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Effects of brown tide (*Aureococcus anophagefferens*) on hard clam *Mercenaria mercenaria* larvae and implications for benthic recruitment

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ABSTRACT: Brown tides of *Aureococcus anophagefferens* occur in shallow mid-Atlantic bays in the USA and attain peak summer densities of ~1000 to 2800 cells μl^{-1}. Blooms coincide with the period of spawning and planktotrophic larval development of the hard clam *Mercenaria mercenaria*, a commercially important bivalve in the region. This laboratory study investigates the effects of *A. anophagefferens* (toxic isolate from Provasoli-Guillard Center for Culture of Marine Phytoplankton [CCMP 1708]) on hard clams throughout their larval development as a function of increasing (1) supplement and (2) contributor to total phytoplankton cell volume in the diet. Brown tide consistently inhibited veliger growth rates in a dose-dependent manner, leading to arrested development in the D-stage; yet mortalities varied greatly among larval batches and were attributed to secondary effects of nutritional stress. Growth of larvae exposed for 2 wk to brown tide at 800 cells μl^{-1} was 89 to 90% less than controls fed *Isochrysis galbana* (clone T-iso) in both the presence and absence of alternate food. No recovery was observed when larvae were returned to the control diet. However, larvae showed variable intrapopulation susceptibility to brown tide when exposed to a mixed suspension of *A. anophagefferens* and *I. galbana* (400 and 50 cells μl^{-1}, respectively). Exposure to low levels of brown tide (50 cells μl^{-1}) resulted in relatively small but significant growth reduction. Larvae had reduced larval clearance rates (ingestion) when exposed to unialgal brown tide, as confirmed by analysis of gut autofluorescence and negative feeding selectivity for *A. anophagefferens* in a mixed suspension. Therefore, primarily through their inhibitory effects on growth, brown tides at ≥200 cells μl^{-1} are expected to cause metamorphic failure of hard clam larval populations. These will lead to extended larval life in the plankton and increased vulnerability to secondary mortality factors. In turn, hard clam larvae are expected to make a negligible contribution to microzooplankton grazing on brown tide.

KEY WORDS: Brown tide · *Aureococcus anophagefferens* · Clam larvae · *Mercenaria mercenaria*

INTRODUCTION

Brown tides of the picoplanktonic alga *Aureococcus anophagefferens* (Pelagophyceae, ~2 µm equivalent spherical diameter [ESD]) have occurred for 2 decades in shallow, temperate estuaries in the mid-Atlantic USA, where they can attain maximum summer densities of ~1000 to 2800 cells μl^{-1} (Gobler et al. 2005). They have appeared in Narragansett Bay, Rhode Island (Sieburth et al. 1988), Long Island bays, New York, (Gobler et al. 2005), the Barnegat/Little Egg Harbor barrier island system, New Jersey (Gastrich et al. 2004) and more recently in Maryland bays (Wazniak & Glibert 2004). Low cell densities have been detected along the Atlantic USA coast from Maine to Florida. *A. anophagefferens* can survive and even persist through the winter at bloom levels (e.g. in 1999–2000 in Great South Bay, New York [records of the Suffolk County Department of Health Services, SCDHS]). Brown tide was reported in Saldaha Bay, South Africa (Probyn et al. 2001) and has since been identified in ship ballast water (Doblin et al. 2004), suggesting a potential risk of further spread of these blooms.

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Brown tides have caused considerable economic losses by adversely affecting wild and cultured populations of bivalves, including the hard clam *Mercenaria mercenaria*, which supports valuable commercial and recreational fisheries and aquaculture in Atlantic North America. Brown tides have caused feeding inhibition of adult suspension-feeding bivalves (e.g. *M. mercenaria* and *Mytilus edulis*), mass mortalities of adult mussels *M. edulis*, growth inhibition and recruitment failure of bay scallops *Argopecten irradians* (reviewed by Bricelj & Lonsdale 1997) and severe (67%) mortalities of 1 mm hard clams (Greenfield et al. 2002). The inability of the hard clam fishery in Great South Bay to recover from overfishing after relaxation of fishing effort may be exacerbated by the chronic recurrence of brown tides (Gobler et al. 2005).

Effects of brown tide are concentration-dependent. Growth rates of juvenile hard clams during an outbreak in Maryland bays ceased at concentrations exceeding 150 cells μl^{-1} (Wazniak & Glibert 2004). Controlled laboratory studies have shown that clearance rates of juvenile *Mercenaria mercenaria* were significantly reduced above a threshold of ~35 cells μl^{-1} of a toxic *Aureococcus anophagefferens* isolate (CCMP 1708) (Bricelj et al. 2001). Growth rates of ~7 mm juveniles were completely suppressed at ≥400 cells μl^{-1} of this isolate in both unialgal and mixed assemblages. However, in the presence of alternate food, these juveniles showed only transient growth reduction at 80 cells μl^{-1}, suggesting acclimation over time to moderate brown tide (Bricelj et al. 2004). Feeding reduction by brown tide in adult bivalves occurs via inhibition of gill lateral cilia responsible for generation of feeding currents (Gainey & Shumway 1991). To date, the bioactive compound responsible for this effect is not known, but its action appears similar to that of the neurotransmitter dopamine and is associated with the alga's extracellular polysaccharide (EPS) layer (Gainey & Shumway 1991).

Brown tide typically occurs in late spring to summer, lasting from 1 to 3 mo and coinciding with the period of spawning and larval development of hard clams in the mid-Atlantic USA (Fig. 1). Despite this coincidence, limited information is available on the effects of brown tide on bivalve larvae (meroplankton). Heterotrophic protists are typically the dominant water column grazers of *Aureococcus anophagefferens* (Lonsdale et al. 1996, Caron et al. 2004). However, in shallow estuaries (<4 m deep), meroplankton could compete with holozooplankton in exerting grazing pressure on the picophytoplankton. Whereas adult *Mercenaria mercenaria* show reduced (~50%) retention efficiency of 2 µm particles (Riisgard 1988a), larvae readily capture particles between ~1 and 4 µm (Gallager et al. 1994, Baldwin 1995). The former indicated that techniques previously

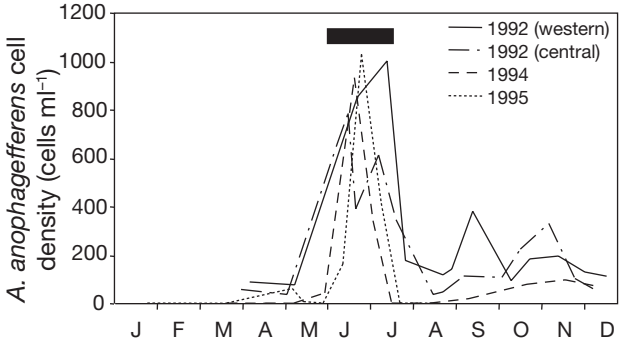

Fig. 1. *Aureococcus anophagefferens* and *Mercenaria mercenaria*. Seasonal occurrence of brown tide in Great South Bay, Long Island, NY during 3 representative years (cell density data from SCDHS — Suffolk County Department of Health Services, NY). No data available between Jan and Mar 1992. Horizontal bar = timing of spawning of hard clams *M. mercenaria* in pre-brown tide years (from Kassner & Malouf 1982)

used by Riisgard (1988b), which did not account for fecal contamination of particle size spectra, grossly underestimated clearance rates of hard clam larvae on picoplankton. Larvae are also likely to be more vulnerable to starvation than juveniles or adults. The only prior study on the effects of brown tide on bivalve larvae was conducted on bay scallops *Argopecten irradians* (Gallager et al. 1989). *A. anophagefferens* (190 to 750 cells μl^{-1}) was found to significantly reduce survival and growth of bay scallop veligers, relative to controls, even in the presence of nutritious algae. However, the effects of harmful algae are known to be highly species-specific.

The main objective of this laboratory study was thus to investigate the density-dependent effects of a toxic isolate of *Aureococcus anophagefferens*, alone or in combination with *Isochrysis galabana* (clone T-iso), on growth and survival of planktotrophic larvae of *Mercenaria mercenaria* throughout development (from D-stage to pediveliger). Short-term effects on larval feeding including gut fullness, clearance rate and feeding selectivity were also determined. It is important to assess the effects of *A. anophagefferens* in mixed phytoplankton assemblages, as this picoplankter can make a variable contribution to total algal biomass depending on site and time (Gobler et al. 2005, Sieracki et al. 2004). Likewise, grazing pressure of bivalve larvae on *A. anophagefferens* is important in understanding selective grazing within the pelagic food web and determining changes in the balance between *A. anophagefferens* growth rate and grazing mortality rate as determinants of bloom dynamics. Experimental results of this study are being incorporated into a numerical simulation model (Hofmann et al. 2006) to assess the influence of brown tide on recruitment suc-

cess of natural hard clam populations in US Atlantic bays, and thus evaluate the potential for rehabilitation of this valuable fishery via restocking efforts.

MATERIALS AND METHODS

Source of larvae and experimental setup. Hard clams *Mercenaria mercenaria*, collected from a native population in St. Mary's Bay, Nova Scotia, were conditioned and induced to spawn at the Institute for Marine Biosciences' Marine Research Station (IMB/MRS). Thirty to 50 clams were conditioned and induced to spawn at any given time. Fertilized oocytes were kept in 0.22 µm-filtered seawater (FSW) overnight and divided among experimental treatments as first-feeding veligers (24 h-old straight-hinged or D-stage larvae). They were maintained in gently aerated 17 l fiberglass conical tanks, covered with PVC lids, filled with ambient 0.22 µm cartridge-FSW at 22°C, a temperature within the optimum range for *M. mercenaria* larvae (Davis & Calabrese 1964) and at 30‰ salinity. Each diet treatment was run in triplicate tanks with a stocking density of 2.5 larvae ml^{-1}. Tanks were drained every 3 d at the time of complete water change, and larvae were collected on a 58 µm Nitex sieve. A subsample of larvae was removed to determine shell lengths (n = 50) and cumulative mortalities (n = 100) using video microscopy with an analog camera (Pulnix model TMC-7DSP) coupled to a compound microscope. All videos were recorded (Sony DVCAM, DSR-V10) and calibrated using a stage micrometer. Shell lengths (SL) were measured as the maximum anteroposterior shell dimension and were determined using image analysis software (Image Pro Plus version 4.5, Media Cybernetics). Dead larvae were retained in the experimental systems to allow determinations of cumulative mortalities over time. The percentage of total live larvae that remained arrested in D-stage was also determined at the end of the experiment.

Growth and survival experiments. Larvae were fed algal isolates obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP): *Isochrysis galbana* (CCMP 1324, clone T-iso, ~4.5 µm ESD) and/or a toxic *Aureococcus anophagefferens* isolate (CCMP 1708, ~2.2 µm ESD) from West Neck Bay, NY. *I. galbana* (T-iso) was chosen as the control diet as it is known to support high growth rates of hard clam larvae (e.g. Tiu et al. 1989) and postlarvae (Wikfors et al. 1992) in unialgal suspensions. Algae were batch-cultured, non-axenically in 20 l aerated carboys using previously described methods (Bricelj et al. 2004). Cell densities were determined with a Beckman-Coulter Multisizer III particle counter fitted with a 50 µm aperture tube and held constant via daily additions of stock cultures in late exponential/early stationary growth phase that had attained *A. anophagefferens* densities of ~40×10^9 cells μl^{-1}. Algae can lose their toxicity over long-term culture (Bricelj et al. 2001); therefore, the toxicity of the CCMP 1708 isolate was confirmed prior to starting larval experiments (i.e. in August 2004). Toxicity was determined from the % inhibition of clearance rate (CR) of juvenile *Mytilus edulis* at 1000 cells μl^{-1} relative to an equivolume control of *I. galbana*, according to the method of Bricelj et al. (2001). This isolate elicited 94.4% reduction in CR of mussels after 3 h of acclimation to the diet. Bricelj et al. (2001) also demonstrated highly significant reduction in CR of mussels exposed to strain CCMP 1708 relative to that obtained with a morphologically identical *A. anophagefferens* isolate (CCMP 1784), and thus attributed this result to differential toxicity between the 2 strains.

Three experiments of 12 to 15 d duration were conducted. In Expt I (cohort produced in September 2004), larvae were offered a baseline diet of *Isochrysis galbana* (50 cells μl^{-1}) supplemented with increasing concentrations of *Aureococcus anophagefferens*: 100, 200, 400 and 800 cells μl^{-1}. Unialgal controls consisted of moderate and high concentrations of *I. galbana* (50 and 150 cells μl^{-1} respectively) to account for concentration-dependent effects. The high-density control was equivalent in biovolume concentration to the highest brown tide treatment (~7×10^6 μm^3 ml^{-1}). A non-fed treatment held in 0.22 µm FSW was also included to determine whether brown tide mimicked the effects of starvation.

Expt II, which used a different larval cohort produced in June 2005, compared the effects of additions of *Aureococcus anophagefferens* cells and cell-free filtrates to a baseline diet of *Isochrysis galbana* (50 cells μl^{-1}). Filtrate volumes added were equal to those added daily with stock suspensions of *A. anophagefferens* cells to the high brown tide treatment (400 cells μl^{-1}). Filtration of cultures was conducted through 0.8 µm Millipore filters (90 mm diameter) under low vacuum (≤10 mm Hg). An additional treatment included the addition of nutrient media used for culturing *A. anophagefferens* prior to inoculation with this picoplankter to exclude the possibility that this could contribute to *Mercenaria mercenaria* toxicity. At the end of Expt II (Day 12), the dry weight (DW) and percent ash content of larvae were determined in the control and high brown tide treatments (for 2 to 3 tanks). A suspension of known density of live larvae was filtered onto pre-combusted, pre-weighed, Whatman GF/C glass fiber filters and rinsed with isotonic ammonium formate solution to remove salts. Filters were dried at 80°C for 24 h, combusted overnight at 480°C and weighed on a Cahn electrobalance.

In Expt III, larvae (produced in July 2005) were exposed to suspensions in which *Aureococcus anophagefferens* contributed varying proportions: 100, 75, 50, 25 and 0% of the total cell volume concentration ($\sim 5 \times 10^6$ µm^3 ml^{-1}), and the remainder was made up by *Isochrysis galbana*. At the end of 15 d, larvae exposed to *A. anophagefferens* at 800 and 400 cells µl^{-1} (100 and 50% of total algal volume respectively) were transferred to an optimum diet of *I. galbana* at 100 cells µl^{-1} for 12 d to assess their capacity for recovery from brown tide. Once most larvae were competent to metamorphose, they were transferred to a downweller (130 µm Nitex screen) inserted in the experimental conical tank with circulation maintained by an airlift. This step was only necessary for larvae fed *I. galbana* throughout development, that were transferred on Day 15 from the start of the experiment when they attained metamorphic competence. The percentage of live larvae that had metamorphosed was determined by the presence of the dissoconch or postlarval shell throughout the experimental period for all treatments.

Feeding experiments. The relative ability to graze upon algal cells was determined in separate experiments in which larvae were held in the same experimental systems but at higher stocking densities (5 larvae ml^{-1}) to allow for measurable, overnight depletion of cells in the suspension. Trial 1 was conducted with larvae produced in March 2005 from broodstock overwintered at MRS; Trial 2 was conducted with the same larval cohort used for the second growth experiment. Clearance rate was determined for 7 to 8 d-old larvae that had been previously grown on *Isochrysis galbana* and were exposed for ~1 h of acclimation to the diet treatments (initial exposure) and for 5 d on diet treatments (repeated measurements, only for Trial 1). Three diet treatments, equal in total algal cell volume, were tested: (1) unialgal *Aureococcus anophagefferens* at 400 cells µl^{-1}; (2) unialgal *I. galbana* at 50 cells µl^{-1} and (3) a mixed suspension of *I. galbana* (25 cells µl^{-1}) and *A. anophagefferens* (200 cells µl^{-1}). The CR were determined from the exponential depletion of cells from the suspension in the static, aerated systems from the equation: $CR = [(\ln Co - \ln Ct)]/N] \times V/t$, where Co and Ct are initial and final cell densities respectively, V = volume of the tank, t = incubation time and N = number of live larvae (Coughlan 1969). Algal densities were determined using a Beckman-Coulter electronic particle counter (duplicate counts, typically ≤1% variation) with parallel control tanks for detection of any settlement and/or algal growth. Incubation times ranged from 16 to 19.5 h.

Contamination by larval feces can potentially occur in particle size-spectra measured by Coulter Counter but does not affect those obtained with flow cytometry (FCM) (Baldwin 1995). Therefore, the ratio of the 2 algae in suspension before and after feeding was also determined independently by FCM to validate results obtained with the particle counter (Trial 2, mixed suspension). Samples fixed with 1% paraformaldehyde were analyzed within a few days of fixation with a FACSort flow cytometer (Becton-Dickinson), and discrimination between *Isochrysis galbana* and *Aureococcus anophagefferens* was achieved by simultaneous measurement of forward light scatter (FSC, relative size) and chlorophyll fluorescence (>650 nm, FL3 detector). Sample volume was not determined, so only the relative proportions of the 2 species before and after feeding were obtained with this method.

Gut contents of individual live larvae were observed qualitatively using a Leica DMRE epifluorescence microscope. Observations were initially made of intact larvae in a well, and then a subsample was transferred onto a glass slide and gently squashed with a cover slip to reduce the effect of depth of field and confirm initial observations. At least 20 to 30 larvae were observed in each unialgal treatment.

Statistical analysis. Final larval sizes and arc sine-transformed cumulative mortalities were compared using single classification ANOVA followed by Tukey multiple comparisons. Differences in linear growth rates among feeding treatments were compared by analysis of covariance (ANCOVA), with time as the independent variable and SL as the dependent variable. Clearance rates between unialgal treatments and between the 2 algae in the binary suspension were also compared using ANOVA. All statistical analyses were conducted using SYSTAT 10.0 (SPSS, Statistical Product and Service Solutions).

RESULTS

Growth and survival experiments

Expt I revealed that when added to a good food source (*Isochrysis galbana*) over 15 d of larval development, *Aureococcus anophagefferens* significantly reduced larval growth at all concentrations tested in a dose-dependent manner (Fig. 2A). Parameters of the linear regressions fitted to the temporal length data are shown in Table 1 and allow estimates of growth rates (slopes). Growth rate of the control treatment at 150 *I. galbana* cells µl^{-1} was significantly higher than that at 50 cells µl^{-1} (8.1 and 7.2 µm d^{-1}, respectively). Therefore, controls in Expt III were run at 100 cells µl^{-1} to approach maximal larval growth rates. Larvae showed positive shell growth rates in all treatments, including the unfed treatment, but growth rates were inversely related to the density of the *A. anophagefferens* supplement and were lowest in the unfed treatment.

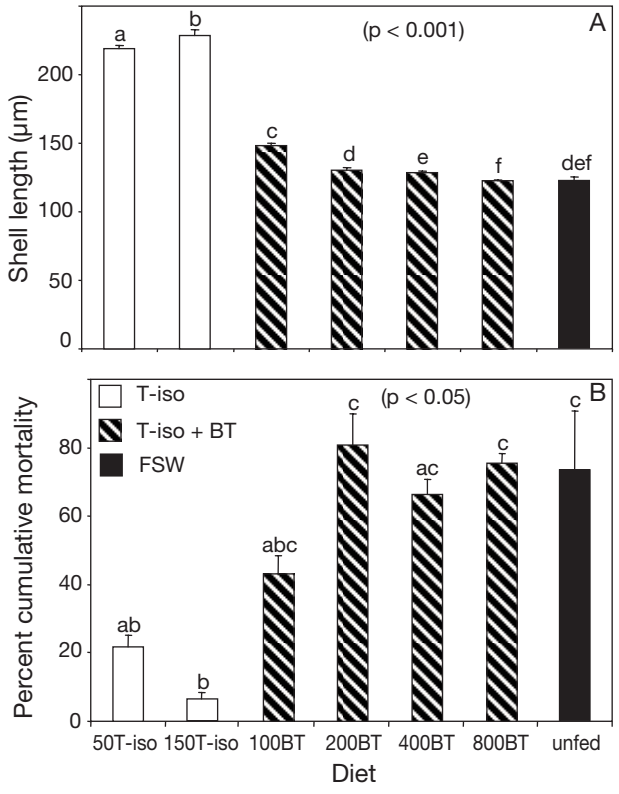

Fig. 2. *Mercenaria mercenaria*. Expt I: effects of a suspension of *Isochrysis galbana* (T-iso control at 50 cells µl^{-1}) supplemented with increasing cell densities of *Aureococcus anophagefferens* (brown tide = BT) from 100 to 800 cells µl^{-1}, compared to unfed hard clam larvae in filtered seawater (FSW). (A) Larval shell length; (B) percent cumulative mortality at 15 d (mean ± SE, 3 tanks). Cell volume of the high-density *I. galbana* control (150 cells µl^{-1}) = T-iso + 800 BT treatment. Different letters indicate statistically significant differences (ANOVA and multiple comparisons)

There was also a progressive non-linear increase in the percentage of live larvae that remained arrested in D-stage with increasing concentration of toxic algae in the mixed suspension, with comparable values at the highest *A. anophagefferens* concentration and unfed treatment (86 and 81%, respectively; Table 1). *A. anophagefferens* at ≥200 cells µl^{-1} also induced significantly higher mortalities than those found in *I. galbana* controls (Fig. 2B). Effects of maximal *A. anophagefferens* additions on larval survival were comparable to those of starvation.

Expt II showed that the inhibitory effect of brown tide on larval growth could be largely attributed to the presence of a toxic metabolite associated with the cellular rather than the dissolved phase of *Aureococcus anophagefferens* cultures. Cell-free filtrates caused a slight (7 %) although statistically significant reduction in growth rate (Fig. 3, Table 1), which may have resulted from some disaggregation of the EPS layer by vacuum filtration. It is noteworthy that even the lowest *A. anophagefferens* concentration (50 cells µl^{-1}) tested in this experiment had a significant deleterious effect on growth (13% reduction relative to the controls). Additions of the Fe-rich nutrient medium to the control diet and used to culture *A. anophagefferens* had no effect on larval growth rate (Table 1) or survival (data not shown). Overall, the larval batch used for this experiment showed improved performance relative to that used in the supplementation trial (Expt I), as larvae attained a higher maximum growth rate (10 µm d^{-1}) and showed no significant differences in cumulative mortalities by the end of 12 d of exposure in any of the treatments (data not shown; range 4 to 16%). Due to the faster growth of these larvae, the experiment was terminated on Day 12. At this time larvae exposed to the *A. anophagefferens* at 400 cells µl^{-1} + T-iso diet had a significantly lower mean dry weight (651 ng ± 30 SE) and significantly higher mean ash content (61.8% ± 0.2 SE) than larvae fed the control diet (1710 ng DW larva^{-1} ± 150 SE; 52.2% ± 0.3 SE) (ANOVA, $p < 0.002$). The appearance of larvae at the end of the experiment was comparable in the unfed treatment (Expt I) and in treatments exposed to unialgal brown tide (800 *A. anophagefferens* cells µl^{-1}, Expt III) or a mixed suspension (800 and 50 cells µl^{-1} of *A. anophagefferens* and *Isochrysis galbana*, respectively, Expt II) (Fig. 4). Larvae showed pale guts and most had not progressed beyond the D-stage (see also Table 1). In contrast, larvae fed the control diet showed densely pigmented, full guts and reached the pediveliger stage of development (Fig. 4A).

When larvae were exposed to varying proportions of the 2 algae (Expt III), growth rate was positively related to the proportion of *Isochrysis galbana* in the diet (Fig. 5A, Table 1). The percentage of live larvae that remained arrested in D-stage increased linearly ($r^2 = 0.95$) with increasing contribution of *Aureococcus anophagefferens* to the suspension (Table 1). This larval cohort showed a lower cumulative mortality on brown tide (maximum mortality 40%; Fig. 5B) than that used in the supplementation trial (maximum mortality 80%). Although in Expt III there was a general pattern of increasing mortalities with increasing proportion of *A. anophagefferens* in the diet, cumulative mortalities were not statistically significant. Reduced susceptibility to starvation is likely due to higher larval physiological condition, as reflected in higher growth rates (10 vs. 8 µm d^{-1} on the control diet). The highest unialgal concentration of *A. anophagefferens* (800 cells µl^{-1}) resulted in a reduction in growth rate relative to the control comparable to the mixed suspension in Expt I (90% in both cases relative to 150 or 100 *I. galbana* cells µl^{-1}) (Table 1).

Table 1. *Mercenaria mercenaria.* Linear regression equations fitted to data from 3 larval growth experiments of the form shell length (SL) (µm) = [*b* × Time (d)] + *a*. Coefficients of determination (r^2) and the % D-stage larvae from total live larvae are also shown. Algal treatments include dietary combinations of *Aureococcus anophagefferens* (BT) and *Isochrysis galbana* (T-iso), where preceding number is concentration in cells µl^{-1} (see 'Materials and methods'). Superscripts indicate results of ANCOVA, where different letters indicate significant differences within each experiment. nd = not determined

Algal treatment (cells µl^{-1})	Growth rate *b* (µm d^{-1})	*a*	r^2	% D-stage mean (SE)
Supplementation trial (Expt I)				
Low control (50T-iso)	7.17^{a}	115.34	0.99	0
High control (150T-iso)	8.08^{b}	115.47	0.98	0
100BT/50T-iso	2.63^{c}	114.34	0.90	33.6 (1.0)
200BT/50T-iso	1.28^{d}	113.04	0.87	61.2 (8.2)
400BT/50T-iso	1.13^{e}	113.16	0.84	71.0 (4.5)
800BT/50T-iso	0.80^{f}	112.08	0.79	86.1 (1.2)
Unfed	0.64^{def}	114.24	0.54	81.3 (4.5)
Filtrate trial (Expt II)				
Control (50T-iso)	10.00^{a}	108.39	0.99	0
BT medium/50T-iso	10.05^{a}	108.37	1.00	nd
BT filtrate/50T-iso	9.31^{b}	109.15	1.00	4.2 (5.5)
BT50/50T-iso	8.68^{c}	107.16	1.00	nd
BT400/50T-iso	3.88^{d}	109.43	1.00	49.1 (5.5)
Varying BT proportions (Expt III)				
100T-iso	9.81^{a}	111.51	0.99	0
75T-iso/200BT	3.95^{b}	107.58	0.98	9.0 (3.6)
50T-iso/400BT	2.04^{c}	109.44	0.98	49.4 (10.0)
25T-iso/600BT	1.78^{c}	109.33	0.95	56.4 (8.8)
800BT	1.01^{d}	109.82	0.98	79.1 (2.9)

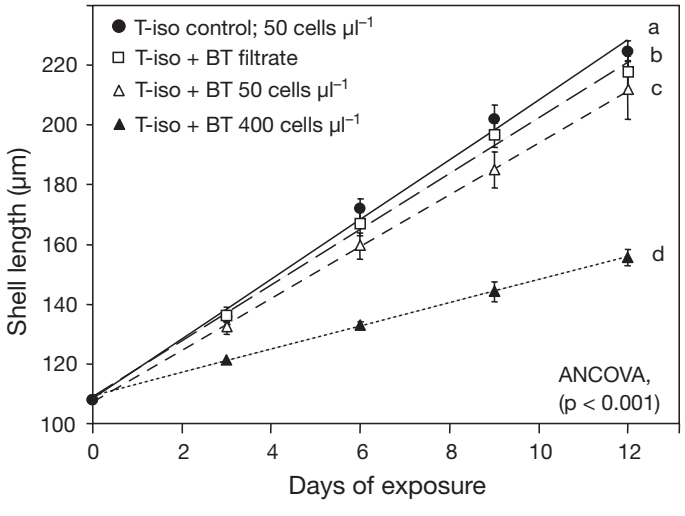

Fig. 3. *Mercenaria mercenaria.* Expt II: effect on hard clam larval shell length (mean ± SE, 3 tanks) of the addition of *Aureococcus anophagefferens* (brown tide = BT) cell-free culture filtrate to a suspension of *Isochrysis galbana* (T-iso; 50 cells µl^{-1}) relative to the effects of particulate addition (50 and 400 *A. anophagefferens* cells µl^{-1}). Different letters indicate statistically significant differences among slopes (Table 1) of linear regression equations fitted to the data (ANCOVA)

During recovery on the control *Isochrysis galbana* diet, larvae previously exposed to the mixed suspension of *Aureococcus anophagefferens* (400 cells µl^{-1}) and *I. galbana* achieved a growth rate (8.6 µm d^{-1}) comparable to those fed the control diet either before or after they started to metamorphose (9.8 and 7.0 µm d^{-1}, respectively) (Fig. 6A). By Day 12 of recovery, larvae previously exposed to unialgal brown tide (*A. anophagefferens* at 800 cells µl^{-1}) had suffered 100% mortality, whereas 20% of larvae previously fed the mixed suspension containing *A. anophagefferens* at 400 cells µl^{-1} survived through Day 12 (Fig. 6B). In contrast, larvae fed *I. galbana* throughout development showed high survival and 67% had completed metamorphosis by Day 12 of recovery.

Feeding experiments

Veliger larvae (7 d old) in Trial 1 (mean SL = 166.8 µm ± 1.8 SE) showed significant reduction (50.3%) in CR following initial exposure to unialgal brown tide (400 *Aureococcus anophagefferens* cells µl^{-1}) relative to an equal cell volume concentration of the control diet, and this reduction was exacerbated after 5 d of exposure (82.2% reduction) (Fig. 7). At this time larvae averaged 217 (unialgal T-iso), 211 (brown tide) and 223 µm (mixed diet). In Trial 2, feeding inhibition of 8 d-old veligers (mean SL = 194.1 µm ± 2.1) was evident sooner (93.8% following 24 h exposure to brown tide; Fig. 7). Percent depletion in unialgal trials averaged between 42 and 54% for T-iso and 5 to 34% for *A. anophagefferens*; in mixed suspensions depletion averaged 39 to 48% for T-iso and 18 to 31% for brown tide.

Exposure to the mixed suspension revealed that CR on *Isochrysis galbana* was consistently higher than that on *Aureococcus anophagefferens*, indicating that clam larvae were capable of selective feeding on *I. galbana*. The ratio of $CR_{T\text{-}iso}/CR_{BT}$ was equal to 1.65 and 2.36 following initial and 5 d exposure respectively to toxic cells in Trial 1, and 2.53 after initial exposure of larvae in Trial 2. Clearance on *A. anophagefferens* after 5 d exposure was also greater when this alga was offered in a mixed suspension than when offered alone. No significant difference was found between the ratios of the 2 algal species in the suspension measured by FCM and Coulter Counter (compared separately for the initial and final suspension, n = 3 tanks, p = 0.46

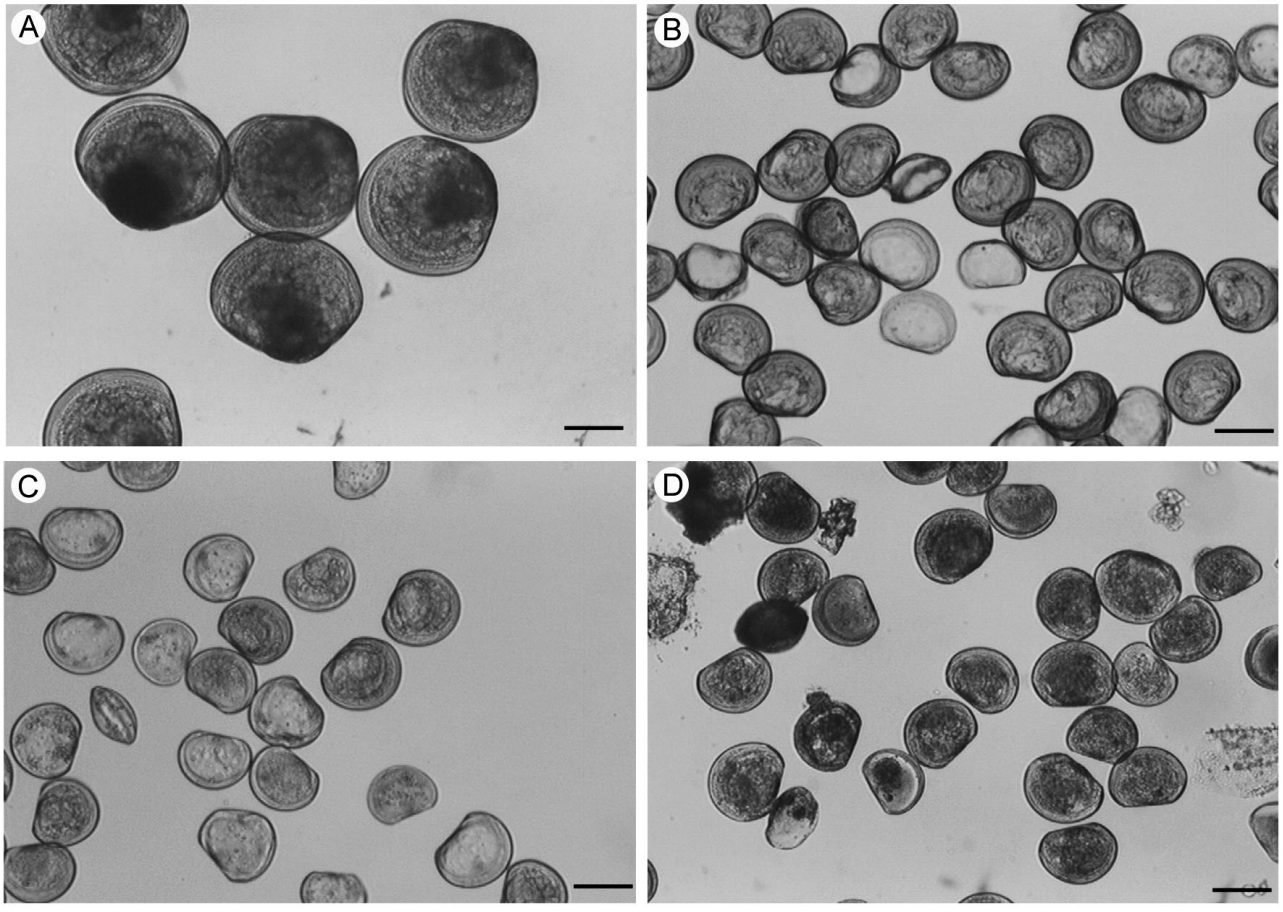

Fig. 4. *Mercenaria mercenaria*. Video micrographs of the final sizes of hard clam larvae after 15 d exposure to various diet treatments: (A) *Isochrysis galbana* 50 cells µl^{-1} (control, Expt I); (B) unfed (Expt I); (C) mixed suspension of *I. galbana* and *Aureococcus anophagefferens* (50 and 800 cells µl^{-1}, respectively) (Expt III); (D) unialgal suspension of *A. anophagefferens* (800 cells µl^{-1}) (Expt III). Scale bars = 100 µm

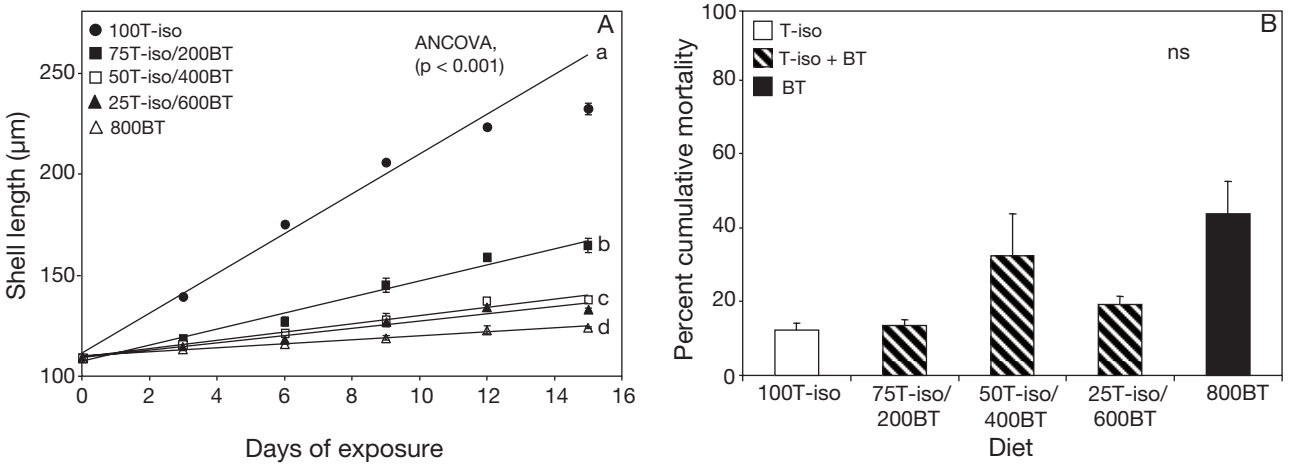

Fig. 5. *Mercenaria mercenaria*. Expt III: effect of increasing proportions of *Aureococcus anophagefferens* (brown tide = BT) in diet (0, 25, 50, 75, 100%) on (A) hard clam larval shell length and (B) percent cumulative mortality after 15 d exposure (mean ± SE, 3 tanks); ns = not significant. Cell densities of *A. anophagefferens* and *Isochrysis galbana* (T-iso) in cells µl^{-1}. Different letters indicate statistically significant differences among slopes (ANCOVA) of linear regression equations fitted to size data (see Table 1). Last data point (Day 15) not included in regression for *I. galbana* control, as larval growth slowed as they neared metamorphosis

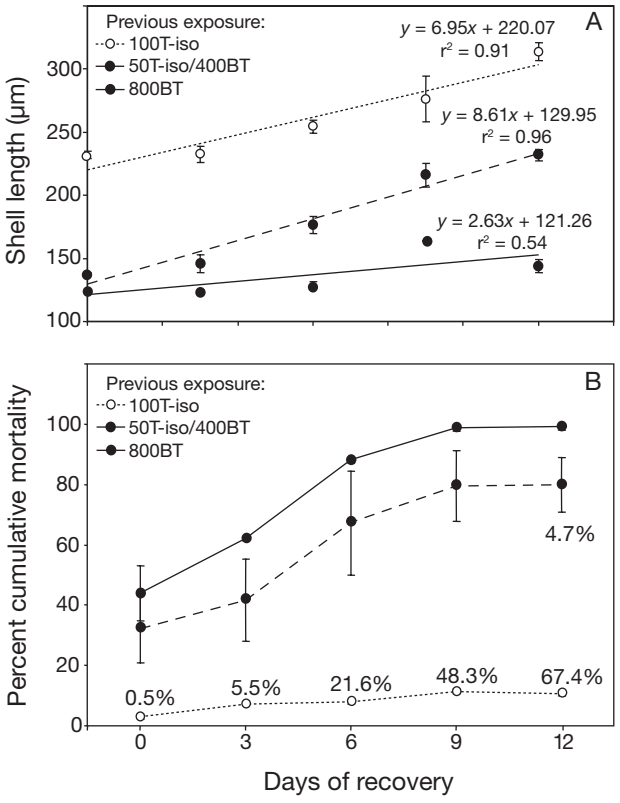

Fig. 6. *Mercenaria mercenaria*. (A) Shell length with fitted linear regression and (B) percent cumulative mortality over 12 d of recovery on a unialgal diet of *Isochrysis galbana* (T-iso) at 100 cells µl^{-1} (mean ± SE, 3 tanks). Hard clam larvae were previously exposed (Expt III, see Fig. 5) for 15 d to a unialgal suspension of *I. galbana*, a 50:50% suspension of *I. galbana* and *Aureococcus anophagefferens*, or a unialgal suspension of *A. anophagefferens* (800 cells µl^{-1}). Some error bars are obscured by symbols. Percentages of live larvae that were dissoconchs (completed metamorphosis) are also shown (no values = no dissoconchs present)

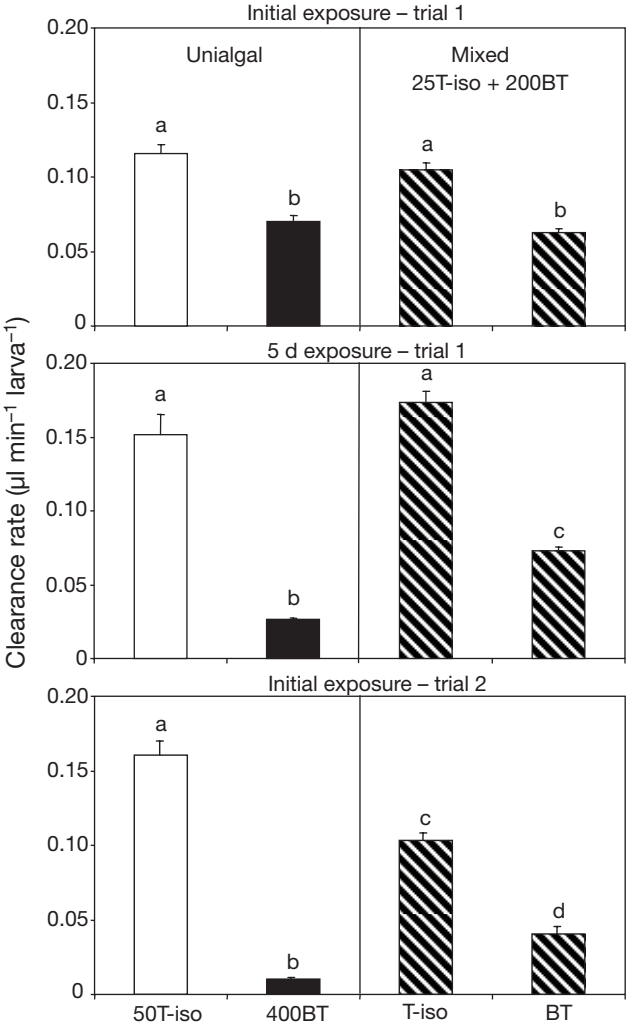

Fig. 7. *Mercenaria mercenaria*. Clearance rates of hard clam veliger larvae exposed to unialgal suspensions (left) of *Isochrysis galbana* (clone T-iso, 50 cells µl^{-1}) or *Aureococcus anophagefferens* (brown tide = BT, 400 cells µl^{-1}), or an equivolume of mixed suspension of *I. galbana* and *A. anophagefferens* at 25 and 200 cells µl^{-1} respectively (right) after initial and 5 d exposure (Trial 1) and initial exposure only (Trial 2). Larvae were fed the control diet (T-iso, 50 cells µl^{-1}) for 7 d or 8 d (Trials 1 and 2, respectively) prior to exposure to the experimental treatments. Letters indicate significant differences ($p < 0.05$) within each panel

and 0.44, respectively), thus validating results obtained with the Multisizer. In Trial 1, mean (±SE) cumulative mortalities at the end of the feeding experiment were 20% ± 3.2, 68% ± 4.6 and 32% ± 7.8 for the T-iso, *A. anophagefferens* and mixed diet, respectively, and thus CR was corrected for the number of live larvae. ANOVA and multiple comparisons tests indicated that mortalities over the 5 d exposure were significantly greater in the unialgal brown tide treatment than in those containing T-iso ($p < 0.05$). Mortalities were negligible (<2%) in all diet treatments in Trial 2.

Observations of gut chl *a autofluorescence* revealed that early veligers showed bright red fluorescence indicative of full guts when fed *Isochrysis galbana* for 5 d (Fig. 8A). In addition, they reached the pediveliger stage of development. In contrast, most larvae showed guts with low fluorescent intensity, comparable to the starved treatment, when fed the unialgal suspension of *Aureococcus anophagefferens* at bloom levels for 5 d (Figs. 8B & C). Fewer larvae were examined from the mixed suspension, but most (8/11) showed relatively full guts. Additional time-series of gut autofluorescence in mixed diets are required to corroborate these preliminary observations.

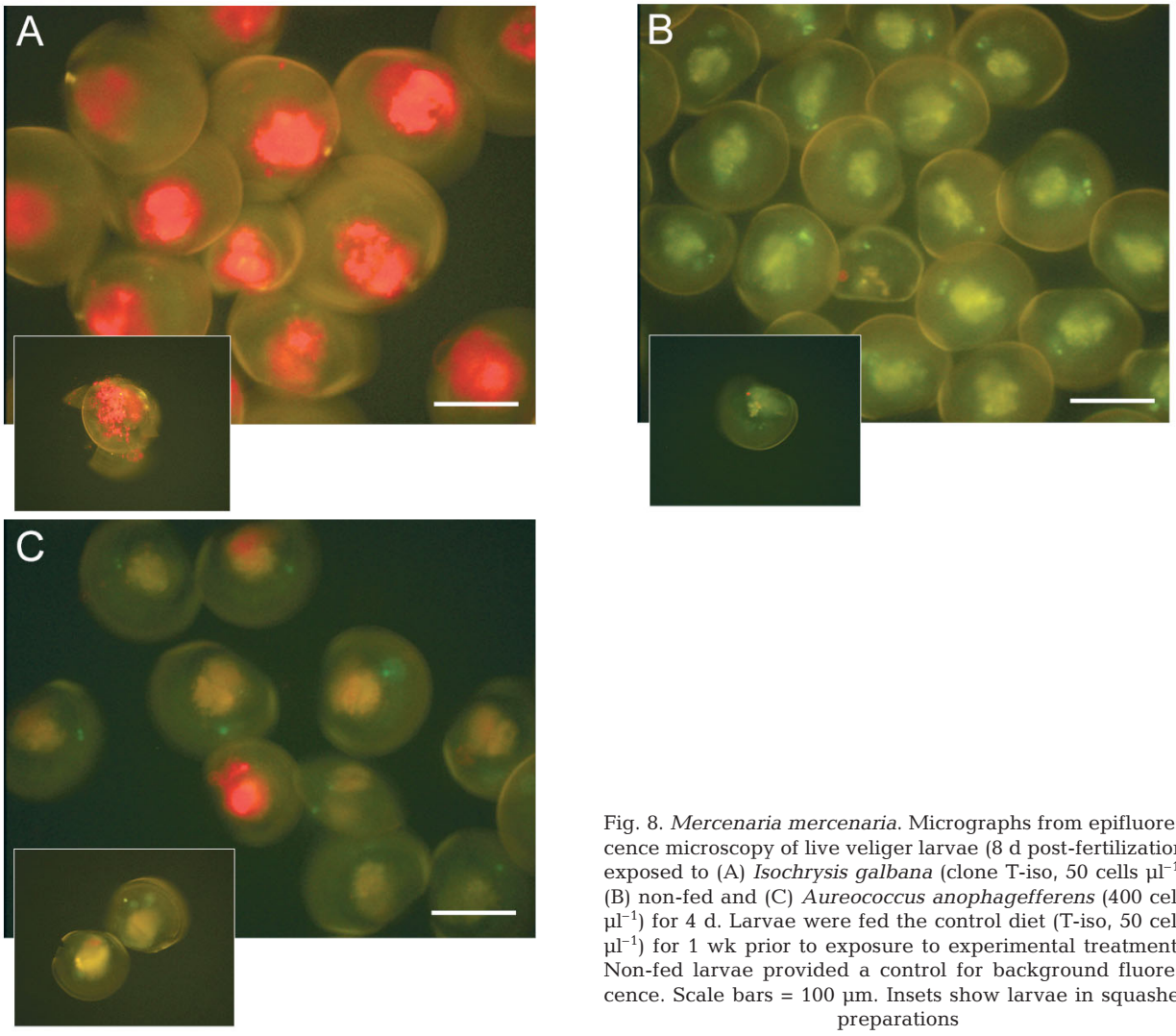

Fig. 8. *Mercenaria mercenaria*. Micrographs from epifluorescence microscopy of live veliger larvae (8 d post-fertilization) exposed to (A) *Isochrysis galbana* (clone T-iso, 50 cells μl^{-1}), (B) non-fed and (C) *Aureococcus anophagefferens* (400 cells μl^{-1}) for 4 d. Larvae were fed the control diet (T-iso, 50 cells μl^{-1}) for 1 wk prior to exposure to experimental treatments. Non-fed larvae provided a control for background fluorescence. Scale bars = 100 µm. Insets show larvae in squashed preparations

DISCUSSION

In 2 wk growth trials, hard clam larvae experienced significantly higher mortalities and lower growth rates in all treatments supplemented with *Aureococcus anophagefferens* than in the 2 *Isochrysis galbana* controls. Cumulative mortalities at 15 d were comparable at brown tide levels ranging from 200 to 800 cells μl^{-1}, and cumulative mortalities were also comparable to those in the non-fed treatment. Adverse effects on growth and survival were time-dependent (detected earlier at higher *A. anophagefferens* densities). Growth rates were inversely related to the concentration of brown tide. It is noteworthy that the lowest *A. anophagefferens* concentration tested in the present study (50 cells μl^{-1}) was sufficient to elicit a small but significant reduction in growth relative to controls (~13% at 12 d of exposure), whereas hard clam juveniles (~7 mm initial SL) were able to acclimate and fully recover after ~2 wk of exposure to a comparable concentration (*A. anophagefferens* at 80 cells μl^{-1}) offered in a mixed suspension with *I. galbana* (Bricelj et al. 2004). Relatively good growth on *A. anophagefferens* cells at 50 μl^{-1} when combined with T-iso indicates that this cell density is below the threshold that elicits marked grazing inhibition of larvae (as reflected by growth results with varying *A. anophagefferens* supplements, Fig. 2B). The reduction in larval growth rates relative to the control was comparable (~90%) in the unialgal *A. anophagefferens* treatment at 800 cells μl^{-1} (Expt III) and in the combination treatment supplemented with *I. galbana* (Expt I), clearly indicating that the adverse effects of brown tide were not related to a lack of available food. It is likely that the presence of alternate food mitigates the effects of low levels of brown tide (≤100 cells μl^{-1}), but our experiments were

not designed to test this hypothesis. Maximum larval growth rates obtained in the present study on *I. galbana* (8 to 10 µm d^{-1}) are comparable to the rate of 10 µm d^{-1} reported for *Mercenaria mercenaria* larvae fed clone T-iso at 1000 cells $µl^{-1}$ by Gallager et al. (1994).

Juvenile (1 mm) *Mercenaria mercenaria* that survived a severe brown tide outbreak resumed rapid growth once the bloom dissipated (Greenfield et al. 2002). In the present study, larvae were unable to recover following 2 wk of exposure to *Aureococcus anophagefferens* at 800 cells $µl^{-1}$. However, 20% of the larval population resumed normal growth during recovery from 2 wk exposure with *A. anophagefferens* at 400 cells $µl^{-1}$ in combination with clone T-iso, indicating intrapopulation differences in susceptibility to brown tide. Much greater variability was also observed in mortalities as determined during recovery of larvae previously exposed to this mixed treatment than in those exposed to the unialgal diets (control and brown tide). Little is known about genetic variation in cohorts of bivalve larvae, but genetically-based differences in susceptibility to toxic algae (*Alexandrium* spp.) have been shown in softshell clam juveniles (Bricelj et al. 2005) and copepods (Colin & Dam 2002a).

In early veligers, gut chl *a* autofluorescence revealed bright red fluorescence indicative of full guts when fed *Isochrysis galbana* (Fig. 8A). In contrast, most larvae showed low gut fluorescent intensity that was comparable to the non-fed treatment, when fed the unialgal suspension of *A. anophagefferens* at bloom levels for 5 d (Fig. 8C), but not at 24 h (data not shown), suggesting that feeding incapacitation is time-dependent and may require a few days of exposure to take full effect. A more detailed time series is required to test this hypothesis.

Suspension-feeding by *Mercenaria mercenaria* veligers includes a series of distinct steps including: (1) capture of cells by pre- and post-oral cirri of the velum; (2) their transport to the mouth along the food groove; (3) concentration of cells into a bolus at the mouth; (4) selection or rejection of cells for entry into the ciliated esophagus; and (5) action of a ciliated sphincter at the junction with the stomach that allows passage of cells into the stomach or their rejection via muscular contractions and reversal of the sphincter cilia (Gallager 1988, Carriker 2001). The mechanism of action of brown tide on larval locomotion and feeding behavior was previously investigated in *Argopecten irradians* (Gallager et al. 1989). These authors found that bay scallop larvae maintained ciliary-driven swimming activity in the presence of *Aureococcus anophagefferens*, but their swimming behavior changed relative to larvae exposed to *Isochrysis galbana*. When larvae were exposed to clone T-Iso, the net-to-gross displacement ratio of swimming larvae dropped relative to a FSW control, reflecting wider and less vertical displacement or 'hovering' behavior. In contrast, no change in this parameter was reported when larvae were offered *A. anophagefferens*, and the larvae showed instead an increase in their absolute vertical velocity along the helical swimming path.

Using radiolabelled algae, Gallager et al. (1989) also demonstrated that bay scallop larvae (both early and late veligers) showed comparable assimilation efficiency for T-iso and *Aureococcus anophagefferens*, but they also showed marked reduction (92 to 99%) in CR in a unialgal suspension of *A. anophagefferens* (1000 cells $µl^{-1}$) relative to an *Isochrysis galbana* control within 1 h of exposure to the diet treatment. The latter agrees with findings obtained for *Mercenaria mercenaria* in the present study, although maximum feeding inhibition of hard clam larvae on brown tide (400 cells $µl^{-1}$) was time-dependent with more pronounced effects observed after 5 d than initial exposure.

When bay scallop larvae were offered a mixed suspension of *Isochrysis galbana*, *Nannochloris atomus* and *Dunaliella tertiolecta*, particle capture efficiency on the preoral cirri, determined using high-speed video microscopy, was 30 to 50% irrespective of algal size (Gallager et al. 1989). However, when *N. atomus* was substituted by *Aureococcus anophagefferens*, capture efficiency was much lower (10 to 13%) than reported for clone T-iso, yet subsequent ingestion of all algal cells was reduced due to rejection from the esophagus of 83 to 94% of algae transported to the mouth, irrespective of algal species. Thus brown tide inhibited ingestion, but not capture, of other nutritious algae present in a mixed assemblage. These observations led Gallager et al. (1989) to suggest that brown tide may interfere with chemosensory function rather than ciliary activity in bivalve larvae, and that the presence of brown tide renders other algal species unpalatable. The mode of toxic action of *A. anophagefferens* differs between larval and adult bivalves, although both suffer feeding impairment in the presence of brown tide. Mean clearance rates measured in the present study for larvae fed *I. galbana* at 50 cells $µl^{-1}$ (~7.2 and 10 µl h^{-1} for larvae 167 and 194 µm SL respectively) were comparable to a value of ~10 µl h^{-1} obtained for 160 µm *Mercenaria mercenaria* larvae using *I. galbana* (T-iso) at the same cell density (Gallager et al. 1994), although lower than those determined by Riisgård (1988b) for 164 µm hard clam larvae fed the same diet (~20 µl h^{-1}).

Based on observations by Gallager et al. (1994), that the bolus formed at the larval mouth breaks up into single cells during the rejection process, our measurements of CR are more likely to represent ingestion

rates, and the consistently higher feeding rates on *Isochrysis galbana* than on *Aureococcus anophagefferens* in the mixed-suspension are interpreted as evidence of selective ingestion rather than differential particle retention. There is conflicting information in the literature, however, on the retention efficiency of picoplankton (0.5 to 3 µm particles) by bivalve larvae. Whereas electronic particle counter data showed maximal clearance for ~3 to 4 µm particles and a sharp decline at both smaller and larger sizes (Riisgård et al. 2000), video and epifluorescence microscopy techniques showed that *Mercenaria mercenaria* larvae effectively clear 1 µm particles (Gallager 1988, Gallager et al. 1994). Furthermore, when exposed to natural seston, oyster *Crassostrea virginica*, larvae were generally found to feed on 2 to 4 µm particles with maximum efficiency (Baldwin 1995), indicating that discrimination between *A. anophagefferens* and *I. galbana* by bivalve larvae would not be expected on the basis of size but that larvae could selectively ingest algae based on their biochemical properties and/or surface properties. Furthermore, deleterious effects of brown tide on larval ingestion and growth found in the present study cannot be attributed to the effects of high algal biomass per se, as volume equivalent concentrations of clone T-iso supported high larval growth rates.

Results of the present study indicated that the harmful effects of brown tide were largely due to intact cells rather than the associated algal filtrate. These results support previous studies showing that the toxic effects of *Aureococcus anophagefferens* in adult bivalves are only elicited by contact with cells and not by dissolved metabolites present in cell-free filtrates (reviewed by Bricelj & Lonsdale 1997). They also agree with the observation that *Aureococcus* culture medium following removal of cells by centrifugation did not elicit toxic effects in metamorphically competent bay scallop larvae (Gallager et al. 1989). Finally, they are consistent with the finding that the bioactive compound produced by brown tide is localized in its EPS layer and was only released into solution following amylase digestion of this layer (Gainey & Shumway 1991).

Jónasdóttir et al. (1998) proposed an experimental framework that would allow discrimination between nutritional insufficiency of an algal diet and toxicity, in which a fitness trait (copepod egg production or hatching success in their study) was determined in mixed suspensions containing variable proportions of a 'suspect' or harmful diet. In the present study, we used shell growth rates as a function of increasing proportions of a good food source (clone T-iso) mixed with *Aureococcus anophagefferens* to provide a test of these effects (Fig. 9). Larval growth rather than survival rate was selected as the best fitness measure, given that growth inhibition by *A. anophagefferens*

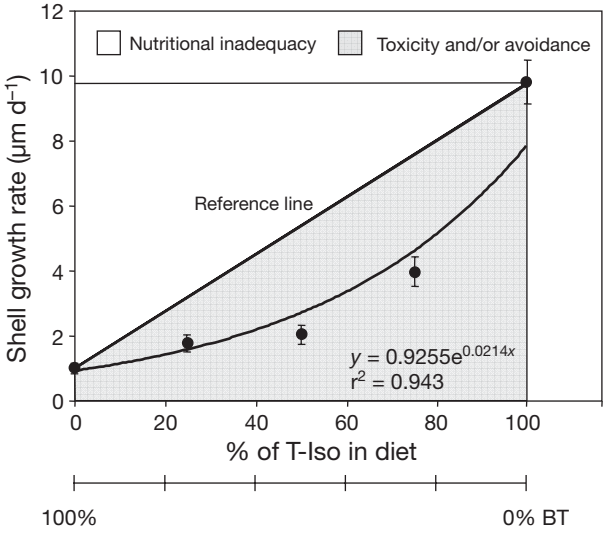

Fig. 9. *Mercenaria mercenaria*. Relationship between shell growth rate of hard clam larvae and relative biovolume contribution of *Isochrysis galbana* (T-iso) and *Aureococcus anophagefferens* in the diet (growth rates = slopes of linear regressions fitted to data from Expt III, in Table 1, ± SE of the slope). Values below reference line indicate that *A. anophagefferens* is toxic and/or elicits avoidance rather than being of poor nutritional value relative to the control diet, based on the scheme proposed by Jónasdóttir et al. (1998) to discriminate between toxicity and nutritional inadequacy

was a consistent, dose-dependent response in all our experiments, whereas larval mortalities were highly variable and thus likely a secondary effect. All growth rate values fell well below the reference line drawn between those on unialgal diets of the 2 algal species (Type 3 response sensu Jónasdóttir et al. 1998) and confirmed that the harmful effects of *A. anophagefferens* are not caused by nutritional insufficiency. This is in agreement with previous findings that an *A. anophagefferens* isolate (CCMP 1784), characterized as non-toxic (i.e. that did not elicit feeding inhibition of juvenile mussels), supported good growth of *Mercenaria mercenaria* juveniles (Bricelj et al. 2004) and larvae (V. M. Bricelj & S. P. MacQuarrie unpubl.). A Type 3 response can also occur when a predator feeds preferentially on the most abundant prey, or when a 'suspect' species causes prey avoidance, effects that can only be distinguished by also determining algal ingestion rates in the mixed suspensions (Jónasdóttir et al. 1998, Colin & Dam 2002b). However, toxic effects of *A. anophagefferens* (CCMP 1708) on *M. mercenaria* larvae are inferred from the fact that growth rate was severely reduced both in unialgal and mixed suspensions containing *A. anophagefferens* at 800 cells µl^{-1} (90 and 88% respectively, Table 1).

Feeding inhibition may result from physiological incapacitation (acting following ingestion) (sensu Ives

1987) or behavioral avoidance/deterrence from cues detected prior to ingestion. Colin & Dam (2003) have associated only the former mechanism to toxicity, in the context of algal species that contain endogenous toxins, such as the PSP-producer *Alexandrium fundyense*. However, these mechanisms may not be mutually exclusive, as behavioral deterrence may be elicited by a detectable toxic compound on the algal cell surface. Thus, the toxic metabolite in *Aureococcus anophagefferens* has been localized in the EPS layer and acts on contact with the adult bivalve gill. Selection against *A. anophagefferens* was demonstrated in the present study when offered in a mixed suspension with *Isochrysis galbana* at equal cell volume concentrations. However, the progressive reduction in CR per larva over time, when hard clam veligers were exposed to unialgal *A. anophagefferens* relative to controls (Fig. 7, Trial 1), suggests that physiological incapacitation occurred over time. Additional experimentation is required to better define the putative chemosensory mechanism(s) involved in feeding inhibition of bivalve larvae by brown tide in unialgal and mixed suspensions, as both mechanisms of grazing inhibition (incapacitation and deterrence) may be occurring in a dose- and time-dependent manner.

Although bivalve larvae (unidentified spp.) were estimated to make a substantial contribution to grazing of picoplanktonic cyanobacteria in Vineyard Sound, MA (Gallager et al. 1994), the present study indicates that severe feeding inhibition will prevent hard clam larvae from exerting grazing pressure on *Aureococcus anophagefferens* at cell densities ≥100 to 200 cells μl^{-1}. Furthermore, our laboratory results provide strong evidence that the persistence of brown tide above this level for ~2 wk (the typical duration of larval development of *Mercenaria mercenaria*) is likely to severely compromise recruitment of benthic postlarvae by slowing down growth, causing starvation and prolonging the time larvae remain in the plankton, thus making them vulnerable to extrinsic mortality factors such as predation and opportunistic pathogens. Thus, our results are consistent with simulations by Powell et al. (2002, 2004) of a larval growth model developed for oyster *Crassostrea gigas* larvae (Bochenek et al. 2001), which inferred that bivalve larvae are very sensitive to even transient changes in food quality. Poor food quality can restrict the range of surviving genotypes as well as affect cohort survivorship via an increase in larval lifespan.

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