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Ecology of phytoplankton communities dominated by *Aureococcus anophagefferens*: the role of viruses, nutrients, and microzooplankton grazing

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Abstract

We investigated the impact of viruses, nutrient loading, and microzooplankon grazing on phytoplankton communities in two New York estuaries that hosted blooms of the brown tide alga *Aureococcus anophagefferens* during 2000 and 2002. The absence of a bloom at one location during 2002 allowed for the fortuitous comparison of a bloom and non-bloom year at the same location as well as a comparison of two sites experiencing bloom and non-bloom conditions during the same year. During the study, blooms were found at locations with high levels of dissolved organic nitrogen and lower nitrate concentrations compared to a non-bloom location. Experimental additions of inorganic nitrogen and phosphorus yielded growth rates within the total phytoplankton community which significantly exceeded control treatments in 83% of experiments, while *A. anophagefferens* experienced significantly increased growth during only 20% of experimental inorganic nutrient additions. Consistent with prior research, these results suggest brown tides are not caused by eutrophication, but instead are more likely to occur when sources of labile DOM are readily available. Microzooplankton grazing rates on the total phytoplankton community during a bloom were lower than grazing rates at a non-bloom site, and grazing rates on *A. anophagefferens* were lower than grazing rates on the total community on some dates, suggesting that reduced grazing mortality may also promote brown tides. Mean densities of viruses during blooms (3×10^8 ml^{-1}) were elevated compared to most estuarine environments and were twice the levels found at a non-bloom site. Experimental enrichment of the natural viral densities yielded a significant increase in *A. anophagefferens* growth rates relative to control treatments when background levels of viruses were low ($<1.7 \times 10^8$ ml^{-1}), suggesting that viruses may promote bloom occurrence by regenerating DOM or altering the composition of microbial communities.

Keywords: Harmful algal blooms; Brown tide; *Aureococcus anophagefferens*; Nutrients; Viruses; Dissolved organic matter; Microzooplankton; Grazing

1. Introduction

Brown tides, caused by the picoplanktonic pelagophyte *Aureococcus anophagefferens*, have plagued mid-Atlantic US estuaries since 1985 (Bricelj and

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Lonsdale, 1997). Although *A. anophagefferens* had been unknown to science prior to 1985, numerous research efforts since then have advanced the understanding of this alga's ecology and physiology. Brown tides are characterized as harmful due to their detrimental effects on eelgrass (*Zostera marina*) beds and shellfish populations. Eelgrass beds suffer during dense brown tides (10^6 cells ml^{-1}) due to reduced light penetration to the benthos. At high densities, *A. anophagefferens* can cause a cessation of feeding and ultimately death of shellfish (e.g. hard clams, bay scallops; Bricelj and Lonsdale, 1997; Bricelj et al., 2001; Greenfield and Lonsdale, 2002). Since some organisms are more adversely affected by *A. anophagefferens* than others (Bricelj and Lonsdale, 1997), chronic bloom occurrence could lead to changes in species composition or biodiversity in affected areas. Although there is severe light attenuation during blooms of *A. anophagefferens*, the shallow-nature and robust mixing of bloom-prone estuaries prevents the occurrence of hypoxic-anoxic conditions (Bricelj and Lonsdale, 1997). To date nutrients, viruses, and microzooplankton grazing have all been advanced as factors that may have a substantial impact on *A. anophagefferens* densities during blooms (Milligan and Cosper, 1994; Bricelj and Lonsdale, 1997; Gastrich et al., 2002; Gobler et al., 2002).

Unlike many phytoplankton, *A. anophagefferens* utilizes organic and inorganic nutrients giving it a competitive edge over other strictly autotrophic phytoplankton (Mulholland et al., 2002). Moreover, inorganic macronutrient enrichment can diminish the likelihood of a bloom proliferation (Keller and Rice, 1989; LaRoche et al., 1997; Gobler and Sañudo-Wilhelmy, 2001). Accordingly, intense brown tides often occur when dissolved organic carbon and nitrogen concentrations are high while inorganic nitrogen levels are reduced (LaRoche et al., 1997; Gobler et al., 2002).

Several studies have suggested that viruses may be an important source of mortality for *A. anophagefferens* during brown tides. Electron micrographs of the first observed brown tide event in Rhode Island and subsequent blooms in New Jersey have revealed the presence of intracellular virus-like particles (VLPs) in *A. anophagefferens* cells (Sieburth et al., 1988; Gastrich et al., 2002), suggesting brown tide populations may experience viral infection and lysis. Additionally, viruses that are capable of lysing *A. anophagefferens* cultures have been isolated from a bloom event (Milligan and Cosper, 1994). To date, the impact of viruses on *A. anophagefferens* field populations has not been accessed.

Microzooplankton grazing may also be an important source of mortality for brown tides. Studies have reported that *A. anophagefferens* is not an adequate nutritional source for some protozoa (Mehran, 1996; Bricelj and Lonsdale, 1997). However, Caron et al. (1989) reported that some cultured protozoan grazers isolated from mid-Atlantic US coastal waters can grow robustly in the presence of brown tide cells. Field observations made during a fall/winter brown tide indicated that microzooplankton grazing rates on *A. anophagefferens* were lower than those on other algae, suggesting reduced grazing pressure might contribute to bloom initiation (Gobler et al., 2002). However, pelagic grazing rates by protozoa on *A. anophagefferens* during a summer bloom event have not yet been reported.

During 2000 and 2002, we investigated the impact of viruses, nutrient loading, and microzooplankton grazing on phytoplankton communities in two New York estuaries which frequently host brown tide blooms. We quantified levels of nutrients, *A. anophagefferens*, bacteria, cyanobacteria, and viruses to evaluate the dynamics of microbial communities and nutritional resources during blooms. We conducted nutrient enrichment experiments to assess the degree to which the growth of *A. anophagefferens* and other phytoplankton were limited by nutrients during blooms. We executed seawater dilution experiments (Landry and Hassett, 1982; Landry et al., 1995) to evaluate microzooplankton grazing rates on *A. anophagefferens* and other phytoplankton during a bloom. Finally, we conducted viral enrichment experiments to assess the impact of increased viral abundance on the growth rates of *A. anophagefferens* and other phytoplankton during blooms. Our approach allowed us to evaluate the relative importance of each top-down and bottom-up process before, during, and after two bloom events in NY waters.

2. Methods

During the summer of 2000, we established a preliminary research campaign aimed toward examining

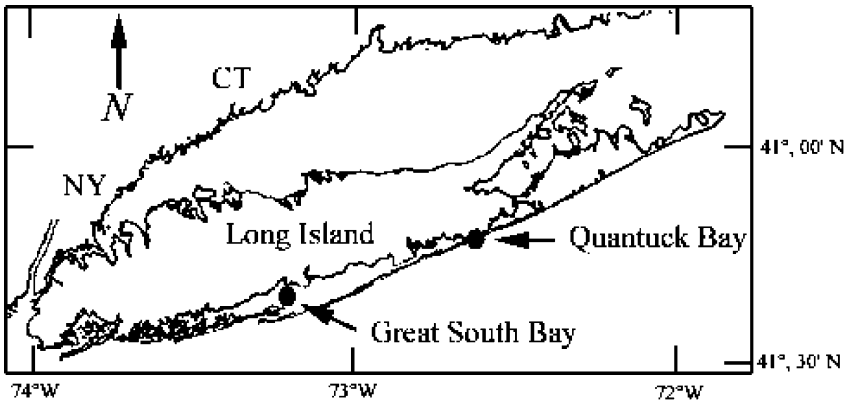

Fig. 1. Study sites in Great South Bay and Quantuck Bay, New York, USA.

the role of viruses in the development of brown tides in New York estuaries. From May through September, we generated a temporal data set from experiments and observations in western Great South Bay, NY (GSB; Fig. 1) during a brown tide event. During 2002, we expanded our research to a second location on the south shore of Long Island (Quantuck Bay, QB; Fig. 1), and broadened the scope of our investigation to examine nutrients and microzooplankton grazing in concert with viral dynamics. Although our two study sites have experienced brown tides more frequently than any other locations in New York (SCDHS, 1985–2002), the absence of a bloom in GSB during 2002 allowed for the fortuitous comparison of a bloom and non-bloom year at the same location (2000 versus 2002) as well as the comparison of two sites experiencing bloom and non-bloom conditions during the same year (2002; QB versus GSB).

Carboys and experimental flasks used for this project were stored in 10% HCl between sampling dates and rinsed liberally with distilled-deionized water before use. At each field location, ~100 l of seawater were collected in polyethylene carboys which were placed in coolers. Measurements of temperature and salinity were obtained with a YSI© 85 probe. Light penetration was estimated with a secchi disk. Samples were transported back to the lab where they were processed immediately.

Whole water was processed to determine concentrations of chlorophyll *a* (size-fractionated), nutrients, *A. anophagefferens*, bacteria, cyanobacteria, and viruses at each field station. Samples of whole water for chlorophyll *a* (chl *a*) quantification were collected using glass fiber filters (GF/F; nominal pore size = 0.7 μm) from triplicate carboys. Chl *a* was also size-fractionated using a 5 μm Nitex© mesh (Gobler and Sañudo-Wilhelmy, 2001). Whole water was filtered through precombusted GF/Fs, stored frozen, and analyzed for nitrate/nitrite (Jones, 1984), ammonium, phosphate and silicate (Parsons et al., 1984), and total dissolved nitrogen (TDN; Valderrama, 1981) from duplicate carboys by standard spectrophotometric methods. DON was calculated as the difference between TDN and dissolved inorganic nitrogen (nitrate, nitrite, ammonium). Triplicate samples for *A. anophagefferens* enumeration were preserved in 1% glutaraldehyde made from a 10% stock solution diluted with 0.2 μm filtered seawater. Triplicate bacteria, cyanobacteria, and virus samples were preserved in 2.5% gluteraldehyde. These samples were quantified within 2 weeks of collection (details below).

To concentrate viruses from seawater for experimental purposes, filtered seawater was made with a peristaltic pump equipped with acid-cleaned polypropylene filter capsules (0.2 μm pore size; MSI Inc.) and acid-washed Teflon tubing (Gobler and Sañudo-Wilhelmy, 2001). The viral size fraction (30 kDa–0.2 μm) was concentrated 50–100-fold with a Amicon M12 ultrafiltration system equipped with a S10-Y30 cartridge (30 kDa mixed cellulose membrane; Wilhelm and Poorvin, 2001) and then post-filtered (0.2 μm; low protein binding filter). A fraction of this volume was microwave sterilized (brought to a near-boil and then cooled three times)

to kill viruses, leaving two stocks of high molecular weight, viral concentrate (HMWVC): one live and one dead. To assess the impact of viruses on algal communities these concentrates were used in 'viral addition experiments' in which live and dead concentrates were added to whole seawater. Since HMW concentrates are known to be enriched in DOM (Amon and Benner, 1994; Gobler and Sañudo-Wilhelmy, 2003; this study, data not shown), the addition of live and dead HMWVC allowed us to contrast the effects of the biological activity of viruses in the presence of elevated DOM (live HMWVC) to those of DOM alone (dead HMWVC). Additions were made at target concentrations of two-times the natural abundance of viruses in 2000 and at four-times background levels in 2002, assuming 50% loss during concentration (Noble et al., 1999). This degree of enrichment was specifically chosen to minimally perturb the microbial community (Noble et al., 1999) and created an enrichment which was consistent with actual changes observed in the water column during this study (Figs. 2–4). Post-experiment enumeration of viral densities (Noble and Fuhrman, 1998; see also later portion of Section 2) in concentrates in 2002 indicated that actual additions were generally within 20% of predicted concentrations (3.3 ± 1.2-fold enrichment = mean ± S.D.). Moreover, parallel laboratory research conducted during 2002 evidenced the presence of viruses capable of lysing multiple isolates of *A. anophagefferens*, *Synechococcus bacillus*, and marine bacteria within live, but not dead, HMWVC (data not shown). This finding is consistent with Noble et al. (1999) who found that live HMWVC additions increased the rate of viral infection within the natural marine bacterial community. Live and dead HMWVC were added to whole water in triplicate, 250 ml polycarbonate flasks, and a volume of <30 kDa seawater equivalent to the concentrates was added to a set of control flasks. Evaluation of viral densities in each treatment (Noble and Fuhrman, 1998; see later portion of Section 2) indicated that additions of dead HMWVC did not enrich viral densities relative to control treatments, providing further evidence that our microwave sterilization technique successfully inactivated viruses in the HMWVC. To ensure nitrogen and phosphorus replete growth during incubations, a filter-sterilized (0.2 μm) nutrient solution was added to each treatment (control, live viral addition, dead

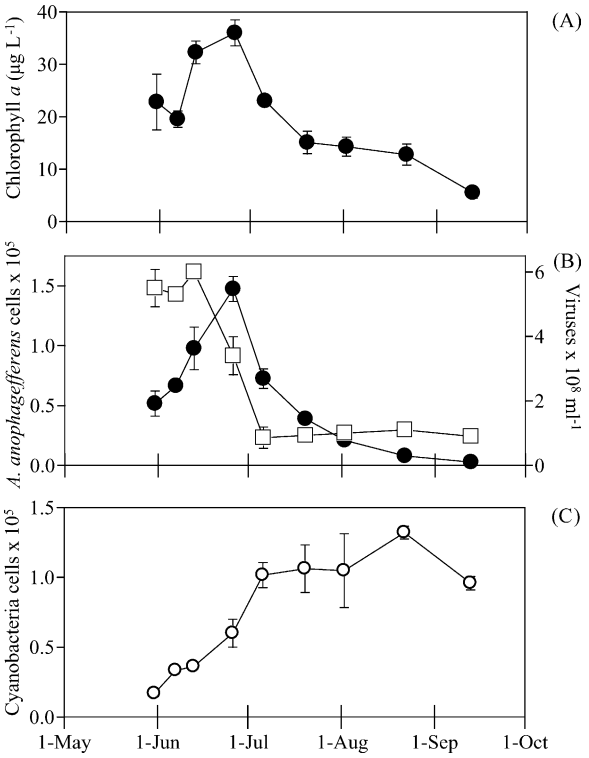

Fig. 2. Variation in (A) Chlorophyll *a*, (B) *Aureococcus anophagefferens* (black circles) and viruses (squares), and (C) cyanobacterial (open circles) cell densities and in Great South Bay, 2000. Error bars represent ± 1 S.D. of triplicate measurements.

viral addition) to create concentrations of 20 μM nitrate and 1.25 μM orthophosphate. Elevated ambient concentrations of silicate (mean = 37 μM) during the course of our experiments assured silicate-replete conditions for diatoms.

In 2002, dilution experiments were conducted to quantify the rates of microzooplankton grazing on algal prey (Landry et al., 1995). Triplicate samples of 100, 75, 50 and 25% experimental dilutions of whole seawater with filtered seawater (0.2 μm) in 250 ml polycarbonate flasks were established. To ensure nutrient-replete growth during these experiments, nitrate and orthophosphate were added to all of the flasks as cited above. Two sets of triplicate controls were established for grazing experiments: One set contained whole seawater without added nutrients and a second set contained all filtered seawater (0.2 μm) to which nutrients were added (Landry et al., 1995). A comparison of whole seawater incubations with

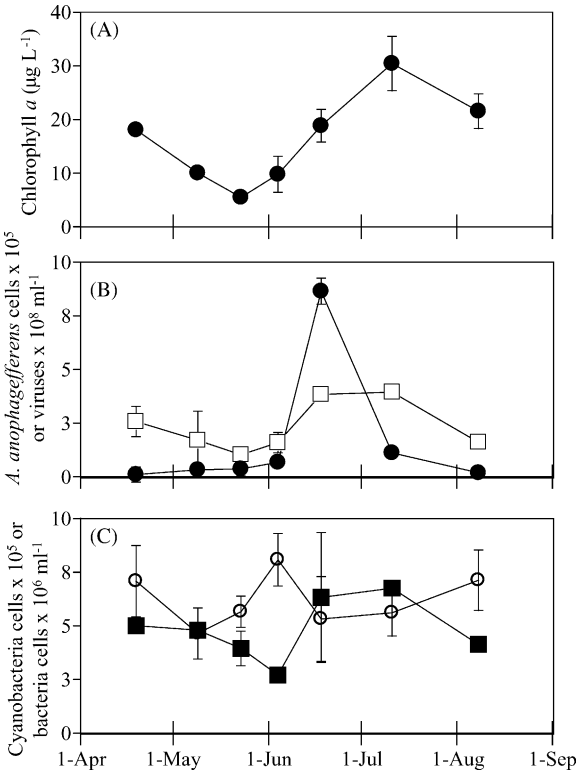

Fig. 3. Variation in (A) chl *a*, (B) *Aureococcus anophagefferens* (circles) and viruses (squares), and (C) bacterial (squares) and cyanobacterial (circles) cell densities in Quantuck Bay, 2002. Error bars represent ± 1 S.D. of triplicate measurements.

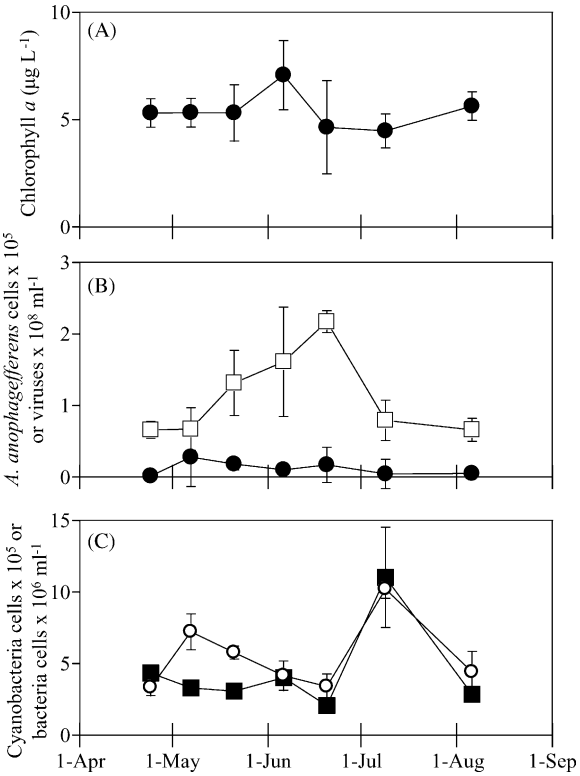

Fig. 4. Variation in (A) chl *a*, (B) *Aureococcus anophagefferens* (circles) and viruses (squares), and (C) bacterial (squares) and cyanobacterial (circles) cell densities in Great South Bay, 2002. Error bars represent ± 1 S.D. of triplicate measurements.

and without nutrients allowed for the evaluation of nutrient loading (N and P additions) on the growth of brown tide and other algal populations during 2002 experiments.

Experimental flasks were incubated for 24–48 h, under neutral density screening in Old Fort Pond (OFP) at the Southampton College, LIU, Marine Station, allowing for simulation of ambient light and temperature conditions of field stations during experiments (Gobler et al., 2002). Aliquots of each flask were removed, processed and preserved for chl *a* and microbial community enumeration after 24 h in 2000 and at 24 and 48 h in 2002. Chl *a* samples were analyzed via standard fluorometric methods (Parsons et al., 1984). *A. anophagefferens* densities were enumerated using a newly developed enzyme-linked immunoabsorbant assay (ELISA) based monoclonal antibody labeling technique (Caron et al., 2003). The monoclonal antibody labeling technique was performed using 96-well microtiter plates and converted to abundance using a preserved culture of *A. anophagefferens*. No significant cross-reactions have been observed with a wide variety of co-occurring algae. Accurate abundances of *A. anophagefferens* can be obtained in natural samples with this technique to a lower threshold concentration of approximately 5×10^3 cells ml^{-1}. Densities of *A. anophagefferens* at or below this threshold in GSB during 2002 and in April 2002 in QB prevented the accurate quantification of experimental growth rates and hence they are not reported.

Cyanobacteria were enumerated by epifluoresence microscopy of autofluorescent cells using a standard Texas Red optical filter set. Two milliliter samples were collected onto 25 mm diameter, 0.20 µm nominal pore-size black polycarbonate filters. Viruses and bacteria were also enumerated by epifluorescence

microscopy. For viruses, 800 μl aliquots of diluted (8–16-fold) sample were collected onto 25 mm diameter, 0.02-μm nominal pore-size Anodisc filters (Whatman) and stained with SYBR Green 1 (Noble and Fuhrman, 1998). Viruses in these samples were enumerated manually using a Leica DMRXA epifluorescent microscope equipped with an appropriate optical filter set (excitation wavelength = 450–490 nm; emission wavelength = 510 nm) and a 10 × 10 ocular grid (calibrated using a stage micrometer). Bacterial particles were likewise enumerated from 2 ml subsamples collected onto 25 mm diameter, 0.20 μm nominal pore-size black polycarbonate filters, stained with acridine orange (Hobbie et al., 1977) and enumerated as above. For all samples, 20 full grids or 200 particles were enumerated to ensure statistical accuracy.

To evaluate growth rates of the total phytoplankton community (based on chl *a*) and *A. anophagefferens* (based on cell densities) during experiments, the following formula was utilized: $\mu = [\ln(Bt/Bo)]/t$, where μ is the net growth rate, Bt is the amount of biomass (cell density or chl *a*) present at the end of the experiments, Bo represents the amount of biomass at the beginning of experiments, and *t* is the duration of the experiment in days. For dilution experiments, the slope of first order linear regressions of dilution of seawater (*x*-axis) and the net growth rates (*y*-axis) were used to establish grazing mortality rates (Landry and Hassett, 1982; Landry et al., 1995). Non-significant regressions were considered failed experiments, and rates gleaned from such regressions are not reported (Landry et al., 1995). Three-point regressions of dilution curves during this study did not indicate saturation of grazing during experiments (Gallegos, 1989). During viral addition experiments conducted in 2000, 24 h algal net growth rates within live, dead, and control treatments were normally distributed and thus were tested via one-way ANOVAs, followed by Tukey multiple comparisons tests (Sokal and Rolf, 1994). During 2002, dilution experiment growth rates were based on changes in biomass after 24 h (Landry and Hassett, 1982; Landry et al., 1995), where as viral and nutrient addition experimental growth rates were based on changes in biomass after 48 h. Differences among algal growth rates among live viral additions, dead viral additions, nutrient addition, and control treatments in 2002 were statically evaluated in a manner identical to the 2000 experiments.

3. Results

3.1. Great South Bay, 2000

During the late spring and early summer of 2000, an intense phytoplankton bloom developed throughout Great South Bay (GSB). Chl *a* levels increased from $\sim$20 μg l^{-1} in late May to a 26 June peak of 36 ± 2.4 μg l^{-1} (Fig. 2A). Following this peak, chl *a* concentrations steadily decreased, varying between 13 and 23 μg l^{-1} during July and August, and being reduced to <6 μg l^{-1} by 20 September. *A. anophagefferens* and pico-cyanobacteria were dynamic populations in GSB during this bloom as brown tide densities steadily increased from 5.1 × 10^5 cells ml^{-1} on 31 May to peak densities of 1.5 × 10^6 cells ml^{-1} on 26 June, while cyanobacterial densities increased from 1.6 × 10^5 to 6.0 × 10^5 cells ml^{-1} during the same period (Fig. 2B). Following the brown tide bloom peak, *A. anophagefferens* densities steadily declined, although concentrations >10^5 cells ml^{-1} were sustained through August (Fig. 2B). During July, August and September, cyanobacteria reached and sustained peak densities of $\sim$1 × 10^6 cells ml^{-1} (Fig. 2C). In contrast to these phytoplankton populations, viruses achieved their annual peak during the initial phases of this bloom, existing at densities >5 × 10^8 viruses ml^{-1} from 31 May to 13 June (Fig. 2B). During mid-June through early July, viral densities dropped nearly seven-fold to <0.9 × 10^8 viruses ml^{-1} on 6 July and remained at $\sim$ 1 × 10^8 viruses ml^{-1} for the remainder of the study (Fig. 2B).

Viral enrichment experiments during the summer of 2000 had little effect on the growth of the total phytoplankton community, as net growth rates of treatments with live and dead HMWVC were not significantly different from control treatments during all five experiments (Table 1). HMWVC additions also had little impact on *A. anophagefferens* net growth rates during the first four experiments conducted (31 May, 7 June, 13 June, 26 June; Table 1). However, live HMWVC enrichments made during the 21 July experiment yielded *A. anophagefferens* net growth rates which were significantly greater than both control treatments and dead HMWVC additions ($P < 0.05$; Tukey test; Table 1). Control and dead HMWVC additions were not significantly

Table 1
The effect of viral additions on the net growth rates of the total phytoplankton community and *A. anophagefferens* in GSB, summer 2000

	Date	Control	Live HMWVC	Dead HMWVC
Total community	31 May	0.09 ± 0.11	−0.05 ± 0.19	−0.03 ± 0.16
	7 June	−0.12 ± 0.06	0.02 ± 0.09	0.06 ± 0.12
	13 June	−0.10 ± 0.02	−0.09 ± 0.11	−0.06 ± 0.07
	26 June	0.08 ± 0.13	0.29 ± 0.26	0.24 ± 0.16
	21 July	0.46 ± 0.05	0.33 ± 0.15	0.39 ± 0.05
A. anophagefferens	31 May	1.1 ± 0.38	0.88 ± 0.17	0.92 ± 0.16
	7 June	0.65 ± 0.04	0.64 ± 0.01	0.72 ± 0.07
	13 June	0.45 ± 0.07	0.27 ± 0.10	0.31 ± 0.18
	26 June	0.51 ± 0.20	0.48 ± 0.18	0.15 ± 0.12
	21 July	−0.06 ± 0.11	**0.23 ± 0.10**	−0.06 ± 0.11

Growth rates are per day. Values represent means ± S.D. ($n = 3$). All treatments were amended with N and P (see Section 2 for further details). Bolded growth rate is significantly greater than other treatments ($P < 0.05$; Tukey test).

different from one another during this experiment (Table 1).

3.2. Great South Bay and Quantuck Bay, 2002

During 2002, high chl *a* levels (>10 μg l^{-1}) were observed in QB from June through August (Fig. 3A), while chl *a* levels at GSB remained low and relatively static (mean ± S.D. = 5.5 ± 0.9 μg l^{-1}; Fig. 4A). Over the course of our study, chl *a* levels were significantly higher at QB (16 ± 8.5 μg l^{-1}) relative to GSB (5.4 ± 0.85 μg l^{-1}; $P < 0.05$; *t*-test; Figs. 3A and 4A). Most of the chl *a* in QB and BSC was <5 μm in size (85 ± 57 and 78 ± 15%). High chl *a* levels in QB were partly due to a brown tide bloom which occurred, as cell densities >10^5 cells ml^{-1} were observed from 4 June through 11 July, with densities peaking at 8.6 × 10^5 cells ml^{-1} on 18 June (Fig. 3B). In contrast, levels of brown tide in GSB were low throughout 2002 (1.2 ± 0.94 × 10^4 cells ml^{-1}) reaching a maximum of only 2.8 × 10^4 cells ml^{-1} on 7 May (Fig. 4B). Corresponding with low and static brown tide levels at GSB were relatively stable viral densities (0.66–2.2 × 10^8 viruses ml^{-1}; Fig. 4B). QB viral densities, on the other hand, increased during the brown tide there and peaked on 11 July at 3.9 × 10^8 viruses ml^{-1} (Fig. 3B). Mean annual viral densities at QB (2.3 ± 1.2 × 10^8 viruses ml^{-1}) were also significantly greater than densities at GSB (1.1 ± 0.50 × 10^8 viruses ml^{-1}; $P < 0.05$; *t*-test; Figs. 3B and 4B).

During 2002, bacteria cell densities at GSB ranged from 2.1 to 11 × 10^6 cells ml^{-1}, while cyanobacteria densities ranged from 3.3 to 10 × 10^5 cells ml^{-1} (Fig. 4C). At QB, bacteria densities ranged from 2.7 to 6.8 × 10^6 cells ml^{-1} and cyanobacteria densities ranged from 4.6 to 8.1 × 10^5 cells ml^{-1} (Fig. 3C). Both populations were slightly higher later in the summer, although densities of these populations in QB did not parallel changes observed in chl *a* or brown tide concentrations. Moreover, there was not a significant difference in cell densities of either bacteria or cyanobacteria between the two sites. At each site during 2002, various physical and chemical parameters such as temperature, salinity, the 1% light depth, and concentrations of phosphate, silica, and ammonium were similar (Table 2). In contrast, mean annual dissolved organic nitrogen (DON) concentrations were significantly higher at QB than GSB ($P < 0.05$; *t*-test; Table 2), while mean annual nitrate concentrations were significantly higher at GSB as compared to QB ($P < 0.05$; *t*-test; Table 2).

Nutrient additions yielded a significant increase in the experimental net growth rates of the total phytoplankton community relative to control treatments during 10 of 12 experiments conducted, with 24 April in GSB and 8 August in QB being the notable exceptions ($P < 0.05$; Tukey test; Table 3). In contrast, during the brown tide in QB, *A. anophagefferens* growth rates were significantly enhanced by these additions in only 1 of 5 experiments analyzed (11 July; $P < 0.05$; Tukey test; Table 3). Moreover, the absolute enhancement of *A. anophagefferens* growth rates were small relative to the increased growth rates observed in the total phytoplankton community (Table 3).

Table 2
Physical and chemical measurements made in GSB and QB, 2002

	Date	*T*	*S*	1% light	NH_4^+	NO_3^-	DON	PO_4^{-3}	Si
GSB	24 April	14	29	5.4	1.0 ± 0.35	1.6 ± 0.01	19 ± 2.6	0.12 ± 0.01	4.2 ± 2.8
	7 May	17	27	3.3	0.55 ± 0.16	1.7 ± 0.81	22 ± 5.3	0.25 ± 0.04	12 ± 5.8
	21 May	16	27	4.1	1.6 ± 0.44	2.2 ± 0.24	33 ± 0.01	0.15 ± 0.03	15 ± 3.5
	6 June	22	27	1.6	0.86 ± 0.59	0.91 ± 0.03	40 ± 4.1	0.34 ± 0.05	28 ± 0.57
	20 June	23	28	2.9	1.4 ± 0.37	0.53 ± 0.18	35 ± 4.1	0.61 ± 0.16	59 ± 8.4
	9 July	26	29	1.6	0.68 ± 0.25	0.79 ± 0.07	25 ± 1.2	0.17 ± 0.06	24 ± 0.6
	6 August	28	30	5.8	0.66 ± 0.03	0.24 ± 0.07	29 ± 0.9	0.23 ± 0.04	56 ± 2.2
	Mean ± S.D.	21 ± 5.2	28 ± 1.1	3.5 ± 1.6	0.96 ± 0.39	1.1 ± 0.71	29 ± 7.5	0.26 ± 0.17	28 ± 21
QB	19 April	20	29	3.4	0.16 ± 0.03	0.22 ± 0.13	38 ± 1.1	0.42 ± 0.21	15 ± 8.0
	9 May	16	30	3.1	0.48 ± 0.08	0.11 ± 0.04	23 ± 5.3	0.35 ± 0.21	14 ± 0.83
	23 May	16	28	3.6	0.92 ± 0.05	0.31 ± 0.04	31 ± 1.5	0.11 ± 0.03	14 ± 6.5
	4 June	20	29	4.1	0.65 ± 0.21	0.57 ± 0.08	37 ± 2.0	0.26 ± 0.04	37 ± 4.2
	18 June	21	26	2.2	0.65 ± 0.09	0.43 ± 0.06	43 ± 0.8	0.28 ± 0.10	47 ± 10
	11 July	24	30	1.9	1.9 ± 0.24	0.67 ± 0.37	42 ± 0.9	0.31 ± 0.10	79 ± 0.44
	8 August	24	29	1.4	0.12 ± 0.16	0.07 ± 0.05	55 ± 7.3	1.0 ± 0.12	110 ± 3.9
	Mean ± S.D.	20 ± 3.4	29 ± 1.2	2.5 ± 1.1	0.69 ± 0.60	0.34 ± 0.23	38 ± 10	0.39 ± 0.28	45 ± 37

T is temperature in °C, *S* is salinity in PSU, 1% light is the 1% light depth calculated from extinction coefficients expressed in meters. All nutrient concentrations are micromolar and are presented as means ± S.D.

Table 3
Net growth rates and grazing mortality rates of the total phytoplankton community and *A. anophagefferens* during nutrient additions HMWVC (high molecular weight viral concentrate) additions, and microzooplankton grazing experiments in GSB (non-bloom site) and QB (bloom site), 2002

	Date	Control	+ Nutrients	+ Live HMWVC	+ Dead HMWVC	Grazing mortality rate
GSB	24 April	0.32 ± 0.02	0.33 ± 0.03	**0.57 ± 0.05**	0.25 ± 0.02	1.1 ± 0.11
	21 May	0.09 ± 0.06	*0.20 ± 0.03*	**0.46 ± 0.05**	**0.49 ± 0.09**	1.2 ± 0.40
	6 June	0.11 ± 0.12	*0.66 ± 0.05*	0.74 ± 0.09	0.96 ± 0.09	ND
	20 June	−0.26 ± 0.04	*0.21 ± 0.02*	0.39 ± 0.09	0.34 ± 0.11	0.72 ± 0.15
	9 July	0.12 ± 0.14	*0.52 ± 0.07*	**0.81 ± 0.05**	**0.77 ± 0.06**	0.84 ± 0.22
	6 August	−0.20 ± 0.03	*−0.01 ± 0.06*	−0.17 ± 0.08	**0.06 ± 0.01**	1.2 ± 0.16
QB	19 April	−0.25 ± 0.03	*0.15 ± 0.03*	0.12 ± 0.03	0.12 ± 0.01	0.25 ± 0.08
	23 May	0.23 ± 0.16	*0.89 ± 0.05*	0.91 ± 0.04	0.90 ± 0.02	0.46 ± 0.08
	4 June	0.04 ± 0.04	*0.78 ± 0.05*	0.76 ± 0.10	0.81 ± 0.02	0.59 ± 0.07
	18 June	−0.26 ± 0.03	*−0.06 ± 0.12*	**0.20 ± 0.05**	**0.19 ± 0.10**	0.76 ± 0.12
	11 July	−0.21 ± 0.02	*0.07 ± 0.02*	0.14 ± 0.04	0.10 ± 0.04	1.6 ± 0.39
	8 August	−0.19 ± 0.03	−0.07 ± 0.09	−0.14 ± 0.05	−0.01 ± 0.04	1.5 ± 0.17
QB-BT	23 May	−0.20 ± 0.08	−0.22 ± 0.15	**0.12 ± 0.02**	0.06 ± 0.02	0.53 ± 0.22
	4 June	0.30 ± 0.02	0.37 ± 0.05	**0.70 ± 0.04**	**0.67 ± 0.04**	ND
	18 June	−0.17 ± 0.03	−0.09 ± 0.07	**0.22 ± 0.00**	**0.22 ± 0.02**	0.15 ± 0.05
	11 July	0.10 ± 0.09	*0.26 ± 0.05*	0.31 ± 0.03	0.30 ± 0.04	0.34 ± 0.11
	8 August	−0.13 ± 0.19	−0.04 ± 0.12	**0.01 ± 0.22**	−0.26 ± 0.36	0.98 ± 0.37

QB-BT refers to the growth response of *A. anophagefferens* during experiments in QB, while GSB and QB rates represent the response of the total phytoplankton community. Rates are per day. ND indicates the grazing rates were not detected due to a non-significant slope of the dilution of seawater v net growth rate regression. Values represent means ± S.D. ($n = 3$) for nutrient and HMWVC addition experiments, and slopes ± standard errors for grazing mortality rates. Italicized values indicate nutrient addition treatments which yielded growth rates significantly greater than control treatments ($P < 0.05$; Tukey test). Bolded values indicate HMWVC additions which yielded growth rates significantly greater than nutrient addition treatments ($P < 0.05$; Tukey test).

Concurrent HMWVC addition experiments yielded several distinct trends within the total phytoplankton community through the summer of 2002. The most common impact of live or dead HMWVC additions on the total phytoplankton community was to yield growth rates which were not different from the nutrient additions, but were significantly greater than unamended control treatments (QB: 19 April, 23 May, 4 June, 11 July, 8 August 6; GSB: 6 June and 20 June; $P < 0.05$; Tukey test; Table 3). A second, but less prominent, trend we observed at both sites was algal growth rates in live and dead HMWVC treatments that were significantly greater than nutrient addition and control treatments, but not different from each other (GSB: 21 May, 9 July; QB: 18 July; $P < 0.05$; Tukey test; Table 3). Finally, two experiments in GSB yielded results that were observed only once each within the total phytoplankton community. During our April GSB experiment, live HMWVC additions yielded growth rates which were significantly greater than all other treatments, while dead HMWVC growth rates were significantly greater than all other treatments during our August GSB experiment ($P < 0.05$; Tukey test; Table 3).

The growth response of *A. anophagefferens* during HMWVC addition experiments differed from the total phytoplankton community during most experiments in QB. In a manner similar to the 2000 bloom results, live viral additions yielded brown tide growth rates which were significantly greater than dead viral additions during two experiments (23 May and 8 August; $P < 0.05$; Tukey test; Table 3). During 4 June and 18 June experiments, *A. anophagefferens* growth rates in live and dead viral treatments were significantly greater than nutrient addition and control treatments, but not different from each other ($P < 0.05$; Tukey test; Table 3). Finally, during the 11 July experiment, live and dead viral additions were greater than control treatments, but not different from the nutrient addition treatment ($P < 0.05$; Tukey test; Table 3).

Grazing mortality rates of the total phytoplankton community in GSB were fairly consistent ($1.0 \pm 0.23\,d^{-1}$) relative to QB which varied 6-fold from 0.25 to $1.6\,d^{-1}$ and averaged $0.87 \pm 0.57\,d^{-1}$ during 2002 (Table 3). Moreover, grazing mortality rates in GSB during the first 2 months of this study ($1.0 \pm 0.26\,d^{-1}$; late April through late June) were twice as high as those in QB ($0.52 \pm 0.22\,d^{-1}$; Table 3). This pattern changed during July and August, when grazing mortality rates in QB (1.6 ± 0.1) were greater than GSB (1.0 ± 0.3). Finally, mean annual grazing rates on *A. anophagefferens* at QB during 2002 were generally lower than those measured on the total phytoplankton community in either system ($0.49 \pm 0.35\,d^{-1}$; Table 3).

4. Discussion

Aureococcus anophagefferens blooms have been disrupting the ecological balance of mid-Atlantic US estuaries for nearly two decades and, to date, a multitude of singular causes have been cited as bloom initiators. Our field study approach allowed us to compare experiments and observations made during bloom and non-bloom periods and facilitated an assessment of biological and chemical factors which may promote bloom onset. Our results confirm some of the conclusions drawn from prior studies of *A. anophagefferens* blooms, specifically that, rather than there being a single universal cause of brown tides, there exist multiple top-down and bottom-up factors which are likely to act individually, as well as in unison, to promote net population growth of this species (Gobler et al., 2002). The relative importance of each of these factors is likely to vary on both temporal and spatial scales of bloom events and is discussed below.

To date, the physical and chemical environments of brown tide-prone estuaries are frequently cited as factors which may be involved in bloom occurrence (Wilson, 1995; Bricelj and Lonsdale, 1997; Gobler and Sañudo-Wilhelmy, 2001). A cursory comparison of QB and GSB during 2002 indicates that the physical and chemical nature of these two estuaries were similar, as levels of temperature, salinity, phosphate, silica, ammonium, and the 1% light depth at each site were not significantly different from one another (Table 2). In contrast, mean annual DON concentrations were significantly higher at QB compared to GSB ($P < 0.05$; *t*-test; Table 2), while mean annual nitrate concentrations were significantly higher at GSB as compared to QB ($P < 0.05$; *t*-test; Table 2). The occurrence of a brown tide in QB where DON was high and nitrate was low relative to GSB is certainly consistent with past research which has demonstrated *A.*

anophagefferens may rely on organic nutrients to form blooms (LaRoche et al., 1997; Gobler et al., 2002; Gastrich et al., 2004) and that eutrophic conditions (high DIN) discourage bloom development (Keller and Rice, 1989; LaRoche et al., 1997; Gobler et al., 2002). Alternatively, the disparity in nutrient concentrations between the stations may be caused, in part, by differing levels of algal biomass. Elevated levels of phytoplankton in QB (Fig. 3A) could have assimilated nitrate and exuded DON (Bronk et al., 1994) at a higher rate than the GSB community which had lower levels of algal biomass (Fig. 4A) and presumably a smaller total N demand and DON exudation rates.

The results of our nutrient enrichment experiments demonstrated that growth rates of the total phytoplankton communities at both sites were enhanced by the addition of inorganic nutrients (N and P) during 10 of 12 experiments conducted in 2002 (Table 3). In contrast, nutrient additions did not alter *A. anophagefferens* growth rates during most experiments (4 of 5 quantified; Table 3). These results evidence a duality among the phytoplankton present during brown tide blooms, with growth rates of non-brown tide algae depending on external inorganic nutrient supply more than brown tide populations (Table 3). This could indicate *A. anophagefferens* populations relied on the ambient DOM for growth during the QB 2002 bloom since DOM is generally more plentiful during bloom events (Gobler et al., 2002), particularly relative to regions not experiencing blooms (Table 2).

Densities of total viruses during the two bloom events we observed (QB 2002: $2.3 \pm 1.2 \times 10^8\,ml^{-1}$; GSB 2000: $2.8 \pm 2.1 \times 10^8\,ml^{-1}$) were more than double and significantly greater than the mean densities observed during the non-bloom data set from GSB 2002 ($1.1 \pm 0.6 \times 10^8\,ml^{-1}$; $P < 0.1$; Tukey test). These differences existed despite similar levels of the most common viral hosts, such as cyanobacteria and bacteria (Wommack and Colwell, 2000) at each location (Figs. 2B, 3C and 4C). The viral densities observed during blooms were also greater than the levels previously reported within most coastal and estuarine environments (as reviewed by Wilhelm and Suttle, 1999; Wommack and Colwell, 2000). This data suggests that *A. anophagefferens* blooms may be associated with elevated viral densities, a scenario which could be due to the presence of *A. anophagefferens* specific viruses, the presence of a bacterial population associated with blooms which are more prone to viral infection than populations which exist during non-bloom conditions, or a greater abundance of algal viruses in general during blooms, since each brown tide event was also associated with higher levels of phytoplankton biomass. It is also possible that the elevated viral populations lead to enhanced bacterial growth rates, but static bacterial abundance due to viral lysis (Noble et al., 1999; Wilhelm et al., 2003). Such a feedback would ultimately lead to elevated concentrations of DOM in the system as well (Middelboe et al., 1996; Gobler et al., 1997; Wilhelm and Suttle, 1999).

During our HMWVC addition experiments we observed a series of contrasting trends in the growth responses of the total phytoplankton community and *A. anophagefferens*. For example, during most experiments conducted in 2000 and 2002, enhancing levels of live or dead HMWVC concentrates did not significantly alter the net growth rates of the total phytoplankton community relative to nutrient enrichment (70% experiments; Tables 1 and 3). Although such a response was also displayed by *A. anophagefferens* during 40% of experiments in which this alga was quantified (Tables 1 and 3), during another 30% of the experiments, net growth rates of *A. anophagefferens* were significantly enhanced by the addition of live HMWVC relative to other treatments (Tables 1 and 3). Live HMWVC additions elicited such a response from the total phytoplankton community in only 6% of experiments (one of 17 experiments; Tables 1 and 3). Moreover, live and dead HMWVC additions enhanced *A. anophagefferens* growth beyond the rates achieved by simple N and P additions during the peak of the brown tide event in QB (4 and 18 June), but did so in the total phytoplankton community in QB on 18 June only when *A. anophagefferens* represented the majority of algal biomass (Fig. 3; Table 3). These results lead to some new hypotheses regarding the potential role of viruses in brown tide dynamics.

Although viruses are generally considered an important source of microbial mortality in aquatic ecosystems, the role of viruses in nutrient regeneration and DOM production has also been well documented (Middelboe et al., 1996; Gobler et al., 1997; Wilhelm and Suttle, 1999). Given that *A. anophagefferens* distinguished itself from the total phytoplankton community by not relying on inorganic nutrients for enhanced growth during experiments (Table 3) but

instead experienced enhanced growth during live and/or dead HMWVC additions (Table 3), it is possible that HMWVC, which are enriched in DOM (Amon and Benner, 1994; Gobler and Sañudo-Wilhelmy, 2003; this study, data not shown), were a nutritional resource which *A. anophagefferens* utilized for growth (Berg et al., 2002; Mulholland et al., 2002). Enhancement of *A. anophagefferens* growth rates during live HMWVC additions significantly beyond those achieved by dead HMWVC additions (Tables 1 and 3) could indicate that live viruses (bacteriophage, cyanophage, algal viruses) regenerated additional DOM (Middelboe et al., 1996; Gobler et al., 1997; Wilhelm and Suttle, 1999) beyond the amount simply present in the HMWVC, which was subsequently utilized by *A. anophagefferens* (Berg et al., 2002; Mulholland et al., 2002). The enhancement of brown tide growth rates by live HMWVC only on dates in which densities of viruses were below annual means ($<1.7 \times 10^8$ ml^{-1}; Tables 1 and 3; Figs. 2 and 4) could indicate a lower threshold of viral densities is required to promote robust *A. anophagefferens* growth.

Although the regeneration of DOM by viruses seems a plausible explanation for the observed enhanced *A. anophagefferens* growth during live, but not dead, HMWVC addition (Tables 1 and 3), there exists alternate hypotheses which also merit consideration. For example, since clonal immunity to viruses has been widely reported among algal populations susceptible to viral infection (Waterbury and Valois, 1993; Tarutani et al., 2000; Schroeder et al., 2002), viral lysis of slower growing clones of *A. anophagefferens* present during a bloom might lead to an enhanced net population growth (Noble et al., 1999). It is also possible that lysis of other microbes, such as cyanobacteria or bacteria, during live HMWVC additions could have benefited *A. anophagefferens*, perhaps by reducing competition for nutritional resources (Noble et al., 1999). The absence of a reduction in brown tide net growth rates during live viral additions, despite the presence of a high titer of *A. anophagefferens*-specific viruses during blooms in 2002 (data not shown) suggests the incubation period used for experiments (48 h) may not have been long enough to allow for lysis of *A. anophagefferens*, which can be up to 5 d when live HMWVC are initially inoculated into laboratory cultures (data not shown). While the precise mechanism is unclear, our data does demonstrate viruses can promote the growth of *A. anophagefferens* during bloom events.

Reduced microzooplankton grazing may also contribute toward the formation of brown tides. During 2002, we observed higher grazing mortality rates in GSB, a non-bloom site, relative to QB which hosted a brown tide (Table 3). These differences were most pronounced during the April through June period, when grazing mortality rates in QB were half of those in GSB (Table 3), suggesting lower grazing rates at QB may have contributed to the initiation of the mixed assemblage phytoplankton bloom there (Fig 3A). These observations are similar to those of Mehran (1996) who documented that community microzooplankton grazing rates on the total phytoplankton community in an estuary during a bloom were half of the rates observed during the previous year when a brown tide did not occur.

Also during our study, grazing mortality rates of *A. anophagefferens* were lower than the rates of the total phytoplankton community in QB during two dates near the peak of the brown tide (18 June, 11 July; Table 3). Lower grazing rates on *A. anophagefferens* relative to the total phytoplankton community have been observed previously during blooms (Gobler et al., 2002) and could be due to or the cause of reduced growth rates of protozoan grazers which typically consume brown tide cells (Caron et al., 1989; Lonsdale et al., 1996; Caron et al., 2004; Sieracki et al., 2004). Markedly increased microzooplankon grazing rates in QB during July and August (three-fold increase over April–June; Table 3) following the peak of brown tide densities could indicate the establishment of a protozoan community which was able to grow and graze robustly in the presence of *A. anophagefferens* (Caron et al., 1989; Caron et al., 2004) and perhaps contributed to reduced brown tide densities at the end of the summer.

5. Conclusions

When considered in light of previous research, our findings corroborate the conclusions of several independent studies and unify them to provide a singular hypothesis about the environmental conditions that promote brown tides. Our observations of the nutrient regime present during a brown tide (ratio of

high organic nutrients to low inorganic nutrients), the nutrient status of phytoplankton during blooms (limitation of non-brown tide algae, but replete growth in *A. anophagefferens*), and the impact of organically enriched HMWVC additions on *A. anophagefferens* growth (live or dead HMWVC additions, on occasion, enhanced brown tide growth) together bolster one of the firmest conclusions about brown tides made to date: they are not caused by eutrophication, but instead are more likely to occur when sources of labile DOM are readily available (Keller and Rice, 1989; LaRoche et al., 1997; Gobler et al., 2002). Our observations of lower rates of microzooplankton grazing on the phytoplankton community, in general, and *A. anophagefferens* in particular during a bloom event are consistent with previous observations made during blooms (Mehran, 1996; Gobler et al., 2002; Caron et al., this issue), and hence supports the conclusion that lowered grazing mortality may promote brown tides. Finally, our observations of viruses suggest they may be an important microbial partner in brown tide occurrence, as both an agent of mortality (Milligan and Cosper, 1994; Gastrich et al., 2002) and a bloom promoter by the regeneration of DOM that increases *A. anophagefferens* population growth or by altering the composition of microbial communities.

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