

Brown tide alga, *Aureococcus anophagefferens*, can affect growth but not survivorship of *Mercenaria mercenaria* larvae

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Abstract

Since the collapse of populations of northern quahogs (hard clam), *Mercenaria mercenaria*, in Long Island bays, brown tide blooms have been proposed to pose a barrier to recovery. We tested whether the brown tide alga, *Aureococcus anophagefferens*, affects survivorship, development or growth in the larvae of *M. mercenaria*. There was no effect of *A. anophagefferens* (clone CCMP1708) on survivorship of hard clam larvae, even at bloom concentrations. Under most experimental conditions, larvae fed a mixed diet of *Isochrysis galbana* (T-Iso) and *A. anophagefferens* or a single species diet of *A. anophagefferens*, developed faster than those fed a single species diet of *Isochrysis*. A mixed diet of *I. galbana* and *A. anophagefferens* either had no effect on larval growth, or produced enhanced growth at moderate cell densities (8×10^4 cells ml⁻¹ of *A. anophagefferens*). Similarly, moderate cell densities of a single food diet of *A. anophagefferens* (1.6×10^5 cells ml⁻¹) generally had no effect on the growth of larvae. When fed bloom concentrations (10^6 cells ml⁻¹) of *A. anophagefferens*, larvae developed faster, but growth was reduced, compared to those fed an equal biovolume of *Isochrysis*. Larvae fed slow growing or near stationary phase cultures of *A. anophagefferens* experienced reduced growth and slowed development. These data suggest a qualitative difference between slow or stationary phase and fast growing cultures of the brown tide alga. They also suggest that impacts of *A. anophagefferens*, when present, are likely to be due to the nutritional quality of this alga as a food source for hard clam larvae, which could have a lasting legacy through ontogeny. Additional studies are needed to test whether our findings apply to more recently isolated strains of *A. anophagefferens*.

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1. Introduction

South shore estuaries of Long Island are historically some of the most productive ecosystems in North America, in terms of both primary productivity and the harvest of shellfish (Lively et al., 1983; COSMA, 1985).

Historically, the composition of the phytoplankton community has been correlated with the success of resident shellfish populations. In the 1950s, green tides dominated by small chlorophytes (2–4 μm, *Nannochloris* sp. and *Stichococcus* sp.) were implicated in the collapse of many Long Island shellfisheries (Ryther, 1954, 1989). During the 1970s the shellfisheries rebounded when a more varied phytoplankton assemblage dominated Great South Bay (Weaver and Hirshfield, 1976; Cassin, 1978; Lively et al., 1983).

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The fishery for the northern quahog or hard clam, *Mercenaria mercenaria*, in Great South Bay was the most successful fishery in the history of New York State. During the 1970s, two out of every three hard clams eaten on the east coast of the United States came from Great South Bay (McHugh, 1991). These peak hard clam landings were followed by a precipitous decline in clam densities, as harvest and natural mortalities greatly exceeded recruitment (Kraeuter et al., 2005). The collapse of *M. mercenaria* populations was followed by a shift in the phytoplankton community starting in 1985, culminating in blooms of the harmful alga, *Aureococcus anophagefferens*, which also appeared in Rhode Island and New Jersey (Sieburth et al., 1988; Gobler et al., 2005). During the 1990s brown tide blooms spread to estuaries in Maryland, and Virginia. Sporadic blooms of *A. anophagefferens* continue in these areas (Gobler et al., 2005), and, at present, *A. anophagefferens* can be found at densities of 10^2 – 10^4 cells ml⁻¹ as part of the background flora in many mid-Atlantic estuaries during the summer (Gobler et al., 2005).

Since the collapse of hard clam populations and the onset of brown tide blooms hard clam populations in Great South Bay have not recovered even though harvest pressure has been greatly reduced, suggesting that the brown tide blooms may pose a barrier to hard clam population recovery; however, cause and effect have not been demonstrated (Bricelj et al., 2001; Greenfield and Lonsdale, 2002; Gobler et al., 2005). Some strains of *A. anophagefferens* are known to slow growth and increase mortality in adult (*Argopecten irradians* and *Mytilus edulis*), juvenile (*M. mercenaria*; Bricelj et al., 2001) and larval bivalves (*A. irradians*; Gallager et al., 1989). *Aureococcus* has been shown to reduce gill function in adult *M. mercenaria*, even at relatively low concentrations ($<4 \times 10^4$ cells ml⁻¹), and well below bloom concentrations (10^6 cells ml⁻¹) (Gainey and Shumway, 1991). Negative effects of brown tide on bivalves has been attributed to reduced feeding caused by a dopamine-like compound in the extracellular polysaccharide-like layer of *A. anophagefferens*, which inhibits gill cilia (Bricelj and Kuenstner, 1989; Draper et al., 1990; Gainey and Shumway, 1991).

In laboratory experiments Bricelj et al. (2001) found that densities of *A. anophagefferens* $>4 \times 10^4$ cells ml⁻¹ (CCMP 1708, a harmful strain of *A. anophagefferens*) significantly reduced the feeding activity of juvenile *M. mercenaria*. Juvenile hard clams placed in Great South Bay during a bloom ($>10^6$ cells ml⁻¹) experienced 66% mortality after 4 weeks (Greenfield and Lonsdale, 2002). In addition, Wazniak and Glibert (2004) found that the

growth rate of juvenile *M. mercenaria* were significantly reduced during a brown tide in Maryland, even at very low concentrations of *A. anophagefferens* (2×10^4 cells ml⁻¹). Adult hard clams seem somewhat more tolerant of *A. anophagefferens* than juveniles, and experience relatively small reductions in growth rate (20%) and no mortality during brown tide blooms (Laetz, 2002). However, adult hard clams can have a significantly reduced condition index when exposed to densities of *A. anophagefferens* 10^5 cells ml⁻¹, relative to clams in nearby waters without high concentrations of the brown tide alga (R.I.E. Newell, personal communication).

Bivalves, such as *M. mercenaria*, are well known ecosystem engineers, and their presence in coastal waters is essential for maintaining functioning ecosystems, and ecosystem health (reviewed in Dame and Olenin, 2005), as well as providing important fisheries resources. The restoration of depleted shellfish populations has therefore become an important environmental issue in many coastal communities. On Long Island The Nature Conservancy has spearheaded efforts to restore hard clam populations by creating sanctuaries of high densities of spawning clams, which will reduce sperm limitation for fertilization (reviewed in Levitan and Petersen, 1995; Levitan, 1998). Larval survival and growth can also pose a significant bottleneck on population dynamics (Schneider et al., 2003), and factors that affect larval growth and survivorship, including food availability and quality, can carry a nutritional legacy through metamorphosis (Padilla and Miner, 2006), impacting juvenile survivorship (reviewed in Moran and Emlet, 2001; Phillips, 2002; Marshall et al., 2003; Marshall and Keough, 2004). Thus, phytoplankton food conditions not only directly impact larvae, but will have a continued legacy affecting later life stages, recruitment and population recovery.

At present it is not known if *A. anophagefferens* has a negative impact on hard clam larvae, and can impede restoration efforts. We conducted a series of laboratory experiments to test whether *A. anophagefferens* affects *M. mercenaria* larval growth, development and survival.

2. Materials and methods

2.1. Algal culture procedures

The brown tide alga, *A. anophagefferens* (CCMP1708) was used for all experiments. This clone was originally isolated from West Neck Bay within the Peconic Estuary during a bloom in July of 1995, and has been shown to inhibit growth, feeding, and survival of juvenile *M. mercenaria* (Bricelj et al., 2001). We used

two culture sources of this isolate (CCMP1708); one we obtained from Bigelow culture bank 1 month before the initiation of our experiments, and the second has been in active culture since its isolation (D. Lonsdale, Stony Brook University). *A. anophagefferens* cultures were grown at 22 °C on a 14 h light:10 h dark cycle, similar to the temperature and photoperiods found in Long Island estuaries during early summer months when *A. anophagefferens* blooms (Gobler et al., 2005), with $\sim 100 \mu\text{E m}^{-2} \text{s}^{-1}$ of light. Cultures were grown using a modified f/2 medium (Guillard and Ryther, 1962; Taylor et al., 2006) prepared from filtered (0.22 μm) Atlantic Ocean seawater collected 10 km southeast of the Shinnecock Inlet, near the east end of Long Island, New York (salinity ~ 33 psu). This medium contained 10^{-8} M selenium as selenite, 5×10^{-6} M citric acid was substituted for EDTA as the metals chelator, and Fe concentrations were 10^{-6} M (Cosper et al., 1993). Semi-complete medium was autoclaved; after cooling, filter-sterilized vitamin stocks were added aseptically to each flask.

New cultures were inoculated with $\sim 2.5 \times 10^5$ cells ml^{-1} of exponentially growing *A. anophagefferens* culture, and were monitored daily through exponential and stationary phases of growth, when cell densities typically exceeded 10^7 cells ml^{-1} . Cell density was estimated by direct cell counts and in vivo fluorescence (Turner Designs model TD-700 fluorometer) measured at the same time each day to account for diel fluctuations in cell fluorescence. Previous research has demonstrated that fluorescence is proportional to cell densities for a variety of cultured phytoplankton species (Fogg and Thake, 1987), including *A. anophagefferens* (Gobler et al., 1997). Daily samples were also preserved with Lugol's iodine for enumeration with a hemocytometer on a compound light microscope. Culture population growth rates were calculated from changes in cell densities or fluorescence using the formula:

$$\mu = \frac{\ln(C_t/C_0)}{t},$$

where μ is the net growth rate, C_0 the amount of biomass (cell density or fluorescence) present at an initial time point and C_t is the amount of biomass at a final time point, t . Growth rates estimated by cell counts and fluorescence were not significantly different (paired t -test, $P > 0.5$). The control alga, *Isochrysis galbana* (Tahitian strain, T-Iso) was cultured with f/2 medium under similar temperature and light conditions as *A. anophagefferens*.

Cell densities of cultures used to feed larvae were counted daily. Two to three replicate samples were counted with a hemocytometer and averaged to estimate cell density. The volume of culture needed to obtain the desired cell number necessary for feeding was then calculated. Cells were distinct and clumps of cells were rare for either *I. galbana* or *A. anophagefferens*, even when they were centrifuged and resuspended to removed excess culture medium.

2.2. Spawning adult clams

To maintain adult hard clams in spawning condition, they were kept at 19 °C in a recirculating temperature controlled tank with natural seawater and fed a marine microalgal concentrate (25% *I. galbana*, 20% *Pavlova*, 20% *Tetraselmis* and 35% *Thalassiosira weissflogii* produced by Reed Mariculture Inc.) administered through a drip bag (Ross Laboratories, Columbus, OH). Clams were spawned using a modification of the procedure described by Deming et al. (1998). The day before spawning, clams were placed dry at 5 °C overnight. The day of spawning, clams were placed individually in 1 l beakers with filtered seawater (0.2 μm) at 22 °C until they began to actively filter. Cycles of warm (30 °C) and cold (10 °C) shocks with filtered seawater were then begun; animals generally spawned within three or four cycles. The seawater containing spawned gametes was filtered (150 μm) to remove mucus and feces, and water containing sperm was diluted 1:1 with filtered seawater. Five to 10 ml of the diluted sperm water was added to the beakers containing unfertilized eggs and were gently stirred for 5 min. Filtered fertilized eggs were rinsed into 4 l glass jars with filtered seawater at an estimated density of 5 eggs ml^{-1} and kept overnight at 22 °C to let the embryos (non-feeding for the first 24 h) develop into larvae. The larvae were then randomly allocated to experimental treatments the following day.

2.3. Culturing larval clams

The techniques for culturing larvae have been proven successful for a variety of bivalves and other molluscs (Strathmann, 1987, Padilla unpublished data). To ensure high survivorship and limited food competition within cultures, larvae were grown at a very low density of 100 l^{-1} .

All containers and glassware (as well as surfaces upon which containers and glassware were in contact) that came in contact with gametes, embryos or larvae were kept "embryologically clean" (never exposed to soaps,

detergents or other chemicals; Strathmann, 1987). All seawater used for spawning adults and culturing larvae was collected fresh from the University Laboratory at Flax Pond, near Stony Brook University, and filtered through a 0.2 μm filter. All water was filtered just prior to use to prevent contamination by protozoa and bacteria.

Preliminary experiments were conducted to determine the appropriate concentrations of the control alga, *I. galbana*, for maximal larval growth and survivorship. In addition, preliminary experiments were also used to determine if the algal culture medium, or antibiotics, which control contamination by bacteria and protozoa that feed on the bacteria, had negative impacts on larval growth, development or survival (Strathmann, 1987).

A density of at least 2×10^4 cells of *Isochrysis* ml^{-1} produced high growth rates and survivorship to metamorphosis for hard clam larvae, and subsequently this concentration was used as a control for all experiments to allow direct comparisons among different experimental trials. Because of the large differences in cell size between *Isochrysis* and *A. anophagefferens* ($\sim 8\times$, v/v), cell densities were adjusted in each experiment to provide equivalent biovolumes of microalgal food.

Algal growth medium had a significant negative impact on hard clam larval survivorship (see Section 3), as is common for many marine invertebrate larvae (Strathmann, 1987). Because large volumes of algal cultures were often required for feeding the appropriate cell densities, if greater than 2 ml of algal culture medium was to be added to larval cultures during feeding (either *Isochrysis* or *A. anophagefferens*), the algal cultures were centrifuged ($G = 1855$ for 25 min; Gobler et al., 1997), the nutrient medium was decanted and the cell pellet was resuspended for feeding as is recommended by Strathmann (1987), and minimal cell clumping was observed as a function of centrifugation.

No negative effect of antibiotics (Sigma–Aldrich Co., #P3664, 5000 units of Penicillin, 5 mg of Streptomycin and 10 mg of Neomycin per milliliter of solution) on larval growth and survivorship (see Section 3) was seen. This antibiotic mixture is used routinely for larval culture to control bacteria and has not been shown to have negative impacts on other larvae (Strathmann, 1987); antibiotics are regularly used in hard clam aquaculture to control bacteria (Castagna, 2001). In addition, these antibiotics have been found to have no negative effect on *A. anophagefferens* (Berg et al., 2002). Therefore, 0.25 ml of the antibiotic mixture was added to each litre of filtered larval culture at the start of each experiment and each time the culture water was changed (twice weekly).

All replicates of all experimental treatments were started with approximately 100 larvae in filtered seawater (~ 800 ml initial volume to allow for volume of algae added daily) in a covered 1 l beaker. Larvae were transferred into clean beakers with clean seawater two times per week. All cultures were kept at a constant temperature and larvae were fed appropriate diets once per day. In each experiment, each treatment had three to five replicates, depending on the number of treatments in the experiment. Larvae were kept at 22 or 26 $^{\circ}\text{C}$ (the range of water temperatures in Great South Bay during July and August when hard clams spawn, Castagna, 2001; Gobler et al., 2004), depending upon the experiment. Experiments were ended when at least 50% of the larvae in each of the replicates of the control *Isochrysis* treatment were at the pediveliger or a more advanced developmental stage. Larvae were considered metamorphosed once they lost their velum.

Survivorship was determined as larvae were transferred into clean water and beakers twice weekly, and the number of individuals at each developmental stage (veliger, pediveliger or metamorphosed juvenile) was recorded. The size of larvae or newly metamorphosed clams at the end of each experiment was measured with a computer assisted imaging system (Image Pro, Media Cybernetics) focused through a dissecting microscope.

2.4. Statistical analyses

Contingency table analyses were used to test for differences in survivorship and development rate among treatments in experiments. Analysis of variance (ANOVA) was used to test for differences in growth among treatments. Tukey post hoc multiple comparison tests were then used to test for pair-wise differences among specific treatments. If replication was very unequal among treatments, non-parametric equivalents of ANOVA were used to test for significance (Zar, 1999).

3. Results

3.1. Effects of controls (nutrient medium, mixed diet, no antibiotics, 22 $^{\circ}\text{C}$)

Differences in growth and survivorship among larvae fed two food treatments with and without algal culture medium were tested: (1) the control alga, *Isochrysis* (2×10^4 cells ml^{-1}), no culture medium, (2) a mixed diet of equal biovolumes of *Isochrysis* (1×10^4 cells ml^{-1}) and *A. anophagefferens* (8×10^4 cells ml^{-1}), no culture medium, (3) *Isochrysis* (1×10^4 cells ml^{-1}) and *A.*

anophagefferens (8×10^4 cells ml^{-1}), culture medium not removed and (4) *Isochrysis* (2×10^4 cells ml^{-1}), plus an equal volume of culture medium as treatment 3.

This experiment lasted 17 days, and survival averaged 70% survival among the replicates without nutrient media ($n = 5$ per each diet treatment, range from 54 to 87%), and averaged 63% survivorship in the replicates with nutrient media ($n = 3$ per each diet treatment, range from 52 to 77%). Overall there was no significant effect of diet on survival (G -test, $P = 0.08$), but there was a significant effect of the addition of nutrient medium on survival (G -test, $P < 0.005$). Larvae grown with nutrient medium had significantly lower survivorship than those without added medium (Fig. 1).

For larvae reared without culture medium, there were significant differences in development time between the two diets (G -test, $P \ll 0.001$). Those reared on a mixed diet developed faster than those on the control *Isochrysis* diet; 40.7% of larvae fed *Isochrysis* had matured to at least the pediveliger stage, while 65.7% of those fed a mixed diet were at least pediveligers. There was no difference in development between diets for larvae grown in the presence of culture medium (G -test, $P > 0.4$), and they developed slower than those reared without medium present (G -test, $P < 0.0001$). Only 28.7% of larvae fed *Isochrysis* and 30.3% fed a mixed diet had reached the pediveliger stage when grown in the presence of culture medium (Fig. 1).

In a two-way analysis of variance to test the effects of nutrient medium and diet on growth, there was a highly significant interaction term ($P = 0.003$), therefore lack of significance of the main effects cannot be taken at face value. We found a significant effect of diet ($P = 0.001$) and no significant effect of nutrient medium ($P = 0.06$) on growth. With a multiple comparison (Tukey) test we found that there was no significant difference in size between larvae fed *Isochrysis* with or without nutrients ($P > 0.6$) and larvae fed a mixed diet without nutrients ($P > 0.5$). However, larvae fed a mixed diet with algal nutrients were significantly smaller than all other larvae ($P < 0.007$) (Fig. 1).

3.2. Effects of mixed and unialgal diet, with and without antibiotics, 26 °C

In a full factorial experiment (four replicate beakers per treatment), we tested the effects of antibiotics (added or not added), and three different diets: (1) the control alga, *Isochrysis* (2×10^4 cells ml^{-1}), (2) a mixed diet of equal biovolumes of *Isochrysis* (1×10^4 cells ml^{-1}) and *A. anophagefferens* (8×10^4 cells ml^{-1}) and (3) a diet of just *A. anophagefferens* (1.6×10^5 cells ml^{-1}).

Survivorship in all replicates of all treatments was $>95\%$ and all but one larva (of ~ 2400) metamorphosed completely when the experiment was terminated 13 days after fertilization.

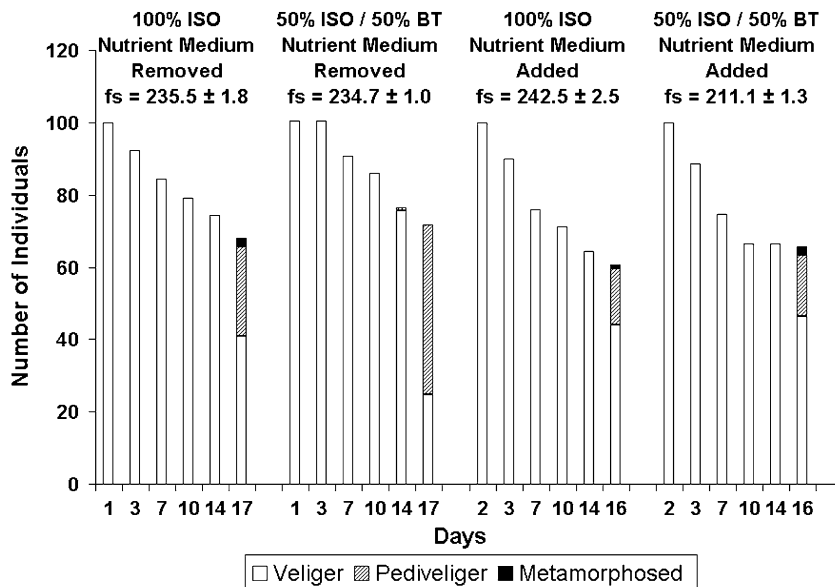


Fig. 1. The effects of algal nutrient medium and a single species diet of *Isochrysis* (ISO) or a mixed diet of 50% (v/v) *Isochrysis* and 50% *Aureococcus anophagefferens* (BT) on survivorship and development and growth of hard clam larvae (grown without antibiotics at 22 °C). Veligers are indicated by white bars, pediveligers by shaded bars and metamorphosed juveniles by solid bars. For each treatment, bars represent the average of five replicates for diet treatments without algal nutrient medium and three replicates for diet treatments with nutrient medium. Days indicates the time since fertilization. fs, the final mean size (μm) \pm standard error of larvae and newly metamorphosed juveniles across replicates.

There was no significant effect of antibiotics on survivorship (G -test, $P > 0.5$) or diet on survivorship (G -test, $P > 0.07$). Prior to the end of the experiment, those larvae with antibiotics developed faster than those without (G -test, $P < 0.001$), and those fed a mixed diet or a single species diet of *A. anophagefferens* developed faster than those fed *Isochrysis* (G -test, $P < 0.001$) (Fig. 2).

When testing for differences in growth among all treatments, we found significant single factor effects of diet (ANOVA, $P < 0.0001$) and antibiotics (ANOVA, $P = 0.01$), as well as a significant antibiotic \times diet interaction (ANOVA, $P = 0.05$). Those with the antibiotics grew more on the mixed diet than on either the *Isochrysis* or *A. anophagefferens* diet. Among treat-

ments, larvae fed a mixed diet in the presence of antibiotics were significantly larger than all other treatments (Tukey multiple comparison test, *Isochrysis* with antibiotics, $P = 0.001$, *A. anophagefferens* with antibiotics, $P = 0.0008$; *Isochrysis* without antibiotics, $P = 0.001$, *A. anophagefferens* without antibiotics, $P = 0.0002$; mixed diet without antibiotics, $P = 0.0158$) (Fig. 2).

3.3. Effects of bloom concentrations, with antibiotics, 22 °C

The effects of bloom concentrations of microalgal food were tested by including three treatments: (1) the control diet, *Isochrysis* (2×10^4 cells ml⁻¹), (2) *A. anophagefferens* at a bloom concentration (1×10^6 cells ml⁻¹) and (3) *Isochrysis* at an equivalent biovolume as the bloom concentration (1.25×10^5 cells ml⁻¹). Unfortunately, we lost two of four replicates of the bloom *Isochrysis* treatment to protozoan contamination, resulting in an unbalanced design.

This experiment lasted 20 days, and survivorship averaged 74.6%, ranging from 58 to 91% among replicates. We found no significant difference in survivorship among the three diet treatments (G -test, $P = 0.42$). Larvae fed bloom concentrations of *Isochrysis* developed at the fastest rate (G -test, $P < 0.05$) (Fig. 3). By the end of the experiment 100% of surviving larvae had metamorphosed (57.6%) or were at the pediveliger stage (42.3%). Of those fed bloom concentrations of *A. anophagefferens*, 41.5% metamorphosed and 38.7% were at the pediveliger stage, and those fed the control concentration of *Isochrysis* developed slightly slower, with 11.5% metamorphosed and 72.9% at the pediveliger stage by the end of the experiment (G -test, $P < 0.05$) (Fig. 3).

There was a significant difference in growth among the diet treatments (Friedman Test, $P < 0.001$). At the end of the experiment there was no difference in larval size between those fed the two different concentrations of *Isochrysis* ($P = 0.506$), but larvae fed bloom concentrations of *A. anophagefferens* were significantly smaller those in the other two treatments ($P = 0.0002$) (Fig. 3). Larvae fed bloom concentrations of *A. anophagefferens* may have had lower nutritional stores than those fed *Isochrysis*. All of the larvae fed *Isochrysis* had conspicuous yellow colored lipid droplets, indicative of lipid stores (Castagna, 2001), while none of the larvae fed bloom concentrations of *A. anophagefferens* had similar conspicuous droplets (Fig. 4). However, the concentrations of lipids in larvae was not quantified.

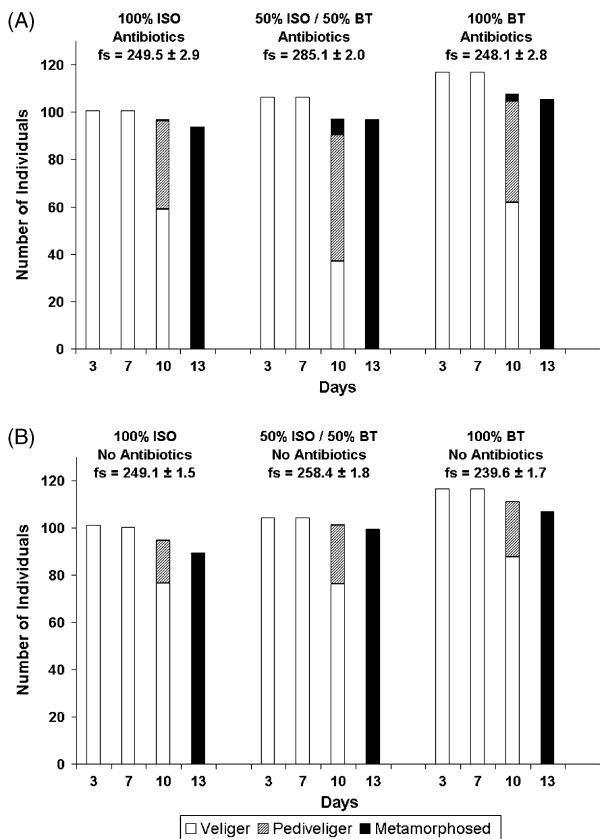


Fig. 2. The effects of single species diets of *Isochrysis* (ISO), *Aureococcus anophagefferens* (BT) or a mixed diet of 50% (v/v) *Isochrysis* and 50% *Aureococcus anophagefferens* (BT) on survivorship and development of larvae grown with antibiotics (A), and without antibiotics (B) (26 °C). Veligers are indicated by white bars, pediveligers by shaded bars and metamorphosed juveniles by solid bars. For each treatment, bars represent the average of four replicates for diet treatments. Days indicates the time since fertilization. fs, the final mean size (μm) \pm standard error of larvae and newly metamorphosed juveniles across replicates.

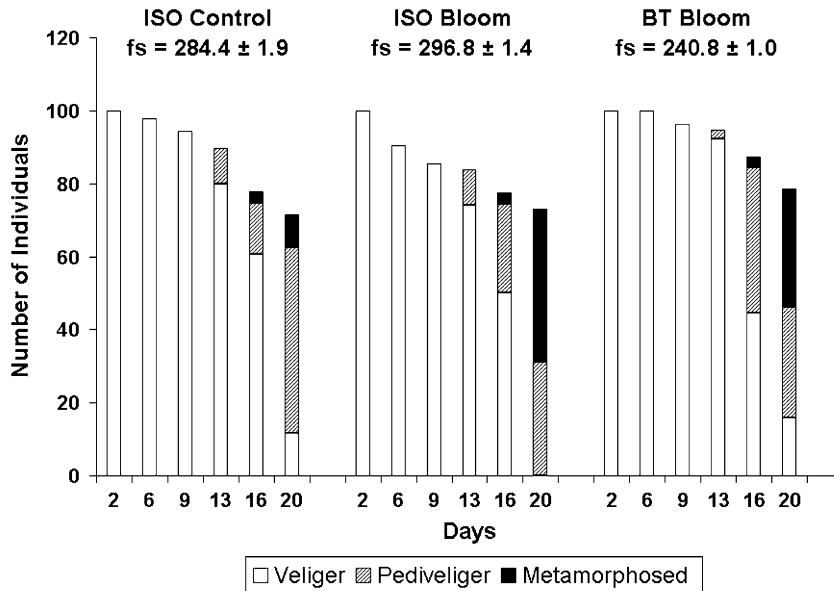


Fig. 3. The effects of single species diets of *Isochrysis* (ISO) at control concentrations (2×10^4 cells ml^{-1}), *Aureococcus anophagefferens* (BT) at a bloom concentration (1×10^6 cells ml^{-1}), and a biovolume of *Isochrysis* equal to the bloom brown tide treatment (1.25×10^5 cells ml^{-1}) on survivorship and development of hard clam larvae (with antibiotics, 22 °C). Veligers are indicated by white bars, pediveligers by shaded bars and metamorphosed juveniles by solid bars. For each treatment, bars represent the average of four replicates for diet treatments. Days indicates the time since fertilization. fs, the final mean size (μm) \pm standard error of larvae and newly metamorphosed juveniles across replicates.

3.4. The effects of culture growth rate, with antibiotics, 22 °C

To test whether the growth rate of *A. anophagefferens* cultures impacts *M. mercenaria* larvae, larvae were reared with three different diet treatments: (1) the control diet of *Isochrysis* (2×10^4 cells ml^{-1}), (2) a slow growing *A. anophagefferens* culture (0.51 ± 0.14 day $^{-1}$; 1.6×10^5 cells ml^{-1}) and (3) a stationary

phase (very slow to no growth) *A. anophagefferens* culture (0.06 ± 0.03 day $^{-1}$) at the same density. For the *A. anophagefferens* cultures, both growth rates were slower than for the cultures used in previous experiments (e.g., 0.90 ± 0.10 day $^{-1}$ in Section 3.3).

This experiment lasted 23 days and overall survivorship averaged 72%, and ranged from 49 to 81% among replicates. There was no significant effect of diet on survivorship ($P = 0.06$). However, there was a sig-

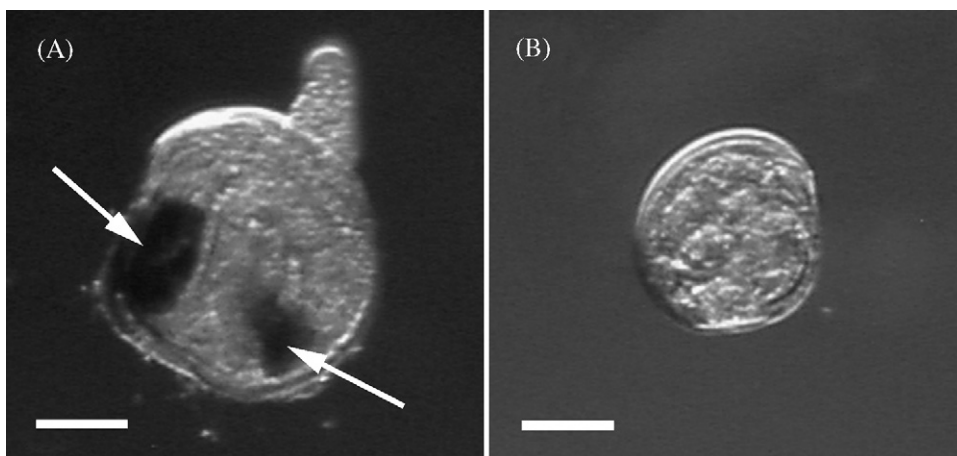


Fig. 4. Hard clam larvae fed *Isochrysis* (A) had conspicuous brown lipid droplets (arrows). Larvae fed bloom concentrations of *Aureococcus anophagefferens* (B) had no visible lipid stores as compared to those fed *Isochrysis*. Scale bars = 100 μm .

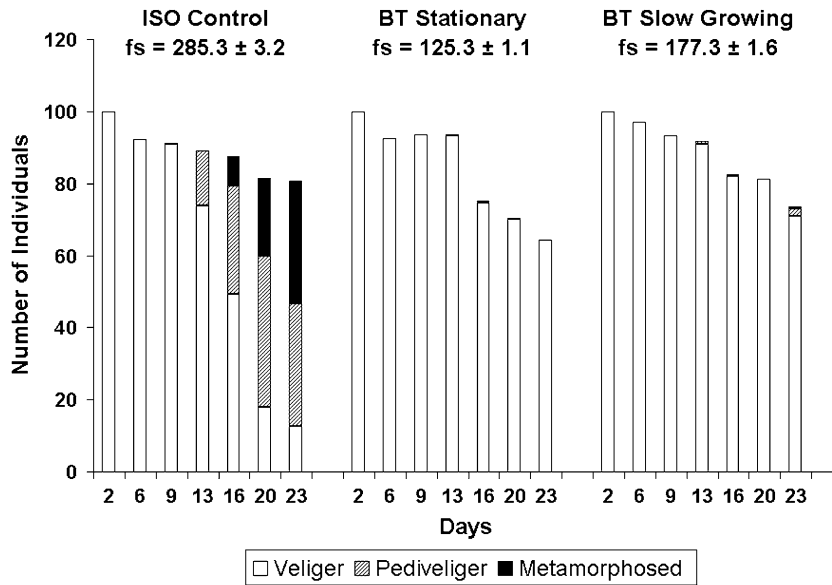


Fig. 5. The effects of single species diets of *Isochrysis* (ISO), stationary phase *Aureococcus anophagefferens* cultures (BT stationary) and slow growing *Aureococcus anophagefferens* (BT slow growing) on survivorship and development of hard clam larvae (with antibiotics, 22 °C). Veligers are indicated by white bars, pediveligers by shaded bars and metamorphosed juveniles by solid bars. For each treatment, bars represent the average of four replicates for diet treatments. Days indicates the time since fertilization. fs, the final mean size (μm) \pm standard error of larvae and newly metamorphosed juveniles across replicates.

nificant effect of diet on development time ($P = 0.006$). At the end of the experiment, of those larvae fed *Isochrysis*, 40% had completely metamorphosed, and an additional 43% were at the pediveliger stage (Fig. 5). Of those fed the slow growing *A. anophagefferens* cultures only 0.7% had metamorphosed and an additional 2.8% were at the pediveliger stage, while none of those fed the stationary phase *A. anophagefferens* cultures even reached the pediveliger stage. This difference in development between the two *A. anophagefferens* treatments was significant (G -test, $P = 0.05$); those fed the slow growing culture developed faster than those fed the stationary phase diet (Fig. 5).

There was also a significant effect of diet on larval growth (ANOVA, $P < 0.0001$). Those fed *Isochrysis* grew larger than those fed slow growing *A. anophagefferens* ($P = 0.0018$), which grew larger than those fed stationary phase *A. anophagefferens* ($P = 0.0019$) (Fig. 5). As with the larvae in the experiment to test the effects of bloom concentrations of *A. anophagefferens*, all of the larvae fed *Isochrysis* had conspicuous yellow colored lipid droplets, while none of the larvae fed either *A. anophagefferens* treatment had similar droplets.

4. Discussion

Survivorship of hard clam larvae was not affected by the brown tide alga, *A. anophagefferens*. Under most

experimental conditions tested, larvae fed a diet of *A. anophagefferens* (either as a sole food or when mixed with *Isochrysis*), larvae developed at a faster rate than those fed the unialgal control diet of *Isochrysis*, even at bloom concentrations (Table 1). When larvae were fed slow growing or stationary cultures, however, development was significantly retarded. Contrary to findings

Table 1

Summary of significant effects of the brown tide alga, *Aureococcus anophagefferens*, on survivorship, development and growth in the larva of the hard clam, *Mercenaria mercenaria*

Treatment	Survivorship	Development	Growth
Culture medium	–	–	= or –
Antibiotics	=	+	+
BT + I, 22 °C	=	+	=
BT + I, 26 °C	=	+	+
BT, 26 °C	=	+	=
Bloom BT, 22 °C	=	+	–
Slow BT, 22 °C	=	–	–
Stationary BT, 22 °C	=	–	–

(=) indicates no effect, (+) indicates a positive effect and (–) indicates a negative effect compared to the control, larvae fed *Isochrysis* (2×10^4 cells ml^{-1}). BT, a diet of the brown tide alga; I, a diet of *Isochrysis* and BT + I, a mixed diet of equal biovolumes of each alga. Bloom concentrations of the brown tide alga (bloom BT) were 10^6 cells ml^{-1} , slow growing brown tide cultures (0.51 ± 0.14 day $^{-1}$, slow BT) were late log phase growth, and stationary brown tide cultures were not appreciably growing (0.03 ± 0.10 day $^{-1}$ stationary BT).

for adult and juvenile *M. mercenaria* (Bricelj et al., 2001; Greenfield and Lonsdale, 2002; Wazniak and Glibert, 2004) and for scallop larvae (Gallager et al., 1989), we found either no effect of *A. anophagefferens* on hard clam larval growth, or enhanced growth (when in a mixed diet) at moderate cell densities (Table 1). We did, however, find evidence to support the hypothesis that *A. anophagefferens* has a nutritional impact on *M. mercenaria* larvae when larvae are fed a single species diet of *A. anophagefferens* at bloom concentrations or slow growing cultures of *A. anophagefferens*. This impact on growth does not appear to be due to a cessation of feeding, as has been suggested for juveniles and adult bivalves, as larvae in all experiments grew (more than doubling in size from fertilized eggs; Castagna, 2001) and those fed a mixed diet had enhanced growth; if *A. anophagefferens* caused larvae to stop feeding, then even on a mixed diet we would expect reduced growth rates, never enhanced growth. Mixed species diets often result in faster growth of larvae (Brown et al., 1993, 1998), especially when the two diet items have complimentary nutritional qualities (Pechenik, 1987; Schioppa et al., 2006).

The negative impacts of slow growing *A. anophagefferens* cultures on larval growth could be due to several different factors. The nutritional quality of microalgal foods can change as a function of their growth rate or growth conditions (Thompson and Harrison, 1992; Brown et al., 1993, 1998; Roncarati et al., 2004). Stationary phase cultures of *A. anophagefferens* may have lower nutritional quality (e.g., have depleted the phosphorus or nitrogen from their growth medium and therefore deficient in cellular phosphorus or nitrogen), which could reduce growth in larvae (Wikfors et al., 1992; Brown et al., 1993; Jones and Flynn, 2005).

Harmful effects of brown tide may also be dependent upon the physiological state and/or growth rate of *A. anophagefferens* cells. The toxicity or harmful effects of many harmful algal species increase under nutrient limitation and/or when they enter stationary phase growth (Bates, 1998; Cembella, 1998; Johansson and Graneli, 1999a,b). While, to date, no toxin has been isolated from *A. anophagefferens*, there is evidence for inhibition of gill ciliary activity in some juvenile and adult bivalves (Gainey and Shumway, 1991); this inhibition may be caused by some compound associated with the extracellular polysaccharide (EPS) sheath surrounding the cells (Sieburth et al., 1988). Phytoplankton are known to release substantially more extracellular dissolved organic carbon in general, and polysaccharides in particular, as they become nutrient

limited and enter stationary phase growth relative to exponential phase growth (Mague et al., 1980; Lee and Fisher, 1992; Mykelstad, 1995). Hence, the negative impact of slow growing *A. anophagefferens* on development and growth of hard clam larvae may be caused by increased EPS production in stationary phase growth. Bricelj et al. (2001) also reported that *A. anophagefferens* cultures in late stationary phase growth were significantly more toxic to blue mussels (*M. edulis*) than were cultures in early exponential phase growth.

If the impacts of a diet of *A. anophagefferens* are nutritional, then even if a brown tide does not have an immediate impact on hard clam larval survivorship, there may be a lasting nutritional legacy (Padilla and Miner, 2006) that impacts post metamorphic survivorship, growth or performance. This nutritional legacy may be especially important for early juvenile stages, which may have high metabolic demands and may not filter an adequate volume of high quality food to fuel growth as well as energetic maintenance costs. Work has shown that size at metamorphosis and lipid concentrations of newly metamorphosed juveniles influences survivorship and subsequent growth (Moran and Emler, 2001; Phillips, 2002; Marshall et al., 2003; Marshall and Keough, 2004; Emler and Sadro, 2006).

4.1. Experimental considerations

We used a single strain of *A. anophagefferens* (CCMP1708), which has previously been shown to inhibit feeding rates of juvenile bivalves (Bricelj et al., 2001). In our experiments we used two culture sources; one we obtained from Bigelow culture bank 1 month before the initiation of our experiments, and one that has been in active culture since its initial isolation (D. Lonsdale, Stony Brook University). Neither culture had an effect on survivorship. Both cultures were used to feed larvae in the first two experiments, which resulted in enhanced development and no effect on growth. It is well known, however, that there are strain differences in nutritional quality and harmful attributes of algae (Chorus and Bartram, 1999; Bricelj et al., 2001; Burkholder et al., 2001, 2005; Wilson et al., 2005). Strains of *Aureococcus* isolated from the first brown tide events in New York (1986) were initially found to inhibit gill cilia in shellfish (Gainey and Shumway, 1991), but have subsequently lost their ability deter feeding in some shellfish (Bricelj et al., 2001). Additional strains of *A. anophagefferens* should be tested for their effects on larvae of *M. mercenaria*, and other species to determine the generality of our findings.

Algal growth medium had a large impact on survivorship, development and growth of *M. mercenaria* larvae. Care should be taken to remove culture medium when conducting such experiments, so that results are not confounded by the negative impacts of the medium itself.

Antibiotics had no negative effects on *M. mercenaria* larvae. An added benefit of using antibiotics, is that they greatly reduce bacteria found in cultures of *A. anophagefferens* (Berg et al., 2002). The harmful effects of some phytoplankton have been known to be influenced by the presence or absence of bacteria (Doucette, 1995). All laboratory studies of the harmful impacts of *A. anophagefferens* on shellfish and zooplankton to date have used non-axenic cultures (reviewed in Gobler et al., 2005); it is possible that observed effects may be affected by the presence of bacteria. By using antibiotics, the effects (or lack of effects) that we observed were therefore most likely due to the alga, *A. anophagefferens*, not an artifact of bacterial contaminants. Previous work on *A. anophagefferens* has found no negative impact of the antibiotics we used on this alga (Berg et al., 2002).

4.2. Implications for restoration and recovery of *M. mercenaria* populations

Since the decline of hard clam populations on Long Island, many different strategies have been attempted to enhance the fishery, including the release of larvae and hatchery reared clams (M. Burns, Islip Shellfish Hatchery, personal communication). These strategies, however, have had limited long term success, have not resulted in the restoration of historic population densities, and have not allowed the fishery to recover to pre 1980s levels. The Long Island Chapter of The Nature Conservancy (TNC) has begun a concentrated effort to restore hard clam populations in Long Island waters, and to restore ecosystem function and health by restoring these important ecosystem engineers. Indeed, experimental evidence and models now suggest that high densities of hard clams may prevent harmful brown tide blooms from developing (Cerrato et al., 2004; E.E. Hofmann, J. Krauter, personal communication).

There will be no recovery of hard clam populations if the larvae that are produced do not grow to metamorphosis, and if juveniles do not flourish and survive to produce reproductive adults in high densities. Changes in the phytoplankton communities of Long Island that have occurred over the past 30 years could be exerting a bottleneck on population recovery that will have to be

overcome. Great South Bay was dominated by moderate sized phytoplankton species during the 1970s (Weaver and Hirshfield, 1976; Cassin, 1978; Lively et al., 1983), however, recent data demonstrate that much of the phytoplankton biomass in these ecosystems during the summer is $<2 \mu\text{m}$ (Gobler et al., 2004; Sieracki et al., 2004). Although Riisgard (1988) found that, in the laboratory, bivalve larvae retain particles $<4 \mu\text{m}$ with a lower efficiency than larger particles, Gallager et al. (1994) found that *M. mercenaria* larvae can feed on the picocyanobacteria *Synechococcus* sp. ($1 \mu\text{m}$), which has been shown to co-bloom (at densities of $10^5 \text{ cells ml}^{-1}$) with brown tide or to dominate phytoplankton biomass ($10^6 \text{ cells ml}^{-1}$) in Long Island bays when *A. anophagefferens* does not bloom (Gobler et al., 2004). Larval growth can be enhanced with mixed algal diets containing *Synechococcus* up to $10^4 \text{ cells ml}^{-1}$, however, larvae grown on single species diets of bloom concentrations of *Synechococcus* had atrophied digestive glands and their growth was halved (Gallager et al., 1994), similar to what was found for larvae grown solely on *A. anophagefferens* at bloom concentrations in this study. In Long Island estuaries *Synechococcus* can now be found at bloom concentrations during the summer ($10^5 \text{ cells ml}^{-1}$, Gobler et al., 2004; Sieracki et al., 2004).

Brown tides most commonly develop during early summer months (late May/early June), when temperatures in mid-Atlantic estuaries approach $15\text{--}20^\circ\text{C}$ (Gobler et al., 2005). Blooms generally persist until temperatures exceed 25°C , at which point densities are greatly reduced, as this species is unable to grow at high temperatures (Casper et al., 1989; Gobler et al., 2005). Presently, in Great South Bay peak spawning in hard clams is mid-summer (Doall and Padilla, unpublished data), when temperatures reach $24\text{--}25^\circ\text{C}$, thus, the likelihood that hard clam larvae experience bloom concentrations of brown tide are low.

Extremely warm summers, with water temperatures above the threshold for brown tide growth ($>25^\circ\text{C}$; Casper et al., 1989; Gobler et al., 2005), may thus benefit hard clam larvae. In contrast, cooler summers, when temperatures do not exceed 25°C , may be most detrimental to larvae, as *A. anophagefferens* may be present for an extended period with stationary phase growth just when larvae are spawned into the water column. Historical temperature and cell count records indicate that both of these scenarios have occurred in Long Island estuaries during the past two decades (SCDHS, 1985–2002). The results of this study indicate that, at present, the brown tide alga does not pose a large mortality risk for hard clam larvae, and thus is not a likely bottleneck for recovery of populations.

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