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Stimulation of the brown tide organism, *Aureococcus anophagefferens*, by selective nutrient additions to in situ mesocosms

Todd M. Kana[a,*], Michael W. Lomas[b], Hugh L. MacIntyre[a,1], Jeffrey C. Cornwell[a], Christopher J. Gobler[c]

[a] *Horn Point Laboratory, University of Maryland Center for Environmental Science, P.O. Box 775, Cambridge, MD 21613, USA*
[b] *Bermuda Biological Station for Research, Inc., St. Georges, Bermuda, Ge01, USA*
[c] *Department of Natural Sciences, Southampton College, Southampton, NY 11968, USA*

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Abstract

The influence of nutrient additions and sediment exchange on *Aureococcus anophagefferens* growth was studied using 2001 mesocosms deployed in situ at the Southampton College Marine Science Center in Long Island, New York. *A. anophagefferens* cell density increased in mesocosms with separate additions of the following materials: urea + glucose and desiccation-stressed *Enteromorpha* tissue. A decrease in *A. anophagefferens* cell density was observed in mesocosms with either no additions (control) or with added nitrate. A treatment containing a sediment layer exhibited an increase in average cell densities, but the increase was not statistically significant ($P = 0.15$). In the 9 day experiment, net growth of *A. anophagefferens* was only observed during the last 3 days, which corresponded to a period of high solar irradiation. Total chlorophyll concentration declined in all treatments during the first 6 days, which corresponded to relatively low daily irradiance, suggesting low-light stress on the phytoplankton assemblage during the initial phase of the experiment. During the ensuing sunny period, a 4–5-fold increase in chlorophyll concentration was observed in the nitrate and urea treatments with lesser increases in the other treatments. *A. anophagefferens* density increased relative to total phytoplankton biomass (Chl basis) in the urea + glucose and *Enteromorpha* treatments. Results are consistent with a prevailing hypothesis that organic nitrogen nutrients favor the growth of *A. anophagefferens*. Specifically, our evidence indicates that *A. anophagefferens* exhibited net population growth under organic N, but not inorganic N nutrient (specifically NO_3^-) loading.
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1. Introduction

There are now close to 20 years of monthly measurements of *Aureococcus anophagefferens* cell counts and water quality parameters in the South-shore and Eastern bays of Long Island, New York (Suffolk County Department of Health). These measurements were started in response to a massive “brown tide” in

* Corresponding author. Tel.: +1-410-221-8481; fax: +1-410-221-8290.
E-mail address: kana@hpl.umces.edu (T.M. Kana).
[1] Present address: Dauphin Island Sea Lab, 101 Bienville Blvd, Dauphin Island, AL 36528, USA

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1985 that had devastating effects on the seagrass and shellfish populations of those economically productive water bodies. Unlike the brown tide caused by another pelagophyte, *Aureoumbra lagunensis,* found in Laguna Madre, Texas, which persisted unabated for years, the Long Island brown tides have occurred sporadically and inconsistently among the many embayments of the affected area. These extensive space- and time-series records from the Long Island bays have proven useful in narrowing down the number of factors that potentially control the blooms. In particular, they helped identify an inverse relationship between nitrate loading via subsurface groundwater discharge and *A. anophagefferens* abundance on a climatic time-scale (12-month average; LaRoche et al., 1997). The suppression of *A. anophagefferens* relative to other phytoplankton by increased nitrate loading from groundwater discharge suggested that *A. anophagefferens* blooms must come about and be supported by alternative nitrogen sources. Dissolved organic nitrogen (DON) was proposed in that study to be the source, insofar as ammonium concentrations were apparently insufficient to support significant biomass accumulation. The importance of DON to *A. anophagefferens* growth was also suggested by physiological evidence of a significant capacity for direct and indirect DON uptake (Dzurica et al., 1989; Berg et al., 1997, 2003) and enzymatic hydrolysis of DON compounds (Berg et al., 2002; Mulholland et al., 2002).

Evidence in favor of the so-called 'DON hypothesis' has been largely circumstantial or correlative. It has not been possible to completely isolate the uptake and utilization of DON by native *A. anophagefferens* from that of other phytoplankton to demonstrate that DON uptake by *A. anophagefferens* is effectively greater than DON uptake by any of the other indigenous phytoplankton, or to demonstrate that the abnormally high bacterial densities associated with *A. anophagefferens* are not responsible for DON hydrolysis and subsequent uptake (but see Berg et al., 2002). The validity of the DON hypothesis currently rests on evidence obtained from a breadth of scales and levels of complexity. In addition to the supportive evidence from climatic-scale behavior and physiological uptake responses, evidence consistent with the hypothesis is available from: (a) time-series data covering event-scale (i.e. blooms) ecosystem behavior demonstrating a relationship between the existence of precursor phytoplankton blooms and brown tide events (Gobler and Sañudo-Wilhelmy, 2001; Seracki et al., 2004); (b) cross-ecosystem correlations between ratios of organic C, N, and P and bloom or non-bloom conditions (Lomas et al., 2001) and (c) bioassay or other nutrient enrichment experiments on natural samples (Keller and Rice, 1989, Gobler and Sañudo-Wilhelmy, 2001; Gobler et al., 2002, Caron et al., 2004). In this study, we designed an experiment to test whether simple and/or complex mixtures of dissolved organic nitrogen and dissolved organic carbon (DOC) added to whole plankton systems would selectively promote net population growth of *A. anophagefferens*.

1.1. Experimental rationale

It has been previously established that *A. anophagefferens* is capable of taking up dissolved organic carbon (DOC, e.g. acetate) as well as DON (e.g. amino acids) and dissolved organic phosphorus (DOP) (e.g. glycerol–phosphate) compounds in culture (Dzurica et al., 1989; Berg et al., 2002). In addition, urea and amino acids, which are functionally categorized within the DON pool, and dissolved peptides have been shown to provide both C and N in the production of *A. anophagefferens* cells (Lomas, 2003; Mulholland et al., 2002). Although a requirement for DOC by *A. anophagefferens* for its growth in nature has not been demonstrated, it is plausible that DOC utilization can confer an advantage to phytoplankton under bloom conditions with associated light limitation. Therefore, in addition to DON sources, we included a strictly organic carbon source (glucose) along with a DON source in selected treatments.

The sources and chemical characteristics of DON and DOC in the bays with brown tide are not fully characterized, but there are several sources that may come into play at different times. These sources include allochthonous inputs from rivers, streams or bordering marshes, inputs from sediment porewater exchange or the benthos, and autochthonous inputs from the turnover of plankton biomass (Gobler and Sañudo-Wilhelmy, 2001). Simulation of some of these potential sources was done using independent additions of either defined chemicals or materials that

deliver complex dissolved organic compounds to the water column. Urea was selected as a defined reduced nitrogen source because it has been shown to be episodically abundant in these bays (Lively et al., 1983; Kaufman et al., 1983) and utilized by *A. anophagefferens* (Lomas et al., 1996). Nitrate was tested in an independent treatment, because it is thought to give a competitive advantage to other phytoplankton species in both Long Island bays (LaRoche et al., 1997) and experimental mesocosms (Keller and Rice, 1989). In addition to these defined sources, two separate sources of complex dissolved organic compounds were tested. One potential source is from sediment. The rationale for testing sediment comes from the fact that brown tides occur in shallow (<2 m deep) bays where benthic fluxes could be a potential source of nutrients (Lomas et al., 2004; MacIntyre et al., 2004). The second complex DOM source used in this study was from the green macroalga, *Enteromorpha* sp. This treatment was inspired by a significant brown tide event that occurred in the fall of 1999 in Great South Bay, Long Island (R. Cerratto, personal communication). In that year, water clarity was unusually high in the summer due to a dense and wide-spread population of the clam, *Mulinia* sp., which cropped the phytoplankton assemblage to low concentrations (R. Nuzzi, personal communication). Increased water clarity resulted in significant growth of benthic macroalgae during the summer. It has been demonstrated that organic material in the green macroalgae *Ulva rigida* is labile and rapidly consumed by bacteria (Castaldelli et al., 2003) and it was suspected that recycling of the nutrients bound in the abundant macroalgae fueled an intense brown tide of 1999, which began in late September and lasted until the bays iced over in January of that winter (Nuzzi and Waters, 2004).

2. Methods

2.1. Mesocosm experimental design

Experimental treatments were conducted in translucent polyethylene film tubes (0.45 m diameter × 1.5 m long) sealed at the bottom by clamping a 30 cm diameter PVC plastic plate or a 30 cm diameter PVC plastic receptacle containing approx. 5 cm deep sediment. The bags were submerged and filled with water at the staging site. At the beginning of the experiment, approximately 10 l of water collected from Quantuck Bay was added to each mesocosm to provide an *A. anophagefferens* seed population. Total volume was ~200 l. The top of each bag had a buoyant collar that floated the mesocosms with the tide. The mesocosms were randomly arranged and tied to a floating dock at the Marine Science Station at Southampton College. The water was mixed intermittently using gentle bubbling through an air stone for 30 min each hour during daylight and 30 min at midnight. The experiment was conducted between 12 June 2001 (day 0) and 21 June 2001 (day 9).

Five treatments were tested: (1) control (no addition), (2) nitrate + phosphate addition, (3) urea + glucose + phosphate addition, (4) sun and desiccation-stressed *Enteromorpha* sp., and (5) sandy sediment collected from Quantuck Bay, a bay with above average frequency of brown tides. Nitrate and urea compounds were added at equivalent N concentrations (40 μM-N final for each dose). Phosphate was added in Redfield ratio (with respect to added N) and it increased the ambient concentrations by 4 μM-P. Glucose was added in an amount of 100 μM-C final concentration (not including ambient glucose) at each addition. Additions of chemical nutrients were made on days 0, 5, and 6. *Enteromorpha* sp. (60 g f.w.) was collected from nearby substrates and placed on a dock in air for 2–3 h of direct sun. The material was then placed in weighted mesh bags and dropped to the bottom of the replicate mesocosms on day 0. Sediment was manually collected from the top 10 cm of a shallow part of Quantuck Bay and placed in the 30 cm diameter PVC cylinders with attached bottoms. To allow the sediment to compact and for resuspension-prone particulates to stabilize or wash away, the containers were set in a shallow area near shore for approximately 18 h before clamping them to the mesocosm bags. The sediment was predominantly sand, with fine particles, shells and organic matter interspersed. All treatments and controls were replicated in triplicate for a total of 15 mesocosms. Water samples were removed daily for chemical constituents and other selected parameters, and at several day intervals for flux measurements. The removal of sample water was <10% of the starting volume.

2.2. Flux measurements

System metabolism was assessed as the rate of oxygen production and dark respiration. Water samples were incubated in 20 ml glass-stoppered test tubes that were suspended in the water column at a depth equal to 50% of the mesocosm depth. Additional samples were kept in darkness at ambient water temperature. Initial and end-point measurements of oxygen concentration were made using membrane inlet mass spectrometry (Kana et al., 1994). Rates were calculated from differences in the measured O_2/Ar ratio, adjusted for the Ar concentration, which was assumed to remain constant at the saturation concentration.

Water column nitrogen uptake rates were measured on samples ($n = 1$) from each mesocosm (except the first time point which was determined on one mesocosm from each treatment) as in Lomas et al. (1996). Briefly, $^{15}NO_3^-$, $^{15}NH_4^+$, ^{15}N-urea, and ^{15}N-glutamic acid (all > 98% enriched) were added to 100 ml samples in polycarbonate bottles to a final concentration of 10 μM-N and incubated for ∼1 h at 60% of surface irradiance. Saturating additions of substrate were used since half-saturation concentrations for Long Island plankton populations have been shown to be very low, < 0.15 μM-N (Lomas et al., 1996) and there was a concern for depletion of tracer substrate and/or isotopic substrate dilution during short incubations (Glibert et al., 1982). The measured saturated rates were used to estimate in situ uptake rates by applying a Michaelis–Menten kinetic type equation parameterized with the measured ambient nutrient concentration and kinetic constants determined previously on phytoplankton populations in Long Island's coastal bays (Lomas et al., 1996, Lomas, unpublished data). The kinetic parameter, k_s, does not vary systematically with respect to *A. anophagefferens* cell density for any of the nitrogen substrates. The ambient concentration of glutamic acid was assumed to be 0.02 μM-N (see Berg et al., 1997).

2.3. Analysis

A. anophagefferens densities were determined on preserved samples by direct count methods employing an epi-fluorescence microscope. *A. anophagefferens* cells were enumerated using an immunofluorescent label technique as described by Anderson et al. (1993). Modifications of the original technique included increasing the amount of primary and secondary antibody used by ∼2-fold. A minimum of 100 cells were counted per sample in at least 10 fields, which yielded a relative standard deviation of 15% for replicate counts of the same sample ($n = 6$). To ensure accurate results, the immunofluorescence technique was compared to counts performed with a hemacytometer on a light microscope. The two techniques yielded statistically identical results using *A. anophagefferens* clone 1708.

Samples for particulate analysis were collected on GF/F filters by vacuum filtration at low pressure. Chlorophyll was measured by fluorometry (Welschmeyer, 1994) after extraction in 90% acetone for 24 h at −4 °C in darkness. Daily irradiance was measured using a LiCor quantum 2π sensor and electronic integrator.

Samples for dissolved inorganic and organic nutrients were collected from particulate sample filtrates and immediately frozen in pre-combusted (450 °C, 5 h) glass scintillation vials (dissolved free amino acids and dissolved monosaccharides) or acid-cleaned HDPE bottles (all others). Dissolved inorganic nitrogen, NO_2^-/NO_3^- and NH_4^+, PO_4^{-3}, and urea were analyzed using standard colorimetric methods (Parsons et al., 1984). Dissolved organic carbon was analyzed by high temperature catalytic oxidation on a Shimadzu TOC-5000 total organic carbon analyzer and calibrated against a glucose standard (Hansell, 1993). Samples for dissolved organic nitrogen and dissolved organic phosphorus were oxidized by persulfate addition and autoclaving (Valderrama, 1981) and then analyzed as NO_3^- and PO_4^{-3}, respectively.

3. Results and discussion

3.1. General patterns

The experiment was carried out during a weather system that brought a 5 day period of cloudy, partly cloudy, and rainy weather (21 mm of rain between 11–18th June, Southampton College rain gauge) followed by 3 days of mostly sunny weather. Daily irradiance during the experiment is shown in Fig. 1A. This weather condition, with reduced daily irradiances in the early phase of the experiment, was

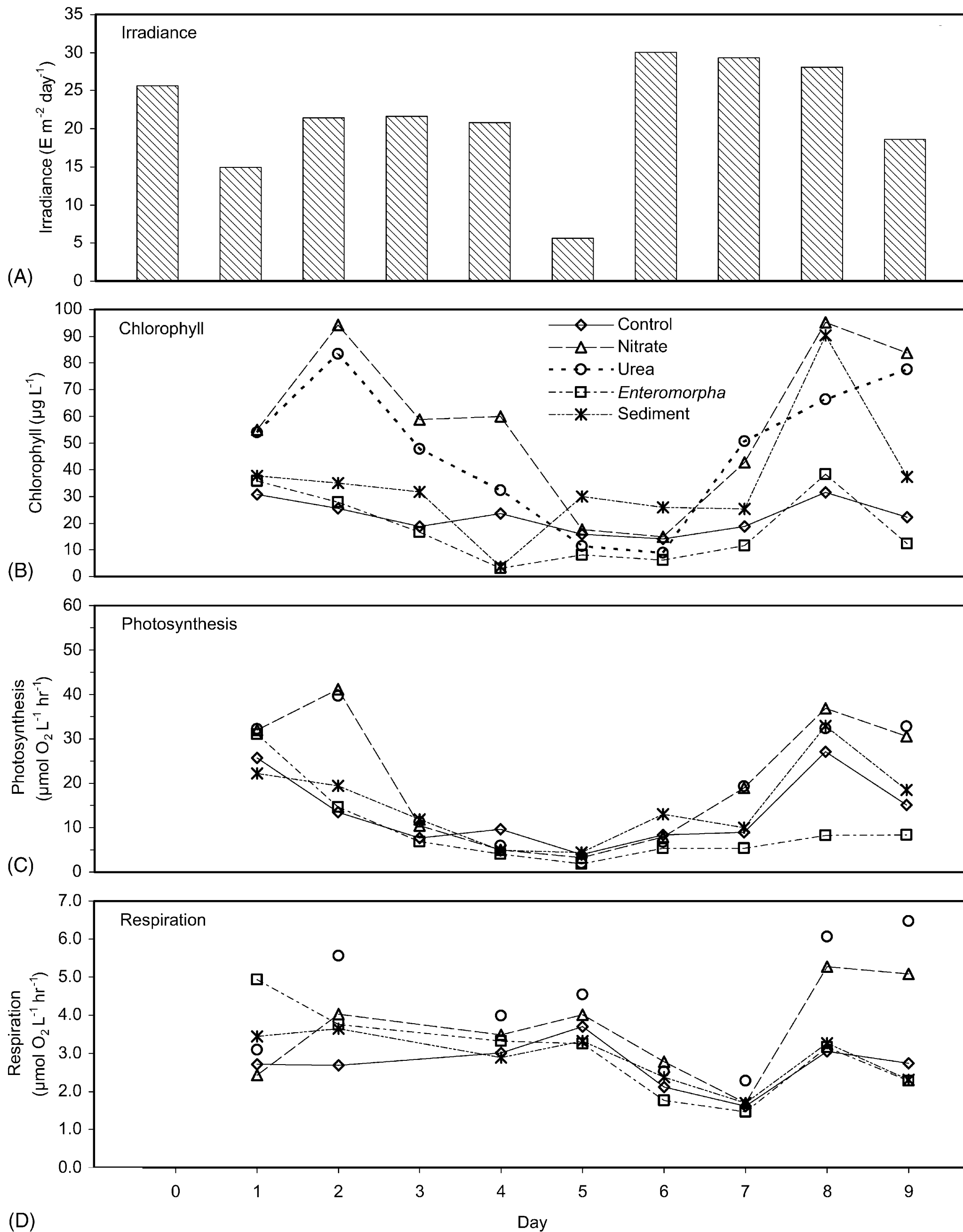

Fig. 1. (A) Twenty four hours integrated irradiance for each day of the study. (B) Average water column chlorophyll concentrations in each treatment. Average coefficients of variation for treatment were: control: 0.65, nitrate: 0.26, urea: 0.19, *Enteromorpha*: 0.31, and sediment: 0.34. (C) Average gross oxygen evolution rates for each treatment. Average coefficients of variation for each treatment were: control: 0.67, nitrate: 0.24, urea: 0.16, *Enteromorpha*: 0.22, and sediment: 0.31. Symbols as in (B). (D) Average oxygen consumption rates in darkness for each treatment. Average coefficients of variation for each treatment were: control: 0.42, nitrate: 0.14, urea: 0.16, *Enteromorpha*: 0.15, and sediment: 0.12. Symbols as in (B).

wholly or partially responsible for the progressive 30–85% decline in chl (Fig. 1B) and 70–95% decline in water column photosynthesis observed among all treatments between days 1 and 5 (Fig. 1C). The onset of the sunny period on day 6 brought about a return to the initial high photosynthesis rates by day 8 in all mesocosm groups except the *Enteromorpha* treatment. Plankton respiration rates remained relatively stable during the first 5 days (Fig. 1D). From days 5 to 7, respiration dropped approximately 50% in all mesocosms, after which it increased. The most significant increases in respiration at the end of the experiment were observed in the nitrate and urea treatments.

3.2. Phytoplankton and A. anophagefferens net growth

Further inspection of the chl data (Fig. 1B) shows that the two treatments with added defined nutrients (nitrate and urea) exhibited measurable increases in phytoplankton biomass by the second day of the experiment whereas the other treatments and control showed no appreciable change during this period. The general trend in chl concentration over the first 5 days of cloudy/rainy weather, however, was downward, except for the sediment treatment, where little change was observed. A period of phytoplankton net growth was observed in all groups starting at day 6, which was the beginning of the sunny period.

A. anophagefferens cell concentrations at the beginning of the experiment averaged ∼8000 cells/ml. During the first 5 days, *A. anophagefferens* cell density decreased by 45% (sediment treatment) to 96% (urea treatment) as shown in Fig. 2. During the later period when phytoplankton biomass (chl) increased in all treatments, *A. anophagefferens* exhibited a statistically significant ($P < 0.05$) increase in density in the urea and *Enteromorpha* treatments between days 6 and 9 of the experiments (Fig. 2C–D). The increase in the average *A. anophagefferens* cell density between days 6 and 9 in the sediment treatment was not statistically significant ($P = 0.15$) (Fig. 2E). Average *A. anophagefferens* cell density remained low in the control and nitrate treatments (Fig. 2A–B) and there was no statistically significant change between days 6 and 9. At the beginning of the experiment, *A. anophagefferens* accounted for ∼1% of the total chl (assuming 0.05 pg chl cell^{-1}; Lomas et al., 1996; Milligan and Cosper, 1997). The fraction of *A. anophagefferens* chl generally diminished during the stress period, but there were substantial fractional gains in the urea, *Enteromorpha*, and sediment treatments during the high growth period (Fig. 2A–E). The *Enteromorpha* treatment had the highest fractional chl in *A. anophagefferens* of 2.7% at the end of the experiment, whereas the highest average densities were observed in the urea + glucose (18,000 cells/ml) treatment at the end of the experiment.

3.3. Dissolved nutrient dynamics

The inorganic nutrient concentrations were all in the low range at the beginning of the experiment ($0.5 < \text{DIN} < 1.0$ μM-N; DIP < 0.5 μM-P). Nitrate remained at trace levels in all mesocosms, except in the nitrate treatment following the second and third additions. The ∼40 μM-N of added nitrate and urea was consumed within 12 h of the initial addition to the respective treatments. Recovery of this added N in the form of PON from the water column is estimated at 25% on day 1 and 50% on day 2 in both treatments. Throughout the 9 days of the experiment, DIN and DIP concentrations varied within the ranges given above in all experimental treatments, except for NO_3^-, which jumped to and remained at ∼30 μM-N following the second addition on day 5. Ammonium concentrations (all measurements were < 1 μM-N) exhibited a progressive decline during the experiment independent of treatment (data not shown), whereas urea concentrations showed an upward trend in all treatments after day 6 (Fig. 3A), with mean concentrations at the end of the experiment being in the range of 1.2–3.8 μM-N. Urea was notably variable among replicate mesocosms with coefficients of variation averaging 85%. DON showed little change or only a modest increase during the first 5 days, but there was an upward trend in DON during the high productivity period (Fig. 3B) and the concentration during the last 3 days was significantly higher (ANOVA, $P < 0.05$) than the earlier period in all treatments except the control. Average DOC concentrations for the groups were variable during the entire experiment, ranging between 300–500 μM-C and they did not exhibit a systematic trend.

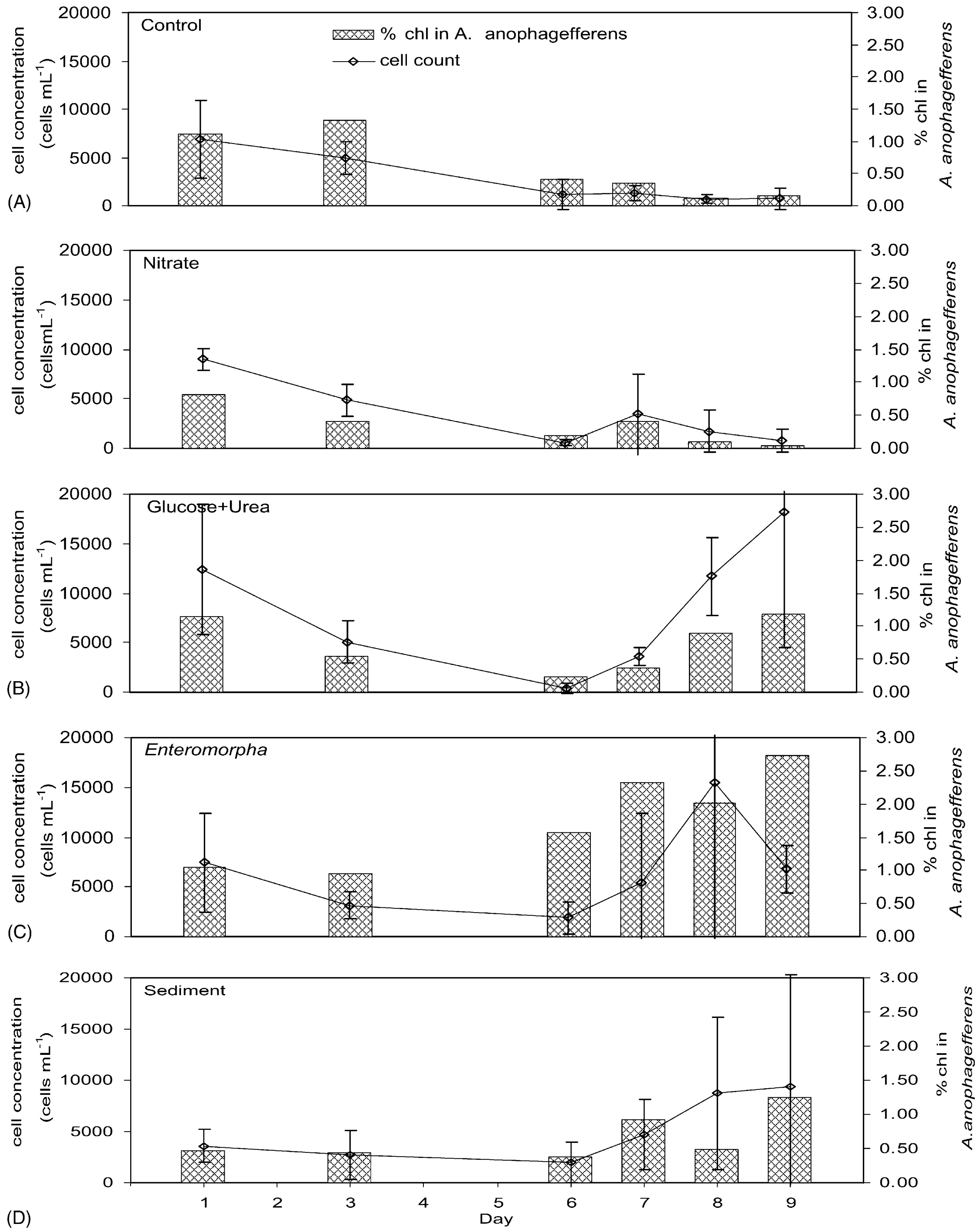

Fig. 2. Time-course of *A. anophagefferens* cell density and percentage of total chlorophyll in *A. anophagefferens*. Diamonds: cell density (error bars = 1 S.D.). Bars: % chl in *A. anophagefferens*.

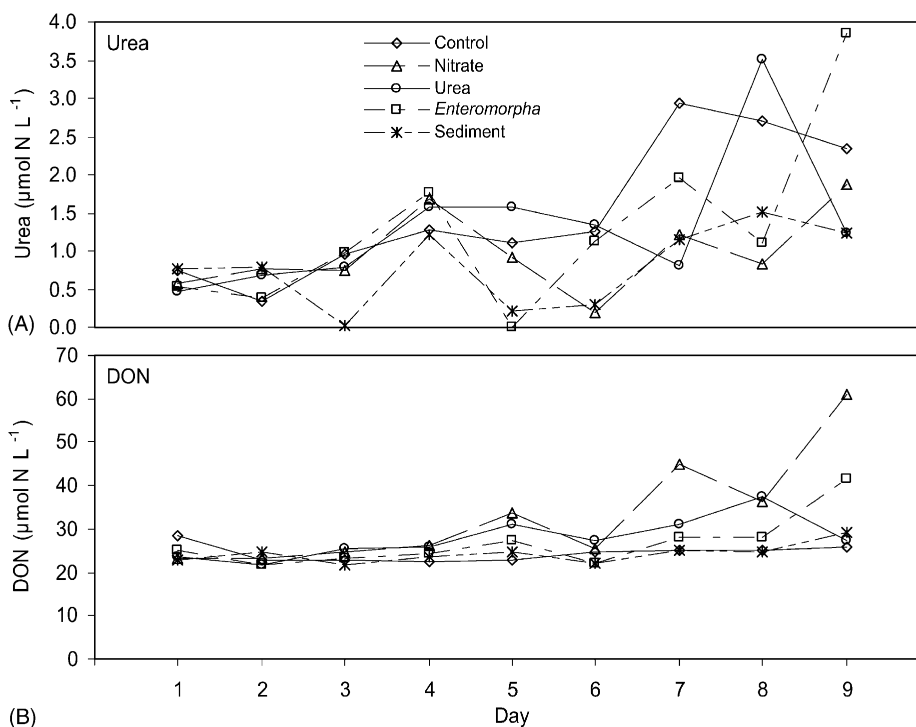

Fig. 3. Time-course of average urea (panel A) and DON (panel B). Panel B symbols as in (A) average coefficients of variation in urea for each treatment were: control: 0.91, nitrate: 0.76, urea: 0.85, *Enteromorpha*: 0.74, and sediment: 0.94. Average coefficients of variation in DON for each treatment were: control: 0.14, nitrate: 0.13, urea: 0.09, *Enteromorpha*: 0.16, and sediment: 0.08.

3.4. Nitrogen uptake

Uptake of NO_3^-, NH_4^+, glutamic acid, and urea was measured in each mesocosm at three separate times (days 1, 5, and 9: Fig. 4). The ambient rate of uptake of the organic nitrogen forms, glutamic acid and urea, did change significantly in some treatments, but in a complex manner (Fig. 4). In the control and NO_3^- treatments, the uptake of glutamic acid increased slightly over time whereas urea uptake decreased slightly over time. The net result was no change in the contribution of organic nitrogen uptake to total measured nitrogen uptake in those treatments. The three other experimental treatments showed significant increases in either glutamic acid uptake (*Enteromorpha* and sediment treatments) or urea uptake (urea treatment), with the net result being an increase in the relative contribution of organic nitrogen uptake to total measured nitrogen uptake (Fig. 4).

The phytoplankton communities in all groups underwent significant changes in total biomass over the course of the experiment. The reductions in chlorophyll during the first 5 days of the experiment may have been due to reduced growth rate from light limitation, loss of phytoplankton through sinking or adhesion to the walls, or a combination of the two factors. Loss by sinking is a possible factor since there was a noticeable accumulation of organic matter on the bottom plates at the end of the experiment. This flocculent and sedimentary material was not quantified during the time-series, but it is likely that much of it consisted of phytoplankton cells that had sedimented during the course of the experiment. The cellular growth rate during the cloudy period was apparently insufficient to overcome losses due to respiration, grazing and other processes. However, rapid increases in chlorophyll concentration were observed in the nutrient treatments when daily irradiance levels were high, indicating that phytoplankton growth likely had been light limited.

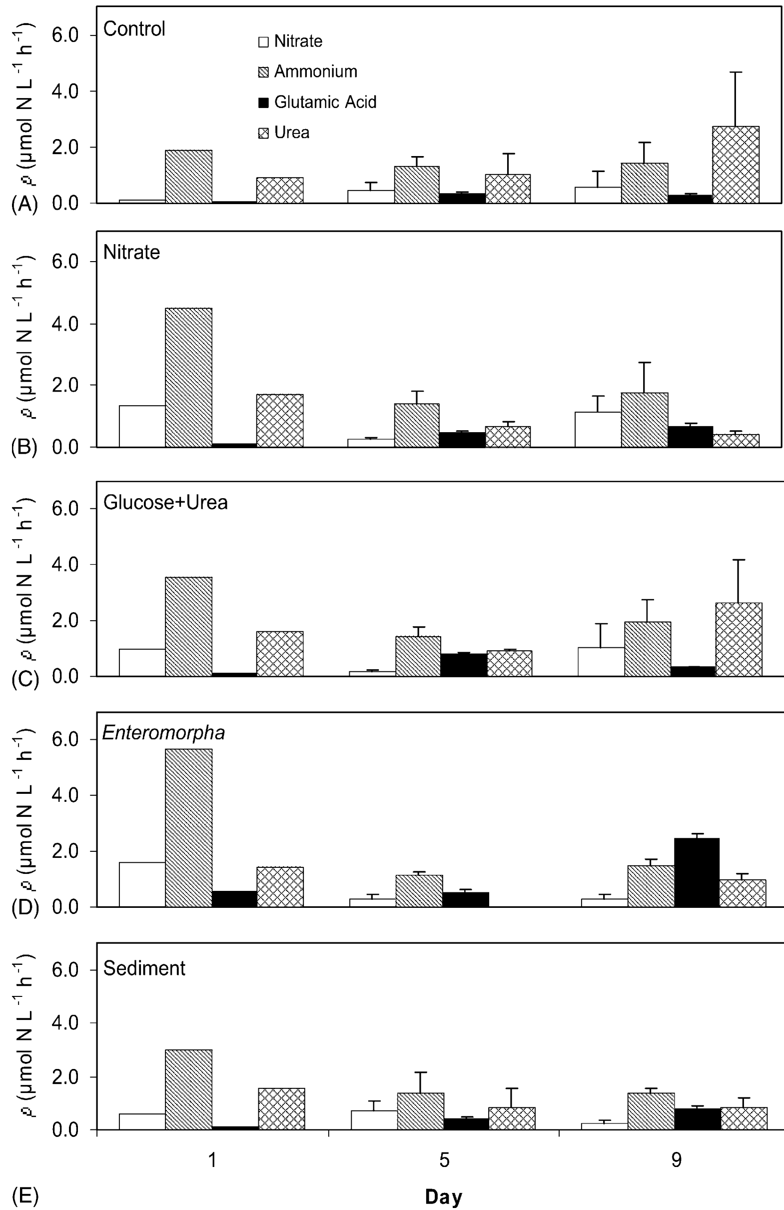

Fig. 4. Uptake rate of nitrate, ammonium, glutamic acid and urea (symbols in panel A) in (A) control, (B) nitrate, (C) urea, (D) *Enteromorpha*, and (E) sediment treatments. Error bars = 1 S.D.

We observed complete removal of the added (40 μM-N) nitrate and urea in the respective treatments within 12 h of the initial nutrient dose (provided during the evening of day 0). The rapid consumption of nitrogen nutrients on the first day indicates that the phytoplankton assemblage had a high nitrogen demand (note the high irradiance on day 0; Fig. 1A). The nitrate and urea that was added on day 0 alleviated this limitation, as shown by the measurable increase in chl concentration by day 2 in those

treatments. An increase in chl was not observed in the other groups indicating that the accessibility of nutrients from the *Enteromorpha* and sediment treatments was initially lower than that for the defined nitrate and urea treatments.

It wasn't until the senenth day of the experiment that phytoplankton exhibited consistent increases in biomass (measured as chl concentration). Days 6 and 7 were cloudless and potential photosynthesis was therefore high. The modest increase in chl and photosynthesis in the control mesocosms during those days indicates that there was sufficient recycling of entrained nutrients to support phytoplankton activity for the duration of the experiment. The *Enteromorpha* treatment did not stimulate chl production or primary production to the extent of the other treatments (though there was a ~4-fold increase relative to the minimum concentration of 1.8 μg chl/l on day 5) and this treatment responded more like the control mesocosms. Thus, the release of nutrients from the added macroalgae was likely limiting. Nevertheless, the *Enteromorpha* treatment did affect *A. anophagefferens* growth (see below). Among the treated mesocosms, the greatest chl accumulation and productivity occurred in the nitrate and urea experiments and the responses of those two treatments were comparable. Thus, the efficiency of utilization of nitrate and urea for overall phytoplankton growth could not be distinguished.

The response of the *A. anophagefferens* population differed in the nitrate and urea treatments, however. In the presence of ~30 μM-N nitrate beginning on day 6, *A. anophagefferens* only showed a transient (1 day) increase in cell density after which there was a 2 day decline in density (Fig. 2) to a level close to the minimum observed in this study. The reason for this decline is unknown and it is surprising in light of evidence from culture studies that indicate that *A. anophagefferens* is capable of growing nearly as fast on nitrate-based media as on urea-based media (Pustizzi et al., 2004; Popels, 2003; MacIntyre et al., 2004). Given the present results, we conclude that the competitive disadvantage of *A. anophagefferens* in nitrate rich waters is due to a secondary factor and not nitrate directly.

The rapid increase in the *A. anophagefferens* populations within the urea, *Enteromorpha*, and sediment treatments during the sunny period was notable. From days 6 to 8, there was an approximately exponential increase in *A. anophagefferens* cell density. During this 2 day period, growth rates of *A. anophagefferens* were 1.68, 1.05, and 0.74 per day for the urea, *Enteromorpha*, and sediment treatments, respectively. Similarly rapid potential growth rates have been observed for natural *A. anophagefferens* in bioassay experiments (Gobler and Sañudo-Wilhelmy, 2001; Gobler et al., 2002). It is seemingly contradictory that rates higher than approximately 1 doubling per day (0.63 per day) are generally not observed for *A. anophagefferens* grown in culture (MacIntyre et al., this issue.), However, the higher temperatures in the mesocosms as compared to those laboratory cultures (25 versus 20 °C) would engender rates of ~0.8 per day at 20 °C if the Q_{10} for growth were 2, which is at the low end for values reported for growth rates (Robarts and Zohary, 1987; Verity, 1981). It remains debatable whether *A. anophagefferens* is handicapped in terms of its maximum growth rate relative to other fast-growing phytoplankton, despite contrary indications from culture studies.

We were unable to identify the factor(s) responsible for the suppression of *A. anophagefferens* under high nitrate loading, or the stimulation in the other three treatments. Although a common factor in the urea, *Enteromorpha*, and sediment treatments is the addition of a nutrient source rich in organic matter, the measurements of DON do not support, on the surface, the hypothesis that DON is responsible for brown tides. In fact, the highest DON concentrations were found in the nitrate treatment from days 7 through 9 (Fig. 3). It will require more detailed analysis of DON compounds to determine whether specific DON molecules are responsible for the differential treatment, or whether some other factor is the cause.

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