), and, in turn, *C. polykrikoides* inhibited growth of the dinoflagellate *Akashiwo sanguinea* and caused morphologically abnormal cells (Yamasaki et al. 2007). All of these attributes likely provide *C. polykrikoides* with competitive advantages over other occurring microalgae and, at least partly, explain the mechanisms of *Cochlodinium* bloom formation.

Although the emergence of *Cochlodinium* blooms and subsequent severe fish kills have been well recorded, the precise toxic mechanisms of this alga are still poorly understood. *Cochldinium* blooms occurring along the coast of Japan were reported to release water-soluble ichthyotoxic substances with characteristics of paralytic shellfish toxins (Onoue et al. 1985) and three toxin fractions: neurotoxic, hemolytic and hemagglutinative (Onoue and Nozawa 1989). In Korean isolates, *C. polykrikoides* has been reported to generate the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Kim et al. 1999), which resulted in the inactivation of transport-related enzyme activities in fish gills, a drop in blood pO_2, and abnormal secretion of gill mucus (Kim et al. 2000). Interestingly, the production of reactive oxygen species (ROS) in two *C. polykrikoides* strains isolated in Japanese waters was very low compared to *Chattonella marina*, a species well-known for ROS production. Fish kills by these two strains were related to biologically active metabolites, such as cytotoxic agents and mucus substances (Kim et al. 2002). Further, the harmful effects of *C. polykrikoides* from the US east coast waters to fish were caused by a labile, extracellular toxic principle produced by actively growing cells (Tang and Gobler 2009).

The interactions between *C. polykrikoides* and zooplankton have been poorly studied. *C. polykrikoides* retarded metamorphosis of the Pacific oyster (*Crassostrea gigas*) from the trochophore stage to the D-shaped larval stage (Matsuyama et al. 2001). The egg production rates and egg viability of the copepod *Acartia omorii* were low when fed *C. polykrikoides* (Shin et al. 2003). On the contrary, the planktonic ciliate *Strombidinopsis* sp. ingested *C. polykrikoides* and grew well (Jeong et al. 2008). In this study, the deleterious effects of *C. polykrikoides* on survival, feeding, and fecundity of the copepod

Acartia tonsa Dana were investigated to assess potential impacts of harmful blooms on lower trophic grazers.

Material and methods

Collection and culture of organisms

The dinoflagellate *Cochlodinium polykrikoides* clone CP1 was isolated from Peconic Bay, Long Island, New York, USA in 2006 (Gobler et al. 2008). The flagellate *Rhodomonas lens* Pascher and Ruttner (CCMP 739) was obtained from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The cultures were maintained in a temperature-controlled incubator at 20℃ with a 14:10 light-dark cycle (approximately 50 μmol photons m^{-2} s^{-1}). The cultures were maintained in exponential growth phase by biweekly dilution with f/2 medium. The length and width of more than 100 cells were measured under a compound microscope. The carbon contents of the two microalgae (Table 2.1) were estimated from their cell volumes (Stoecker et al. 1994).

The copepod *Acartia tonsa* was collected from Stony Brook Harbor, Long Island Sound, New York, USA, with a 202-μm mesh plankton net. The population was continuously cultured in 20-L tanks at a density of 20 to 50 ind. L^{-1}. The copepods were offered *R. lens* at a carbon concentration of approximately 500 μg C L^{-1} and maintained at 20℃ with a 12:12 light-dark cycle (approximately 1 μmol photons m^{-2} s^{-1}). The low irradiance level minimized the potential effects of light on copepods and algal growth during experiments. Half of the copepod culture medium was refreshed twice a week with 0.2-μm filtered sea water (FSW).

Table 2.1. Characteristics of two algae used in the experiments

Alga	Length (μm)	Width (μm)	Equivalent spherical diameter (μm)	Carbon content (pg $cell^{-1}$)
Cochlodinium polykrikoides	34±4.7	27±4.1	28.2	1816
Rhodomonas lens	11±1.2	7.0±1.0	7.97	39.5

Survival experiments

A life table experiment was performed to compare survivorship of *A. tonsa* when fed *C. polykrikoides* at five concentrations ranging from 900 to 4700 μg C L^{-1} (500 to 2600 cells mL^{-1}). My experimental concentrations were within the range of *C. polykrikoides* densities observed in the field (Gobler et al. 2008). Copepod survivorship in FSW and two *R. lens* solutions (900 and 2200 μg C L^{-1}) were used as the controls. Approximately 400 female *A. tonsa* were transferred into a 5-L plastic container and acclimated in 0.2-μm filtered seawater for 24 h. For each treatment, 20 – 48 healthy females were

transferred individually into 6-well tissue culture plates. Each well was filled with 13 mL of the food medium and one *A. tonsa*. The copepods were checked every 12 h until they all died. Approximately 80% of the food medium was refreshed daily. All experiments in this study were conducted in a temperature-controlled incubator at 20^{o}C with a 12:12 light-dark cycle (approximately 1 μmol photons m^{-2} s^{-1}).

An acute toxicity experiment was conducted to elucidate stage susceptibility of copepods to *C. polykrikoides*. The organisms from the first naupliar stage (N1), the fourth naupliar stage (N4), the first copepodite stage (C1), the fourth copepodite stage (C4), adult females, and eggs were exposed to a series of *C. polykrikoides* solutions ranging from 0 to 4700 μg C L^{-1} (0 to 2600 cells mL^{-1}). Each treatment had four replicates. The organisms (n = 8 – 12) were individually held in tissue culture plates filled with *C. polykrikoides* solutions. After 24 h, the copepods were observed under a dissecting microscope.

The mode of harmful effects of *Cochlodinium polykrikoides* on copepods was explored using two 48-h bioassay experiments. Healthy female *A. tonsa* were exposed to either *C. polykrikoides* live culture, frozen and thawed culture, culture filtrate (0.2 μm), or 0.2-μm filtered seawater (the control). The culture density was 2200 μg C L^{-1} (1200 cells mL^{-1}). The procedures were the same as described above. Another experiment was designed to investigate whether the toxic reaction of copepods was dependent on physical contact with *C. polykrikoides* cells. The experiment was performed using cages made from polyethylene centrifuge tubes (50 mL) with sealed nylon-mesh bottoms. The mesh sizes were 100-μm and 5-μm for treatment 1 and treatment 2, respectively. Cages with 100-μm mesh would permit the passage of *C. polykrikoides* cells while the 5-μm mesh did not, which was verified by microscopy. Each treatment had four replicates. Healthy females (n = 8 – 12) were transferred into each cage. The cages in treatment 1 and treatment 2 were immersed in a 4-L culture of *C. polykrikoides* at a concentration of 2200 μg C L^{-1} (1200 cells mL^{-1}). The cages with 5-μm mesh immersed in 4-L of 0.2-μm filtered seawater were used as the control. Copepod mortality was compared after 48 h.

Feeding experiments

Active adult copepods with intact appendages were transferred into 2-L beakers with 0.2-μm filtered seawater for 24 h prior to the feeding experiments. Six food concentrations of *C. polykrikoides* and *R. lens* ranging from 150 to 1500 μg C L^{-1} were used to determine copepod ingestion rates. I used 3 or 4 replicates of 250-mL bottles for each experimental diet and concentration. The bottles without copepods were used as the controls. Ten active adult females were transferred into each bottle. The bottles were placed on a plankton wheel and rotated at 1 rpm for 24 h at 20^{o}C with a 12:12 light-dark cycle (approximately 1μmol photons m^{-2} s^{-1}). At the beginning and end of the experiment, samples for cell densities were taken. Algal densities were approximated by measuring *in vivo* chlorophyll fluorescence with a Turner AU-10 fluorometer. Actual cell densities were quantified on Lugol's iodine preserved samples. *In vivo* fluorescence of *C. polykrikoides* and *R. lens* was significantly linearly related to algal concentration (the

regression coefficients: r = 0.997 and 0.999, respectively, for both, $P < 0.001$, unpublished data). The significant relationships between fluorescence and algal concentration provided a rapid and simple measurement to monitor algal concentration during this experiment. The ingestion rates (I) were calculated according to the equation described by Båmstedt et al. (2000):

$$I = \frac{V \times ln\frac{C_t^{'}}{C_t}}{t \times n} \times \frac{C_{0+}C_t}{2}$$

where C_0 and C_t are the food concentrations at the beginning and end of the experiment; $C_t^{'}$ is the final food concentration in the controls; V is the volume of the bottles; t is the duration of the experiment; n is the number of copepods.

Egg production and egg hatching experiments

Egg production rates and hatching success were measured at algal concentrations of 18, 90, 180, 360, 540, and 1080 μg C L^{-1}. Approximately 300 healthy adult *A. tonsa* were transferred to each of 6 containers filled with 5-L of the appropriate diet suspension and acclimated for 24 h. Approximately 80% of the diet medium was refreshed daily. Two healthy female *A. tonsa* were then transferred from the container into a dish filled with 50-ml food solution. A 200-μm mesh was fixed above the bottom to minimize egg cannibalism. All eggs and nauplii were enumerated after a 24-h incubation. There were seven replicates for each treatment. Eggs were placed individually in 1-mL wells of a multi-depression dish contained within a closed plastic box. Distilled water was added to the bottom of the box to reduce evaporation from the wells. Fresh FSW was added to the wells. Eggs were observed once a day for 2-3 days. The measurements in the *C. polykrikoides* treatments ran for 10 days or until all copepods in the containers were dead. The measurements in *R. lens* treatments only ran for 1 day.

Copepod egg sizes were measured when exposed to *C. polykrikoides* and *R. lens* at concentrations of 90, 180, 360, 540, and 720 μg C L^{-1} during the 10-day period. Approximately 600 healthy adult *A. tonsa* were transferred to each of 5 containers filled with 10-L of the appropriate diet suspension. Copepod eggs were collected by 60-μm mesh and 80% of the food solutions were refreshed every day. At least 15 eggs from a sample were measured under a compound microscope using the 100× magnification to determine the mean egg diameter.

Statistical analyses

Survivorship curves were compared using the Gehan-Wilcoxon test (Pyke and Thompson 1986). Lethal median concentration (LC_{50}) was determined by applying a probit analysis. One-way ANOVAs followed by Tukey multiple comparison tests were used to compare means of different treatments in bioassay experiments. A two-level nested ANOVA was used to test the effects of algal species and concentration on ingestion rates. A two-way ANOVA was used to analyze the effects of algal species and

concentration on egg production rates and hatching success. A three-way ANOVA was used to analyze the effects of algal species, concentration, and exposure time on egg sizes (Sokal and Rohlf 1995). All statistical analyses were conducted using SPSS 16.0 statistical package.

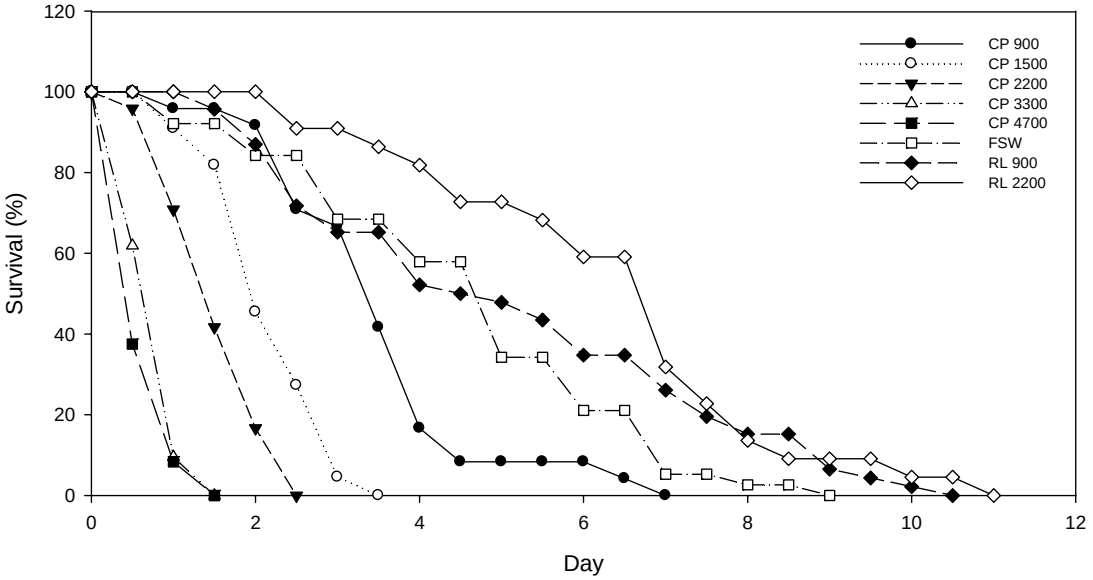

Fig. 2.1. Survivorship of *Acartia tonsa* when exposed to five *Cochlodinium polykrikoides* solutions (CP 900 µg C L^{-1}, CP 1500 µg C L^{-1}, CP 2200 µg C L^{-1}, CP 3300 µg C L^{-1}, and CP 4700 µg C L^{-1}), two *Rhodomonas lens* solutions (RL 900 µg C $L^{-1}$and RL 2200 µg C L^{-1}), and 0.2 µm-filtered seawater (FSW).

Results

Survival experiments

Life table experiments revealed that survivorship of female *A. tonsa* was significantly reduced when fed *C. polykrikoides* compared to those starved or fed non-toxic *R. lens* (Fig. 2.1 and Table 2.2). Survivorship of female *A. tonsa* significantly decreased with increasing *C. polykrikoides* concentrations (Fig. 2.1 and Table 2.2). Female *A. tonsa* experienced rapid mortality at high (3300 and 4700 µg C L^{-1}, or ~ 1800 and 2600 cells mL^{-1}) and intermediate (1500 and 2200 µg C L^{-1}, or ~ 800 and 1200 cells mL^{-1}) concentrations of *C. polykrikoides* with 100% of individuals expiring within 1.5 and 3.5 days, respectively (Fig. 2.1). Survivorship of female *A. tonsa* fed *C. polykrikoides* was

moderately improved at the low concentration of 900 μg C L^{-1} (~ 500 cells mL^{-1}) with individuals surviving 7 days (Fig. 2.1). All of these survival times were significantly shorter than those in FSW and in the *R. lens* control treatments (Fig. 2.1 and Table 2.2).

Mortality of *A. tonsa* from early nauplii to adult females significantly increased with increasing *C. polykrikoides* concentrations after a 24-h exposure ($P < 0.001$ for all, one-way ANOVA, Fig. 2.2). In contrast, egg hatching was not affected by *C. polykrikoides* ($P > 0.05$, one-way ANOVA, Fig. 2.2). LC_{50} values indicated that the susceptibility of *A. tonsa* to *C. polykrikoides* decreased with development, especially from early copepodite to adult stage (Fig. 2.3). Early nauplii of *A. tonsa* were approximately four times more sensitive to *C. polykrikoides* than adult females after 24-h exposure, with LC_{50}s of 607 μg C L^{-1} (334 cells mL^{-1}, 95% confidence interval: 399 – 877 μg C L^{-1}, 220 – 483 cells mL^{-1}) and 2511 μg C L^{-1} (1383 cells mL^{-1}, 95% confidence interval: 1769 –3602 μg C L^{-1}, 974 – 1983 cells mL^{-1}), respectively.

Table 2.2. Gehan-Wilcoxon test results of survivorship curves in the life table experiment. Arrow indicates whether the survivorship curve indicated by the column header is greater (up arrow) or less (down arrow) than that indicated in the row header. Significant differences are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and ns (not significant). CP = *Cochlodinium polykrikoides*; RL = *Rhodomonas lens*; FSW = 0.2-μm filtered seawater; The numbers indicate algal carbon concentrations (μg C L^{-1}).

	CP 900	CP 1500	CP 2200	CP 3300	CP 4700	FSW	RL 900	RL 2200
CP 900	----							
CP 1500	***↑	----						
CP 2200	***↑	**↑	----					
CP 3300	***↑	***↑	***↑	----				
CP 4700	***↑	***↑	***↑	ns	----			
FSW	*↓	***↓	***↓	***↓	***↓	----		
RL 900	*↓	***↓	***↓	***↓	***↓	ns	----	
RL 2200	***↓	***↓	***↓	***↓	***↓	**↓	ns	----

Mortality of *A. tonsa* exposed to the frozen and thawed *C. polykrikoides* culture was significantly reduced to half of that exposed to the live culture (Fig. 2.4), but was significantly higher than that in FSW (Table 2.3). Copepods in the 0.2-μm culture filtrate had significantly increased survivorship compared to those in the live culture and their mortality did not significantly differ from that in FSW (Fig. 2.4 and Table 2.3). Copepod mortality in the cages with 5-μm nylon mesh and immersed in *C. polykrikoides* live

culture was significantly lower than that in the cages with 100-μm nylon mesh; however, it was significantly higher than that in FSW (Fig. 2.4 and Table 2.3).

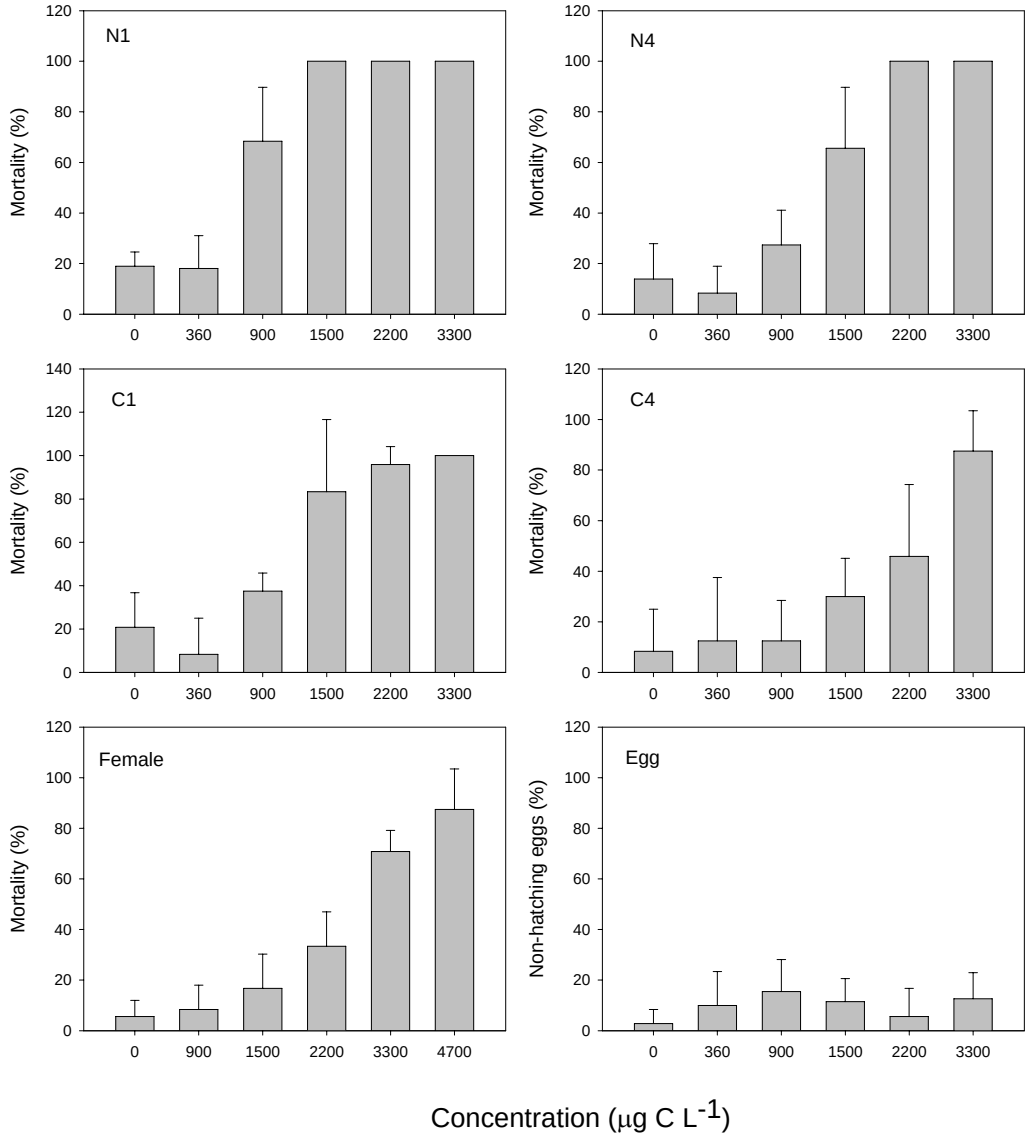

Fig. 2.2. Stage-specific mortality (mean ± SD) of *Acartia tonsa* when exposed to *Cochlodinium polykrikoides* for 24 h. N1: the first naupliar stage, N4: the fourth naupliar stage, C1: the first copepodite stage, C4: the fourth copepodite stage.

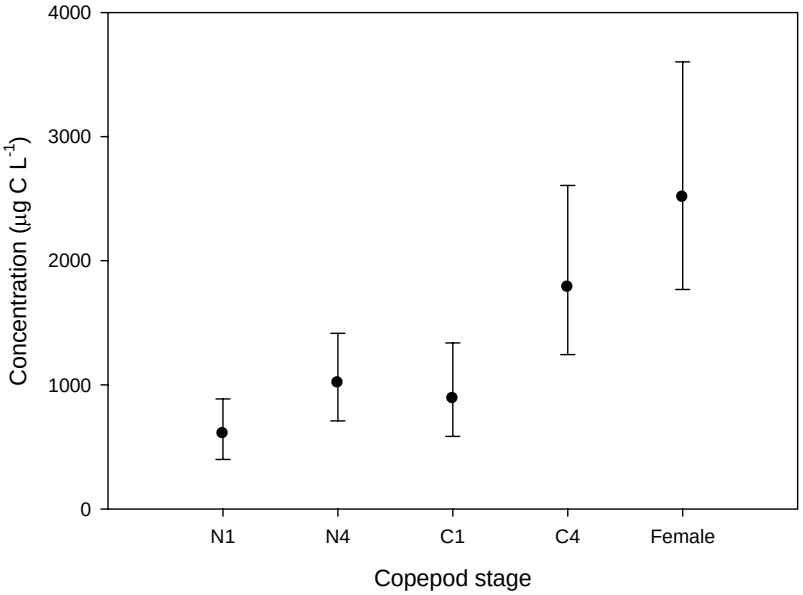

Fig. 2.3. 24-h LC_{50} and 95% confidence intervals for five development stages of *Acartia tonsa* when exposed to *Cochlodinium polykrikoides*. N1: the first naupliar stage, N4: the fourth naupliar stage, C1: the first copepodite stage, C4: the fourth copepodite stage.

Table 2.3. Results of Tukey multiple comparison tests for mortality in two bioassay experiments. Healthy females were (a) exposed to live *Cochlodinium polykrikoides* (LCP) culture, frozen and thawed CP culture (FTCP), 0.2-µm filtered CP culture (FCP), and 0.2-µm filtered seawater (FSW); or (b) placed in cages covered with 5 or 100 µm nylon mesh and immersed in live CP culture or 0.2-µm FSW. Significant differences are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and ns (not significant).

(a)				(b)		
	LCP	FTCP	FCP		100 in CP	5 in CP
FTCP	***			5 in CP	***	
FCP	***	***		5 in FSW	***	*
FSW	***	***	ns			

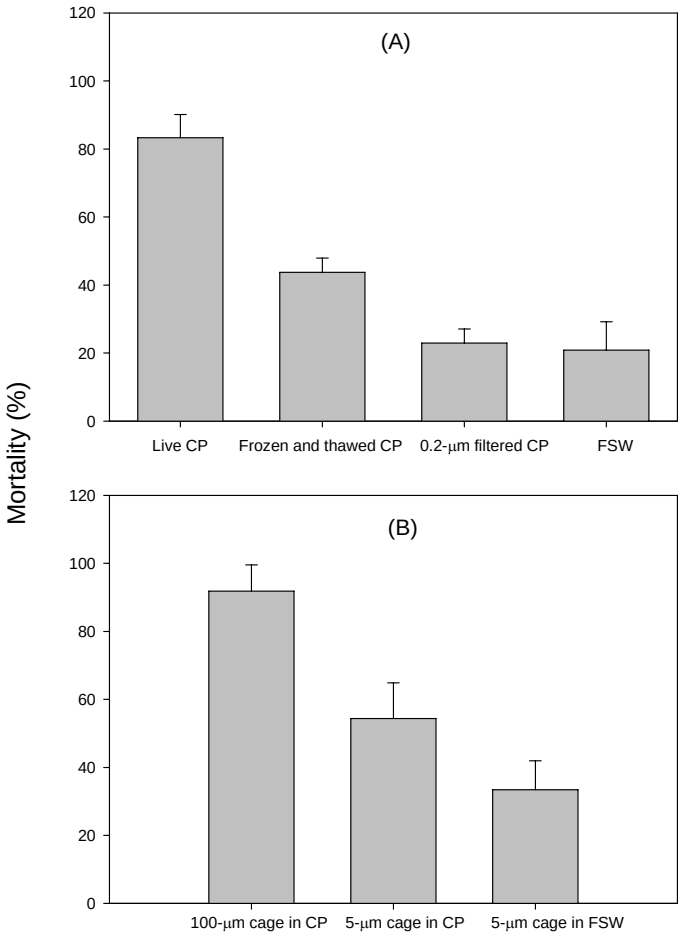

Fig. 2.4. Bioassay experiments of *Acartia tonsa* conducted for 48 h. (A) Percent mortality (mean ± SD) when exposed to either *Cochlodinium polykrikoides* live culture, frozen and thawed culture, culture filtrate (0.2-μm), or 0.2-μm filtered seawater. (B) Percent mortality (mean ± SD) in the cages that were covered by 100- μm or 5-μm nylon mesh and immersed in *C. polykrikoides* live culture or 0.2-μm filtered seawater.

Feeding experiments

The ingestion rates of *Acartia tonsa* were significantly affected by algal species ($F_{1, 29}$ = 10.2347, $P < 0.01$, two-level nested ANOVA) and algal concentration ($F_{10, 29}$ = 2.9841, $P < 0.05$, two-level nested ANOVA). The ingestion rates of *A. tonsa* fed *Cochlodinium polykrikoides* were 25 – 60% lower than ingestion rates on *R. lens* (Fig. 2.5). The

ingestion rates on *C. polykrikoides* and *R. lens* by *A. tonsa* significantly increased with their increasing concentration ($F_{10, 29}$ = 2.9841, P < 0.05, two-level nested ANOVA, Fig. 2.5). Their maximum daily ingestion rates were 3.15 and 6.18 µg C ind.$^{-1}$ d^{-1}, respectively (Fig. 2.5).

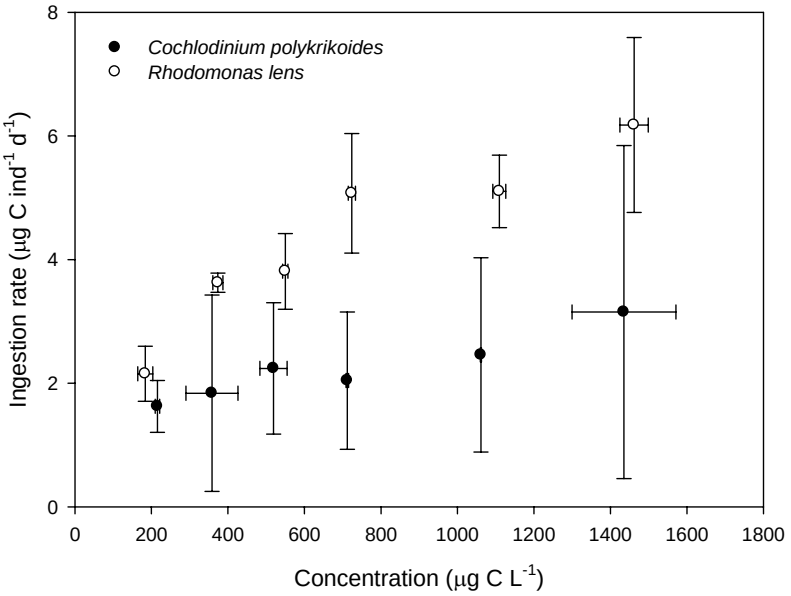

Fig. 2.5. Ingestion rates (mean ± SD) of *Acartia tonsa* when fed either *Cochlodinium polykrikoides* or *Rhodomonas lens*.

Egg production and egg hatching experiments

The two-way ANOVA showed that egg production rates of *Acartia tonsa* after a 1-day exposure were significantly affected by algal species ($F_{1, 60}$ = 13.9295, P < 0.001), algal concentration ($F_{5, 60}$ = 8.0195, P < 0.001), and their interactions ($F_{5, 60}$ = 13.8806, P < 0.001). Egg production rates of *A. tonsa* increased progressively with increasing *Rhodomonas lens* concentration (Fig. 2.6). In contrast, egg production rates of *A. tonsa* moderately increased with increasing *Cochlodinium polykrikoides* concentrations from 18 to 180 µg C L^{-1}, then were greatly reduced by the high concentrations of *C. polykrikoides* (180 – 1080 µg C L^{-1}, Fig. 2.6). *C. polykrikoides* supported higher egg production rates of *A. tonsa* than *R. lens* at low algal concentrations (18 – 180 µg C L^{-1}), while egg production rates of *A. tonsa* fed *C. polykrikoides* were greatly lower than those fed *R. lens* at high concentrations (360 – 1080 µg C L^{-1}, Fig. 2.6). The two-way ANOVA showed that egg hatching success of *A. tonsa* was significantly affected by algal species ($F_{1, 48}$ = 30.8405, P < 0.001), but not by algal concentration ($F_{51,48}$ = 2.2991, P = 0.06). Egg hatching rates of *A. tonsa* were very high (82 – 100%) when fed *R. lens*, except the values

at the lowest concentration (18 μg C L^{-1}, Fig. 2.6). Egg hatching success was very low ranging from 20% to 43% when fed *C. polykrikoides* (Fig. 2.6).

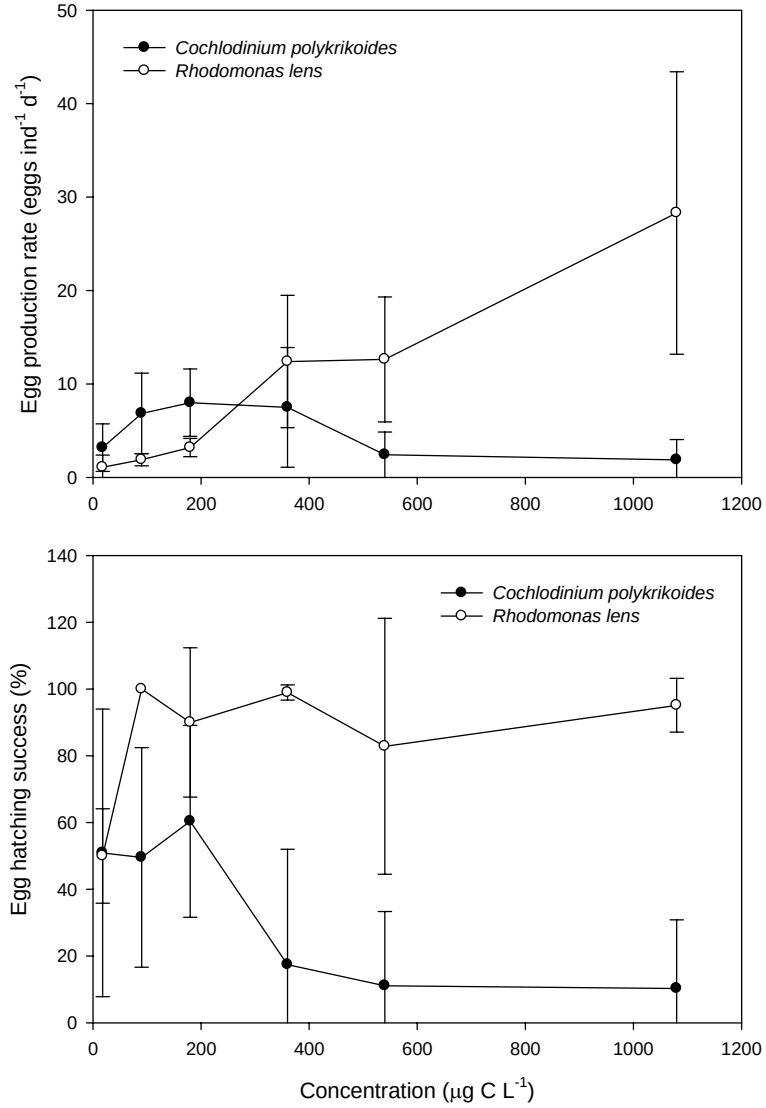

Fig. 2.6. Egg production rates (mean ± SD) of *Acartia tonsa* and hatching success (mean ± SD) when fed either *Cochlodinium polykrikoides* or *Rhodomonas lens* for 1 day as a function of algal concentration.

The two-way ANOVA showed that exposure time did not significantly change egg production rates ($F_{26, 155}$ = 1.5491, P = 0.055, Fig. 2.7) and hatching rates ($F_{26, 142}$ = 1.3164, P = 0.165, Fig. 2.8) of *Acartia tonsa* when fed *Cochlodinium polykrikoides*. The moderate concentrations of *C. polykrikoides* (90 – 360 µg C L^{-1}) supported higher egg production rates of *A. tonsa* than the lowest concentration (18 µg C L^{-1}) and the higher concentrations (540 and 1080 µg C L^{-1}, Fig. 2.7). Egg production of *A. tonsa* when fed *C. polykrikoides* at 90 µg C L^{-1} persisted during the entire experiment (10 d). In contrast, egg production of *A. tonsa* only persisted for several days at the lowest and two highest concentrations of *C. polykrikoides*. *C. polykrikoides* at 1080 µg C L^{-1} reduced *A. tonsa* egg production to zero within two days (Fig. 2.7). The hatching successes of *A. tonsa* eggs when fed *C. polykrikoides* at 18 and 90 µg C L^{-1} were higher than other concentrations. *C. polykrikoides* at 1080 µg C L^{-1} reduced *A. tonsa* egg hatching success to zero within 1 d (Fig. 2.8).

The three-way ANOVA showed that *A. tonsa* egg sizes were significantly affected by algal species ($F_{1, 2370}$ = 89.337, P < 0.001), algal concentration ($F_{4, 2370}$ = 7.273, P < 0.001), and exposure time ($F_{9, 2370}$ = 2.35, P < 0.001, Fig. 2.9). Egg sizes of *Acartia tonsa* when fed *Cochlodinium polykrikoides* were lower than those fed *Rhodomonas lens* at each experimental concentration. The average egg sizes of *A. tonsa* when fed *C. polykrikoides* and *R. lens* for all concentrations were 76.40 µm and 77.60 µm, respectively. Egg sizes of *A. tonsa* when fed *C. polykrikoides* decreased from 77.30 µm to 75.96 µm with increasing concentrations from 90 µg C L^{-1} to 720 µg C L^{-1}. In contrast, egg sizes of *A. tonsa* remained constant (77.34 – 77.89 µm) when fed non-toxic *R. lens* from 90 µg C L^{-1} to 720 µg C L^{-1}. The trend of egg sizes over time when fed either *C. polykrikoides* or *R. lens* was not clear (Fig. 2.9).

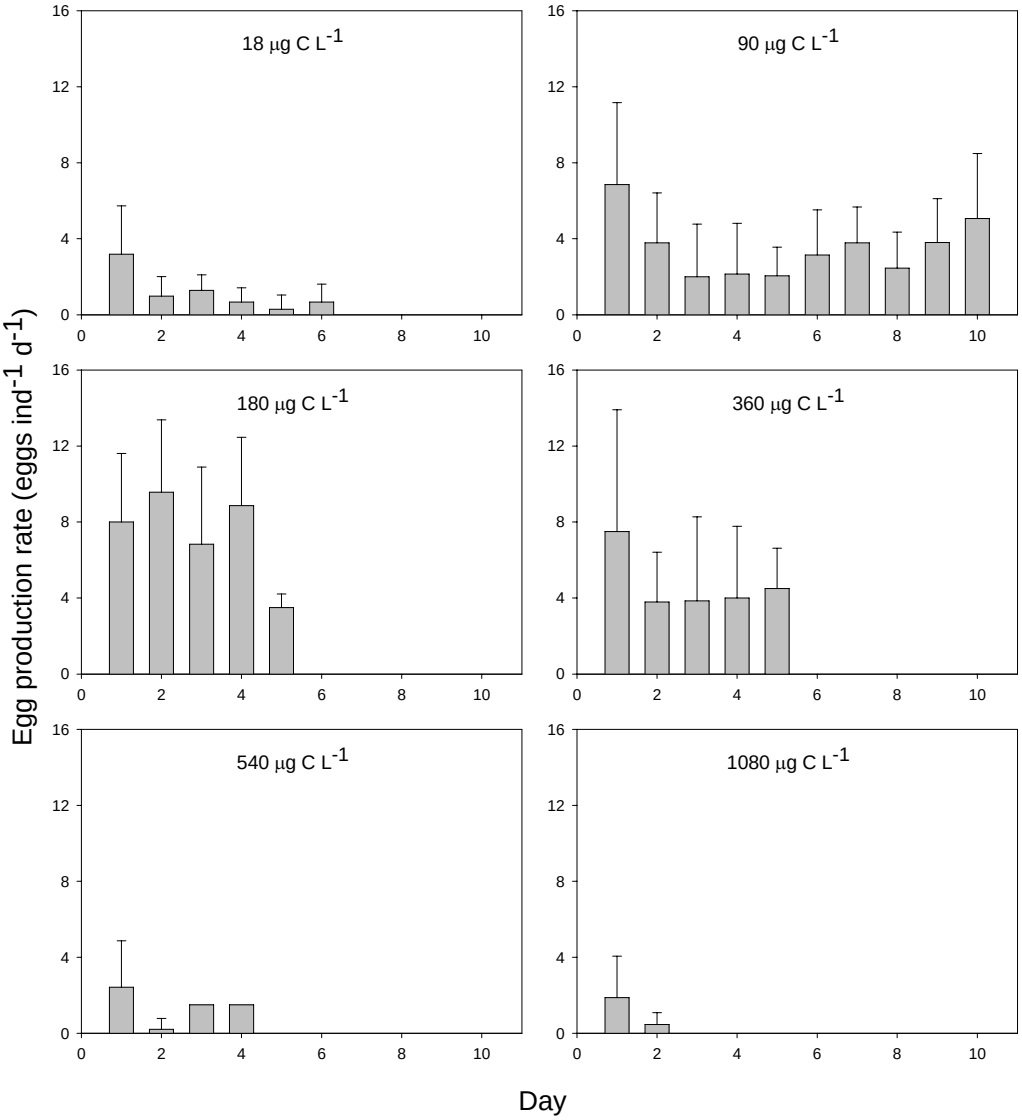

Fig. 2.7. Egg production rates (mean ± SD) of *Acartia tonsa* when fed *Cochlodinium polykrikoides* as a function of exposure time.

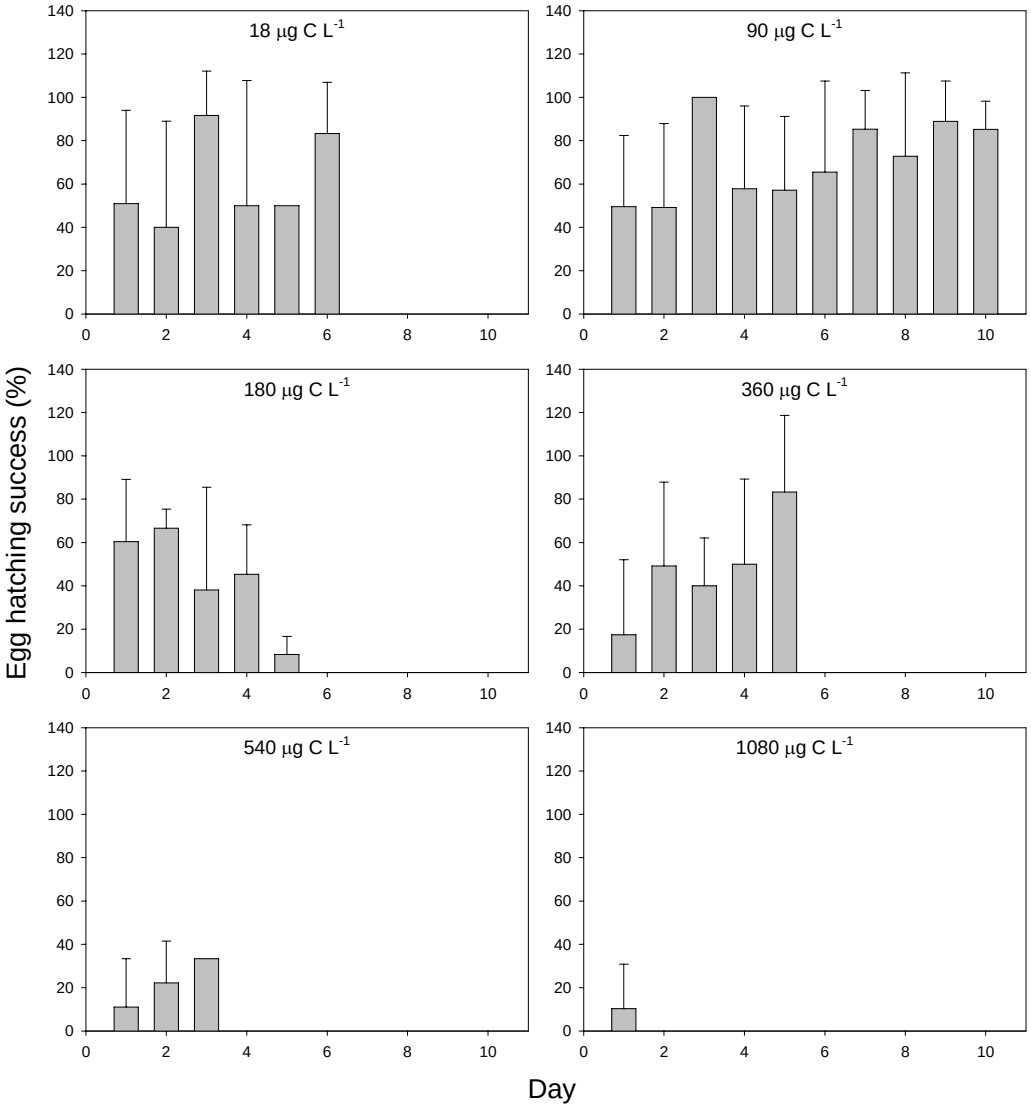

Fig. 2.8. Egg hatching success (mean ± SD) of *Acartia tonsa* when fed *Cochlodinium polykrikoides* as a function of exposure time.

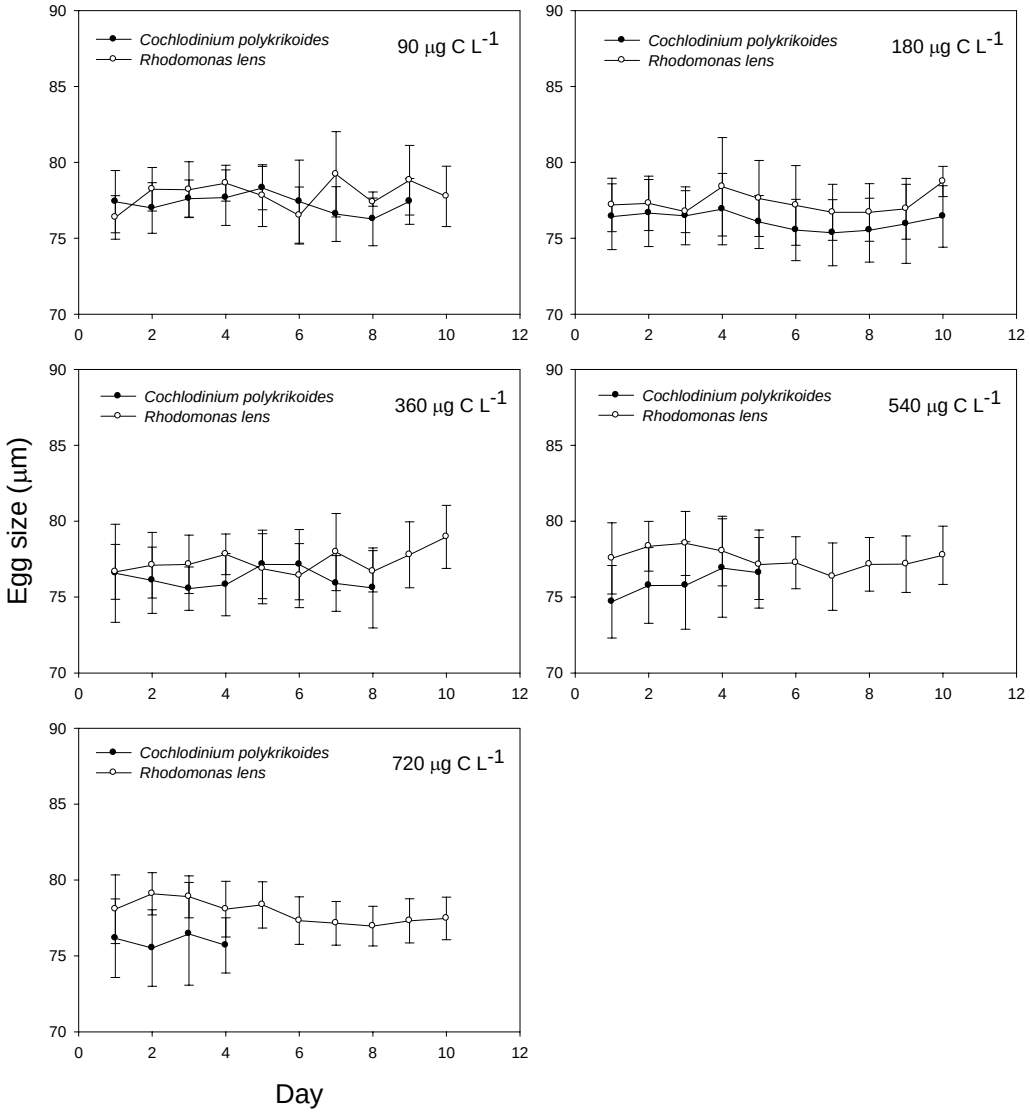

Fig. 2.9. Egg sizes (mean ± SD) of *Acartia tonsa* when fed either *Cochlodinium polykrikoides* or *Rhodomonas lens* as a function of exposure time.

Discussion

My study showed that the dinoflagellate *Cochlodinium polykrikoides* had comparable or more deleterious impacts on copepods compared to other well-known toxic dinoflagellates. Copepods completely expired within 1.5 and 3.5 day, respectively, at high (3300 and 4700 μg C L^{-1}, ~ 1800 and 2600 cells mL^{-1}) and intermediate (1500 and 2200 μg C L^{-1}, ~ 800 and 1200 cells mL^{-1}) concentrations of *C. polykrikoides*.

Approximately 50% of *Acartia clausi* were dead during a 7-day exposure to the toxic strain *Alexandrium minutum* at a density of 650 cells mL^{-1} (Barreiro et al. 2007). The dinoflagellate *Karenia brevis* cultured at densities ranging from 1.8×10^4 to 2.1×10^4 cells mL^{-1} led to approximate 80% mortality of *A. tonsa* over a period of 5 days (Prince et al. 2006). Cohen et al. (2007) reported that the *K. brevis* culture at a density of 1×10^4 cells mL^{-1} caused approximately 10% mortality in *Temora turbinata*, 3% mortality in *A. tonsa*, and 1% mortality in *Centropages typicus* after a 24-h exposure. *Karlodinium corsicum* (as *Gyrodinium corsicum*) at concentrations around 1500 μg C L^{-1} killed approximate 50% *A. grani* after 6 days and all copepods after 12 days (da Costa et al. 2005). During blooms lasting one to two months in US eastern coast waters, typical densities of *C. polykrikoides* were $>10^3$ cells mL^{-1}, frequently 10^4 cells mL^{-1} (Gobler et al. 2008, Mulholland et al. 2009). Since lethal concentrations of *C. polykrikoides* for *A. tonsa* are lower than their densities during the blooms, exposure to high densities of toxic *C. polykrikoides* cells for such extended periods may cause substantial mortalities within *A. tonsa* populations. The population dynamics of copepods are sensitive to variation in mortality, as a relatively small increase in female mortality can considerably change population growth by reducing recruitment. Even before blooms occur, the moderate densities of *C. polykrikoides* ($\sim10^2$ cells mL^{-1}) may lead to adverse effects on zooplankton, such as reduced feeding and fecundity. Thus, toxic blooms may reduce secondary production and further lead to food restriction for consumers at higher trophic levels.

Extrapolating laboratory experiments to the natural environment can be complex. Rapid evolution of resistance may shape the interactions between zooplankton and toxic algae. Some studies have shown that grazer populations that have experienced recurrent HABs can evolve local adaptations to toxic algae (Hairston et al. 1999, Colin and Dam 2004). An artificial selection experiment showed that copepods evolved resistance to toxic algae over only 2-5 generations (Colin and Dam 2004). The rapid evolution of resistance may be an important feedback mechanism to minimize the potential deleterious effects of toxic algae on zooplankton. In New York, *C. polykrikoides* blooms only occur in eastern Long Island waters (Gobler et al. 2008). The copepod population used in this research was collected from Stony Brook Harbor, Long Island Sound, where no *C. polykrikoides* blooms have been observed. Thus, the adverse consequences may be maximized assuming there is no zooplankton gene flow between bloom and non-bloom areas. Another potential factor is the complexity of plankton. Toxic algae rarely bloom in nature in the absence of other phytoplankters. Ingestion of *C. polykrikoides* with other concurrent phytoplankters or heterotrophic prey may dilute potential adverse effects on copepods. Some zooplankton has the ability to actively select a non-toxic diet (Turner and Tester 1997). The adverse effects of *C. polykrikoides* may be reduced by the presence of other occurring microalgae (Tang and Gobler 2009).

Stage-specific effects of HABs on zooplankton have rarely been considered in prior studies of the interactions between harmful algae and zooplankton. The present results showed the resistance of *Acartia tonsa* to *Cochlodinium polykrikoides* increased with development. Early nauplii of *A. tonsa* were four times more sensitive to *C. polykrikoides*

than adult females. These results are similar to the previous studies on stage-specific variations in sensitivity of copepods to toxic chemicals. The nauplii of *Tigriopus brevicornis* were two to four times more sensitive to three insecticides and two metals than the adults (Forget et al. 1998). The nauplii of *A. tonsa* were 28 times more sensitive to an organic pesticide (cypermethrin) than adults after 96 h of exposure (Medina et al. 2002). The greater sensitivity of copepod early life stages to toxic algae may be related to their relatively larger surface per unit volume, which may promote a greater diffusive flux of phycotoxins into the copepod body. Another possible explanation is that the later stages may have a greater ability to detoxify. Copepods may transfer toxins into fecal pellets and/or eggs, or eliminate them through excretion in dissolved form (Guisande et al. 2002). More developed metabolic systems in adults (Mauchline 1998) may improve detoxification abilities of copepods. Regardless, the studies on stage-specific effects of HABs on zooplankton may be necessary to understand their true impact on planktonic ecosystems. The investigation of all life stages also provides a more appropriate tool for predicting potential toxicity of harmful algae to copepod populations. Interestingly, live *C. polykrikoides* cells did not inhibit *A. tonsa* egg hatching. Tang and Dam (2001) reported a similar result that marine diatom exudates did not have negative effects on *A. tonsa* egg hatching.

Mortality of *Acartia tonsa* exposed to the frozen and thawed *Cochlodinium polykrikoides* culture was significantly lower than that in the live culture. The freezing and thawing treatment destroyed *C. polykrikoides* cells (personal observation). This result indicated that harmful effects were mainly dependent on the viability of *C. polykrikoides* cells. Similar results were observed on the lethal effects on fish by *C. polykrikoides* natural bloom waters (Gobler et al. 2008, Mulholland et al. 2009) and pure cultures (Tang and Gobler, 2009). Copepod mortality exposed to the frozen and thawed *C. polykrikoides* culture was still significantly higher than that in FSW, which suggested that some harmful compound(s) remained after this treatment. The extracellular secretion and continuous accumulation of polysaccharides in *C. polykrikoides* medium were considered as one of the causes of fish kills (Kim et al. 2002). An extensive exocellular organic fibrillar matrix and a closely enclosing organic envelope surround the *C. polykrikoides* cells of our strain (Gobler et al. 2008). The freezing and thawing treatment may not completely eliminate the harmful effect of such polysaccharides. Direct contact with those polysaccharides or other harmful compounds located on the *C. polykrikoides* cell surface may be responsible for the death of some copepods in this treatment. Another possibility is that some harmful compounds in *C. polykrikoides* may be released when cells are broken. The result from my second bioassay supported the above explanations. The mortality of copepods in the cages with 5-μm nylon mesh and immersed in *C. polykrikoides* live culture was significantly lower than that in the cages with 100-μm nylon mesh. The separation from harmful compounds in *C. polykrikoides* cells or on cell surfaces by the 5-μm nylon mesh may account for the improved survival of copepods. Yamasaki et al. (2007) observed that cell contact with *C. polykrikoides* inhibited the growth of another dinoflagellate *Akaskiwo sanguinea* and caused morphologically abnormal cells. This result indicated some harmful compounds located on *C.*

polykrikoides cell surface, but I still do not have evidence to exclude the possibility of the presence of harmful compounds in cells. Interestingly, the freezing of *C. polykrikoides* culture did not show toxic to juvenile fish (*Cypinodon variegates*) (Gobler et al. 2008, Tang and Gobler 2009). This dissimilarity is probably due to the differences in the physiology of these organisms, such as different binding sites and tolerance to harmful compounds. This harmful fraction may impact on lower trophic copepods, but not vertebrate fish.

Another harmful principle may be the dissolved, highly reactive, labile compounds released by live *Cochlodinium polykrikoides* cells. The complete lack of the harmful effects of the 0.2-μm culture filtrate suggested that *C. polykrikoides* cells did not release water-soluble harmful compounds or that released compounds were very unstable. The second bioassay experiment supported the latter explanation. The mortality of copepods in the cages with the 5-μm nylon mesh and immersed in *C. polykrikoides* live culture was higher than that in the FSW. This result suggested that some water soluble harmful compounds released by *C. polykrikoides* cells may pass through the 5-μm nylon mesh and affect copepods. Kim et al. (1999) reported that reactive oxygen species (ROS) generated from *C. polykrikoides* was responsible for oxidative damage leading to fish kills. Tang and Gobler (2009) also reported that the ichthyotoxicity of *C. polykrikoides* could be caused by non-hydrogen peroxide, highly reactive, labile compounds such as ROS-like chemicals. Thus, I propose that multiple harmful compounds produced by *C. polykrikoides* are responsible to their deleterious effects on copepods.

Cochlodinium polykrikoides significantly reduced ingestion rates of *Acartia tonsa* when compared to non-toxic *Rhodomonas lens*. Two possible mechanisms, behavioral rejection and physiological incapacitation, have been postulated to explain such reduced feeding due to harmful algae (Ives, 1987). I did not directly test which mechanism was responsible for the reduced feeding by *C. polykrikoides*. Higher mortality of *A. tonsa* when exposed to *C. polykrikoides* than in FSW suggested that poisoning rather than starvation was the main mechanism for copepod death. Therefore, the physiological incapacitation may, at least partially, explain the reduced feeding of *A. tonsa* by *C. polykrikoides*. My present experiments, however, did not directly rule out the possibility of feeding deterrents. Copepod feeding is shaped by prey size, motility, and quality (Berggreen et al. 1988, Hansen et al. 1994, Mauchline 1998). The equivalent spherical diameters (ESD) for *C. polykrikoides* and *R. lens* were 28.2 μm and 7.97 μm, respectively. The optimal particle size for feeding by *A. tonsa* females was 14.8 μm (Berggreen et al. 1988). Clearance rates of *A. tonsa* females were nearly equal when fed the flagellate *R. baltica* (ESD: 6.91 μm) and the dinoflagellate *Scripsiella faröense* (ESD: 19.0 μm, Berggreen et al. 1988). Thus, it is reasonable to assume that the effect of size difference in this study was limited because the two algae used in this study were very similar to *R. baltica* and *S. faröense* in size. Egg sizes of *A. tonsa* when fed *C. polykrikoides* were smaller than *R. lens*. To my knowledge, this is the first report that toxic algae reduced copepod egg size. Cooney and Gehrs (1980) reported that there was a direct positive relationship between egg size and naupliar size in the calanoid copepod

Diaptomus clavipes. Thus, copepod population fitness may be reduced by toxic algae since larger nauplii usually have lower mortality rates or matured more rapidly than smaller nauplii (Mauchline 1998). I do not know the mechanism by which a *C. polykrikoides* diet yielded smaller eggs of *A. tonsa*. The ingestion rates of *A. tonsa* on *C. polykrikoides* were 25 – 60% lower than values on *R. lens*. The lack of adequate nutrition and (or) physiological incapacitation would lead to impaired gametogenesis and spawning failure in copepods.

The present results clearly showed that the red tide dinoflagellate *Cochlodinium polykrikoides* at my experimental concentrations caused deleterious consequences for the copepod *Acartia tonsa*. Is *C. polykrikoides* really a toxic alga? Harmful effects of algae on zooplankton can be explained by the absence of essential nutrients or the presence of toxins (Turner and Tester 1997, Colin and Dam 2002b, Prince et al. 2006). One of major challenges in algae-grazer interactions is to separate potential toxic effects from nutritional inadequacy or deficiency. *C. polykrikoides* at high concentrations (≥ 900 μg C L^{-1} or 500 cells mL^{-1}) significantly reduced survivorship of female *A. tonsa* compared to those starved in FSW. The lethal effects suggested that *C. polykrikoides* was a toxic prey for *A. tonsa* at high concentrations. Recently, the mixed diet approach has been developed to discern whether the suspect prey is beneficial, nutritionally inadequate, or toxic to grazers (Colin and Dam 2002b). In another study, I conducted mixed diet experiments at four concentrations (100 μg C L^{-1}, 200 μg C L^{-1}, 600 μg C L^{-1}, and 1000 μg C L^{-1}) and three durations (1 d, 3 d, and 5 d). The results showed that harmful effects on *A. tonsa* at 1000 μg C L^{-1} were caused by *C. polykrikoides* toxicity. However, the nutritional value of *C. polykrikoides* was greater than or equal to the standard diet of *R. lens* at 100 μg C L^{-1} and 200 μg C L^{-1}. These results showed that the nutritional value of *C. polykrikoides* to *A. tonsa* ranged from beneficial to toxic with increasing cell density. The density-dependent nutritional value of this alga suggests that *C. polykrikoides* can be nutritious or toxic for *A. tonsa* depending on ambient concentrations.

Chapter 3 Density-dependent nutritional value of *Cochlodinium polykrikoides* to *Acartia tonsa**

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Introduction

One long-standing ecological question in aquatic sciences is why a major fraction of dense phytoplankton blooms in aquatic environments, generally dominated by diatoms or dinoflagellates, is ungrazed and sinks out of the euphotic zone (Litchman and Klausmeier 2008). The low grazing pressure on these blooms has been attributed to the inability of herbivore populations, mainly copepods, to take advantage of the blooms due to the latter's long development time, ranging from weeks to months (Mauchline 1998), relative to fast algal reproductive rates. An alternative explanation is that predation, especially cannibalism, constrains the cohort size of copepods (Ohman and Hirche 2001). On the other hand, the nutrition hypothesis argues that not all algae are good food sources due to nutritional inadequacies, morphological defenses, and/or chemical defenses. These traits are known to depress herbivore feeding and negatively affect herbivore fitness (Miralto et al. 1999, Prince et al. 2006). The nutritional value of algae is usually considered species-specific and varies greatly in terms of digestion resistance, biochemical composition, and toxin production (Sterner and Schulz 1998). Some genera of phytoplankton such as *Rhodomonas*, *Chlamydomonas*, and *Scenedesmus* are typically considered as high-quality food sources for zooplankton (Sterner and Schulz 1998, Koski et al. 2008). Many biochemical components in algae including certain vitamins, amino acids, and fatty acids are nutritionally important for zooplankton success (Jónasdóttir 1994). Element imbalances can reduce phytoplankton quality and limit zooplankton growth (Litchman and Klausmeier 2008). Incomplete digestion, possibly due to thickened cell walls or increased extracellular mucilage, can also contribute to the low quality of some algae (Sterner and Schulz 1998). When algae produce toxins, grazers are often deleteriously affected due to impaired feeding, physiological dysfunction, depressed growth and reproduction, and reduced population fitness (Landsberg 2002, Prince et al. 2006). Therefore, the negative effects of algae on zooplankton may be explained by both the absence of essential nutrients and the presence of toxins. A major challenge in

understanding the nutritional ecology of zooplankton is separating potential toxic effects of prey from their nutritional inadequacy (Colin and Dam 2002b).

The mixed-diet technique has been developed to discern whether a given phytoplankton species is beneficial, nutritionally inadequate, or toxic to grazers (Jónasdóttir et al. 1998). This approach is based on the premise that grazer responses, such as clearance rate, egg production rate, and egg hatching success, are linearly related to the proportion of good and poor prey in a mixed diet. Grazers are offered sole diets of the suspected prey (the treatment), a well-known good prey (the control), and mixed diets. A reference line is drawn connecting the responses of the grazer feeding on the 100% suspect and 100% control prey. If the responses of the grazer with the suspected prey are higher than or similar to values with the control prey, the suspect prey is likely to be a nutritionally beneficial food. If the responses of the grazer fed the suspected prey are lower than values with the control prey, deleterious effects due to either toxicity or nutritional insufficiency are suggested. If the responses of the grazers with mixed diets fall along the reference line, the suspect prey has no nutritional value since the responses of the grazers are entirely determined by the dilution of the control prey. If the grazer responses fed mixed diets fall above the reference line, the suspect prey has some nutritional value. And, if the values of the grazer with mixed diets fall below the reference line, the suspect prey is toxic because it detracts from the beneficial effects of the control prey.

Using mixed-diet experiments, Colin and Dam (2002b) investigated whether several algae that had been previously reported to have harmful effects on grazers were in fact toxic to the copepod *Acartia tonsa*. The experiments performed at a concentration of 250 μg C L^{-1} indicated only a highly toxic *Alexandrium* sp. strain was toxic to female *A. tonsa* and other algae (low toxicity *Alexandrium* sp. strain, *Heterosigma carterae*, *Thalassiosira rotula*, and *Phaeodactylum tricornutum*) could not be considered toxic (Colin and Dam 2002b). The red tide dinoflagellate *Karenia brevis* is usually considered to be toxic, but mixed-diet experiments at a single food concentration showed it was only nutritionally inadequate for *A. tonsa* (Prince et al. 2006, Speekmann et al. 2006). Although the diatoms *P. tricornutum* and *T. rotula* produce polyunsaturated aldehydes (PUA), mixed-diet experiments at 240 μg C L^{-1} showed that *P. tricornutum* did not have any effects on the copepod *Temora longicornis* and *T. rotula* had a beneficial effect (Koski et al. 2008). Variability between results of mixed-diet experiments and previous reports may not only reflect differences among copepod species, but also imply that using a single food concentration in experiments does not adequately reflect the nutritional value of an alga to zooplankton.

In this study I used the mixed-diet approach to determine whether *Cochlodinium polykrikoides* was beneficial, nutritionally insufficient, or toxic to the calanoid copepod *Acartia tonsa*. *A. tonsa* is an abundant species in many neritic and estuarine environments. The copepod is common in US estuaries where *C. polykrikoides* blooms occur, and is capable of consuming *C. polykrikoides* (Jiang et al. 2009). Based on egg production rate, egg hatching success, and naupliar recruitment rate of *A. tonsa*, mixed-diet experiments

showed that the nutritional value of *C. polykrikoides* ranged from beneficial to deleterious with increasing cell density. Long-term survival experiments also supported this conclusion. This density-dependent nutritional quality provides new insights into the ecological effects of putatively harmful algae and their bloom dynamics, and may explain some aspects of plant-herbivore interactions, in general.

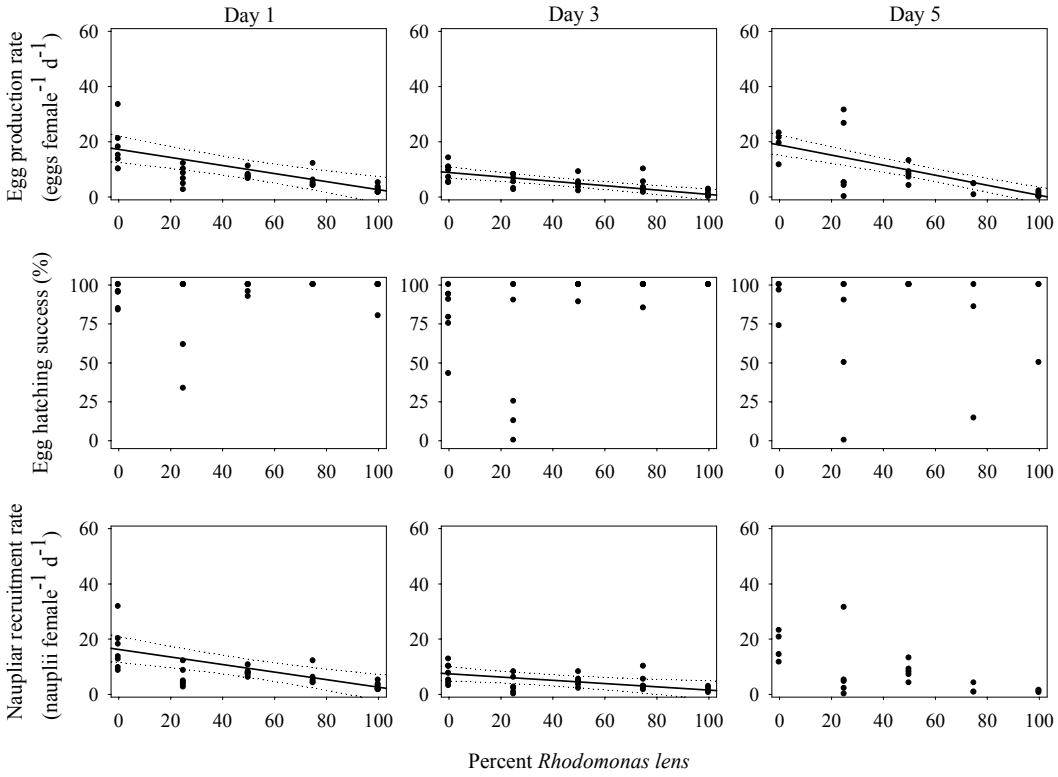

Fig. 3.1. Performances of *Acartia tonsa* vs. the percent carbon of *Rhodomonas lens* at the total carbon concentration of 100 µg C L^{-1}. The linear regression line (solid line) and 95% confidence limits (dotted line) are set for the performance with 0% and 100% *R. lens* when they are significantly different.

Materials and methods

Collection and culture of organisms

The dinoflagellate *Cochlodinium polykrikoides* clone CP1 was isolated from Peconic Bay, Long Island, New York, United States of America in 2006. The flagellate *Rhodomonas lens* Pascher and Ruttner (CCMP 739) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The cultures were maintained in a temperature-controlled incubator at 20°C with a 14 h light:10 h dark cycle (approximately 50 µmol photons m^{-2} s^{-1}). The cultures were maintained in

exponential growth phase by biweekly dilution with autoclaved f/2 medium prepared with 0.2-µm filtered seawater (FSW, salinity 30). The carbon contents of *C. polykrikoides* and *R. lens* were 1816 and 39.5 pg cell^{-1}, respectively (Jiang et al. 2009).

The copepod *Acartia tonsa* was collected from Stony Brook Harbor, New York, with a 202-µm mesh plankton net. The population was continuously maintained in 20-L tanks at 20℃ with a 12 h light:12 dark regime. Copepods were offered *Rhodomonas lens* at a near saturating concentration of 500 µg C L^{-1} (Mauchline 1998) every day. Half of the seawater in the copepod culture was replaced with fresh FSW twice a week.

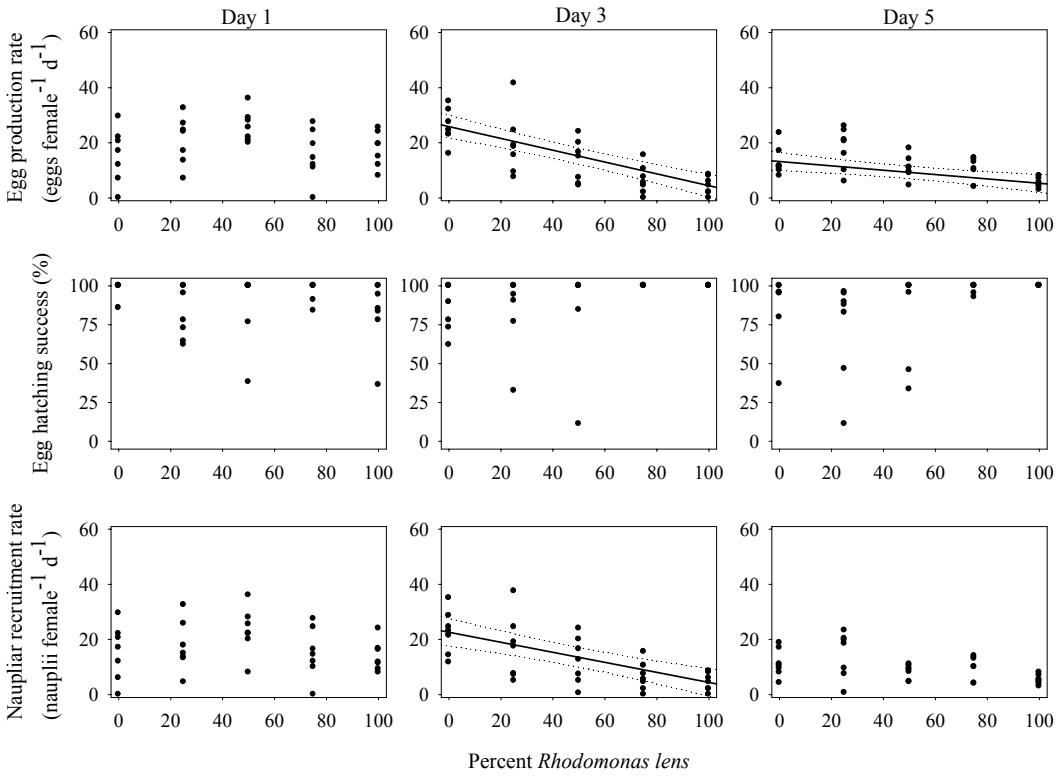

Fig. 3.2. Performances of *Acartia tonsa* vs. the percent carbon of *Rhodomonas lens* at the total carbon concentration of 200 µg C L^{-1}. Regression line and 95% confidence limits as Fig. 3.1.

Mixed-diet experiments

The experiments were performed at 4 concentrations of total algal carbon: 100, 200, 600, and 1000 µg C L^{-1}. The corresponding densities of *Cochlodinium polykrikoides* were 55, 110, 330, and 550 cells mL^{-1}, which represented their densities from initiation to the development of blooms in the natural environment (Gobler et al. 2008). For each concentration, the carbon fractions of *C. polykrikoides* in diets were nominally 100%,

75%, 50%, 25%, and 0%. Each experimental algal suspension was prepared by diluting algal cultures at the concentration of approximately 1800 µg C L^{-1} (1000 cells mL^{-1} for *C. polykrikoides* and 45600 cells mL^{-1} for *Rhodomonas lens*) with FSW. On day 0, 150 *Acartia tonsa* adults were isolated from culture and kept in a 2-L beaker containing sole diets of *C. polykrikoides* or *R. lens*, or mixed diets. Approximately 80% of the algal suspension was changed daily. Although *C. polykrikoides* has been reported as a mixotrophic alga when fed picoplankton (Jeong et al. 2004), my initial study with 50% *C. polykrikoides* and 50% *R. lens* at 600 µg C L^{-1} showed that *C. polykrikoides* did not feed on *R. lens* since the ratio of two species did not significantly change after 24 h (paired two sample *t*-test, $t = 1.4887$, df = 3, $P = 0.2333$, author's unpubl. data). Another feeding experiment of *A. tonsa* with 50% *C. polykrikoides* and 50% *R. lens* at 600 µg C L^{-1} showed that *A. tonsa* did not selectively feed on *R. lens* (paired two sample *t*-test, $t = 1.4910$, df = 3, $P = 0.2327$, author's unpubl. data).

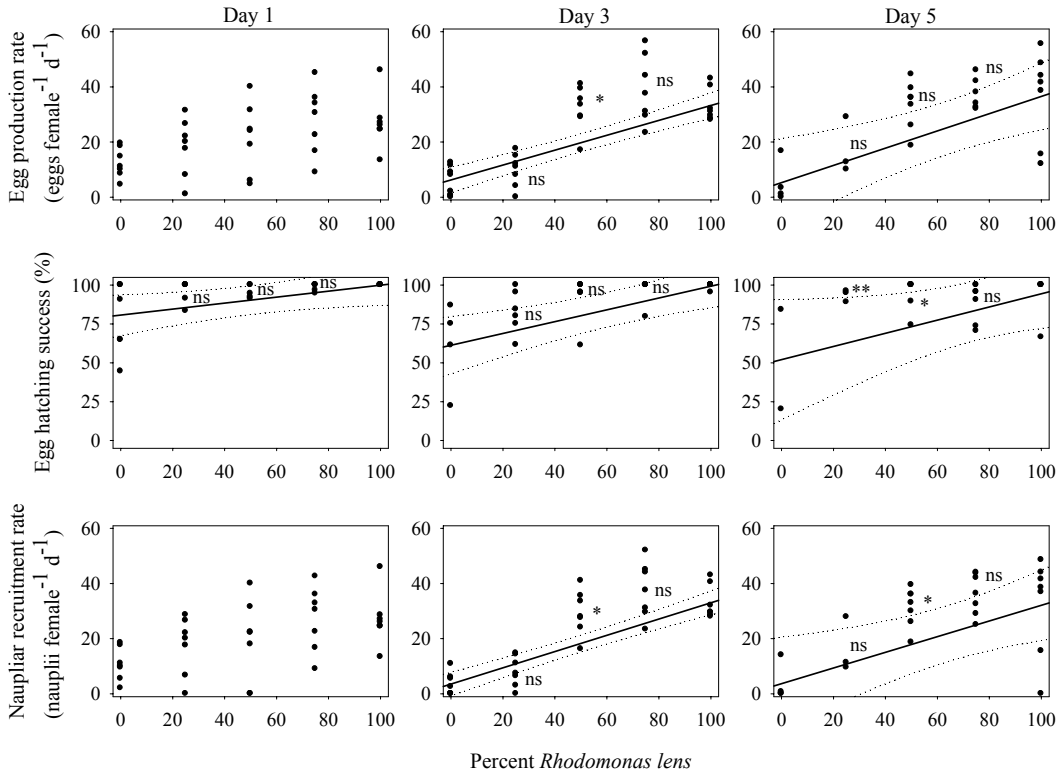

Fig. 3.3. Performances of *Acartia tonsa* vs. the percent carbon of *Rhodomonas lens* at the total carbon concentration of 600 µg C L^{-1}. Regression line and 95% confidence limits as Fig. 1; Significant differences between the observed means on mixed diets and the predicted means from the regression line are indicated by asterisks (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$) and ns (not significant).

Copepod performances were assessed by 3 functional responses: egg production rate, egg hatching success, and naupliar recruitment rate. Egg production rate (eggs female^{-1} d^{-1}) and egg hatching success (%) of *Acartia tonsa* for each treatment were measured on days 1, 3, and 5. The experiments at 1000 µg C L^{-1} did not persist beyond 3 d due to the massive mortality of *A. tonsa* in the 100% *Cochlodinium polykrikoides* treatment. Two healthy females were transferred into 5 to 7 replicated glass dishes filled with 50 mL of algal suspension. A 202-µm mesh was fixed above the bottom to minimize egg cannibalism. All eggs and nauplii were quantified after 24 h. Eggs were then incubated in 1-mL wells of a multi-depression dish filled with FSW. The dishes were contained within a closed plastic box with distilled water added to the bottom of the box to minimize evaporation from the wells. Eggs were examined daily for 2 to 3 d. All experiments in this study were performed at 20°C with a 12 h light:12 h dark cycle. The irradiance level was approximately 1 µmol photons m^{-2} s^{-1} to minimize the potential effects of light on copepods and algal growth during experiments. Naupliar recruitment rate (nauplii female^{-1} d^{-1}) was calculated by multiplication of egg production rate and the proportion of hatched eggs.

Survival experiment

A 10-d experiment was carried out to compare survivorship of *Acartia tonsa* when fed *Cochlodinium polykrikoides* and *Rhodomonas lens* at 4 carbon concentrations ranging from 180 to 1800 µg C L^{-1} (100 to 1000 cells mL^{-1} for *C. polykrikoides*). Approximately 300 *A. tonsa* females were transferred into a 2-L beaker and acclimated in FSW for 24 h. For each treatment 16 to 44 healthy females were transferred individually into 15-mL wells of 6-well tissue culture plates. Each well was filled with 1 female and 13 mL of the experimental algal suspension. The copepods were examined and 80% of the algal suspension was refreshed daily.

Statistical analyses

Egg production rate, egg hatching success, and naupliar recruitment rate on 5 diet treatments were compared by one-way ANOVA for each carbon concentration and exposure time, respectively. Multiple comparisons among the fractions were made using the Tukey post hoc test for equal sample sizes or the Gabriel post hoc test for slightly unequal sample sizes. The original data was transformed to meet the assumptions of ANOVA when necessary. A linear regression line and 95% confidence limits were set for the responses of copepods fed 100% *Cochlodinium polykrikoides* and 100% *Rhodomonas lens* when their means were significantly different. The linear regression line was treated as the reference line in mixed-diet experiments. *T*-tests were used to compare differences between the predicted means from the reference line and the observed means on each mixed diet. The overall difference between the observed data on 3 mixed diets and the reference line was compared using Fisher's procedure of combining probabilities from 3 independent *t*-tests. Survivorship curves of copepods when fed two algae were compared using the Gehan-Wilcoxon test. Statistical analyses were conducted using SPSS 16.0 statistical package.

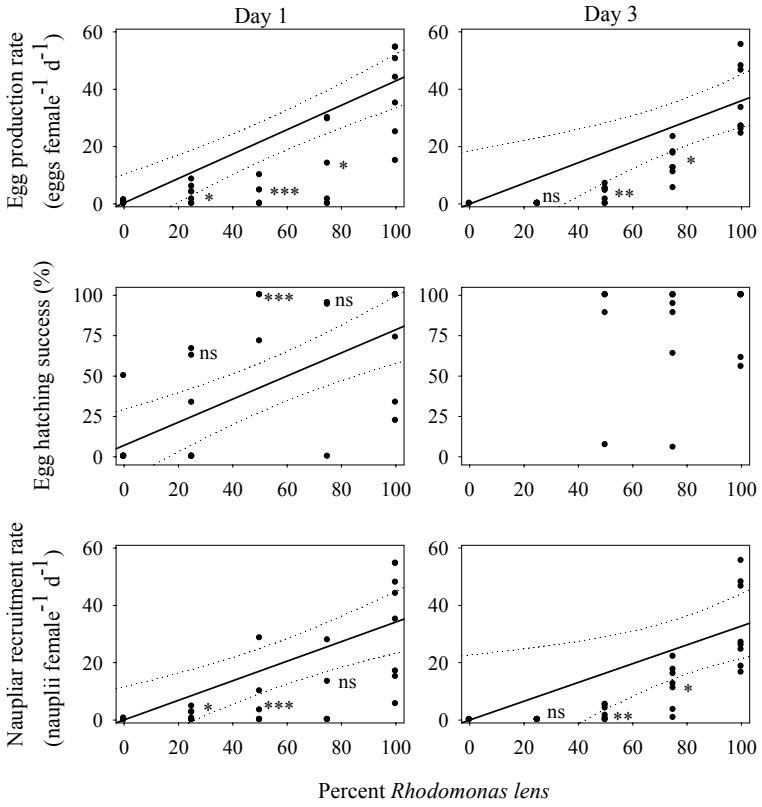

Fig. 3.4. Performances of *Acartia tonsa* vs. the percent carbon of *Rhodomonas lens* at the total carbon concentration of 1000 µg C L^{-1}. Regression line and 95% confidence limits as Fig. 3.1; Statistical symbols as Fig. 3.3.

Results

When *Acartia tonsa* females were fed 100% *Cochlodinium polykrikoides* at 100 µg C L^{-1}, egg production rates on days 1, 3, 5 and naupliar recruitment rates on days 1, 3 were significantly higher than the control *Rhodomonas lens* (Fig. 3.1, one-way ANOVA with post hoc tests, $P < 0.05$ for all). Similarly, copepod responses when fed 100% *C. polykrikoides* at 200 µg C L^{-1} were significantly improved (e.g., egg production rates on days 3, 5 and naupliar recruitment rate on day 3, Fig. 3.2, one-way ANOVA with post hoc tests, $P < 0.05$ for all). In contrast, all responses of *A. tonsa* when fed 100% *C. polykrikoides* at 600 µg C L^{-1} were significantly reduced compared with the controls (Fig. 3.3, one-way ANOVA with post hoc tests, $P < 0.05$ for all) except egg production rate and naupliar recruitment rate on day 1. The overall egg production rates of *A. tonsa* when fed mixed diets on day 3, egg hatching success on day 5, and naupliar recruitments rates on days 3, 5 were significantly above the reference lines connecting the two monoculture

diets at 600 µg C L^{-1} (Fig. 3.3, Fisher's procedure of combining probabilities, df = 6, $P <$ 0.05 for all), while the overall egg production rate on day 5 and egg hatching success on days 1, 3 were not (Fisher's procedure of combining probabilities, df = 6, $P > 0.05$). Furthermore, all egg production and naupliar recruitment rates with mixed diets at 1000 µg C L^{-1} were significantly below the references lines (Fig. 3.4, Fisher's procedure of combining probabilities, df = 6, $P < 0.01$ for all), except for the egg hatching success on day 1 which was significantly above the reference line (Fisher's procedure of combining probabilities, df = 6, $P < 0.01$).

Table 3.1. Nutritional value of *Cochlodinium polykrikoides* to *Acartia tonsa* inferred from the mixed-diet experiments with *Rhodomonas lens*. EPR: egg production rate; EHS: egg hatching success; NRR: naupliar recruitment rate; + +: more beneficial; +: equal beneficial; -: nutritional insufficient; --: no nutrition; ---: toxic

Concentrations (µg C L^{-1})	Time (day)	EPR	EHS	NRR
100	1	+ +	+	+ +
	3	+ +	+	+ +
	5	+ +	+	+
200	1	+	+	+
	3	+ +	+	+ +
	5	+ +	+	+
600	1	+	--	+
	3	-	--	-
	5	--	-	-
1000	1	---	-	---
	3	---		---

The nutritional value of the dinoflagellate *Cochlodinium polykrikoides* to the copepod *Acartia tonsa* decreased from beneficial to deleterious with increasing *C. polykrikoides* concentration (Table 3.1). The nutritional value of *C. polykrikoides* was more beneficial than or equal to *Rhodomonas lens* at 100 and 200 µg C L^{-1}. In contrast, *C. polykrikoides* was nutritionally inadequate or had no nutritional value to *A. tonsa* relative to *R. lens* at 600 µg C L^{-1}. The nutritional value of *C. polykrikoides* to *A. tonsa* became toxic at 1000 µg C L^{-1}.

Survivorship of *Acartia tonsa* females when fed *Cochlodinium polykrikoides* was significantly higher than *Rhodomonas lens* at 180 µg C L^{-1} (Fig. 3.5; Gehan-Wilcoxon

test, df = 1, $P < 0.05$) and 540 μg C L^{-1} (Fig. 3.5; Gehan-Wilcoxon test, df = 1, $P < 0.01$). In contrast, survivorship of *A. tonsa* when fed *C. polykrikoides* was significantly lower than *R. lens* at 900 μg C L^{-1} (Fig. 3.5; Gehan-Wilcoxon test, df = 1, $P < 0.05$) and 1800 μg C L^{-1} (Fig. 3.5; Gehan-Wilcoxon test, df = 1, $P < 0.001$).

Discussion

Ecological significance of density-dependent nutritional value

My results showed *Cochlodinium polykrikoides* had variable nutritional effects on the copepod *Acartia tonsa* over concentrations ranging from 100 to 1000 μg C L^{-1}. Contrary to expectation, *C. polykrikoides*, which has been reported as a harmful red-tide alga (Gobler et al. 2008, Tang and Gobler 2009, 2010), was more beneficial to *A. tonsa* than the flagellate *Rhodomonas lens* at low concentrations. Harmful algae are typically considered universally deleterious to target organisms (Landsberg 2002), even though harmful effects often vary with growth stage, inorganic nutrients, organic matter, temperature, salinity, light, and grazers (Granéli and Flynn 2006). My results, however, clearly showed that *C. polykrikoides* was a nutritious alga for grazers at low densities, which challenges the traditional view on harmful algae. On the other hand, *C. polykrikoides* was toxic to *A. tonsa* at the highest concentration of 1000 μg C L^{-1} (550 cells mL^{-1}). Jiang et al. (2009) found that survivorship of *A. tonsa* females was significantly reduced when fed *C. polykrikoides* monocultures at high concentrations (≥ 900 μg C L^{-1}, 500 cells mL^{-1}) compared to copepods starved in filtered seawater. These results along with my current findings using mixed diets indicate the deleterious mode of this alga is related to cellular toxicity rather than nutritional insufficiency. There is no doubt that *C. polykrikoides* is highly deleterious to a variety of marine organisms at high densities, but its effects on ecosystems at low densities are likely different than those observed at high densities.

This study demonstrates that the ecological effects of putatively harmful algae in natural systems can be density-dependent. Typical densities of *C. polykrikoides* in US eastern coast waters during blooms have been > 10^3 cells mL^{-1}, frequently exceeding 10^4 cells mL^{-1}, with bloom events persisting for approximately one month during late summer (Gobler et al. 2008). Harmful effects of *C. polykrikoides* on copepods may only occur at high densities during blooms. In contrast, the alga may serve as a good nutritional resource and support copepod production when its cell densities are low. Such density-dependent nutritional value may shed light on the controversy regarding the interaction between diatoms and copepods (Miralto et al. 1999). Diatoms, which were traditionally considered an ideal food sources for copepods, have been reported to cause impaired recruitment of copepods, especially when fed high concentrations of diatoms (Miralto et al. 1999). The nutritional inadequacy hypothesis argues that reduced egg production or hatching of copepods fed diatoms was due to the deficiency in some mineral or lipid (Jones and Flynn 2005). The toxicity hypothesis states that the negative

effects on copepods were specifically related to the production of polyunsaturated aldehydes (PUA) by diatoms (Miralto et al. 1999). One important but often overlooked factor, cell density, may contribute to the diatom-copepod controversy. Most laboratory experiments and some field observations (Miralto et al. 1999) showing harmful effects on zooplankton were conducted at high diatom concentrations. In the context of my results, I hypothesize that a density-dependent nutritional value of diatoms may account for the observed discrepancies of diatom-copepod interactions.

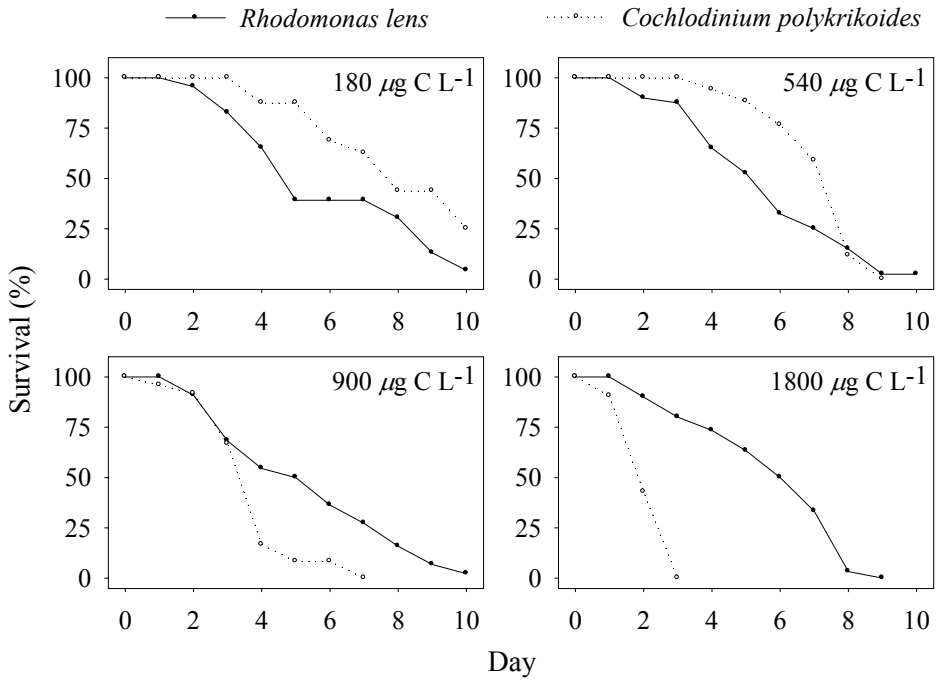

Fig. 3.5. Survivorship of *Acartia tonsa* when exposed to either *Cochlodinium polykrikoides* or *Rhodomonas lens* at 4 concentrations.

Density-dependent nutritional quality of algae may provide some insights into the formation of monospecific *Cochlodinium polykrikoides* blooms. The maximum growth rate of *C. polykrikoides* is approximately 0.4 d^{-1} (Kim et al. 2004), which is comparable to some dinoflagellates but slower than most diatoms and flagellates (Smayda 1997). Hence, killing zooplankton during early stages of bloom development would be a dangerous strategy for *C. polykrikoides* since this would facilitate the dominance of fast growing competitors within algal community (Flynn 2008). Although supporting grazers at low densities of *C. polykrikoides* would depress its populations, grazers also control the population size of fast-growing algae. Several attributes of *C. polykrikoides*, such as mixotrophy (Jeong et al. 2004), allelopathy (Tang and Gobler 2010), and resistance to algicidal bacteria (Imai and Kimura 2008), may elevate its population density and

facilitate bloom formation. Once a bloom population with high cell densities is established, *C. polykrikoides* gains advantages with competing algae and subsequent killing grazers would benefit *C. polykrikoides*, particularly since its allopathic effects on competing algae are also maximal under elevated cell densities (Tang and Gobler 2010). Thus, the harmful effects of *C. polykrikoides* to grazers do not contribute to the bloom initiation, but become increasing important as blooms develop and likely contribute towards bloom maintenance as its nutritional value switches from beneficial to deleterious with increasing cell density. In addition to the support from my empirical study, the competition-predation hypothesis is also consistent with model simulations of algal blooms, which indicate that the ability of an alga to kill a generalist zooplankton predator can only be considered advantageous when the alga has strong competitive advantages with regard to substrate affinity and/or maximum growth rates (Flynn 2008). Testing the competition-predation hypothesis, in combination with some important factors in trophic interactions (e.g., grazing deterrence, nutrient regeneration by zooplankton) and other traditional hypotheses (e.g., nutrient-uptake adaptations, allelopathy, and turbulence effects, Smayda 1997, Flynn 2008), will enable us better understand bloom formation of slow-growing dinoflagellates, such as *C. polykrikoides*.

Proposing the competition-predation hypothesis puts forward a new question regarding how *Cochlodinium polykrikoides* cells at low densities avoid being completely decimated by grazers before they gain a window of opportunity for bloom formation. My preliminary observations suggest that *C. polykrikoides* cells can detect the presence of grazers and increase cell chain length. Chain formation in *C. polykrikoides* could be an effective defense by creating predator-prey size mismatch. Additionally, since the swimming speeds of the dinoflagellates *Gymnodinium catenatum* and *Alexandrium affine* increased by 1.5 times from single cells to chains of 4 cells (Fraga et al. 1989), chain formation in *C. polykrikoides* could increase motility and subsequent escape ability. Thus, induced chain formation by grazers may help *C. polykrikoides* to avoid grazing even when their nutritional value is beneficial to zooplankton at low densities.

Possible mechanisms of density-dependent nutritional value

The mechanism(s) of density-dependent nutritional quality of *Cochlodinium polykrikoides* is not clear. The nutritional value of phytoplankton indicated by zooplankton performances is an overall balance between positive factors (e.g., nutritional compounds such as fatty acids), and negative factors (e.g., toxins). Production of fatty acids and toxins (harmful compounds) by microalgae is greatly variable, even on a daily or hourly scale (Sterner and Schulz 1998, Granéli and Flynn 2006). Toxin production in some dinoflagellates is positively related to cell density (Granéli and Flynn 2006). *C. polykrikoides* cells should have had equally nutritional value at the beginning of the experiments since they were diluted from the same culture at approximately 1000 cells mL^{-1}. After the dilutions, *C. polykrikoides* cells may respond to density changes and thus production of fatty acids and harmful compounds may change with cell densities and/or growth rate, although this likelihood is small given the slow growth of this species and the short duration between transfers (24 h). A more plausible explanation is that

zooplankton responses to harmful compounds are dose dependent. *C. polykrikoides* would not have deleterious effects on *Acartia tonsa* when cell concentrations are below a threshold value. I hypothesizes that the amount of nutritional components in *C. polykrikoides* probably exceeds *Rhodomonas lens* and thus zooplankton perform better when fed *C. polykrikoides* at low concentrations. Jónasdóttir (1994) reported that egg production of the copepods *A. tonsa* and *A. hudsonica* was positively correlated with some specific fatty acids [20:5(n-3), 22:6(n-3), and 18:0]. The relative concentrations of fatty acids 20:5(n-3) and 18:0 to total fatty acids were 17.5% and 2.1% in *C. polykrikoides* (Dorantes-Aranda et al. 2009), which were higher than those in *R. lens* (Jónasdóttir 1994). These two fatty acids may contribute to the high nutritional value of *C. polykrikoides* for *A. tonsa* at lower cell densities. On the other hand, deleterious effects on zooplankton occur when *C. polykrikoides* concentration exceeds a critical level and further increase with increasing cell concentrations. The potential modes of toxicity in *C. polykrikoides* include the production of reactive oxygen species (ROS, Kim et al. 1999, Tang and Gobler 2009) and the production of mucus polysaccharides (Kim et al. 2002), both of which are extracellular and would increase in total toxicity with increasing cell densities. Hence, the nutritional value of some algae, such as *C. polykrikoides*, which is inherently nutritious but also produces harmful compounds, may frequently range from beneficial to deleterious with increasing cell density. Given the known variations in production of fatty acids, toxins, or harmful compounds among algal clones and species (Granéli and Flynn 2006), one must take care in extrapolating prior chemical composition data of algae to the present study. Investigations of production of these compounds by both *C. polykrikoides* and other algae at different cell densities with respect to the physiological responses of copepods are expected to provide more insight regarding the mechanisms influencing the density-dependent nutritional value of phytoplankton.

Some zooplankton exposed to recurrent HABs can rapidly evolve and adapt to toxic algae (Colin and Dam 2004). The copepods used in this study came from Stony Brook Harbor, Long Island Sound, where no *Cochlodinium polykrikoides* blooms have been observed. Thus, it is unlikely that these copepods have evolved resistance to any putative *C. polykrikoides* toxins. Some copepods compensate for low food quality by increasing the quantity of food consumed (Mauchline 1998). Although ingestion rates (μg C copepod^{-1} d^{-1}) of *Acartia tonsa* on *C. polykrikoides* were reduced 40 – 60% relative to *Rhodomonas lens* when food concentrations increased from 350 to 1500 μg C L^{-1}, there was no significant difference in ingestion rates between the two diets at approximately 200 μg C L^{-1} (Jiang et al. 2009). Thus, the high quality of *C. polykrikoides* to *A. tonsa* relative to *R. lens* at low concentrations was not due to higher ingestion rates.

Interpreting mixed-diet experiments

My findings regarding the density-dependent nutritional value of phytoplankton suggests that algal food quality should be assessed using multiple concentrations. Previous studies using mixed-diet experiments (Colin and Dam 2002b, Prince et al. 2006, Speekmann et al. 2006) were performed only at a single food concentration. The results showed that several toxic algae were nutritionally insufficient for copepods (Colin and

Dam 2002b, Prince et al. 2006, Speekmann et al. 2006). However, similar experiments performed under a wide range of environmentally-realistic prey densities may alter this conclusion. Usually, a food-limiting concentration is used in mixed-diet experiments (Jónasdóttir et al. 1998, Colin and Dam 2002b), which is appropriate when toxins are intracellular and grazer performances are influenced by how much of the toxin they ingest. Within this context, the experimental concentrations for algae with intracellular toxins must be below the feeding saturation point. However, the modes of toxicity for many harmful algae depend on extracellular toxins, exudates, or cell surface contact (Landsberg 2002). In these cases, the toxic reactions of grazers are influenced by the concentration of the toxic algae in the environment and not by how many toxic algae they ingest. Thus, the experimental abundances of these algae would not be limited by the feeding saturation point. As discussed above, the toxic modes of *C. polykrikoides* are extracellular reactions (Kim et al. 1999, Kim et al. 2002, Tang and Gobler 2009). Even in the case of intracellular toxins, if toxin production is density-dependent (Granéli and Flynn 2006), the concentrations above the feeding saturation point should also be examined. Toxin amounts ingested by grazers could differ, even if ingestion rates were constant.

My results indicated that egg hatching success was a less sensitive indicator of the nutritional condition of copepods than egg production rates in mixed-diet experiments. Egg production rates of *Acartia tonsa* fed *Cochlodinium polykrikoides* were higher than when fed *Rhodomonas lens* at the concentration of 100 μg C L^{-1}, while the differences in hatching success were not significant. Also, the toxic effects of *C. polykrikoides* at 1000 μg C L^{-1} were more evident for egg production rates than egg hatching success. Furthermore, egg hatching success of *A. tonsa* was more variable than egg production rates in my experiments. Some extremely low values of egg hatching were observed when copepods were fed mixed diets. In some cases, egg hatching success was not statistically reliable because low egg production did not provide adequate sample numbers for hatching experiments. Alternatively, events, such as unfertilized eggs, may influence hatching results. Copepod behavioral changes including prey switching when feeding on mixed diets (Mauchline 1998) may also contribute to higher variation in the responses of *A. tonsa* compared to mono-specific diets.

The size, motility, and quality of prey all influence copepod feeding (Berggreen et al. 1988, Mauchline 1998). The equivalent spherical diameters (ESD) of *Cochlodinium polykrikoides* and *Rhodomonas lens* are 28.2 and 7.97 μm (Jiang et al. 2009). The optimal particle size for feeding by *Acartia tonsa* females is about 15 μm (Berggreen et al. 1988). Clearance rates of *A. tonsa* females were nearly equal when fed on the flagellate *R. baltica* (ESD: 6.91 μm) and the dinoflagellate *Scripsiella faröense* (ESD: 19.0 μm, Berggreen et al., 1988). Thus, the effects of prey size on feeding of *A. tonsa* should have been minimal since the two algae used in the present study were very similar in size to *R. baltica* and *S. faröense* (Berggreen et al. 1988). Although copepods may actively select for particular prey (Mauchline 1998), I did not observe significant prey selection by *A. tonsa* when fed 50% *C. polykrikoides* and 50% *R. lens* at 600 μg C L^{-1} (author's unpubl.

data). Given that prey selection was only examined within this treatment, care should be taken when extrapolating this result to all treatments.

My results may challenge the traditional view that harmful algae are chronically deleterious to ecosystems. The nutritional value of the red tide dinoflagellate *Cochlodinium polykrikoides* to the copepod *Acartia tonsa* ranged from beneficial to deleterious with increasing cell densities. Therefore, the ecological roles of *C. polykrikoides* during bloom and non-bloom periods may be distinctly different. Density-dependent nutritional quality also suggests that supporting grazers may benefit slow-growing *C. polykrikoides* at low densities since grazers may keep fast-growing algae in check. Once *C. polykrikoides* gains a competitive advantage at high concentrations, its effect on grazers may then switch to deleterious, which leads to monospecific blooms. Testing my results under field conditions is expected to bring more insights into the complexity of such planktonic interactions.

Chapter 4 Grazers and vitamins shape chain formation in *Cochlodinium polykrikoides*

Introduction

Almost all species have evolved adaptations for interactions with other species. While prey develop numerous defenses to avoid predation, predators evolve means to breach these defenses resulting in an evolutionary "arms race" of adaptation and counteradaptation (Vermeij 1994, Brodie and Brodie 1999, Agrawal 2001). Selection on prey is often stronger than on predators due to the "life-dinner principle" which argues that it is worse to lose life than to miss a dinner (Brodie and Brodie 1999). Reciprocal adaptations between species regulate population dynamics, shape community structure, affect biogeochemical cycles, and drive genetic diversification (Agrawal 2001, Pohnert et al. 2007, Hay 2009). In aquatic environments, phytoplankton have developed morphological and chemical defenses against predation. Many phytoplankton have protective external structures, such as siliceous or calciferous shells, spines, and horns (Litchman and Klausmeier 2008). Thickened cell walls, increased extracellular mucilage, or fast gut passage of some phytoplankton can lead to incomplete digestion and subsequent imbalances in lipids or unknown compounds important for the reproductive success of herbivores (Dutz et al. 2008). A wide variety of harmful compounds are produced by more than 200 algal species from 20 genera to deter grazing or directly kill grazers (Landsberg 2002). Compared to constitutive defenses, induced defenses by the presence or action of predators may be an effective way to minimize the cost of defense (Agrawal 2001). Since zooplankton grazing usually varies both on temporal and spatial scales, evolution of inducible defenses should be favored compared to constitutive defenses. Zooplankton grazing can induce more toxin production in phytoplankton (Selander et al. 2006). When attacked by zooplankton, some phytoplankton release volatile chemicals to serve as directional cues for predators of zooplankton, such as seabirds, reef fishes, harbor seals, and whale sharks, which lessens grazing mortality of zooplankton on phytoplankton (Hay 2009). Presence of grazers also promotes colony formation in some phytoplankton, reducing grazing pressure due to size mismatch (Lürling 2003, Long et al. 2007).

Colony formation occurs in some species of cyanobacteria, Bacillariophyceae, Charophyceae, Chlorophyceae, Pavlovophyceae and Prymnesiophyceae (Beardall et al. 2009). These colonies are formed by assemblages of fully differentiated and morphologically identical cells of the same genotype. The colony structures are diverse including one-dimensional filaments (chains), two-dimensional mats (plates), or three-

dimensional cylinders, spheres, or amorphous structures (Beardall et al. 2009). Hessen and Van Donk (1993) discovered that the cladoceran *Daphnia magna* released dissolved chemicals that stimulated colony formation in *Desmodesmus subspicatus* (formerly known as *Scenedesmus subspicatus*). Such induced colony formations have been documented in the freshwater green algae *Scenedesmus* and *Desmodesmus* (Hessen and Van Donk 1993, Lürling and Van Donk 1997, Lürling 2003), and the marine prymnesiophyte *Phaeocystis* (Tang 2003, Long et al. 2007). Colony induction may be non-predator specific. For example, colony formation in *S. acutus* was evoked by cladocerans, rotifers, and copepods (Lürling and Van Donk 1997). Two protozoa and one copepod all stimulated colony enlargement in *P. globosa*, although the extent of colony enlargement varied with predator type (Tang 2003). A recent study, however, showed that size-specific feeding induced consumer-specific, but opposing, morphological transformations in *P. globosa* (Long et al. 2007). Ciliates that consumed single cells of *P. globosa* enhanced colony formation, while copepods that fed on colonies suppressed colony formation (Long et al. 2007).

Some species in the Dinophyceae are considered as pseudocolonial organisms relative to the colonial organisms discussed above (Beardall et al. 2009). They form chains as a result of a series of incomplete cell divisions, resulting in the total number of flagella not matching the number of nuclei in chains (Beardall et al. 2009). Chains of some dinoflagellates swim faster than individual cells (Fraga et al. 1989), which may affect predator-prey interactions. However, it has not been investigated whether the chain formation in these pseudocolonial organisms can be induced by the presence of grazers as in colonial organisms. The aim of the present study was to test the hypothesis that the presence of zooplankton grazers would induce chain formation in *Cochlodinium polykrikoides*. I compared chain structures in a field population (presence of grazers) and a cultured population (absence of grazers), investigated chain length following grazer addition, and explored the relationship between chain length and grazer abundance. Given the variation of chain length in cultured cells through its natural growth cycle even without grazers, I further tested the hypothesis that some nutrients would influence chain formation in *C. polykrikoides* using culture-based nutrition amendment experiments. Resolving factors which influence chain formation provides new insights into *C. polykrikoides* bloom dynamics and improves my understanding of plant-animal interactions.

Materials and methods

Collection and culture of organisms

The dinoflagellate *Cochlodinium polykrikoides* clone CP1 was isolated from Peconic Bay, Long Island, New York, USA in 2006 (Gobler et al. 2008). The culture was grown in f/2 medium under a standard incubation condition (20°C with a light/dark cycle of 14 h/10 h at approximately 50 μmol photons m^{-2} s^{-1}). The copepod *Acartia tonsa* was

collected from Stony Brook Harbor, Long Island Sound, NY, with a 202-µm mesh plankton net. The copepod population was continuously cultured in 20-l tanks at a density of 20 to 50 ind. L^{-1}. Copepods were daily offered the flagellate *Rhodomonas lens* Pascher and Ruttner (CCMP 739) at a carbon concentration of approximately 500 µg C L^{-1}.

Chain formation in field and cultured populations

Field sampling was conducted every other day in Old Fort Pond, Shinnecock Bay, NY, from 29 August to 6 October 2008. The water depth was approximately 1.5 m and seawater was vertically well-mixed. Seawater samples (120 mL) were preserved in 5% Lugol's iodine for enumeration of *Cochlodinium polykrikoides*. At least 400 *C. polykrikoides* cells and their chain lengths were recorded using a Sedgewick Rafter counting chamber under a compound microscope. All field samples of *C. polykrikoides* were analyzed within one month after the samplings to minimize the effects of preservation on chain intactness, although I observed long chains did not significantly break up even after one-year preservation without violent shaking. Twenty to 50 L of seawater was filtered onto a 64-µm mesh and preserved in 5% formalin buffered with hexamethylentetramin for determination of *Acartia tonsa* stage-specific abundance. At least 100 *A. tonsa* adults and copepodites were counted under a dissecting microscope.

I also examined chain length of *Cochlodinium polykrikoides* in a cultured population. Approximately 50 mL of an exponentially growing *C. polykrikoides* was transferred to a 2-L flask containing 1.5 L of autoclaved f/2 medium. The initial cell density of *C. polykrikoides* was approximately 10 cells mL^{-1}, which represented a cell density prior to a field bloom. The culture was kept at 20°C under a light/dark cycle of 14 h/10 h at approximately 50 µmol photons m^{-2} s^{-1} and lasted for 60 days. A sample (20 mL) was preserved daily in 5% Lugol's iodine for enumeration of *C. polykrikoides* cells and their chain lengths. All samples of *C. polykrikoides* from the laboratory experiments were counted within 12 h.

Grazer exposure experiment

A grazer exposure experiment was performed to directly test the hypothesis that copepods release chemical cues to induce chain formation in *Cochlodinium polykrikoides*. An exponentially growing culture of *C. polykrikoides* was diluted with sterile 0.2-µm filtered seawater (FSW) and randomly divided into 4 treatments with 6 replicates. Each replicate had 200 mL of *C. polykrikoides* culture in a 250-mL flask. The cell density of *C. polykrikoides* in the experiment was 100 cells mL^{-1}, which represented a cell density during bloom initiation (Gobler et al. 2008). The first treatment was the control with no addition. To produce grazer exudates, *Acartia tonsa* females were maintained in sterile FSW at a density of 30 ind. L^{-1} for 24 h. The solution from the incubation was filtered through 0.2-µm GF/F filters and these filtered exudates were stored for 24 h under the standard incubation condition or administered immediately after filtration. One ml of fresh or stored exudate was added to flasks twice daily to create the second and third treatments, respectively. Healthy *A. tonsa* females were added to the fourth set of the

treatment flasks at a density of 30 ind. L^{-1}. After a 48-h incubation of all treatments under the standard condition, samples (20 ml) from each flask were fixed in 5% Lugol's iodine for examination of chain length of *C. polykrikoides*.

Copepod grazing experiment

Ingestion rates of *Acartia tonsa* on different cell types of *Cochlodinium polykrikoides* were investigated to determine whether chain formation can reduce grazing mortality. Active adult copepods were acclimated in a 2-L beaker with 0.2-μm FSW for 24 h prior to the feeding experiment. The experiment was performed using 200 mL of *C. polykrikoides* suspension at a food concentration of approximately 400 μg C L^{-1} (220 cells mL^{-1}) with 5 replicates and 3 controls. The experimental bottles were rotated on a plankton wheel at 1 rpm and 20°C for 1 h. The short incubation would ensure that the change of *C. polykrikoides* cell types was due to the copepod feeding rather than the induced chain formation. The grazer density was 50 ind. L^{-1} (10 copepods per bottle), which was within the copepod abundance (adults and copepodites) in my field site. Samples for cell densities were taken at the beginning and end of the experiment and preserved in 5% Lugol's iodine. Ingestion rates on different cell types were calculated using the equations of Båmstedt et al. (2000).

Nutrient amendment experiments

Nutrient amendment experiments were performed to test whether nutrients would affect chain formation in *Cochlodinium polykrikoides*. An exponentially growing culture of *C. polykrikoides* was diluted with sterile FSW and randomly divided into 6 treatments with 6 replicates. Each replicate had 200 mL of *C. polykrikoides* culture in a 250-mL flask with an initial density of 100 cells mL^{-1}. The first treatment was the control without adding any nutrients. Trace metals, nitrate, phosphate, vitamins, and f/2 working solution were, respectively, added into other 5 treatments. The composition and concentration of each nutrient followed the f/2 medium recipe (Guillard 1975). After 48 h, chain lengths of *C. polykrikoides* were quantified under a compound microscope.

After an increased chain length of *Cochlodinium polykrikoides* was observed in a mixed-vitamin treatment, I further determined which vitamin(s) contributed to the increased chain length. An exponentially growing *C. polykrikoides* was diluted by f/2 medium without vitamins and randomly divided into 8 treatments with 8 replicates. In each replicate, 200 mL of *C. polykrikoides* culture was maintained in a 250-ml flask with an initial density of 100 cells mL^{-1}. The first treatment was the control without any vitamins. Vitamins B_1, B_7, and B_{12} were added to other treatments both individually and collectively. The final concentrations of vitamins B_1, B_7, and B_{12} were 1.00×10^{-4}, 5.00×10^{-7}, and 5.00×10^{-7} g L^{-1}, respectively, following the f/2 medium recipe (Guillard 1975). Chain lengths of *C. polykrikoides* were investigated after 48 h.

Data analysis

Chain length data from each sampling were pooled for the field population and the cultured population, respectively, and their frequency distributions were compared using a *G*-test. Chain length in grazer and nutrient addition experiments and ingestion rates on different cell types were compared by one-way ANOVA followed by a Tukey post hoc test, respectively. Linear regressions were used to explore the relationship between *Cochlodinium polykrikoides* chain length and *Acartia tonsa* abundance.

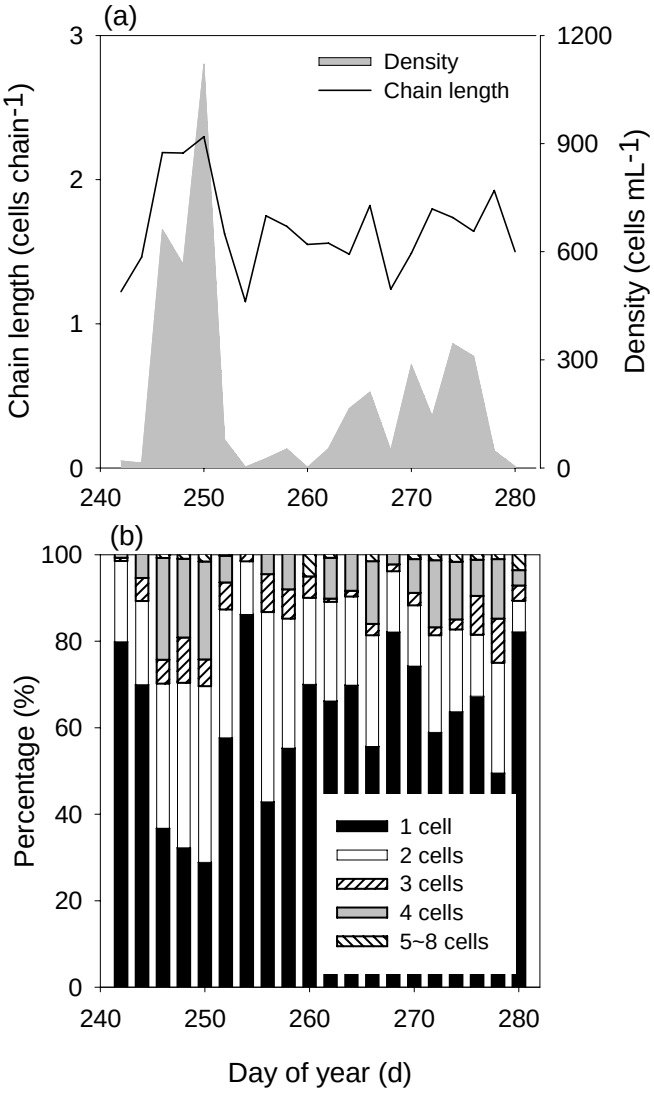

Fig.4.1. Temporal changes of (a) chain length, cell density, and (b) chain structure of *Cochlodinium polykrikoides* in Old Fort Pond, Shinnecock Bay, NY, in 2008.

Results

Chain formation in the field and cultured populations

A bloom of *Cochlodinium polykrikoides* was observed from late August to early October in Old Fort Pond (Fig. 4.1a). *C. polykrikoides* achieved an initial peak in cell densities (564 to 1120 cells mL^{-1}) in early September. The cell density of *C. polykrikoides* was low during the second peak (50 to 350 cells mL^{-1}) which occurred from 20 September to 2 October. The chain structure of *C. polykrikoides* varied during the field bloom. The chain length of *C. polykrikoides* ranged from 1.15 to 2.30 cells $chain^{-1}$ in the field population (Fig. 4.1a). Single cells were most abundant, followed by 2 cells, 4 cells, and 3 cells. Chains with 5 – 8 cells were also observed in the field bloom, although their percentages were very small (Fig. 4.1b). The chain length of *C. polykrikoides* in the field bloom was significantly correlated with the abundance of *Acartia tonsa* (the sum of adults and all copepodites, $r^2 = 0.3580$, $F_{1,17} = 9.479$, $P = 0.0068$, Fig. 4.2).

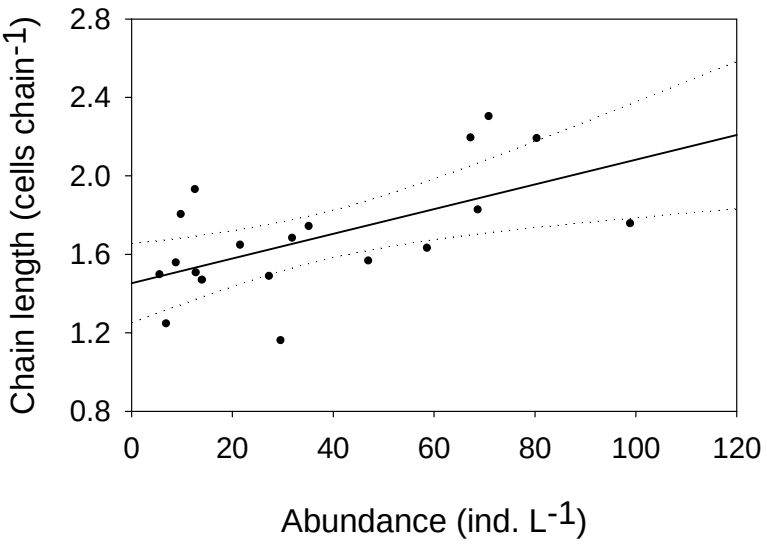

Fig. 4.2. Relationship between chain length of *Cochlodinium polykrikoides* and abundance of *Acartia tonsa* adults and copepodites in Old Fort Pond, Shinnecock Bay, NY, in 2008. The linear regression (solid line) and 95% confidence limits (dotted lines).

After inoculation into fresh medium from a stock culture, the density of *Cochlodinium polykrikoides* exponentially increased from 12.5 to 418.8 cells mL^{-1} during the first 33 d (Fig. 4.3a). The population entered into a relatively stationary phase on day

34 (Fig. 4.3a). The chain length of *C. polykrikoides* ranged from 1.05 to 2.10 cells chain^{-1} in the cultured population (Fig. 4.3a). The chain structure of *C. polykrikoides* gradually switched from 2 cells to single cells. The 4-cell chains occurred during the first 35 d and diminished thereafter. The chains with 5 – 8 cells were not observed in the cultured population (Fig. 4.3b).

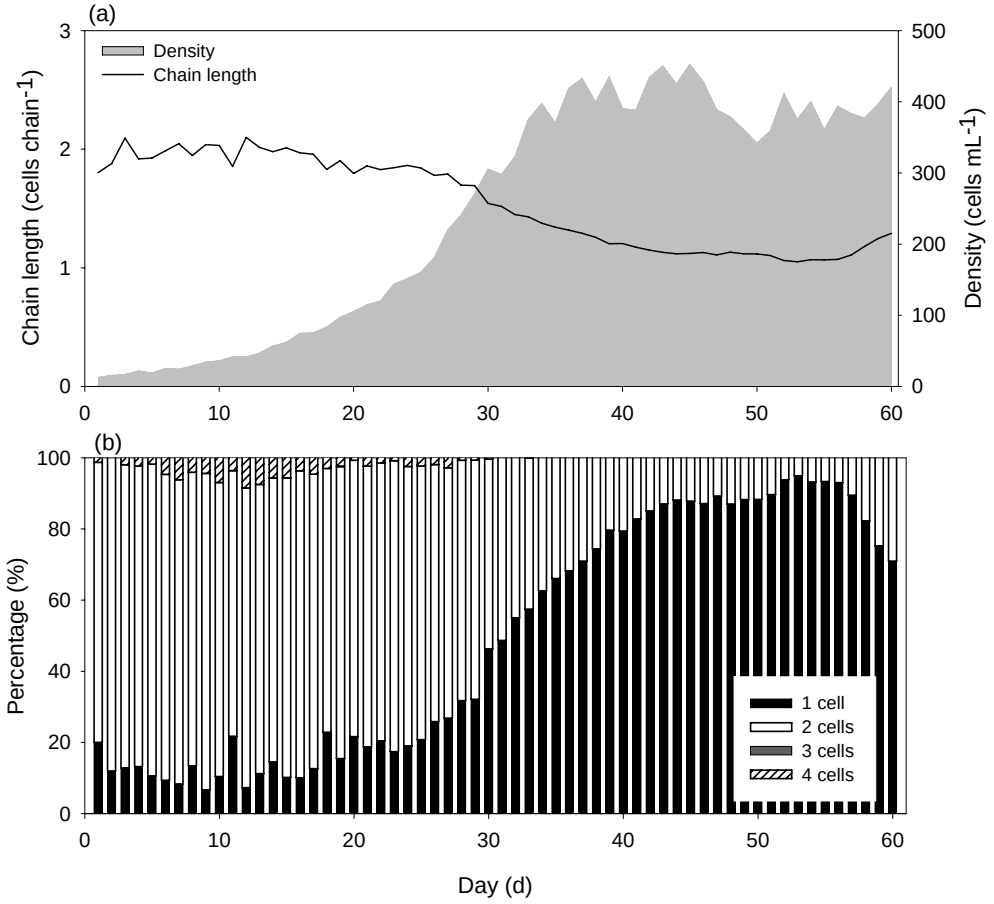

Fig. 4.3. Temporal changes of (a) chain length, cell density, and (b) chain structure of *Cochlodinium polykrikoides* during a 60-d incubation of an isolated culture.

The chain structure of *Cochlodinium polykrikoides* in the field bloom was significantly different from the culture bloom (*G*-test, G_7 = 7490, P < 0.001). The percentages of 1 and 2 cells of *C. polykrikoides* were higher in the cultured population than in the field population. In contrast, the percentages of other chain types (>2 cells) were lower in the cultured population than in the field population (Fig. 4.4). The chain

length of *C. polykrikoides* in the cultured and field populations was 1.32 and 1.88 cells chain^{-1}, respectively.

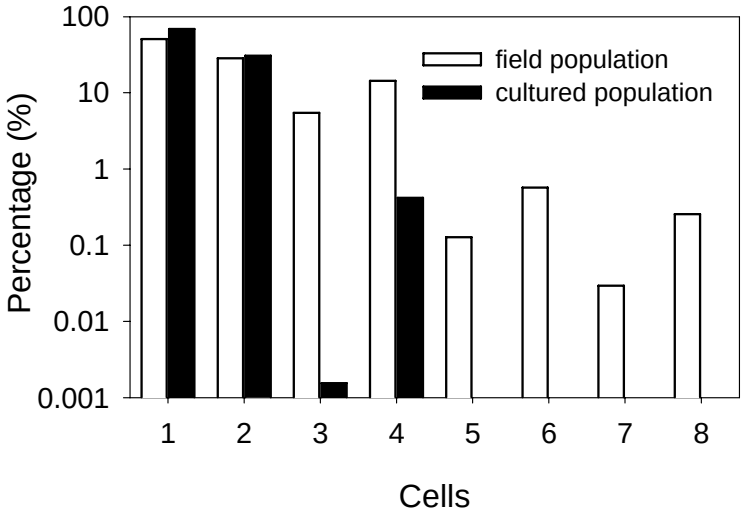

Fig. 4.4. Frequency distribution of pooled chain structure in the field and cultured populations of *Cochlodinium polykrikoides*.

Grazer exposure experiment

The exposure of *Cochlodinium polykrikoides* to adult females or fresh exudates (0-h storage) of *Acartia tonsa* significantly enhanced its chain length from 1.44 (SD ± 0.038) to 1.59 (SD ± 0.044) and 1.60 (SD ± 0.027) cells chain^{-1}, respectively (Tukey post hoc test, $P < 0.001$ for both, Fig. 4.5). In contrast, the stale *A. tonsa* exudates after the 24-h storage did not significantly increase the chain length of *C. polykrikoides* (Tukey post hoc test, $P = 0.163$).

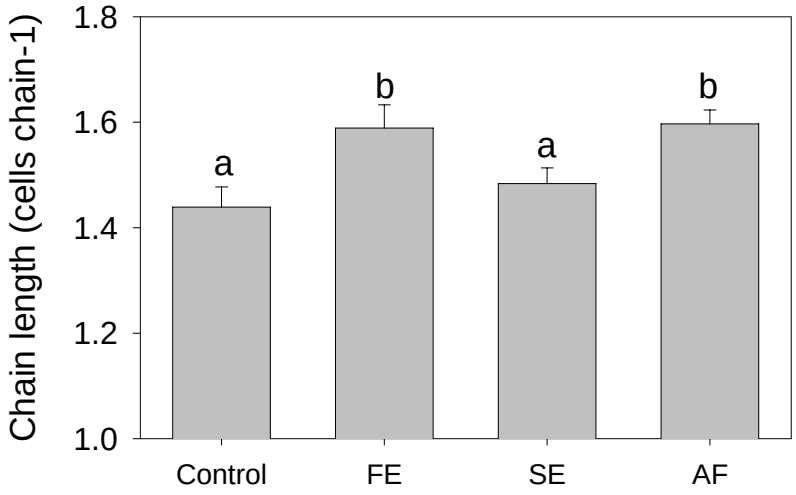

Fig. 4.5. Effects of grazers on chain length (mean + SD) of *Cochlodinium polykrikoides* after a 48-h incubation. Adding the fresh (0 h storage) exudates (FE) and stale (24 h storage) exudates (SE) of the copepod *Acartia tonsa*, and adult females; Letters indicate significant differences among treatments.

Copepod grazing experiment

The experimental suspension of *Cochlodinium polykrikoides* consisted of three types of cells (single cell: 45 ± 3.7 µg C L^{-1}; 2-cell chain: 243 ± 17 µg C L^{-1}; 4-cell chain: 127 ± 9 µg C L^{-1}). The ingestion rate on 4-cell chains was lower than on single cells (Tukey post hoc test, $P = 0.022$) and 2-cell chains (Tukey post hoc test, $P = 0.053$, Fig. 4.6). The difference between ingestion rates on single cells and 2-cell chains was not significant (Tukey post hoc test, $P = 0.88$).

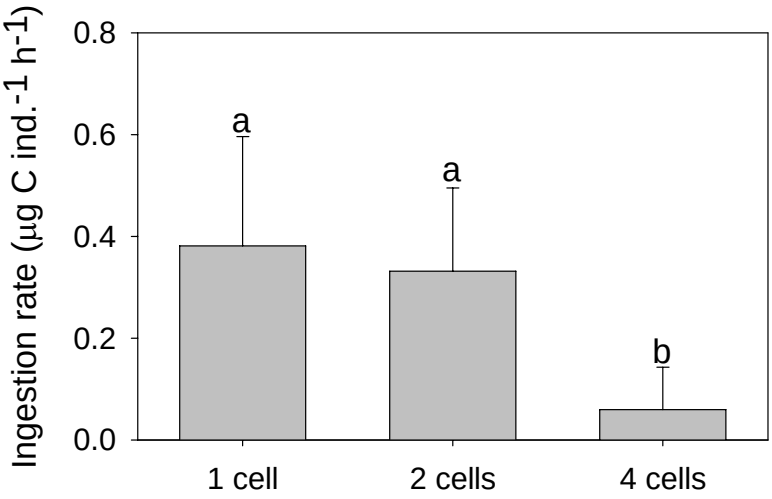

Fig. 4.6. Ingestion rates (mean + SD) of adult females of *Acartia tonsa* on 3 dominant cell types (1, 2, and 4 cells) of *Cochlodinium polykrikoides*. Letters indicate significant differences among treatments.

Nutrient addition experiments

None of the additions of trace metals, nitrate, and phosphate significantly enhanced the chain length of *C. polykrikoides* (Tukey post hoc test, $P > 0.05$ for all, Fig. 4.7a). However, the additions of vitamins either solely or with other nutrients (the f/2 treatment) significantly increased the chain length of *C. polykrikoides* from 1.46 (SD ± 0.058) to 1.60 (SD ± 0.050) and 1.61 (SD ± 0.028) cells chain^{-1}, respectively (Tukey post hoc test, $P < 0.001$ for both). The chain length of *C. polykrikoides* was significantly increased after adding vitamins B_1, B_7, and B_{12} both singly and collectively (Tukey post hoc test, $P < 0.001$ for all, Fig. 4.7b), but did not significantly differ among the vitamin treatments (Tukey post hoc test, $P > 0.35$ for all).

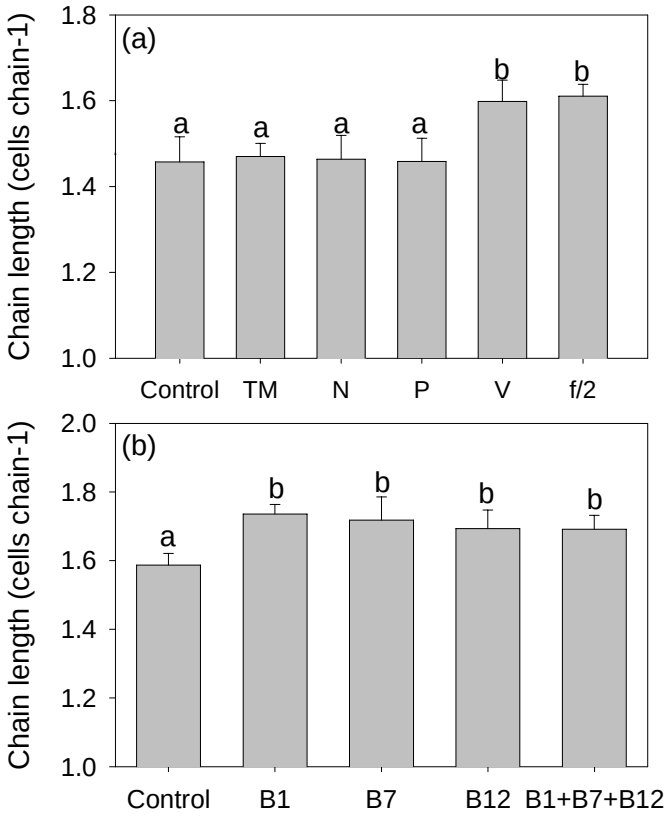

Fig.4.7. Effects of nutrients on chain length (mean + SD) of *Cochlodinium polykrikoides* after a 48-h incubation. (a) Nutrient amendment experiment: adding trace metals (TM), nitrate (N), phosphate (P), vitamins (V), and f/2 medium; (b) Vitamin amendment experiment: adding vitamin B_1, vitamin B_7, and vitamin B_{12} both singly and collectively. Letters indicate significant differences among treatments.

Discussion

My results show that the presence of grazers can induce chain formation in *Cochlodinium polykrikoides*. Chain length of *C. polykrikoides* was significantly increased when exposed to adults and fresh exudates of the copepod *Acartia tonsa*. In addition, the mean chain length of the wild *C. polykrikoides* population facing chronic predation pressure was 42% higher than the cultured population without predators. Furthermore, the chain length of the wild *C. polykrikoides* population was positively correlated to the copepod abundance. Induced chain formation is likely an effective defense against grazing for *C. polykrikoides*. Ingestion rates of copepods on 4-cell long chains of *C.*

polykrikoides were lower than on single cells and 2-cell short chains suggesting chain formation can reduce population mortality due to grazers. Although there was no significant difference between ingestion rates on single cells and 2-cell short chains of *C. polykrikoides* in the feeding experiment, ingestion rates on single cells would likely be higher if food concentrations were normalized. Copepod ingestion exponentially increases with increasing food concentrations (Mauchline 1998) and the concentration of single cells (45 ± 3.7 μg C L^{-1}) was approximately 5-fold lower than 2-cell chains (243 ± 17 μg C L^{-1}) in the present experiment. While ingestion rates on different cell types would ideally be compared at the same concentration, adjusting the ratio of cell types in a suspension of *C. polykrikoides* was not possible.

Reduced grazing mortality by chain formation in *Cochlodinium polykrikoides* is likely due to a prey-predator size mismatch. Clearance rates of *Acartia tonsa* females peak at 14 μm (equivalent spherical diameter, ESD), then decline with increasing food size (Berggreen et al. 1988). The length and width of a single cell of *C. polykrikoides* was 34 and 27 μm, respectively (Jiang et al. 2009). Mean length of *C. polykrikoides* chains was 44.9 μm for the cultured population and 63.9 μm for the field population. Exposure to adults and fresh exudates of *A. tonsa* for 2 d increased the mean chain length from 48.9 to 54.4 μm. The calculated ESD of single cells, two-cell chains, and four-cell chains of *C. polykrikoides*, were approximately 33, 42, and 52 μm, respectively. Thus, chain formation in *C. polykrikoides* would reduce clearance rate of *A. tonsa* by increasing algal size substantially beyond the ideal food size (14 μm) for this species (Berggreen et al. 1988). In addition to creating a size mismatch, induced chain formation in *C. polykrikoides* may also affect grazing mortality via behavioral changes. Chain formation may increase swimming speed of *C. polykrikoides*, since the swimming speeds of two dinoflagellates *Gymnodinium catenatum* and *Alexandrium affine* increased by 1.5 times by increasing from single cells to 4-cell chains (Fraga et al. 1989). Successful prey escape depends on their remote detection and motility (Titelman and Kiørboe 2001, Kiørboe et al. 2009). Assuming no change in predator detection, chain formation would increase escape ability of *C. polykrikoides* by increasing swimming speeds. In some cases, however, enhanced swimming speed increases encounter rate between algal prey and predators (Titelman 2001). Predation risk is mainly determined by the probability of successful prey escape and the rate of encounter between predator and prey. Although the net consequence of chain formation on predation risk is not available without qualifying these processes, I would offer behavioral changes caused by chain formation as a candidate explanation for the reduced grazing mortality, which should be explored in the future studies.

Physical contact with grazers was not necessary for the induced chain formation in *Cochlodinium polykrikoides* since both the fresh *Acartia tonsa* exudates and live adults significantly increased chain length. Hence, it seems that *C. polykrikoides* detects the chemical cues of potential grazers and initiates chain formation, a putative defense system. Success of induced plasticity is dependent on the predictability of a changing environment (Agrawal 2001). Zooplankton grazing varies greatly with time and space

due to their heterogeneous distribution and composition. Degradable chemical cues are more likely to reflect the real-time risk of grazing. The stale *A. tonsa* exudates after the 24-h storage did not induce chain formation in *C. polykrikoides*. A similar phenomenon was observed in a *Desmodesmus – Daphnia* interaction (Lürling and Van Donk 1997). These results suggest that the chemical cues released by aquatic grazers are not persistent. The rapid degradation ensures the reliability of the chemical cues since they reflect the actual not the past risk of grazing. Increased chain length in *C. polykrikoides* when exposed to live *A. tonsa* is due to both induced chain formation and selective feeding on single cells. Interestingly, chain length of *C. polykrikoides* when exposed to live adults was not significantly higher than that exposed to fresh exudates of *A. tonsa*, suggesting selective feeding did not obviously contribute to the increased chain length in this 48-h incubation. The ingestion rates of *A. tonsa* on *C. polykrikoides* at 400 cell mL^{-1} were high (1.42-2.33 µg C ind^{-1} h^{-1}) during the first 6 h and subsequently declined to very low values (0.13-0.22 µg C ind^{-1} h^{-1}, author's unpubl. data). Thus, low feeding rates of *A. tonsa* may explain the limited contribution of selective feeding to increased chain length in *C. polykrikoides* when exposed to live females for 48 h. Regardless, the increased chain length in *C. polykrikoides* when exposed to fresh *A. tonsa* exudates firmly indicated the induced chain formation by copepods. Copepod abundance only explained approximately 36% of the variation in chain length during a field bloom of *C. polykrikoides*, implying other unknown factors may contribute to chain formation in *C. polykrikoides*. Except for grazers, the natural population of *C. polykrikoides* experiences complicated physical environments compared to the cultured population. Intermediate turbulence facilitates chain formation in the dinoflagellate *Alexandrium catenella* relative to high turbulence and no turbulence (Sullivan et al. 2003). Thus, unmeasured hydrographic flow fields in this study may account for some variation of chain size during a bloom of *C. polykrikoides*. In addition, another unmeasured, but potentially important, factor, nutrients, may also contribute to partial variation of chain size of *C. polykrikoides* in the field.

The chain structure of *Cochlodinium polykrikoides* varied in the cultured population as a function of growth stages, with chains being almost completely absent in the stationary growth stage. The pH in the *C. polykrikoides* cultures remained fairly constant ranging from 7.8 to 8.8 and did not significantly relate with chain length (author's unpubl. data). Thus, the variation of chain length in the cultured population implies that nutrients might influence chain formation. Nutrient addition experiments indicated that chain formation of *C. polykrikoides* was stimulated by B-vitamins and not by inorganic nutrients or trace metals. Further experiments demonstrated that vitamins B_1, B_7, B_{12} were each capable of increasing the chain length of *C. polykrikoides*. B-vitamins are involved in multiple biochemical pathways and serve as enzyme cofactors and antioxidants in algal metabolism (Croft et al. 2006). A compilation of 306 species reveals that >50% algae require B_{12}, while 22% required B_1 and 5% required B_7 to grow (Croft et al. 2006). I have found *C. polykrikoide*s had an absolute requirement for B_{12} and B_1 for growth (author's unpubl. data). Trace amounts of these vitamins in estuaries which host *C. polykrikoide*s blooms can influence phytoplankton productivity, succession, and their

interactions with other organisms (Gobler et al. 2007). To my knowledge, the present study is the first report on the role of vitamins in algal chain formation. While the mechanism by which B-vitamins facilitate chain formation in *C. polykrikoides* is unknown, B vitamins may function as cofactors for enzymes involved in this process. Given the universal response to all vitamins and the well known heterotrophic nature of dinoflagellates, B-vitamins may also serve as an organic carbon source to *C. polykrikoides* and thus enhancing its cellular carbon supply, irrespective of photosynthesis. Regardless of the mechanism, these results clearly demonstrate that while grazers can induce chain formation, an ample supply of B-vitamins is also required for *C. polykrikoides* to undergo chain formations.

Induced chain formation by grazers may influence the population dynamics of *Cochlodinium polykrikoides*. The nutritional value of *C. polykrikoides* to *Acartia tonsa* ranged from beneficial to deleterious with increasing concentration from 100 to 1000 μg C L^{-1} (Jiang et al. in press). In view of this density-dependent nutritional value, I have proposed that slow-growing *C. polykrikoides* might be palatable to predators when their densities are low, but kill predators when they obtain advantages over competitors at high densities. A crucial question is how *C. polykrikoides* can avoid being completely grazed down at low densities prior to bloom formation. Induced chain formation by grazers provides *C. polykrikoides* with a morphological defense against grazing, irrespective of cell densities. Although *C. polykrikoides* cells are nutritionally beneficial to copepods at low densities, copepods would not over-graze them due to their ability to form chains. Thus, the induced chain formation may be especially important for the persistence of *C. polykrikoides* prior to bloom formation.

Since the resources which may be allocated to all traits are limited, defenses against predation may lead to a fitness cost in other traits (Litchman and Klausmeier 2008). A trade-off between colony formation and growth has often been proposed for phytoplankton (Litchman and Klausmeier 2008), although this expected negative relationship has not been observed in many phytoplankton (Lürling and Van Donk 1997, 2000, Tang et al. 2008). Since photon absorption per unit pigment can be higher in single cells than in colonies (Beardall et al. 2009), chain formation in *C. polykrikoides* may reduce the specific light absorption coefficient for pigment molecules due to the "package effect". Another possible cost of chain formation is the increased diffusive limitation of nutrients. The thickness of a diffusion boundary layer around all objects in a fluid is positively related to the size of objects. When the concentrations of nutrients are low, diffusive limitation is more likely in colonies than in single cells (Beardall et al. 2009). In addition, the biochemical process of chain formation would cause additional energy expenditure. Finally, chain formation in *C. polykrikoides* may increase the risk of infection with pathogens, which may easily spread from one cell to others in a chain. These possible costs of chain formation may impose an evolutionary constraint on responses to natural selection favoring chain formation in *C. polykrikoides*. This constraint may partially explain the dominance of single cells and 2-cell chains and the low degree of the morphological plasticity of *C. polykrikoides* in cultures where predators

are absent. The principle of economy of design implies that unused structures of organisms may be reduced and lost since it is costly to develop and maintain them (Agrawal 2001). The strain CP1 of *Cochlodinium polykrikoides* has been maintained in the laboratory without grazing pressure for 3 years after isolation. Thus, the costs of the chain formation may outweigh the benefits for *C. polykrikoides* cultures. My observation supported the theoretical predication to some degree. The mean chain length of *C. polykrikoides* in the cultured population was 42% lower than the field population. The natural population had more long chains (>2 cells) than the culture of *C. polykrikoides*. Similarly, the maximal chain lengths of *Skeletonema costatum* were lower in the batch culture than in the natural population (Takabayashi et al. 2006). However, I must keep in mind that the natural and cultured populations experience different exposures of not only grazers but also other ecological factors, such as turbulence and nutrients, which may also affect chain formation but have not been measured in the present study.

Understanding the adaptations of species to interactions with other species is an important goal of ecology and the study of evolution. Although the dinoflagellate *Cochlodinium polykrikoides* forms chains with multiple cells in both the field and cultured populations, the field population displayed a greater morphological plasticity. Grazer addition experiments and the positive relationship between the chain length and grazer abundance in the field suggest that the presence of grazers induced chain formation in *C. polykrikoides*. The chemical cues of *Acartia tonsa* were water soluble and degradable, which may ensure the reliability of the chemical cues since they reflect the actual not the past risk of grazing. In addition, chain formation in *C. polykrikoides* was also enhanced by B-vitamins. Chain formation in *C. polykrikoides* may serve as a morphological defense since long chains suffered lower grazing mortality than single cells. This ecological strategy may permit *C. polykrikoides* cells to avoid being completely grazed during non-bloom periods.

Chapter 5 Population dynamics of *Acartia tonsa* during a *Cochlodinium polykrikoides* bloom

Introduction

Zooplankton populations affect primary production, structure food webs, modulate fishery production, and shape biogeochemical cycles in the ocean. Traditionally, zooplankton dynamics have been thought to be controlled by the variability of physical processes, particularly unpredictable fluctuations, at all spatial and temporal scales ranging from ocean circulation to turbulence (Daly and Smith 1993). On the other hand, zooplankton dynamics are also regulated by food resources, with phytoplankton dynamics strongly influencing zooplankton production and biomass (Martin 1965, Lonsdale 1981). These factors directly impact birth and death rates independently of zooplankton density. However, evidence for density-dependent regulation that constrains the population size within certain limits is emerging. Egg mortality of *Temora longicornis* in Long Island Sound was regulated by an unknown density-dependent force (Peterson and Kimmerer 1994). Cannabalism has been reported to control populations of *Calanus finmarchicus* (Ohman and Hirche 2001, Ploude et al. 2009).

Growth and mortality rates of zooplankton together regulate their population fluctuations and standing stocks, although two processes have been investigated asymmetrically. The majority of marine zooplankton studies have focused on factors relating to population growth, including feeding, metabolism, development, egg production and egg hatching. This emphasis on growth processes in marine zooplankton studies probably originates to some extent from the view of a bottom-up controlled marine ecosystem structure (Frank et al. 2007), where zooplankton biomass and production are determined by phytoplankton availability. However, some recent studies have shown that intra- or inter-specific predation was the primary agent of zooplankton mortality (Ohman et al. 2001, Hirst et al. 2007, Ploude et al. 2009). In addition, methodological easiness and precision also contribute to massive studies on population growth. Zooplankton feeding, metabolism, development, egg production and hatching can be accurately measured by short-term incubations. In contrast, mortality estimates in marine field populations are complicated and not robust (Ohman and Wood 1995, Aksnes et al. 1997). Top-down controls on zooplankton populations are multiple, including predation, parasitism, and senescence, and variable in the field and it is almost impossible to precisely simulate those processes in incubation experiments. Furthermore, the assumptions of current techniques for estimating mortality are difficult to meet, particularly at sea. For example, the vertical life table approach requires constancy in the

parameters, while the horizontal life table approach assumes negligible effects of advection (Aksnes et al. 1997). Although usually overlooked, mortality is clearly important for understanding a range of ecological phenomena, from behavioral and life history traits, to population dynamics, to ecosystem functions (Ohman and Wood 1995).

Many studies of zooplankton dynamics are performed during phytoplankton blooms comprised of nutritious algae (Ohman et al. 2001, Hirche et al. 2001). In contrast with these blooms, harmful algal blooms (HABs) usually cause negative impacts on aquatic ecosystems. HABs have increased in frequency, duration, and distribution in recent decades due to eutrophication, species dispersal or introduction, altered food webs due to increased aquaculture and overfishing, climate changes, and positive feedback mechanisms (Sunda et al. 2006, Heisler et al. 2008, Buskey et al. 2008). Zooplankton grazing may affect initiation of HABs and transfer toxins along food webs (Turner and Tester 1997, Buskey et al. 2008). Although some field investigations have been conducted, focusing on harmful impacts of blooms on zooplankton feeding, egg production, and egg hatching (Jansen et al. 2006, Badylak and Phlips 2008), none of the studies have quantitatively assessed the population dynamics of zooplankton during HABs, especially some vital rates.

The goal of this study was to determine the relative importance of a harmful alga and density-dependent regulation in controlling a zooplankton population during a HAB event. A high-frequency (sampling interval 2 d) time series of *Acartia tonsa* was investigated during a bloom of *Cochlodinium polykrikoides*. I focused on the early life stages of copepods where peak mortality occurs (Ohman et al. 2008). My results showed that *C. polykrikoides* was the main causative agent for embryonic mortality of *A. tonsa* and copepod-density-dependent birth rate was the secondary force for the population regulation.

Materials and methods

Population abundance

Field sampling was conducted every other day in Old Fort Pond, Shinnecock Bay, NY, from August 29 to October 18, 2008. Old Fort Pond is a semi-enclosed system with a narrow channel connecting to Shinnecock Bay (Fig. 5.1). The water depth was approximately 1.5 m and seawater was vertically well mixed. Seawater temperature and salinity were measured by a thermometer and a refractometer. Whole seawater samples (120 mL) were preserved in 5% Lugol's iodine for enumeration of *Cochlodinium polykrikoides*. Zooplankton samples were collected by filtering 20 to 50 L of seawater through a 64-µm mesh and the contents on the mesh were preserved in 5% formalin buffered with hexamethylentetramin.

At least 400 cells or 20 mL of seawater sample were counted using a Sedgewick Rafter counting chamber under a compound microscope to determine *Cochlodinium*

polykrikoides density. At least 100 individuals for each copepod stage or a whole zooplankton sample were counted under a dissecting microscope to determine stage-specific abundance of *Acartia tonsa*.

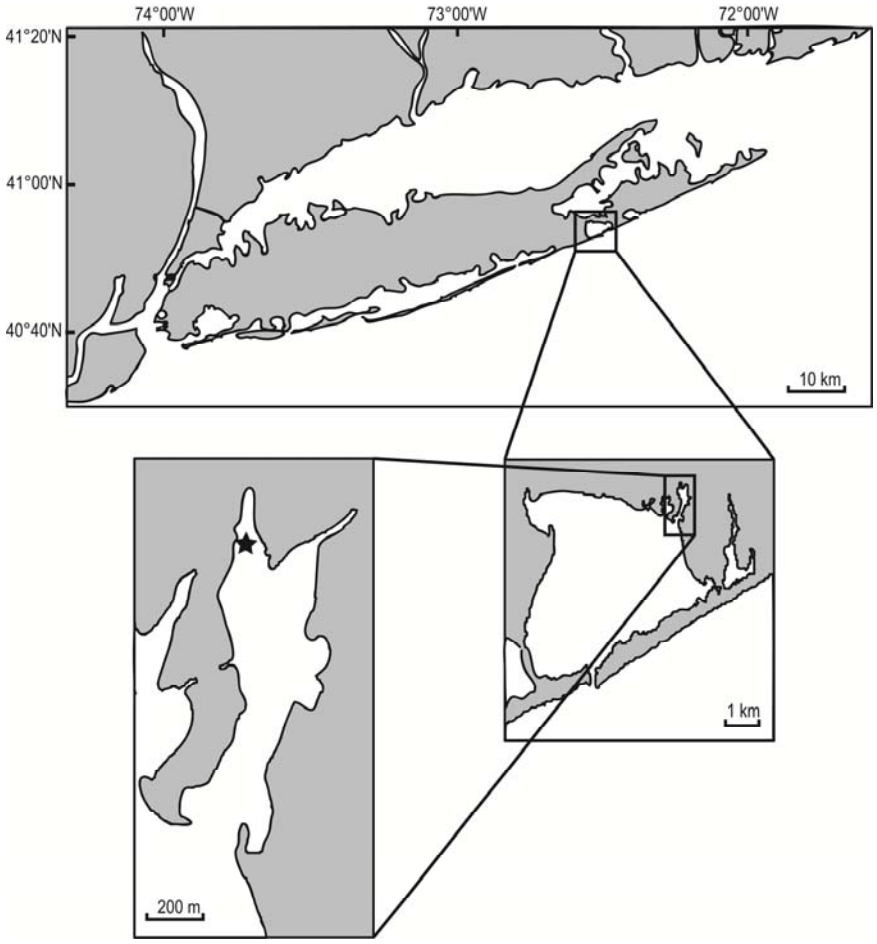

Fig. 5.1. Sampling site in Old Fort Pond, Shinnecock Bay, New York.

Egg production rates and hatching success

Live copepods were collected using a 200-µm zooplankton net and transferred to the laboratory within 1.5 h. Two healthy females of *Acartia tonsa* were transferred into a 5-cm diameter glass dish filled with 50 mL of 64-µm filtered, ambient seawater. A 200-µm mesh was fixed above the bottom of a dish to minimize egg cannibalism. Experiments were conducted in a temperature-controlled incubator at ambient temperature and light cycle with 5 – 10 replicates. All eggs and nauplii were counted after a 24-h incubation.

Eggs were individually transferred into 1-mL wells of a multi-depression dish contained within a closed plastic box. Distilled water was added to the bottom of the box to reduce evaporation from the wells (Lonsdale and Levinton, 1985). One mL of 64-µm filtered ambient seawater was added to the wells. Eggs were observed daily for 2 to 3 d.

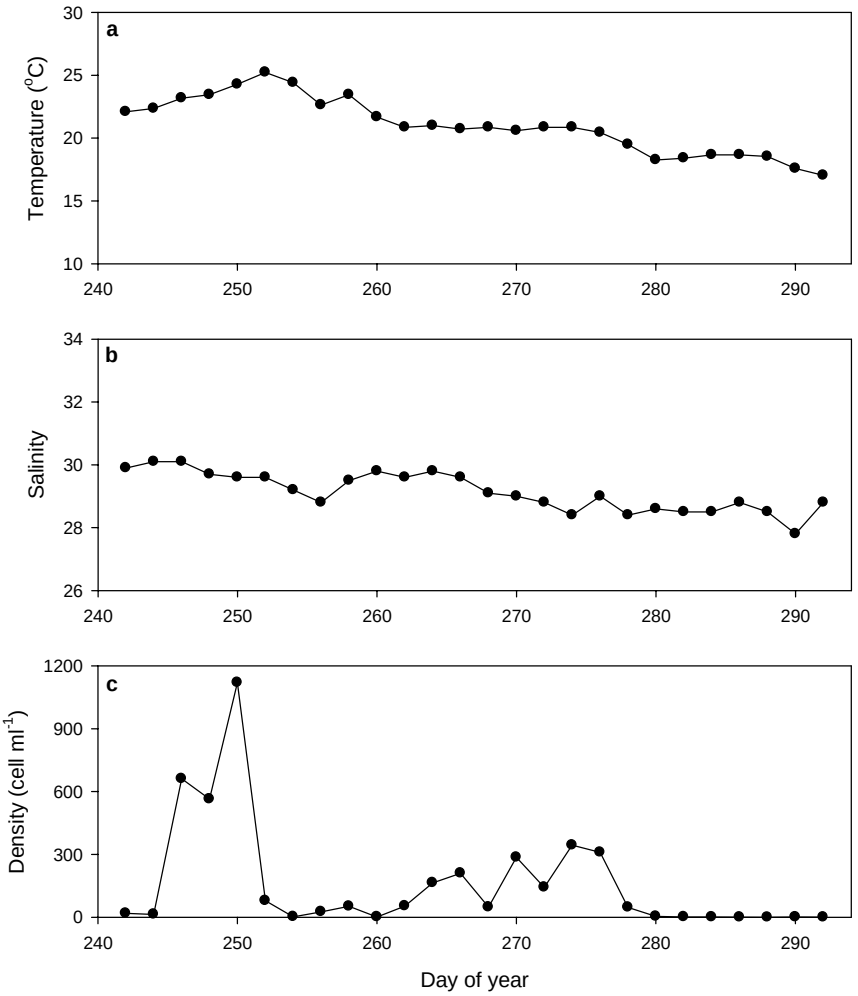

Fig. 5.2. Time series of (a) water temperature, (b) salinity, and (c) cell density of *Cochlodinium polykrikoides* in Old Fort Pond from August 29 to October 18 2008.

Gut fluorescence, ingestion, and grazing pressure

Copepod feeding in the field was estimated using gut fluorescence (Båmstedt et al. 2000). Fifty healthy adults of *Acartia tonsa* were transferred into a beaker filled with 1 L of 200-µm filtered ambient seawater with 5 replicates. The beakers were incubated for 3

h under ambient conditions. Copepods were quickly filtered through a 200-µm mesh and gently rinsed using distilled water. Copepod samples were extracted in 7 mL of 90% acetone for 24 h. The chlorophyll *a* content was measured with a Turner AU-10 fluorometer. Although my protocol required an additional incubation compared to the suggested procedures (Båmstedt et al. 2000), it avoided pigment destruction when sorting frozen samples.

The ingestion rate of *Acartia tonsa* (I, µg C ind.$^{-1}$ d^{-1}) in the field was estimated using the equation:

$$I = kG$$

where k (min^{-1}) is the gut clearance coefficient; and G (ng chl *a* ind.$^{-1}$) is gut fluorescence (Båmstedt et al. 2000). A temperature-dependent value of k for *A. tonsa* was estimated from Calliari et al. (2009). The grazing pressure (*GP*, %) of *A. tonsa* on *Cochlodinium polykrikoides* was estimated by dividing the ingestion rates by the *C. polykrikoides* carbon biomass. A carbon to chlorophyll a ratio of 81.43 (author's unpubl. data) was used for *C. polykrikoides cells*.

Embryonic mortality and birth rates

The recruitment rate of *Acartia tonsa* ($R_{e(t)}$, eggs L^{-1} d^{-1}) was calculated by the multiplication of egg production rate and female abundance at time t. The instantaneous embryonic mortality (the combined egg-through-the-second-naupliar-stage, egg-N2) of *A. tonsa* as a function of time ($m_{e(t)}$, d^{-1}) was calculated using the following equation (Ohman and Hirche 2001):

$$m_{e(t)} = \frac{-\ln\left(\dfrac{N_{n(t)} m_{n(t)}}{R_{e(t-a_{e(t)})}(1-e^{-m_{n(t)}a_{n(t)}})}\right)}{a_{e(t)}}$$

where $N_{n(t)}$ (ind. L^{-1}) is the abundance of the combined first 2 naupliar stages at time t; $m_{n(t)}$ is the mortality of the first 2 naupliar stages at time t; $R_{e(t-a_{e(t)})}$ is the egg recruitment at time $t - a_{e(t)}$; $a_{e(t)}$ is the embryonic duration at time t; and $a_{n(t)}$ is the duration of the first 2 naupliar stages at time t. The value for $m_{n(t)}$ was estimated by the single negative exponential mortality model (Hirche et al. 2001). The values of $a_{e(t)}$ and $a_{n(t)}$ were calculated using Belehradek's equation:

$$a = \alpha(T + \beta)^{-2.05}$$

where a is duration time in days and T is seawater temperature. The coefficients (α and β) *of A. tonsa* were from McLaren et al. (1969) for eggs and Leandro et al. (2006) for naupliar stages. The percentage of mortality at egg hatching was calculated by the ratio of

mortality attributable to the production of nonviable eggs to the total embryonic mortality. Such combined stages reflect mortality between the production of eggs and the end of N2.

The birth rate of *Acartia tonsa* ($b_{(t)}$, d^{-1}) was calculated using the following equation (Ohman and Hirche 2001):

$$b_{(t)} = \ln\left(1 + \frac{R_{e(t)}}{2N_{f(t)}}\right)$$

where $R_{e(t)}$ is egg recruitment rate, and $N_{f(t)}$ is the abundance of adult females at time t. Estimations of grazing pressure, embryonic mortality, and birth rate were made through day 278 because the temperature obviously decreased and *C. polykrikoides* cells began to disappear from then on.

Statistical analyses

A cross-correlation function was used to evaluate the strength and direction of time-lagged relationships between time series data. Correlation and regression analyses were used to explore relationships between variables. All statistical analyses were conducted using the SPSS 16.0 statistical package.

RESULTS

Seawater temperature increased slowly from day 240, peaked on day 252, and thereafter generally declined (Fig. 5.2a). The mean temperature was 20.1°C (SD ± 2.2°C) during the study. Variation in salinity was low with a mean of 29.1 (SD ± 0.6, Fig. 5.2b). The cell density of *Cochlodinium polykrikoides* was highly variable (Fig. 5.2c). Although the cell densities were high, ranging from 564 to 1,120 cells mL^{-1}, during the peak from day 246 to 250, cell densities were relative low (<400 cells mL^{-1}) on most sampling days of the investigation (Fig. 5.2c). The abundance of *Acartia tonsa* nauplii showed considerable variability with several peaks (Fig. 5.3). Adults and late-stage copepodites peaked on day 256, then gradually dropped to an undetected level, especially after day 278 (Fig. 5.3).

The egg production rate of *Acartia tonsa* ranged from 0 to 34.4 eggs $female^{-1}$ d^{-1}, with a mean of 12.5 eggs $female^{-1}$ d^{-1} (SD ± 9.8 eggs $female^{-1}$ d^{-1}, Fig. 5.4a). The egg hatching success varied between 55.6 and 100% with a mean of 85.2% (SD ± 11.6%). The gut fluorescence (ng chl *a* ind^{-1}) of *A. tonsa* adults peaked on day 250, followed by a substantial decline on day 252, then a general increase until day 266, and finally another decrease (Fig. 5.4c). The ingestion rate of *A. tonsa* on *C. polykrikoides* exhibited the same temporal variation (Fig. 5.4d).

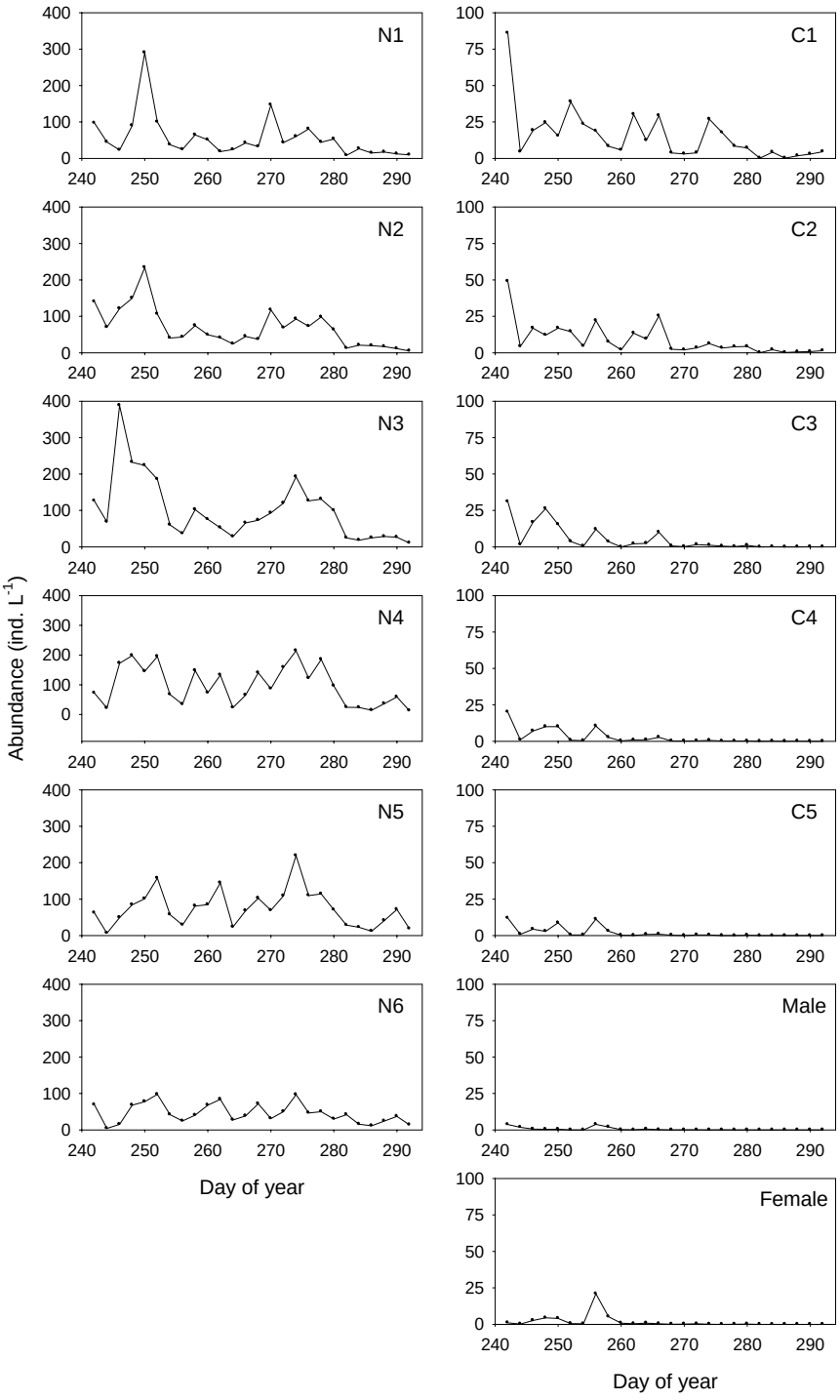

Fig. 5.3. Abundance of *Acartia tonsa* each stage in Old Fort Pond from August 29 to October 18 2008.

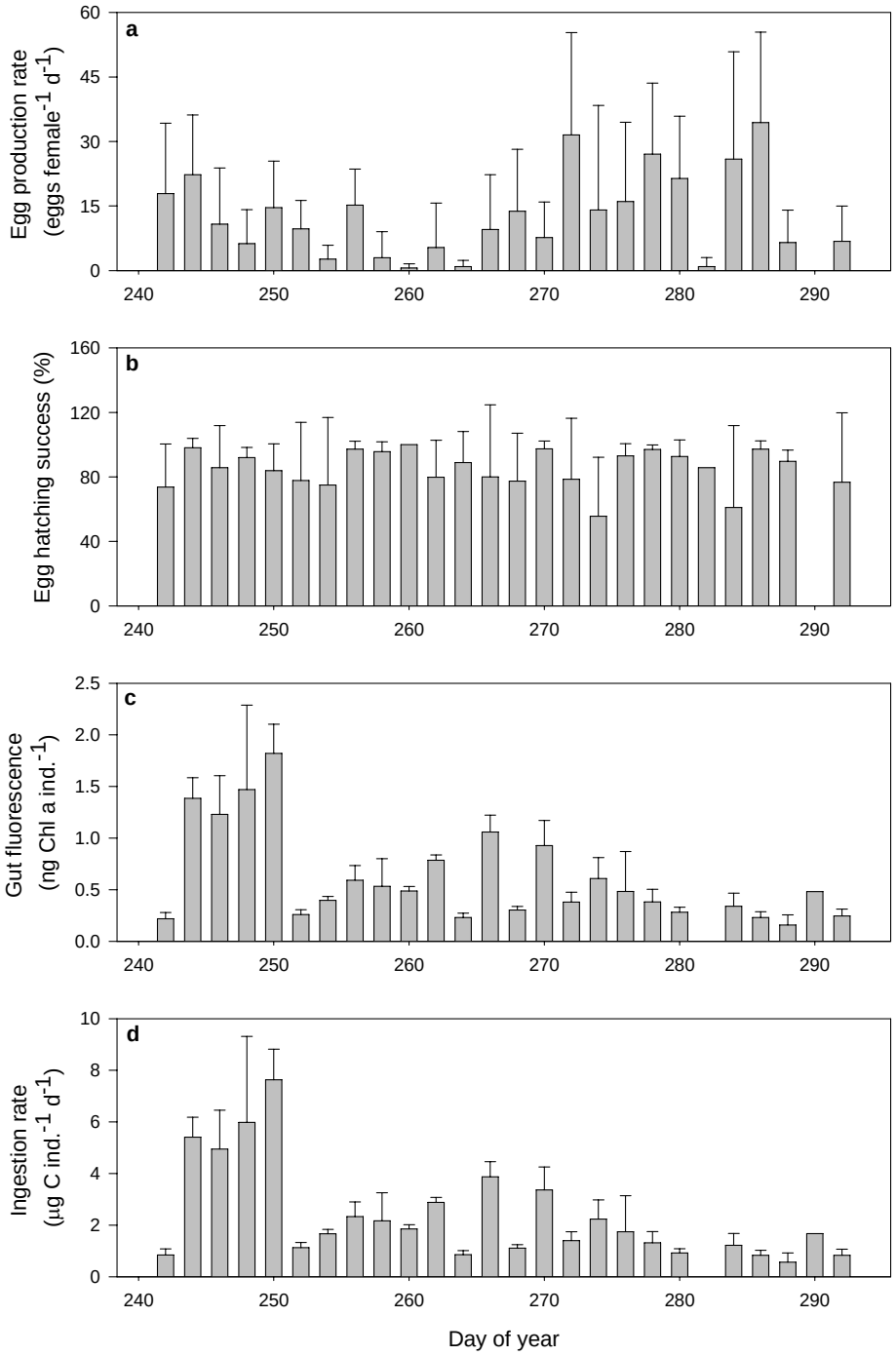

Fig. 5.4. (a) Egg production rate (mean ± SD), (b) egg hatching success, (c) gut fluorescence, and (d) ingestion rate of *Acartia tonsa* in Old Fort Pond from August 29 to October 18 2008

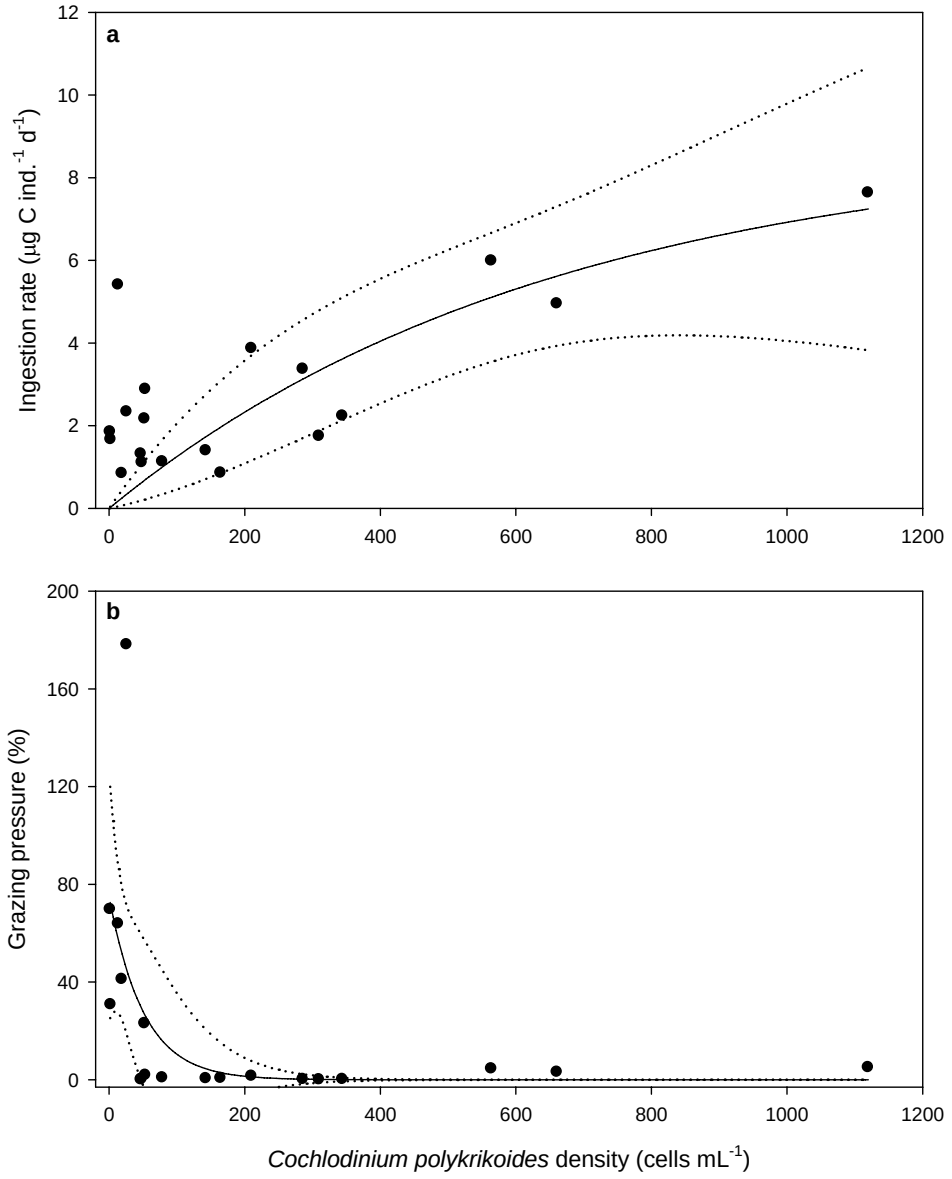

Fig. 5.5. (a) Ingestion rate and (b) grazing pressure of *Acartia tonsa* as a function of cell density of *Cochlodinium polykrikoides* in Old Fort Pond. The regression lines (solid lines) and 95% confidence limits (dotted lines) are shown.

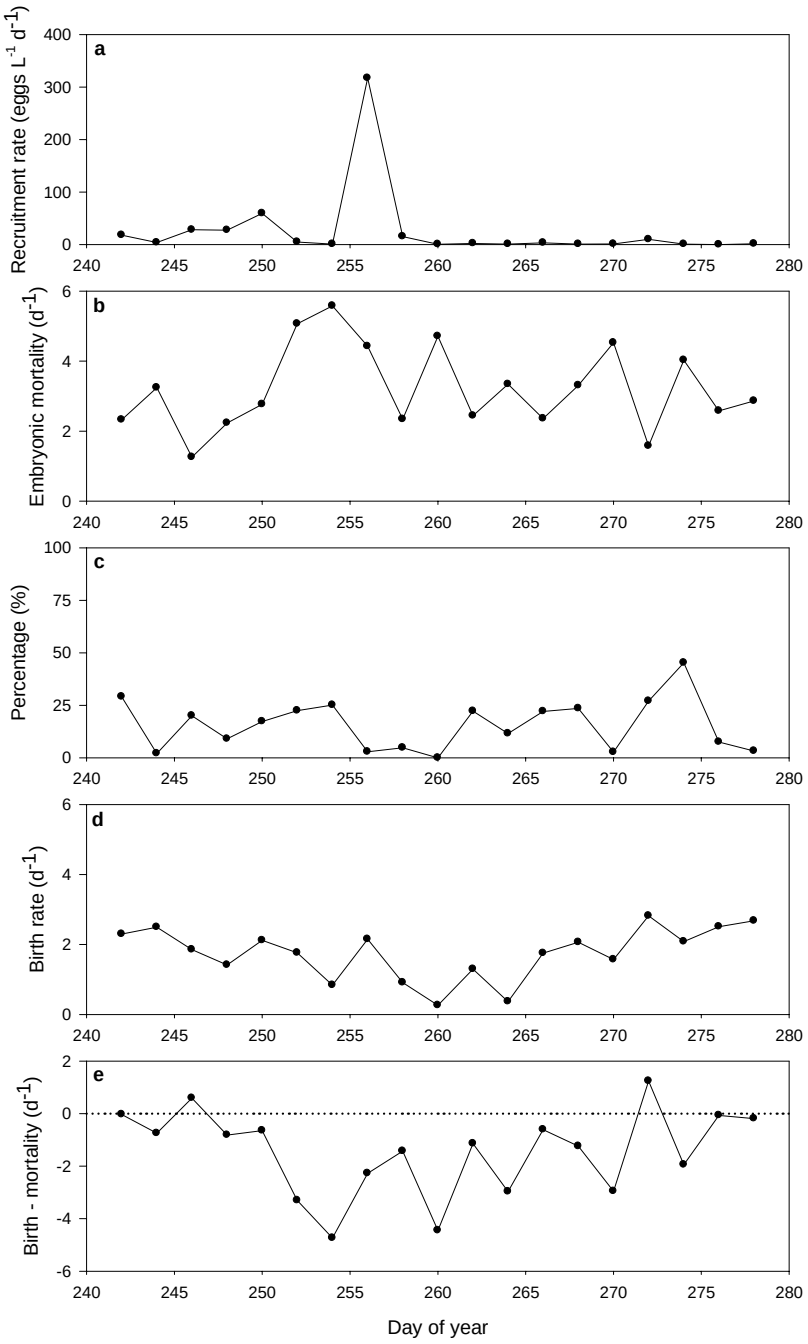

Fig. 5.6. (a) Recruitment rate, (b) embryonic mortality, (c) percentage of mortality at egg hatching, (d) birth rate, and (e) difference between birth rate and embryonic mortality of *Acartia tonsa* in Old Fort Pond during the bloom of *Cochlodinium polykrikoides*.

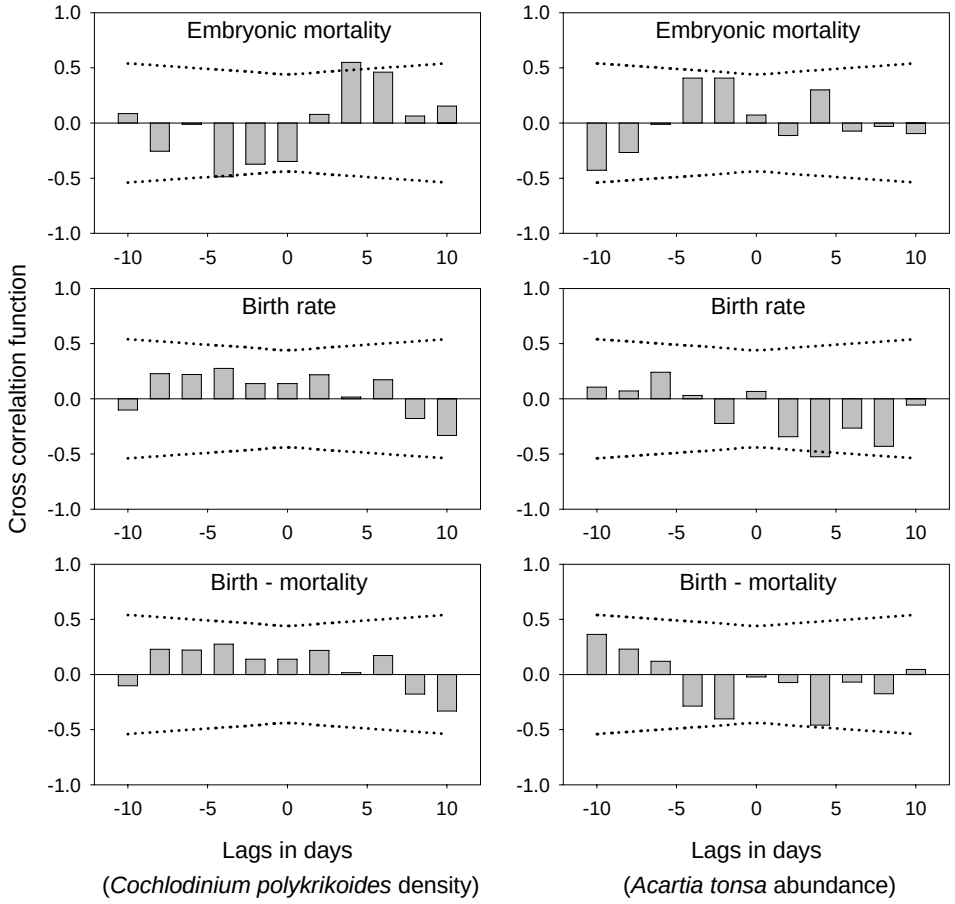

Fig. 5.7. Cross-correlation function (CCF) and 95% confidence limits (dotted lines) for population parameters of *Acartia tonsa* and time series of cell density of *Cochlodinium polykrikoides* (left) and adult abundance of *A. tonsa* (right).

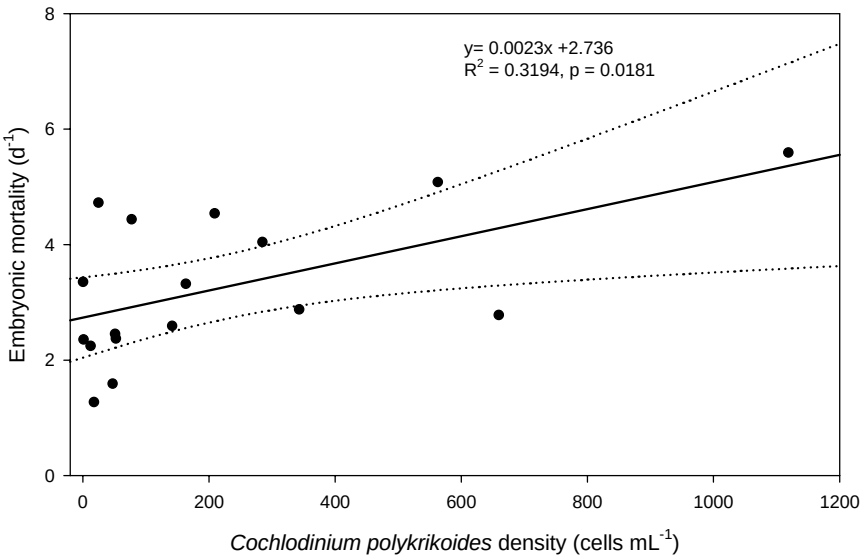

Fig. 5.8. Dependence of embryonic mortality of *Acartia tonsa* on cell density of *Cochlodinium polykrikoides* with a lag of 4 days. The regression line (solid line) and 95% confidence limits (dotted lines) are shown.

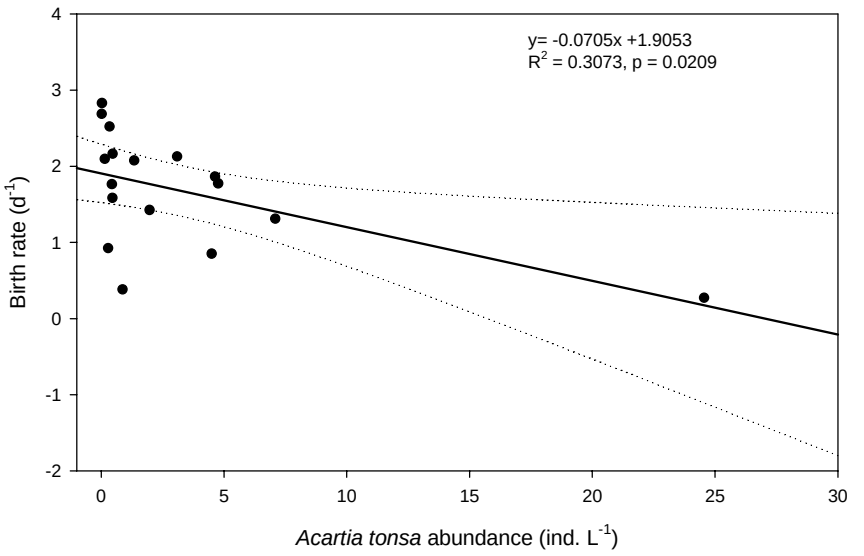

Fig. 5.9. Dependence of birth rate on adult abundance of *Acartia tonsa* with a lag of 4 days. The regression line (solid line) and 95% confidence limits (dotted lines) are shown.

The ingestion rate (I) of *Acartia tonsa* was significantly correlated with the cell density (C) of *Cochlodinium polykrikoides* (Pearson correlation coefficient $r = 0.764$, n = 19, $P < 0.01$). The regression between the two variables was marginally significant ($r^2 = 0.1965$, $F_{1,17} = 4.1563$, $P = 0.0573$) using the type II functional response model (Fig. 5.5a),

$$I = 8.8344(1 - e^{-0.0015C})$$

The daily grazing pressure of the *A. tonsa* population was 30.8 ~ 178.1% of *C. polykrikoides* biomass at low densities (<40 cells mL^{-1}), and decreased to 0.03 ~ 4.98% at high densities (>100 cells mL^{-1}, Fig. 5.5b). Grazing pressure (GP) decreased with increasing cell density of *C. polykrikoides* ($r^2 = 0.3563$, $F_{1,17} = 9.4085$, $P = 0.007$) with an exponential decay (Fig. 5.5b):

$$GP = 74.94e^{-0.0197C}$$

The recruitment rate of *Acartia tonsa* varied considerably with time (Fig. 5.6a). It peaked on day 256 with a value of 317 eggs L^{-1} d^{-1}, then declined to almost 0 eggs L^{-1} d^{-1} (Fig. 5.6a). The embryonic mortality of *A. tonsa* ranged from 1.26 to 5.58 d^{-1}, with a mean of 3.21 d^{-1} (SD ± 1.21 d^{-1}, Fig. 5.6b). Mortality attributable to the production of nonviable eggs was a small fraction of total embryonic mortality, ranging from 0 to 45.2% with a mean of 15.7% (SD ± 12.1%, Fig. 5.6c). Birth rates ranged from 0.26 to 2.82 d^{-1}, with a mean of 1.75 d^{-1} (SD ± 0.74 d^{-1}, Fig. 5.6d).

The results of the cross-correlation function showed that embryonic mortality of *Acartia tonsa* was correlated with cell density of *Cochlodinium polykrikoides* with a lag of 4 d, while birth rate and the difference between embryonic mortality and birth rate were not (Fig. 5.7). On the other hand, birth rate was correlated to abundance of *A. tonsa* adults with a lag of 4 d, while embryonic mortality and the difference between them were not (Fig. 5.7). There were significant relationships between population parameters with abundance of *A. tonsa* adults and juveniles (the 5th copepodites, C5) with a 4-d lag (data not shown). Embryonic mortality of *A. tonsa* was significantly correlated with cell density of *Cochlodinium polykrikoides* with a lag of 4 d (Fig. 5.8, $F_{1,15} = 7.04$, $P = 0.0181$). Birth rate was significantly correlated to abundance of *A. tonsa* with a lag of 4 d (Fig. 5.9, $F_{1,15} = 6.65$, $P = 0.0209$).

Table 5.1 Instantaneous mortality (d^{-1}) in early life stages in broadcast-spawning copepods. HLT: horizontal life table; VLT: vertical life table; Ns: naupliar stage;

Species	Location	Method	Stage	Mortality	Agent	Reference
Acartia tonsa	Old Fort Pond, NY	HLT	egg-N2	3.21 ± 0.74	Harmful alga	this study
Calanus finmarchicus	St Lawrence estuary	VLT	egg-N3	0.659 ± 0.317	Intrapredation	Plourde et al. 2009
Calanus finmarchicus	Norwegian Sea	HLT	egg-N2	1.76	Intrapredation	Ohman and Hirche 2001
Calanus finmarchicus	Georges Bank	VLT	egg-N3	0.34	Intra- and interpredation	Ohman et al. 2008
Calanus finmarchicus	Georges Bank	VLT	egg-N2	0.49 ± 0.06	Intrapredation	Ohman et al. 2002
Calanus finmarchicus	North Sea	HLT	N1	0.34	predation	Eiane and Ohman 2004
Calanus finmarchicus	North Sea	HLT	N2	0.26	predation	Eiane and Ohman 2004
Calanus helgolandicus	English Channel	VLT	egg-N1	1.3 ± 0.307	Interpredation	Hirst et al. 2007
Calanus helgolandicus	Off La Jolla, California	VLT	N3	0.09 – 2.71	Unknown factor	Fager 1973
Temora longicornis	Long Island Sound	HLT	egg	0.46 – 5.3	Density-dependent factor	Peterson and Kimmerer 1994

Discussion

Algal-controlled embryonic mortality

Although impacts of harmful algae on zooplankton have been extensively investigated, this is the first study to determine birth and death rates of a zooplankton population exposed to an HAB event. Myr results show that the population dynamics of *Acartia tonsa* in Old Fort Pond during a bloom of *Cochlodinium polykrikoides* were primarily regulated by two factors: algal-density-dependent mortality and copepod-density-dependent birth rate. The mean of embryonic mortality of *A. tonsa* was 3.21 d^{-1} (SD ± 1.21 d^{-1}) during the bloom, which is higher than rates of copepods in marine systems without HABs (Table 5.1).

My estimated embryonic mortality encompasses both biological and physical loss terms. The results show that *Cochlodinium polykrikoides* was the main source of embryonic mortality of *Acartia tonsa* during the bloom. Compared with all *C. polykrikoides* blooms in the past 6 years (Gobler et al. 2008), the bloom in 2008 was moderate, with a mean cell density of 218 cells mL^{-1} (SD ± 290 cells mL^{-1}). Such a moderate bloom may not seriously impact *A. tonsa* adults. Using cultured cells, my previous studies have shown that *C. polykrikoides* significantly reduced adult survival of *A. tonsa* at high cell densities (>500 cells mL^{-1}, Jiang et al. 2009), but supported good survival at low densities (Jiang et al. in press). However, a moderate bloom of *C. polykrikoides* can negatively affect early life stages of copepods. Early nauplii (N1) of *A. tonsa* (24h LC_{50} at 334 cells mL^{-1}) were approximately 4 times more sensitive to *C. polykrikoides* cultures than adults (24h LC_{50} at 1,383 cells mL^{-1}, Jiang et al. 2009). The embryonic mortality rate of *A. tonsa* increased with increasing cell density of *C. polykrikoides*. Furthermore, the population size of *A. tonsa* was impaired since the differences between birth rate and embryonic mortality were always negative during the *C. polykrikoides* bloom. These results suggest that including early life stages of zooplankton in HAB monitoring programs and research, may be necessary to more fully understand their impacts on ecosystems and provide a more appropriate tool for predicting potential toxicity.

Populations and communities of organisms are simultaneously influenced by top-down (consumer-driven) and bottom-up (resource-driven) controls, although their relative roles usually vary within and among systems (Hunter and Price 1992, Frank et al. 2007). Traditionally, consumers refer to agents that depress population growth, for example predators and pathogens (Hunter and Price 1992). As a dominant alga in the field, *C. polykrikoides* is a bottom-up resource for *A. tonsa*. However, embryonic mortality of *A. tonsa* increased with increasing *C. polykrikoides* density. Thus, a bottom-up food resource alga, to some degree, can control herbivores in a top-down manner. Such a quasi top-down control by a resource suggests that trophic levels may be influenced by multiple, reticulate forces. Therefore, the traditional, linear view on of top-down and bottom-up forces in ecosystems likely over-simplifies population dynamics and community structure in natural systems.

Other potential sources of mortality

Cannibalism has been reported as a major agent for density-dependent mortality in many copepods (Table 5.1). However, cannibalism was not likely the primary agent for embryonic mortality of *Acartia tonsa* in this study since mortality was not correlated with the abundance of adults or adult-C5 combinations. *A. tonsa* is a typical omnivore, evolving two feeding strategies: passive suspension and active ambushing (Lonsdale et al. 1979, Kiørboe et al. 2009). Switching between herbivory and carnivory depends on the relative abundance of prey items (Anraku and Omori 1963, Gifford and Dagg 1988). The mean cell density of *Cochlodinium polykrikoides* was 218 ± 290 cells mL^{-1} (396 ± 526 µg C L^{-1}), while the mean abundance of N1 and N2 for *A. tonsa* was 69.2 ± 62.9 ind. L^{-1} (2.21 ± 2.01 µg C L^{-1}) and 85.5 ± 51.5 ind. L^{-1} (2.74 ± 1.65 µg C L^{-1}), respectively. The dominance of *C. polykrikoides* cells (98.8% in carbon concentration) may have resulted in low cannibalism of *A. tonsa* in Old Fort Pond. Thus, density-dependent mortality due to cannibalism may not have contributed significantly to embryonic mortality of *A. tonsa* during the *C. polykrikoides* bloom. Feeding on smaller size algal species (*Pseudoisochrysis* sp., approximately 5 µm in diameter) might not elicit active ambushing feeding by *A. tonsa*, while larger cells might influence predatory feeding in some manner (Lonsdale et al. 1979). The length of single cell of *C. polykrikoides* is approximately 34 µm. Chain formation (2 ~ 8 cells) is common in natural *C. polykrikoides* populations and thus, greatly increases the effective length of *C. polykrikoides* (Jiang et al. in review). The large size and motility of *C. polykrikoides* could interfere with the feeding mode of *A. tonsa* and release cannibalism pressure on eggs and nauplii.

Predation by other invertebrates on eggs and nauplii of *A. tonsa* was not likely to have influenced embryonic mortality in Old Fort Pond. Since late-stage copepodites and adult *A. tonsa* comprise the large majority of mesozooplankton biomass in summer and fall in Long Island waters (Lonsdale et al. 1996), predation by other copepods on *A. tonsa* was likely minimal. Although some meroplanktonic larvae, such as polychaetes, barnacles, and tintinnids, account for 20 ~ 40% abundance of micrometazoan zooplankton (Lonsdale et al. 1996), these organisms are not likely to feed on eggs and nauplii of *A. tonsa* due to the prey-predator size mismatch (Hansen et al. 1994). The lobate ctenophore *Mnemiopsis leidyi* is an ecologically important gelatinous predator in Long Island estuaries (McNamara et al. 2010), and can feed on eggs and nauplii of *A. tonsa*. However, populations of *M. leidyi* peak in June and almost disappear in late August (McNamara et al. 2010) and I also observed the very low abundance or the absence of *M. leidyi* during this study, suggesting predation by *M. leidyi* may not influence embryonic mortality of *A. tonsa* in this study.

Losses due to advection were likely low during this study. Old Fort Pond is shallow, well-mixed, and experiences persistent, south winds during summer months which maximize its residence time, as evidenced by the dense algal blooms it experiences through the summer and fall (Gobler et al. 2007, 2008). The sinking of eggs to sediments and subsequent consumption by benthic organisms or death due to harmful sediment

conditions, such as hypoxia, could have been a source of embryonic mortality of *A. tonsa* in Old Fort Pond because the average water depth is 2 m. Typical sinking velocities of *A. tonsa* eggs range from 13 to 24 m d^{-1} (Miller and Marcus 1994). Assuming no vertical water movement, *A. tonsa* eggs could settle on the sediments before hatching in Old Fort Pond. However, the persistent wind mixing of this system would return copepod eggs into the water column (Marcus 1996), and greatly lessen egg loss due to sinking.

Copepod-density-dependent birth rate

I present evidence of copepod-density-dependent birth rate of *Acartia tonsa* in Old Fort Pond. In support of my field findings, a laboratory study showed that egg production rate of *A. tonsa* decreased with increasing adult stocking density (Peck and Holste 2006). Density-dependent birth rates may be a mechanism of population regulation via negative feedback in *A. tonsa*. Density-dependent regulation is usually considered to be a mechanism of reducing future competition for resources, especially food resources (Walker 1979, Murray 1994). It is a challenge to explain why copepods exhibited this relationship during an algal bloom when food was presumably abundant. Reproduction and growth of some food-replete cladocerans have been reduced with increasing population density (Matveev 1993, Burns 1995, Lee and Ban 1999). High population density in these cladocerans may depress feeding activities and subsequently reduce growth and reproduction (Matveev 1993, Burns 1995). This potential relationship did not, however, hold for *A. tonsa* in Old Fort Pond, where ingestion rate was not related to population abundance (Pearson correlation coefficient $r = 0.104$, n = 19, $P = 0.673$). The density-dependent birth rate of *A. tonsa* may be caused by chemicals released by copepods. Chemical substances released by high densities of four *Daphnia* species caused a depression of growth and reproduction (Burns 1995). Another explanation for the reduction of birth rate at high densities could be physical interference between individuals. For example, the reduction of egg production in the harpacticoid *Amphiascoides* sp. (Walker 1979) and the calanoid *Centropages typicus* (Miralto et al. 1996) at high densities was caused by physical disturbance between individuals, rather than released chemicals.

Embryonic mortality of *Acartia tonsa*, however, did not show evidence of copepod-density-dependency in Old Fort Pond. The copepod-density-independent embryonic mortality in *Acartia tonsa* was likely due to the high biomass of *Cochlodinium polykrikoides*. Feeding behavior of *A. tonsa* may be affected by the density of *C. polykrikoides*. The presence of *C. polykrikoides* would also impair physiological status of *A. tonsa* via producing harmful compounds (Jiang et al. 2009). Since gut fluorescence of *A. tonsa* was high during my study, active feeding on *C. polykrikoides* may reduce cannibalism. Copepod-density-dependent mortality of *Calanus finmarchicus* eggs through N3 was only promoted at low ambient phytoplankton biomass and not significant at high biomass (Plourde et al. 2009). An alternative explanation for the density-independent embryonic mortality is that the density of *A. tonsa* in Old Fort Pond may have not reached a critical density for such regulation. Antonovics and Levin (1980) argued that organisms regulate populations via reduced growth at moderate density and

increased mortality at high density. The density-dependent mortality of *C. finmarchicus* early life stages has been only observed at high population densities (Ohman et al. 2002, Plourde et al. 2009).

Delayed population regulation

Cochlodinium polykrikoides influenced embryonic mortality of *Acartia tonsa* with a 4-d delay. My estimated embryonic mortality includes any death from eggs to the second naupliar stage (N2). *C. polykrikoides* may impact early life stages of *A. tonsa* via direct and indirect modes. Since N1 and N2 of *A. tonsa* are non-feeding stages, a possible toxicity mode is that *C. polykrikoides* releases harmful compounds that directly reduce naupliar survival (Jiang et al. 2009). Alternatively, harmful compounds produced by *C. polykrikoides* may have deleterious consequences during gametogenesis and spawning of *A. tonsa*, for example as reflected in reduced egg size (Jiang et al. 2009). An impaired reproductive process in *A. tonsa* could lead to low-quality nauplii which may be less apt to survive. I do not know the relative importance of these two alternative toxicity modes in embryonic mortality. *A. tonsa* is an opportunistic copepod that transforms food energy into egg production just 9.5 h after ingestion (Tester and Turner 1990). Development times for eggs, N1, and N2, are 1.27, 0.99 and 1.09 d, respectively, at 20°C (McLaren et al. 1969, Leandro et al. 2006). Thus, the response time from ingestion to death of N2 in *A. tonsa* is approximately 3.8 d via this indirect mode, which is comparable with the observed 4-d delay between cell density of *C. polykrikoides* and embryonic mortality. Even in the event of direct toxicity, the response time of nauplii to released harmful compounds may result in a delayed relationship between cell density of *C. polykrikoides* and embryonic mortality, although it would be less than 4 d.

Density-dependent birth rates of *Acartia tonsa* also showed a 4-d delay in Old Fort Pond. Many density-dependent regulations are characterized by lags and complex dynamic behavior (Turchin 1990). The mechanism(s) responsible for the delayed, density-dependent birth rates in copepods are unknown. Many processes including detecting density change, modulating hormone metabolism, and altering production investment, may contribute to the lagged response of birth rate to adult abundance in copepods.

Grazing pressure

Outbreaks of HABs are determined by the balance between bottom-up factors affecting their growth and top-down factors affecting their losses. Top-down controls mainly include zooplankton grazing (Turner and Tester 1997, Buskey 2008), algicidal bacteria, and viral lysis (Chambouvet et al. 2008). The daily grazing pressure of *Acartia tonsa* (30.8 to 178.1% of the biomass of *Cochlodinium polykrikoides*) at low densities (<40 cells mL^{-1}) in Old Fort Pond is comparable with or greater than the maximum daily growth rate of *C. polykrikoides* (~35%, Kim et al. 2004). However, grazing pressure exponentially declined to negligible levels (<5%) when *C. polykrikoides* exceeded a

density of 100 cells mL^{-1}. Thus, *A. tonsa* is capable of controlling initiation of *C. polykrikoides* blooms but would have little influence on bloom maintenance.

Gut fluorescence values of *Acartia tonsa* during the bloom of *Cochlodinium polykrikoides* were comparable to studies in marine ecosystems without HABs (Calliari et al. 2009 and references therein). The significant increase in ingestion rate with increasing cell density indicated that *A. tonsa* actively fed on *C. polykrikoides* cells in the field. Ingestion rates of *A. tonsa* on *C. polykrikoides* are higher during a field bloom than my previous laboratory studies, which used *C. polykrikoides* cultures and *A. tonsa* populations from non-bloom area (Jiang et al. 2009). My common garden experiments have shown that copepods in bloom areas have evolved more resistance to *C. polykrikoides* and their conspecifics from non-bloom areas. Thus, local adaptation could result in higher feeding of copepods on *C. polykrikoides* in Old Fort Pond. In addition, the higher ingestion of *A. tonsa* in the field is likely due to the dilution effect by concurrent organisms. The *C. polykrikoides* bloom water was less toxic to juveniles of fish and bay scallops than pure culture (Tang and Gobler 2009). Finally, ingestion rates on *C. polykrikoides* in the field may be overestimated using the gut fluorescence method, especially at low cell densities, since I assumed the all gut fluorescence was contributed by *C. polykrikoides* cells.

Chapter 6 Rapid gain and loss of resistance to *Cochlodinium polykrikoides* in *Acartia tonsa*

Introduction

Although ecological and evolutionary processes are traditionally assumed to occupy different timescales, a wave of recent studies has demonstrated their overlap and reciprocal interplay, e.g. eco-evolutionary dynamics, in many biological contexts (Thompson 1998, Post and Palkovace 2009). Evolution occurring on ecological timescales affects population persistence, drives speciation or extinction (Hendry et al. 2007), alters community structure, and shapes ecosystem function (Post and Palkovace 2009). Ecological variables, such as spatial habitat heterogeneity, dispersal (Grant et al. 2007), and phenotypic plasticity (Ghalambor et al. 2007), influence the potential for evolution. Some of the most striking examples of eco-evolutionary dynamics are from predator-prey interactions. Prey can develop numerous defenses to avoid predation and vice versa, creating an evolutionary arms race of adaptation and counteradaptation (Vermeij 1994, Brodie and Brodie 1999, Post and Palkovace 2009). Many empirical and theoretical studies show that selection by predators on prey is much stronger than selection by prey on predators (Vermeij 1994, Brodie and Brodie 1999). The life-dinner hypothesis argues that predators experience weaker selection than prey because it is worse to lose life than to miss a dinner (Brodie and Brodie 1999). This asymmetry in selection would result in faster evolution of prey than predators, leaving predators lagging behind in an arms race. However, a recent study on asymmetric coevolution between garter snakes and newts demonstrated that in over one-third of sampled localities predators were sufficiently resistant to prey toxicity, but prey were not too toxic to be ingested by local predators, making predators ahead of prey in that particular arms race (Hanifin et al. 2008).

An arms race is likely to occur between harmful algae and grazers. Harmful algal blooms (HABs) have increased in frequency, duration, and distribution in recent decades. A wide variety of harmful compounds are produced by more than 200 algal species from 20 genera (Landsberg 2002), which may exert strong selective pressures on aquatic grazers. In the context of such dangerous prey, grazers are under stronger selection to breach algal defenses, otherwise they would risk losing not only their dinner but also their life. An increase in nutritionally poor or even toxic cyanobacteria in eutrophic lakes caused evolution of resistance in the cladoceran *Daphnia* within the time span of little more than a decade (Hairston et al. 1999). The rapid evolutionary change in the *Daphnia* population is due to the evolution of reduced phenotypic plasticity, showing a reduced

degree of sensitivity to cyanobacteria (Hairston et al. 2001). Copepods (Colin and Dam 2002a, 2004) and clams (Bricelj et al. 2005) have evolved greater resistance to toxic *Alexandrium* in populations that are frequently exposed to toxic *Alexandrium* blooms. The resistance to paralytic shellfish poisoning (PSP) in clams is caused by a natural mutation of a single amino acid residue, which greatly reduces the binding affinity of PSP toxins to Na^+ channels (Bricelj et al. 2005). Rapid evolution of resistance to harmful algae in grazers would have the potential to alter population, community and ecosystem responses to HABs. Long-term effects of harmful algae on aquatic organisms may be very different from those predicted from bioassays using non-adapted individuals. Adaptive evolution of resistance could provide a means to mitigate HABs using adapted grazers. On the other hand, the transfer of toxins through the food web might be strengthened due to enhanced ingestion of harmful algae, which may pose a more serious risk to higher trophic organisms and humans.

A crucial unexplored question, whether evolutionary change of grazer resistance is reversible, would shape the ecological consequences of adaptive evolution of resistance to harmful algae in grazers. Aquatic grazers are usually opportunistic herbivores, feeding on numerous algae and protozoa. HABs rarely persist for the whole year, which implies grazers periodically experience selection and relaxation to HABs. Loss and reversal of adaptive traits are a widespread phenomenon. Reverse evolution can occur on small evolutionary time scales and lessen the effects of past history by promoting adaptation (Teotónio and Rose 2001). A limited number of studies on ecological restoration have shown evolutionary reversals following relaxation of an environmental stressor, such as recovery of melanism in peppered moths with mitigation of air pollution (Teotónio and Rose 2001), loss of metal resistance in oligochaetes after the cleanup of contamination (Levinton et al. 2003), and adaptive reversal of acid tolerance in copepods with lake recovery from sulfur induced acidification (Derry and Arnott 2007).

The dinoflagellate *Cochlodinium polykrikoides* produces multiple harmful compounds, seriously affecting copepod feeding, egg production, and survival (Jiang et al. 2009). In addition to chemical defense, *C. polykrikoides* can form chains that lessen grazing mortality (author's unpubl. data). From the perspective of an arms race, copepods are hypothesized to evolve means to breach the chemical and morphological defenses of *C. polykrikoides*. In this study, I investigated the potential for evolutionary response in copepods to *C. polykrikoides*. Evidence for the evolution of resistance was tested in two ways. Resistance of *A. tonsa* populations from bloom and non-bloom areas was compared using a common garden experiment. In addition, copepods from a non-bloom area were subjected to artificial selection of resistance to *C. polykrikoides* for four generations. Both avenues of research demonstrated rapid adaptation of copepods to this alga. To test whether evolved resistance is reversible, the exposure to *C. polykrikoides* was halted and the resultant evolutionary trajectory was tracked for another four generations. My results provide the empirical evidence of the intrinsic capacity for reversal of grazer resistance to harmful algae. Rapid adaptation to harmful algae and loss of resistance in copepods

highlight the need for evolutionary consequences of grazers to be considered in understanding and management of harmful algal blooms.

Materials and methods

Common garden experiment

The dinoflagellate *Cochlodinium polykrikoides* clone CP1 was isolated from Peconic Bay, Long Island, New York, United States of America in 2006. The flagellate *Rhodomonas lens* Pascher and Ruttner (CCMP 739) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The algal cultures were maintained in a temperature-controlled incubator at 20°C with a 14h light: 10 h dark cycle (approximately 50 μmol photons m^{-2} s^{-1}). The cultures were incubated in exponential growth phase with f/2 medium.

Six populations of *Acartia tonsa* were collected from 18 to 25 September, 2009, from known bloom areas (Old Fort Pond, OFP; Great Peconic Bay, GPB; Cold Spring Pond, CSP) and non-bloom areas (Stony Brook Harbor, SBH; Mt Sinai Harbor, MSH; Great South Bay, GSB) in Long Island waters (Fig. 6.1A) using a 202-μm mesh plankton net. Seawater samples (100 mL) were preserved in 5% Lugol's iodine for enumeration of *Cochlodinium polykrikoides* using a Sedgewick Rafter counting chamber. Chlorophyll *a* concentration was measured using a standard fluorometric method. For each site, approximately 500 adult copepods were picked out and their eggs were collected to set up a laboratory culture with the cohort size of approximately 2000 individuals. Copepods were offered *Rhodomonas lens* at a carbon concentration of approximately 600 μg C L^{-1} and maintained at the standard conditions (20°C, 14:10 light-dark cycle, approximately 1 μmol photons m^{-2} s^{-1}). Half of the seawater in the copepod culture medium was replaced with 0.2-μm filtered seawater (FSW, salinity 30) twice a week. Copepods were reared under these conditions for two generations prior to the common garden experiment. The breeding of two generations in a common environment would sufficiently eliminate both maternal effects and environmental variance in most cases (Futuyma 1998).

Egg production rates of *Acartia tonsa* were measured when feeding on *Cochlodinium polykrikoides* and *Rhodomonas lens* at 400 μg C L^{-1} under the standard conditions since *C. polykrikoides* at this concentration depressed egg production rates of *A. tonsa* (Jiang et al. 2009). Adult copepods were acclimated for 24 h to ensure that egg production rates reflected the experimental algal suspension. Copepod resistance (*R*) to *C. polykrikoides* was calculated as the ratio of egg production rates feeding on *C. polykrikoides* (EPR_{CP}) to mean egg production rates on *R. lens* (EPR_{RL}): $R = EPR_{CP}/EPR_{RL}$ (Brodie and Brodie 1999, Hairston et al. 1999, 2001).

Artificial selection experiment

A culture of *Acartia tonsa* from Stony Brook Harbor (non-bloom area) was split into two lines, the selection line and the control line. Copepods in the control line were offered *Rhodomonas lens* at 600 μg C L^{-1} during the whole experiment, while copepods in the selection line were offered a mixed diet of 50% *Cochlodinium polykrikoides* and 50% *R. lens* at a total carbon concentration of 600 μg C L^{-1} because pure *C. polykrikoides* culture at this concentration is lethal to *A. tonsa* (Jiang et al. 2009). Resistance to *C. polykrikoides* in copepods of each line in each generation was quantified using egg production bioassays as described for the common garden experiment. After four generations, when evolved resistance to *C. polykrikoides* was evident in the selection line, their diet was switched to 100% *R. lens*, and copepod resistance in both the control and selection line was measured after another four generations.

Evolutionary rates

Rates of divergence (*h*, haldanes) between any two of six populations were calculated as differences between the population means in units of standard deviation using the formulation: $h = (x_1/s_p - x_2/s_p)/g$, where x_1 and x_2 are mean resistance for each of two populations, s_p is the pooled standard deviation, and g is the number of generations separating the populations (Hendry and Kinnison 1999). Geographically extensive blooms of *Cochlodinium polykrikoides* have occurred in Long Island eastern waters since 2004 (Gobler et al. 2008, author's unpubl. data for blooms in 2008 and 2009). The blooms (cell density >100 cells mL^{-1}) usually last for about one month and seawater temperatures range from 20 to 25°C (Gobler et al. 2008), which implies that approximately two generations of *Acartia tonsa* are exposed to blooms every year (Mauchline 1998). Thus, g was assumed to be 12 generations.

The evolutionary rate (*h*) in the artificial selection experiment was estimated by a linear regression between mean resistance in the unit of standard deviation and generations (Hendry and Kinnison 1999). The slope of the regression line was an average rate of evolution or loss of resistance to harmful algae in copepods.

Results

Common garden experiment

The cell densities of *Cochlodinium polykrikoides* ranged from no detectable cells in the non-bloom areas (SBH, MSH, and GSB) to approximately 3000-6000 cells ml^{-1} in bloom areas (OFP, GPB, and CSP, Fig. 6.1B) in late September, 2009. The chlorophyll *a* concentrations in the bloom areas were also higher than in the non-bloom areas and differed among sampling sites (Fig. 6.1B). Copepod egg production differed among the copepod populations (two-way ANOVA, $F_{5,135} = 3.21$, $P = 0.009$), and food sources (two-way ANOVA, $F_{1,135} = 11.17$, $P < 0.001$), and there was a significant interaction between these two variables (two-way ANOVA, $F_{5,135} = 6.51$, $P < 0.001$). When copepods fed on *Rhodomonas lens*, egg production rates did not differ significantly

among most copepod populations (Hotchberg's GT2 test, $P > 0.1$ for all) with the exception of a slight difference between OFP and GSB (Hotchberg's GT2 test, $P = 0.04$). In contrast, the egg production rates of the copepods from the bloom areas fed *C. polykrikoides* were significantly higher than those from the non-bloom areas (Hotchberg's GT2 test, $P < 0.05$ for all) (Fig. 6.1C). The egg production rates of all copepod populations from the non-bloom areas were dramatically reduced when feeding on *C. polykrikoides* relative to *R. lens* (*t*-test, $P < 0.001$ for SBH, MSH, and GSB). The egg production rates were not significantly different for copepods from OFP and GPB feeding on *C. polykrikoides* versus *R. lens* (*t*-test, $P > 0.05$ for both), but were slightly reduced in the CSP population (*t*-test, $t = 2.32$, $P = 0.03$). The resistance to *C. polykrikoides* significantly differed among the copepod populations (one-way ANOVA, $F_{5,66} = 12.38$, $P < 0.001$, Fig. 6.1D). All copepods from the bloom areas displayed significantly elevated resistance compared to those from the non-bloom areas (Hotchberg's GT2 test, $P < 0.01$ for all, Fig. 6.1D). There was no significant difference in copepod resistance among the bloom areas (Hotchberg's GT2 test, $P > 0.1$ for all among OFP, GPB, and CSP) or the non-bloom areas (Hotchberg's GT2 test, $P > 0.1$ for all among SBH, MSH, and GSB).

Artificial selection experiment

The egg production rates of the copepods in the control line when feeding on *Rhodomonas lens* were significantly higher than those feeding on *Cochlodinium polykrikoides* (*t*-test, $P < 0.001$ for all generations) and were relatively unchanged over generations (Fig. 6.2A). In the selection line, the egg production rates of the copepods feeding on *C. polykrikoides* gradually increased during the first four generations (Fig. 6.2B) and did not significantly differ from those feeding on *R. lens* in generations 3 and 4 (*t*-test, $p = 0.70$ and 0.49), then declined from generation 5 to the end (Fig. 6.2B). The resistance to *C. polykrikoides* was higher in the selection line than that in the control line (*t*-test, $P < 0.05$ for the generations 1-4) and gradually increased during the first four generations (Fig. 6.2C). By the fourth generation of selection, resistance of the selection line was approximately three times that of the control line (Fig. 6.2C). After the food source was changed back to *R. lens* at generation 5 in the artificial selection experiment, copepods in the selection line were still more resistant to *C. polykrikoides* than those in the control line (*t*-test, $P < 0.001$ for generation 5 and $P = 0.042$ for generation 6), but the resistance gradually declined (Fig. 6.2C). At generations 7 and 8, copepod resistance in the selection line and control line was no longer significantly distinguished from that in the control line (*t*-test, $P = 0.34$ for generation 7 and $P = 0.75$ for generation 8).

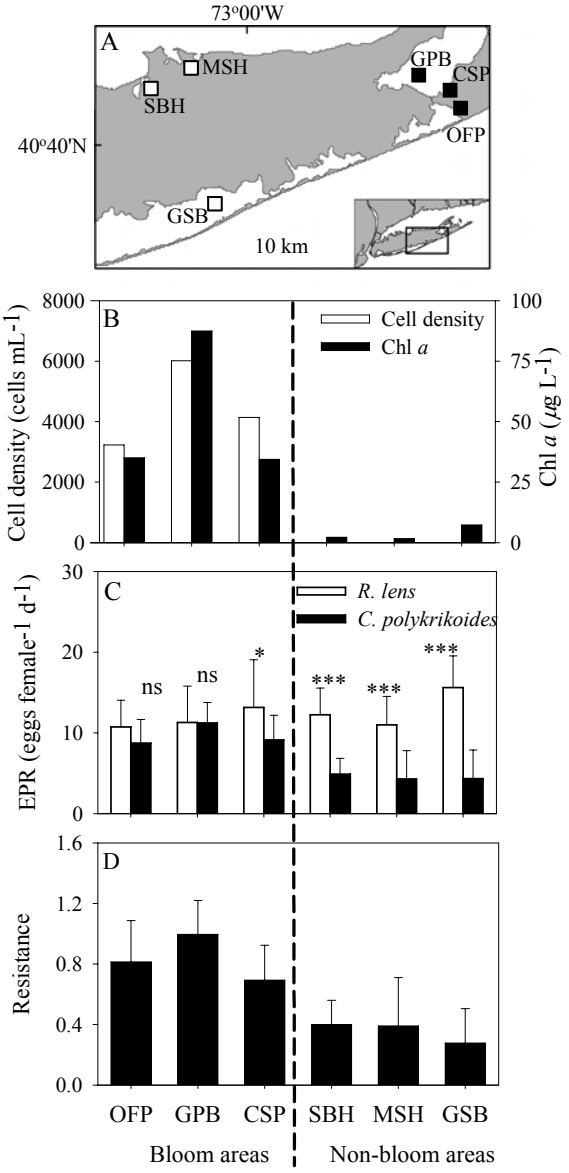

Fig. 6.1. Results of the common garden experiment for six populations of *Acartia tonsa*. (A) Sampling sites in bloom areas (Old Fort Pond, OFP; Great Peconic Bay, GPB; Cold Spring Pond, CSP) and non-bloom areas (Stony Brook Harbor, SBH; Mt Sinai Harbor, MSH; Great South Bay, GSB) of Long Island waters; (B) Cell density of *Cochlodinium polykrikoides* and Chl *a* concentration; (C) Egg production rates of *A. tonsa* feeding on *C. polykrikoides* and *Rhodomonas lens*; (D) Resistance to *C. polykrikoides*. The resistance was indicated by the ratio of egg production rates of *A. tonsa* feeding on the two algal species. Error bars represent standard deviations.

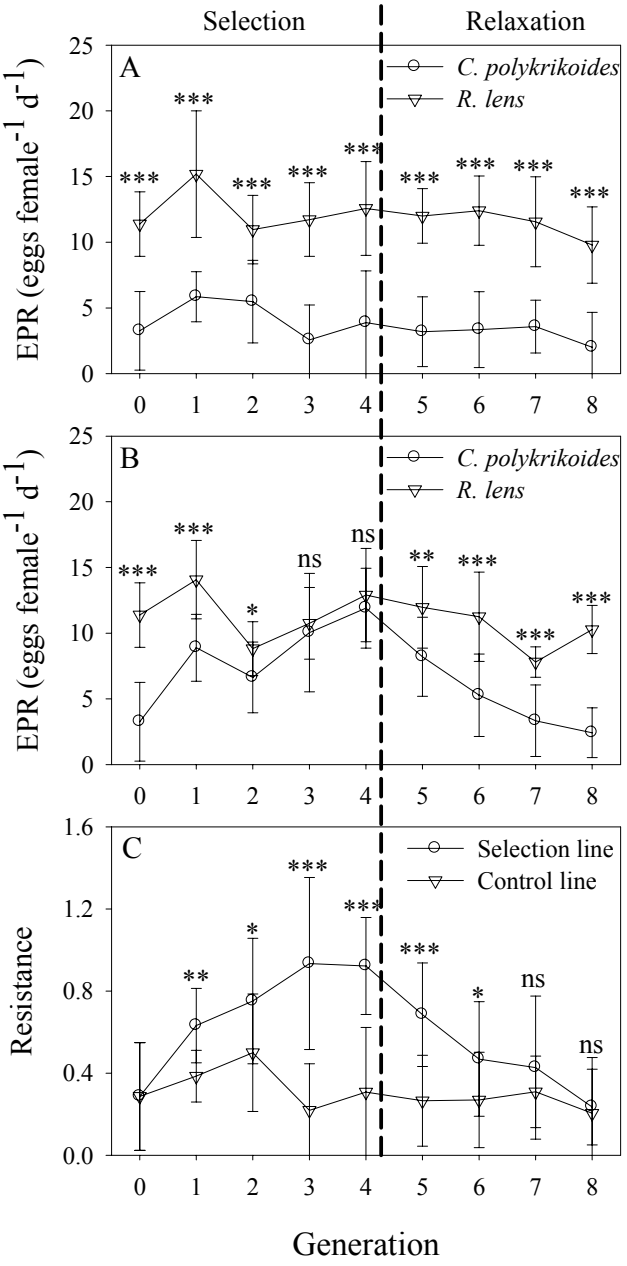

Fig. 6.2. Egg production rates of *Acartia tonsa* feeding on *Cochlodinium polykrikoides* and *Rhodomonas lens* (A) in the control line and (B) in the selection line with 4-generation selection and relaxation, and (C) resistance to *C. polykrikoides* in the artificial selection experiment. Error bars represent standard deviations.

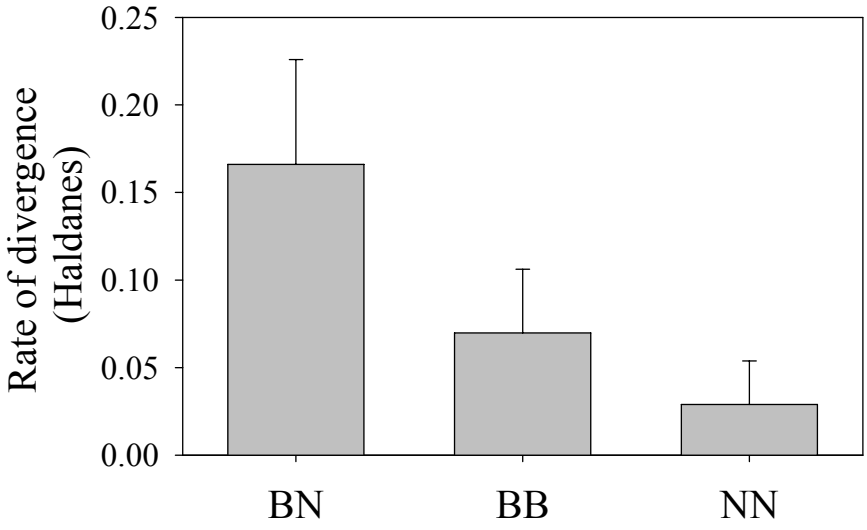

Fig. 6.3. Rates of divergences in the populations of *Acartia tonsa*. BN: rates between bloom and non-bloom areas; BB: rates among bloom areas; NN: rates among non-bloom areas; Error bars represent standard deviations.

Evolutionary rates

The rates of divergence of copepod populations between the bloom and the non-bloom areas (0.17 ± 0.06 haldanes) were significantly higher than those among the bloom areas (0.07 ± 0.04 haldanes, Hotchberg's GT2 test, $P = 0.04$) and the non-bloom areas (0.03 ± 0.02 haldanes, Hotchberg's GT2 test, $P = 0.006$). The rates of divergence of the copepod populations among the bloom areas were slightly higher than those among the non-bloom areas (Fig. 6.3), but the difference was not significant (Hotchberg's GT2 test, $P = 0.716$). In the selection line of the artificial selection experiment, the linear regressions between the mean resistance in units of standard deviation and the generations were significant before ($F_{1,4} = 21.49$, $P = 0.02$) and after the relaxation of the selection ($F_{1,4} = 71.16$, $P = 0.003$, Fig. 6.4). There was no significant difference between the rates of evolution (mean: 0.57, 95% CI: 0.19 – 0.96 haldanes) and loss (mean: -0.62, 95% CI: -0.85 – -0.39 haldanes) of resistance to *C. polykrikoides* in *A. tonsa*.

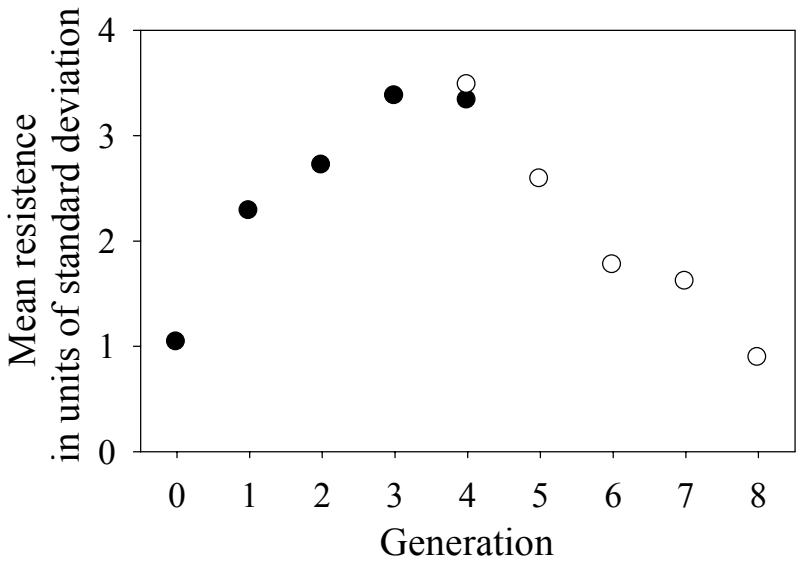

Fig. 6.4. Mean resistance of *Acartia tonsa* in unit of standard deviation with generations in the artificial selection experiment. The slopes of selection (filled circle) and relaxation (open circle) represent evolutionary rates of the gain and loss of resistance, respectively.

Discussion

Acartia tonsa populations in eastern bays of Long Island were much more resistant to *Cochlodinium polykrikoides* than their conspecifics in nearby non-bloom waters. The biogeography of *C. polykrikoides* along Long Island has provided an ideal chance to test whether historical exposure to HABs would shape phytoplankton-zooplankton interactions. Harmful *C. polykrikoides* blooms have become annual events in Long Island eastern waters since 2004 (Gobler et al. 2008). The many semi-enclosed bays along the narrow island likely result in numerous population mosaics of *A. tonsa*. The biogeographic variation in the blooms results in differential exposure of geographically-separate populations of copepods. Thus, there appears to be a selection difference caused by the historical occurrence of *C. polykrikoides* on *A. tonsa* from east to west along Long Island.

My results on local adaptation of *Acartia tonsa* to harmful *Cochlodinium polykrikoides* are consistent with previous studies on *A. hudsonica* resistance to toxic *Alexandrium* spp. (Colin and Dam 2002a, 2004), but also provide evidence that adaptative divergence can occur on a finer scale. *A. tonsa* resistance to *C. polykrikoides* differed significantly on a scale of 10s of km along Long Island compared to 100s of km

in *A. hudsonica* along the Atlantic coast of the USA (Colin and Dam 2002a, 2004). Local adaptation in copepods on such a fine scale may be due to both restricted gene flow and high selection intensity. Gene flow of copepods between bloom areas and non-bloom areas may constrain local adaptation to *C. polykrikoides*. *A. tonsa* is traditionally characterized by its potential for long-distance passive dispersal (Mauchline 1998) and presumably high gene flow. Recent studies of DNA sequence variation in *A. tonsa* from estuaries on the Atlantic coast of the USA, however, indicate that dispersal of *A. tonsa* was restricted between different estuaries, resulting in highly significant genetic differentiation and geographic isolation (Caudill and Bucklin 2004). Even on the scale of a single estuary, the evidence of DNA sequencing has demonstrated that two cryptic species of *A. tonsa* were reproductively isolated (Chen and Hare 2008). The spatially discontinuous nature of bays, coves, and tributaries in Long Island may provide geographical barriers and facilitate geographic isolation. Restricted gene flow due to geographic barriers would promote evolution of resistance to *C. polykrikoides* in copepods in Long Island eastern waters. On the other hand, moderate gene flow may also facilitate local adaptation since it increases fitness by increasing genetic variation and disrupting unfavorable genetic correlations (Hendry et al. 2007). High genetic diversity can provide copepods a broad spectrum of genotypes with different environmental optima that can buffer against ecosystem changes in the event of an HAB occurrence. The rapid evolution of resistance in copepods may also relate to high selection intensity by *C. polykrikoides*. Typical cell densities of *C. polykrikoides* range from 10^3 to 10^4 cells ml^{-1}, sometimes even exceeding 10^5 cells ml^{-1}, during blooms (Gobler et al. 2008). Given that copepods rapidly evolved resistance when fed *C. polykrikoides* at 300 μg C L^{-1} (165 cells ml^{-1}) in the artificial selection experiment, selection intensity by *C. polykrikoides* blooms would very strong in the field, despite the transient nature of blooms (1 to 2 months in duration). Thus, both geographical barriers and dense algal blooms likely promote local adaptation of *A. tonsa* to *C. polykrikoides*.

Copepod populations from bloom areas have evolved resistance to *Cochlodinium polykrikoides* within six years since the occurrence of the first bloom. The estimated rate of divergence among copepod populations between bloom and non-bloom areas is high, and comparable to other reported examples of rapid evolution (Hendry and Kinnison 1999). The results from the artificial selection experiment further provide strong evidence that *C. polykrikoides* acts as a selective force that can rapidly cause evolutionary change in copepod populations. The rapid gain of resistance in *Acartia tonsa* to *C. polykrikoides* is comparable with previous studies on adaptive responses of aquatic organisms to harmful algae (Hairston et al. 1999, Colin and Dam 2004, Bricelj et al. 2005). Although the ultimate cause of rapid evolution in my experiments is not clear, I offer the following explanations. Adaptive phenotypic plasticity may contribute to the evolution of resistance to harmful algae (Ghalambor et al. 2007). Freshwater grazers, *Daphnia galeata*, evolve resistance to cyanobacteria through reduced phenotypic plasticity (Hairston et al. 2001). Since evolution of plasticity is based on genetic variation for plasticity per se or on variation among individuals in the phenotype expressed in a new environment, I might expect selection either to reduce the reaction norms (to make egg production rates of *A.*

tonsa less sensitive to harmful *C. polykrikoides* while keeping mean egg production rates when fed *C. polykrikoides* and *Rhodomonas lens* constant), or to decrease the harmful effects of *C. polykrikoides* on egg production rates of *A. tonsa* while leaving egg production rates on *R. lens* unaffected. The second explanation would be that populations of *A. tonsa* may maintain a degree of standing genetic variation with low frequencies of resistant genotypes. When harmful *C. polykrikoides* blooms occur, selection on resistant genotypes can generate adaptation. A change in genotype frequency has been confirmed as the mechanism of clam resistance to toxic *Alexandrium* (Bricelj et al. 2005). An alternative mechanism is that resistant alleles may occur in related species or populations, and occasional hybridization incorporates the adaptive alleles into populations where selection favors their fixation. Finally, mutation may generate new alleles subsequent to a selective challenge. Some of these novel variants may have positive fitness effects, and thereby spread through a population. Although the last two explanations should not account for the evolution of resistance to *C. polykrikoides* in the artificial selection experiments, they cannot be excluded from possible hypotheses for evolved resistance in copepods from the bloom area.

Artificial selection experiments demonstrated that copepods in the selection line, which initially evolved high resistance to *Cochlodinium polykrikoides*, displayed reverse evolution back to their original state after selection was relaxed. The gradual loss of resistance in copepods implies the changes were genetically based. There was a lag time, although only two generations, before resistance was completely lost, further demonstrating that the loss of resistance was a genetic process. Phenotypic variance in resistance was not reduced by the initial selection, suggesting that sufficient genetic variation remained to allow recovery. Reversion to low resistance in the absence of *C. polykrikoides* could be explained by an evolutionary cost of resistance in copepods. Evolutionary trade-offs are basic elements in life-history theory due to an economic limitation in which resources allocated to one trait are not available for others. Resistance to *C. polykrikoides* may be negatively related to population fitness during non-bloom periods due to a trade-off between resistance to *C. polykrikoides* and other life history-traits.

Both gain and loss of resistance in copepods occurred within several generations in the artificial selection experiment. The selection and relaxation conditions in nature may differ substantially from the artificial selection experiment. The rates of evolution and loss of resistance in natural copepod populations depend on many factors, such as strength and constancy of selection and relaxation, effective population size, and the degree of population isolation (Teotónio and Rose 2001). Extrapolation of the rates of evolution upon exposure to *C. polykrikoides* and subsequent non-exposure observed in the artificial selection experiment would yield incorrect predictions for natural populations (Futuyma 1998). Although absolute rates estimated from artificial selection experiments may not be directly comparable to the likely rates of evolutionary change in nature, they at least suggest that evolution and reversal of resistance in copepods occur on the same time scale.

The process of reversal of resistance has potential ecological consequences since reversal can occur in a few generations. In bloom areas, loss of resistance might occur during non-bloom periods because resistant genotypes may be poor competitors with non-resistant conspecifics. Although evolution of resistance to harmful algae may improve zooplankton control on harmful algal blooms, rapid loss of resistance would constrain the grazing control of field zooplankton populations, which may partly explain frequent reoccurrence of HABs (Avery and Dam 2007). Maintaining higher resistance in a controlled grazer population by continuously feeding them harmful algae and releasing them into the field upon bloom initiation perhaps could provide a feasible biological approach to mitigate HABs, such as the annual blooms of *C. polykrikoides* which have occurred in eastern bays of Long Island since 2004. The approximately ten-month period between blooms likely provides a long relaxation for resistance loss in *A. tonsa* populations. On the other hand, rapid loss of resistance may reduce exposure risk of toxins to higher trophic organisms since fewer toxins would enter the food web via grazers. Rapid gain and loss of resistance to harmful algae in grazers underscore the importance of considering the evolutionary history of grazers when modeling phytoplankton-grazer interactions, particularly in the context of the rapid spread of harmful algal blooms. It is important to assess the extent of the potential for natural selection and the potential for genetic recovery.

The bloom-forming dinoflagellate *Cochlodinium polykrikoides* is an abundant food source for the copepod *Acartia tonsa* during the bloom periods. Multiple compounds produced by *C. polykrikoides* including compounds similar to reactive oxygen species (Tang and Gobler 2009), however, harmfully affect feeding, egg production, and survival of *A. tonsa* (Jiang et al. 2009). *C. polykrikoides* also can form chains to avoid copepod feeding (Chapter 4). The chemical and morphological defenses of *C. polykrikoides* may create strong selection for resistance in *A. tonsa*. This study has demonstrated that *A. tonsa* exhibits evolutionary changes in response to *C. polykrikoides*. *A. tonsa* in eastern bays of Long Island were more resistant than their conspecifics in nearby non-bloom areas. Both gain and loss of resistance can occur in *A. tonsa* within a few generations. These evolutionary changes on ecological scales would shape interactions between harmful algae and grazers, which may provide new insights for understanding and management of HABs.

Chapter 7 Dissertation summary

Prey have developed numerous defenses to avoid predation and vice versa, creating an evolutionary arms race of adaptation and counteradaptation (Vermeij 1994, Brodie and Brodie 1999, Post and Palkovace 2009). Many ecological and evolutionary processes involved in predator-prey interactions overlap on similar timescales and reciprocally interact, which affects population persistence, drives speciation or extinction (Hendry et al. 2007), alters community structure, and shapes ecosystem function (Post and Palkovace 2009). Ecological and evolutionary interactions between *Cochlodinium polykrikoides* and the copepod *Acartia tonsa* are likely to result in an arms race (Fig. 7.1).

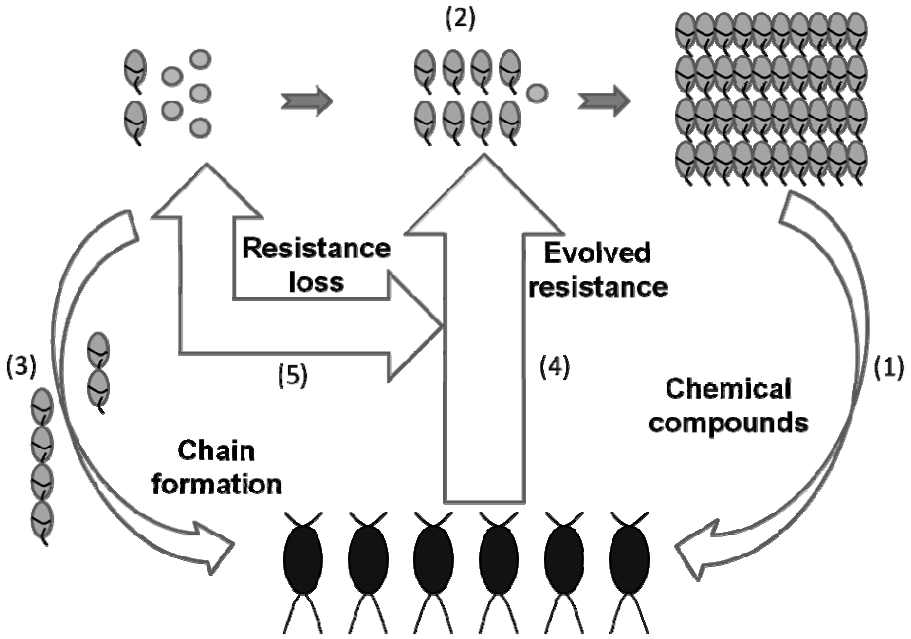

Fig. 7.1. An arms race between *Cochlodinium polykrikoides* and *Acartia tonsa*. (1) *C. polykrikoides* produces multiple harmful compounds at high cell densities, which can reduce *A. tonsa* survival, feeding, and reproduction; (2) *C. polykrikoides* is beneficial to *A. tonsa* at low cell densities; (3) *C. polykrikoides* can form chains to reduce grazing mortality; (4) *A. tonsa* can rapidly evolve resistance to *C. polykrikoides* during bloom periods; (5) The resistance to *C. polykrikoides* can also be rapidly lost during non-bloom periods.

Survivorship of female *Acartia tonsa* was significantly reduced with increasing *Cochlodinium polykrikoides* concentrations from 900 to 4700 μg C L^{-1} (500 – 2600 cells mL^{-1}). Copepods completely expired within 1.5 days at *C. polykrikoides* concentrations of 3300 and 4700 μg C L^{-1}(1800 and 2600 cells mL^{-1}, respectively), which are within the range of bloom densities of this alga. Stage-specific mortality of *A. tonsa* showed copepod susceptibility to *C. polykrikoides* decreased with developmental stage. Two bioassay experiments suggested that copepod mortality was due to multiple harmful compounds produced by *C. polykrikoides* (Fig. 7.1). Ingestion rates of *A. tonsa* fed *C. polykrikoides* were 25 – 60% lower than ingestion rates on non-toxic *Rhodomonas lens* when the food concentrations ranged from 150 to 1500 μg C L^{-1}. *C. polykrikoides* supported higher egg production rates of *A. tonsa* than *R. lens* at the low algal concentrations (18 – 180 μg C L^{-1}), while egg production rates of *A. tonsa* fed *C. polykrikoides* were significantly less than those fed *R. lens* when the concentrations increased from 360 to 1080 μg C L^{-1}. Egg hatching success of *A. tonsa* fed *C. polykrikoides* ranging from 90 to 1080 μg C L^{-1} was low (20 – 43%) compared to the values on *R. lens* (83 – 100%). Egg sizes of *A. tonsa* fed *C. polykrikoides* were significantly lower than those fed *R. lens*. All of these deleterious consequences may lead to *A. tonsa* population collapses during *C. polykrikoides* blooms (Chapter 2).

However, *Cochlodinium polykrikoides* is not always harmful to *Acartia tonsa* and its ecological effects may be distinctly different during bloom and non-bloom periods (Fig. 7.1). Based on egg production rate, egg hatching success, and naupliar recruitment rate of *A. tonsa*, mixed-diet experiments indicated *C. polykrikoides* was nutritionally insufficient or had no nutritional value to *A. tonsa* at 600 μg C L^{-1} (330 cells mL^{-1}), and was toxic at 1000 μg C L^{-1} (550 cells mL^{-1}) when compared with non-toxic flagellate *Rhodomonas lens*. Contrary to expectation, the nutritional value of *C. polykrikoides* to *A. tonsa* at 100 and 200 μg C L^{-1} (55 and 110 cells mL^{-1}) was greater than or equal to *R. lens*. The density-dependent nutritional value of *C. polykrikoides* to *A. tonsa* was also demonstrated in the long-term survival experiments. Survivorship of *A. tonsa* fed *C. polykrikoides* was lower than when fed *R. lens* at 900 and 1800 μg C L^{-1}. In contrast, *C. polykrikoides* supported higher survivorship of *A. tonsa* than *R. lens* at 180 and 540 μg C L^{-1}. The nutritional value of *C. polykrikoides* to *A. tonsa* decreased from beneficial to deleterious with increasing cell density (Chapter 3).

Although *Cochlodinium polykrikoides* is beneficial to *Acartia tonsa* at low cell densities, chain formation may prevent its cells from being completely grazed down. Field populations of *C. polykrikoides* displayed a significantly larger variation in chain length compared to laboratory cultures without grazers. Chain length of *C. polykrikoides* was significantly increased when exposed to *Acartia tonsa* adults for 48 h or to fresh (<24 h post-isolation) exudates of *A. tonsa*. Chain length of *C. polykrikoides* was correlated with *A. tonsa* abundance in the field. These results suggest that dissolved chemical cues released by *A. tonsa* induce chain formation in *C. polykrikoides* (Fig. 7.1). Ingestion rates of *A. tonsa* on the 4-cell chains of *C. polykrikoides* were lower than on single cells, suggesting chain formation may be an effective anti-grazing defense. Finally,

nutrient amendment experiments demonstrated that vitamins (B_1, B_7, and B_{12}) enhanced the chain length of *C. polykrikoides* both singly and collectively, while trace metals and inorganic nutrients did not, showing vitamins may also influence chain formation in this species (Chapter 4).

The *Cochlodinium polykrikoides* bloom in Old Fort Pond, Shinnecock Bay, NY, in 2008, was moderate with a mean cell density of 218 cells mL^{-1} (SD ± 290 cells mL^{-1}). However, embryonic mortality (the combined egg-through-the-second-naupliar-stage, egg-N2) of *Acartia tonsa* was higher than birth rate on most sampling days, resulting in the consistent decline in copepod abundance. Embryonic mortality was correlated with cell density of *C. polykrikoides* with a 4-d delay. Contrary to expectation, there was no significant relationship between embryonic mortality and adult abundance of *A. tonsa*. In contrast, birth rate was negatively dependent on adult abundance of *A. tonsa*, but not on cell density of *C. polykrikoides*. Therefore, the population dynamics of *A. tonsa* was regulated by algal-density-dependent mortality and copepod-density-dependent birth rate (Chapter 5).

In the light of an arms race, copepods were hypothesized to have evolved a means to breach chemical and morphological defenses of *Cochlodinium polykrikoides* (Fig. 7.1). Six years after the first occurrence of *C. polykrikoides* blooms in eastern bays of Long Island, *A. tonsa* populations from bloom areas were significantly more resistant to *C. polykrikoides* than conspecifics from nearby non-bloom areas. The *A. tonsa* population taken from a non-bloom area gradually increased the resistance with time when exposed to *C. polykrikoides*. Copepod resistance in the selection line was approximately 3 times that of the control line after 4 generations. Following a two-generation relaxation of selection, the elevated resistance in *A. tonsa* was completely lost. *C. polykrikoides* blooms in eastern bays of Long Island diverged *A. tonsa* populations from their conspecifics in nearby non-bloom waters at a high rate (0.17 ± 0.06 haldanes) from 2004 to 2009. The rates of reversal and evolution of resistance in *A. tonsa* to *C. polykrikoides* were comparable. Rapid gain and loss of resistance to harmful algae highlight the need for the evolutionary responses of grazers to be considered in understanding and management of harmful algal blooms (Chapter 6).

The watery arms race between *Cochlodinium polykrikoides* and *Acartia tonsa* may provide some valuable insights into the mechanisms of *C. polykrikoides* bloom formation. *C. polykrikoides* cells can detect the presence of copepods and increase cell chain length creating predator-prey size mismatch. The induced chain formation may help *C. polykrikoides* to avoid grazing during non-bloom periods when their nutritional value is beneficial to zooplankton at low densities. High nutritional value may also provide a window of opportunity for the initiation of *C. polykrikoides* blooms. Since *C. polykrikoides* grows slower than most diatoms and flagellates (Kim et al. 2004, Smayda 1997), killing copepods would not contribute to bloom initiation since this would facilitate the dominance of fast growing competitors within the algal community (Flynn 2008). Although supporting copepods at low densities of *C. polykrikoides* would depress its populations, copepods also control the population size of fast-growing algae. Multiple

harmful compounds produced by *C. polykrikoides* at high cell densities do not contribute to the bloom initiation, but become increasing important as blooms develop and likely contribute towards bloom maintenance. Harmful effects of *C. polykrikoides* during bloom periods exert strong selective pressures on copepods. The rapid evolved resistance to harmful *C. polykrikoides* in copepods would result in increased grazing pressure on *C. polykrikoides* cells, which may partly facilitate the termination of *C. polykrikoides* blooms. However, the evolved resistance in copepods would be quickly lost due to the periodical occurrence of *C. polykrikoides* blooms. The present results, in combination with other attributes of *C. polykrikoides*, such as mixotrophy (Jeong et al. 2004), allelopathy (Tang and Gobler 2010), and resistance to algicidal bacteria (Imai and Kimura 2008), enable us to better understand bloom formation of slow-growing dinoflagellates such as *C. polykrikoides*.

Although the present work has answered some questions about the interactions between *Cochlodinium polykrikoides* and *Acartia tonsa*, these results put forward additional new questions for future studies. We know the density-dependent nutritional value in *C. polykrikoides* (Chapter 3), but we do not know whether it is a universal phenomenon for harmful algae. We know some chemical compounds regulate interactions between *C. polykrikoides* and *A. tonsa* (Chapter 2, 4), but we do not know their structures, specific modes of actions, and the synthesis processes in organisms. We know the chemical and morphological defenses to grazers in *C. polykrikoides* (Chapter 2, 4), but we do not know whether these defenses are directly driven by the presence of grazers or are just metabolic byproducts. We know the rapid gain and loss of resistance in copepods to *C. polykrikoides* (Chapter 6), but we do not know the molecular or physiological mechanisms of theses evolutionary processes. We know adaptation and counteradaptation in organisms shapes interactions between harmful algae and grazers, but we do not know how these processes affect ecosystem function, speciation and extinction. All of these topics offer fruitful future avenues of research which would generate a better understanding of the interactions of harmful algae and zooplankton in general, and between *C. polykrikoides* and *A. tonsa* in particular.

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