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Note

Effects of media N : P ratio on extracellular polymeric substances (EPS) production of the brown tide-forming alga, *Aureoumbra lagunensis*

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Aureoumbra lagunensis formed a high density, persistent bloom in the hypersaline Laguna Madre of Texas from 1990–1997; this bloom is often referred to as the "Texas brown tide". A lack of effective grazing on *A. lagunensis* by both protozoa and copepods is thought to have contributed to the extraordinary persistence of this bloom (Buskey and Stockwell 1993, Buskey and Hyatt 1995). *A. lagunensis* produces a mucus layer of extracellular polymeric substances (EPS) outside its cell wall (DeYoe et al. 1997). The thick EPS layer surrounding *A. lagunensis* reduces grazing by protozoa (Liu and Buskey 2000) and helps the cell resist digestion after being consumed by copepods (Bersano et al. 2002). In this study we examine the effects of altered media N : P ratio on the production of EPS by *A. lagunensis* to provide insight into possible interactions between nutrient limitation, EPS production and grazing inhibition, and to improve understanding of the role of these processes in the unusual persistence of the brown tide bloom.

The excretion of EPS by phytoplankton is well documented (e.g., Myklestad et al. 1972, Myklestad 1974, Decho 1990). EPS is composed primarily of carbohydrates, mainly polysaccharide, although the exact composition appears to be dependent on the cells producing it, their nutrient conditions and the age of the cells (Myklestad et al. 1972, 1989, Myklestad 1977, 1995, Lancelot 1984, Decho 1990).

Previous studies have shown that many phytoplankton species release more EPS under nutrient limited conditions (Myklestad & Haug 1972, Myklestad et al. 1972, 1989, Ignatiades & Fogg 1973). The production of EPS has also reported being influenced by nutrient ratios, mainly the N : P ratio in the culture media or natural water (Myklestad & Haug 1972, Myklestad 1977, Obernosterer & Herndl 1995, Penna et al. 1999). Different species, however, react differently to nutrient ratios. For example, the diatom *Chaetoceros affinis* released significantly more extracellular polysaccharide under severe P-limitation than under severe N-limitation (Myklestad & Haug 1972, Myklestad 1977). In a recent study, Penna et al. (1999) reported that *Nitzschia closterium* produces more extracellular polysaccharide under phosphorus limitation, whereas *Skeletonema costatum* and *Chaetoceros* sp. released more carbohydrates at low N : P ratio. In general, phytoplankton appears to produce more EPS during the stationary phase (e.g., Guillard & Wangersky 1958, Myklestad & Haug 1972, Myklestad 1977), although other experimental evidence suggests higher EPS production during the rapid growth phase (Myklestad et al. 1989). Thus, it is difficult to separate the influence of nutrient limitation and the aging of the cultures to the EPS production rates of phytoplankton growing in batch culture. Continuous culture can overcome this problem by allowing the population to reach and maintain a steady state at controlled growth rates with continuous nutrient supply. Here we report the exudation of carbohydrates by *Aureoumbra lagunensis* (Stockwell, DeYoe, Hargraves, et Johnson) in a series of nitrogen-limited and phosphate-limited continuous culture experiments with various growth rates.

All experiments were conducted in a light and temperature controlled environmental chamber at 22°C under continuous white fluorescent light ($130\,\mu Ein\,m^{-2}\,s^{-1}$) using a continuous culture system described in Liu et al. (2001). One-liter polycarbonate cell culture bottles with internal stirrers (Nalgene) were used as growth chambers and growth media was supplied from a polycarbonate carboy through Teflon tubing using a peristaltic pump. The growth chamber, connection tubing and media supply container were acid cleaned and autoclaved before each experiment. *A. lagunensis* was grown in ammonia-limited (5 chemostats) or phosphate-limited (4 chemostats) modified f/2 medium (Buskey et al. 1998) made from 0.2 μm filtered and microwave-sterilized seawater with a salinity of 32 PSU (Table 1). Concentrations of ammonia and phosphate, as well as nitrate and silicate, were measured in the inflow and outflow for each experiment using a Lachat Quikchem 8000 ion analyzer (Zellweger Analytics, Milwaukee, WI). *A. lagunensis* cell density was monitored by drawing one-ml from

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Table 1. Nitrogen and phosphorus concentrations in the in flow and out flow media of all chemostat experiments conducted. Concentrations of nitrate+nitrite and silicate were also measured, but not listed here. Concentrations of nitrate+nitrite were always very low.

Growth rate	Limiting nutrient	Nutrient in Media (μM)		Nutrient left (μM)	
(d^{-1})		NH_4^+	PO_4^{-3}	NH_4^+	PO_4^{-3}
0.133	NH_4^+	53.81	25.44	0.13	0.17
0.225	NH_4^+	53.81	25.44	0.30	2.96
0.277	NH_4^+	53.81	25.44	0.69	1.92
0.409	NH_4^+	53.81	25.44	1.38	6.83
0.522	NH_4^+	38.71	17.43	0.47	15.87
0.162	PO_4^{-3}	52.98	0.02	3.05	0.09
0.235	PO_4^{-3}	52.98	0.02	1.48	0.01
0.342	PO_4^{-3}	52.98	0.02	11.92	0.01
0.487	PO_4^{-3}	52.98	0.02	32.86	0.03

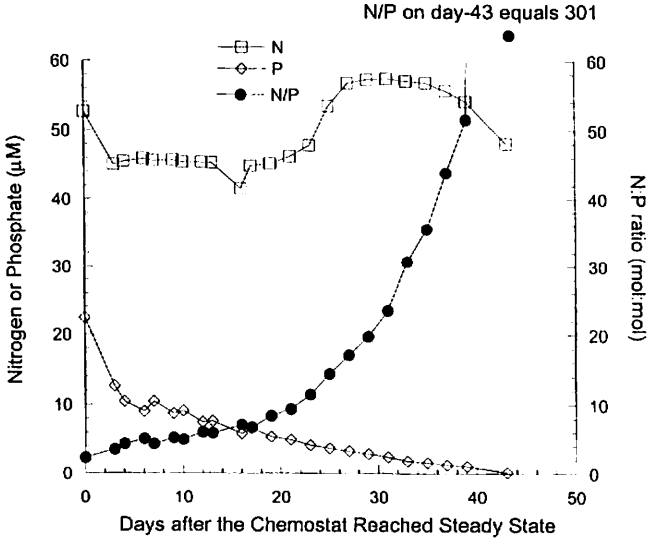

Fig. 1. Changes of inorganic nitrogen (ammonium, nitrate and nitrite) and phosphate concentrations and N:P ratio in the medium introduced to the growth chamber of a chemostat designed to grow experimental alga in a medium gradually transit from N-limited to P-limited conditions.

the growth chamber daily at approximately the same time and counting cells using a Z1 Coulter Counter. When the biomass in the chemostat reached steady state, cells were harvested for carbohydrate and EPS analysis, as well as for cellular carbon, nitrogen and phosphorus contents and chlorophyll *a* concentrations (see Liu et al. 2001).

We have also conducted one chemostat experiment (μ=0.34 d^{-1}) in which the input medium was gradually shifted from N-limitation to P-limitation. In this experiment, *A. lagunensis* was first grown in ammonia-limited modified f/2 media as described above. After it reached steady state, phosphate depleted medium (modified f/2 without phosphate enrichment) was siphoned into the ammonia-limited media reservoir, resulting in a continuous increase of N:P ratio in the media (Fig. 1). Since the inorganic nitrogen (ammonium+nitrate and nitrite) remained relatively constant, and the phosphate concentration decreased, the resulting N:P ratio in the media increased from the initial 2.35 to 301 at the end of the experiment. Nutrient concentration in the inflow and outflow media and cell density, total, dissolved and particulate carbohydrates, EPS and other cellular properties were measured every 2–4 days.

Particulate organic carbon (POC) and nitrogen (PON) were determined by filtering triplicate 50-ml subsamples of the culture onto precombusted GF/C filters and using a Carlo Erba Instruments, CHN–EA1108 elemental analyzer. Particulate organic phosphorus (POP) was measured colorimetrically using the method of Solorzano & Sharp (1980) calibrated to KH_2PO_4 as a standard (see Liu et al. 2001 for details). Chlorophyll *a* concentrations were measured using a Turner Designs fluorometer. The cellular carbon, nitrogen and phosphorus contents of *A. lagunensis* were calculated from POC, PON, and POP concentrations divided by cell concentrations. Bacteria contamination was ignored in our calculations of the *A. lagunensis* cellular C, N, P contents because they accounted only about 2% of the carbon biomass in the culture and were mostly not retained by GF/C filters (see Liu et al. 2001).

Total carbohydrate (unfiltered culture), and carbohydrate in dissolved (filtrate of 0.4 μm porosity polycarbonate membrane filter) and particulate (material retained by 0.4 μm filter) portions were determined spectrophotometrically with the phenol-sulfuric acid method (Dubois et al. 1956, see Liu & Buskey 2000a for detailed description). Media blanks were also determined, and results showed that carbohydrates in all media were not significantly higher than those in distilled and deionized waters. Particulate carbohydrates were determined after the filter was soaked for 20 h in 5 ml of 80% sulfuric acid according to Passow et al. (1994). Comparison of a glucose standard in water solution with hydrolysis procedures produced identical values. EPS was also measured spectrophotometrically using alcian blue as a dye-binding assay that stains both carboxyl and sulfated polysaccharides, but not neutral sugars (Passow & Alldredge 1995). In this study we refer to this analysis as the measurement of mucus acidic polysaccharides. Carbohydrates were normalized to glucose concentration and acidic polysaccharides were normalized to gum xanthan equivalent.

Cellular carbon, nitrogen and phosphorus quotas of *A. lagunensis*, and concentrations of carbohydrates and mucus acidic polysaccharides normalized to cell densities in each chemostat are reported in Table 2. Among 5 ammonia limited chemostats, both total carbohydrates and acidic polysaccharide mucus increased with growth rate, except at the lowest growth rate. There is no clear relationship between the release of acidic polysaccharides and *A. lagunensis* growth rate under P-limited experiments. Average total carbohydrate per cell was approximately the same for *A. lagunensis* growing under ammonium and phosphate limited chemostat cultures. In contrast, the acidic polysaccharides mucus associated with *A. lagunen-*

Table 2. Cell densities, carbon, nitrogen and phosphorus contents, and concentrations of carbohydrates and acidic polysaccharides for all chemostat experiments (Cellular C, N and P contents has been reported in Liu et al. 2001). Data shown are the means ± standard errors.

Growth rate (d^{-1})	Limiting nutrient	Cell density (10^6 cell ml^{-1})	Carbon content (fmol $cell^{-1}$)	Nitrogen content (fmol $cell^{-1}$)	Phosphorus (fmol $cell^{-1}$)	Total carbohydrates (pg glucose equiv. $cell^{-1}$)	Acidic polysaccharides (pg xanthan equiv. $cell^{-1}$)
0.133	NH_4^+	1.66	494.2±30.0	22.19±0.31	1.364±0.028	10.11±0.68	17.33±2.92
0.225	NH_4^+	1.76	527.9±10.7	24.77±0.67	1.481±0.002	5.79±0.65	9.78±0.85
0.277	NH_4^+	2.07	525.1±1.4	25.23±0.22	1.674±0.005	8.30±0.92	10.25±1.76
0.409	NH_4^+	1.29	745.5±12.2	41.32±1.60	2.201±0.029	12.30±0.38	19.06±2.56
0.522	NH_4^+	0.543	1144±11	76.36±1.14	2.196±0.104	21.59±1.56	33.53±4.89
Average						11.62±2.71	17.99±4.30
0.162	PO_4^{-3}	0.901	1102±4	66.3±0.7	0.177±0.010	10.87±3.45	10.56±0.52
0.235	PO_4^{-3}	1.06	935.1±2.1	49.2±0.1	0.282±0.053	10.67±0.50	6.10±0.54
0.342	PO_4^{-3}	0.327	1085±6	126.1±1.3	0.412±0.064	10.94±0.13	10.69±1.61
0.487	PO_4^{-3}	0.164	856.0±2.8	97.4±0.9	0.458±0.015	11.38±0.99	6.59±0.68
Average						10.96±0.15	8.49±1.24

sis was twice as high for cells growing in nitrogen limited conditions than those in the phosphate limited chemostats (Table 2). If we normalize the measurements of EPS to cellular carbon contents, it is more apparent that *A. lagunensis* produced more carbohydrates or polysaccharide mucus in ammonium limitation than in phosphate limitation (Fig. 2).

The results from the nutrient gradient experiment agreed with the findings from other chemostat experiments. As the medium N : P ratio increased, *A. lagunensis* cellular carbon and nitrogen contents did not change greatly while phosphorus content decreased logarithmically. As a result, cellular P : N and P : C decreased in the same fashion while cellular N : C ratio remained relatively constant (Fig. 3). Total carbohydrates remained constant through a range of media N : P ratio from 2.3 to 52 (Fig. 4a). At the same time, carbohydrates measured in the dissolved portion showed a decrease, whereas the carbohydrate in the particulate portion increased slightly, and then decreased again in media with N : P ratio >50 (Fig. 4b, c). Acidic polysaccharides mucus measured with the alcian blue dye-binding assay showed a 3/4 decrease for *A. lagunensis* growing in high N : P conditions compared to that in low N : P conditions (Fig. 4d).

Our measurements of total carbohydrates and alcian blue stained acidic polysaccharides mucus concentration for *A. lagunensis* growing under N and P limited chemostats showed that, on the per cell base, total carbohydrates were about the same under ammonium or phosphate limitation, whereas the alcian blue stainable acidic polysaccharides mucus were approximately twice as high under N-limitation compared to that under P-limitation (Table 2). A most likely scenario is that, while the exudation of carbohydrates by *A. lagunensis* increased in N-limited cultures, its cellular carbohydrate storage was depleted, resulting in a relatively constant level of total carbohydrates in the growth chamber. This hypothesis is supported by the rusults of the N : P gradient experiment where

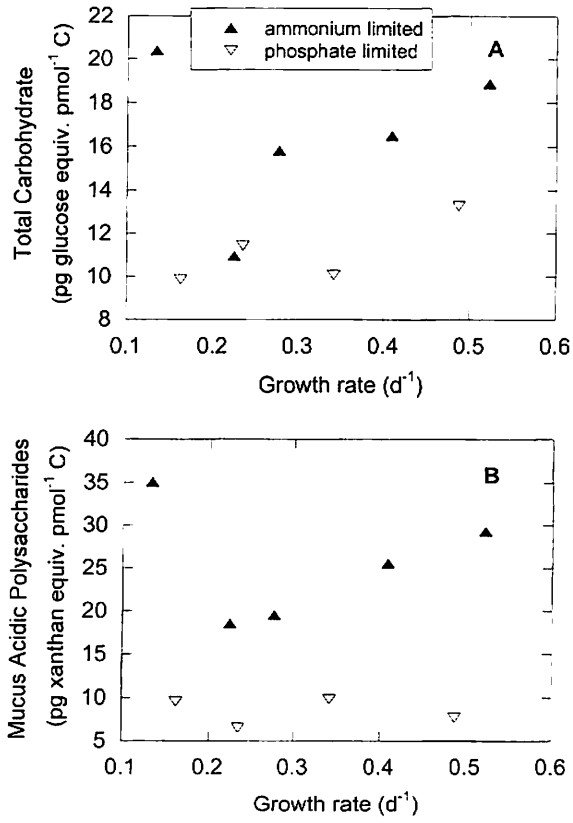

Fig. 2. Total carbohydrates (A) and acidic polysaccharides mucus (B) per carbon biomass plotted against growth rate for *A. lagunensis* growing in ammonium and phosphate limited chemostats.

both dissolved carbohydrates (which are a part of exudated carbohydrates) and alcian blue stainable acidic polysaccharides mucus were higher when *A. lagunensis* was nitrogen limited (Fig. 4). Myklestad (1977) observed the continued release of

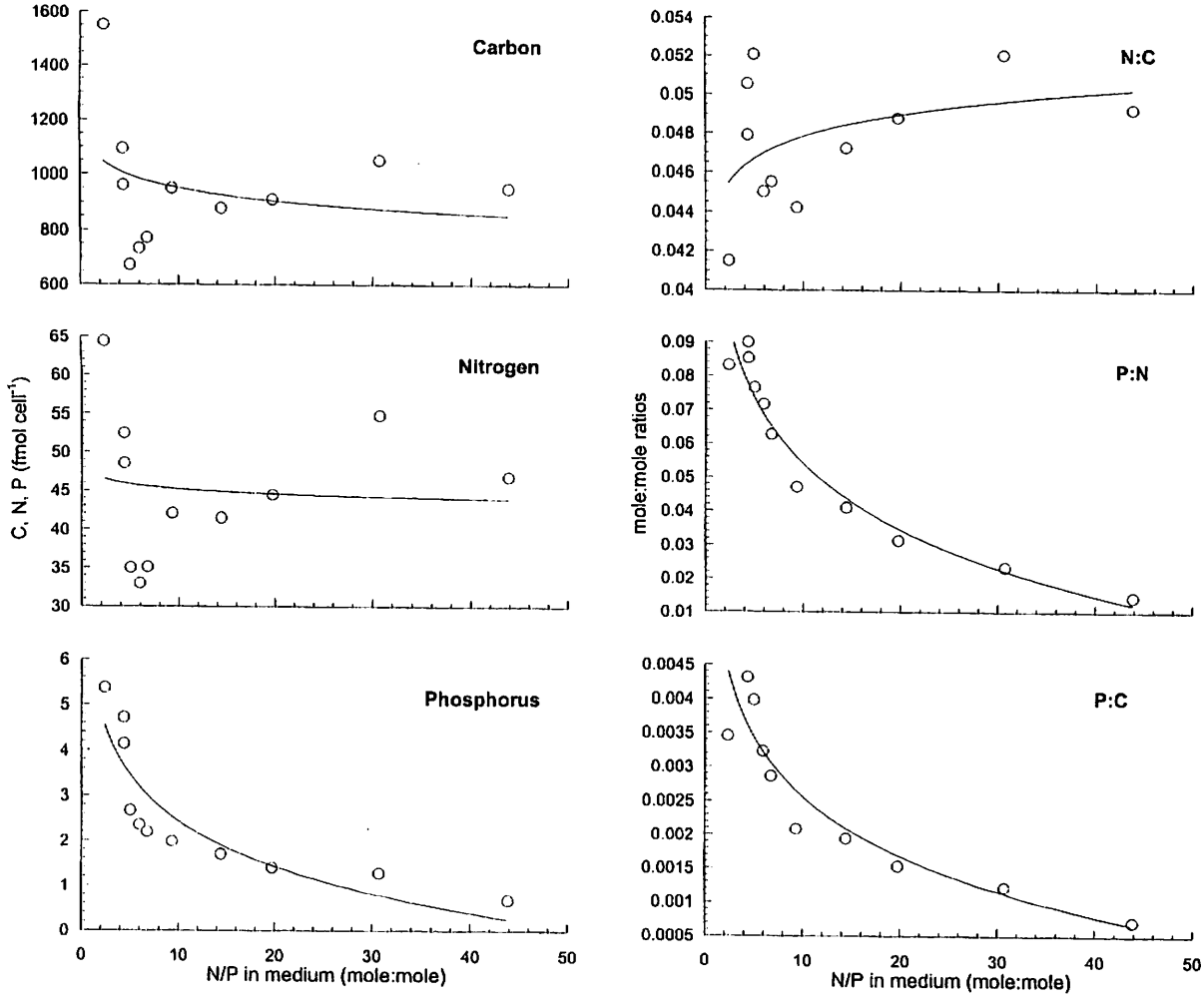

Fig. 3. Changes in *A. lagunensis* cellular carbon, nitrogen and phosphorus contents and ratios with media N : P ratio. Curves are logarithm fitting. Data for the highest media N : P ratio at the end of the experiment were not shown, but fit the displayed trend.

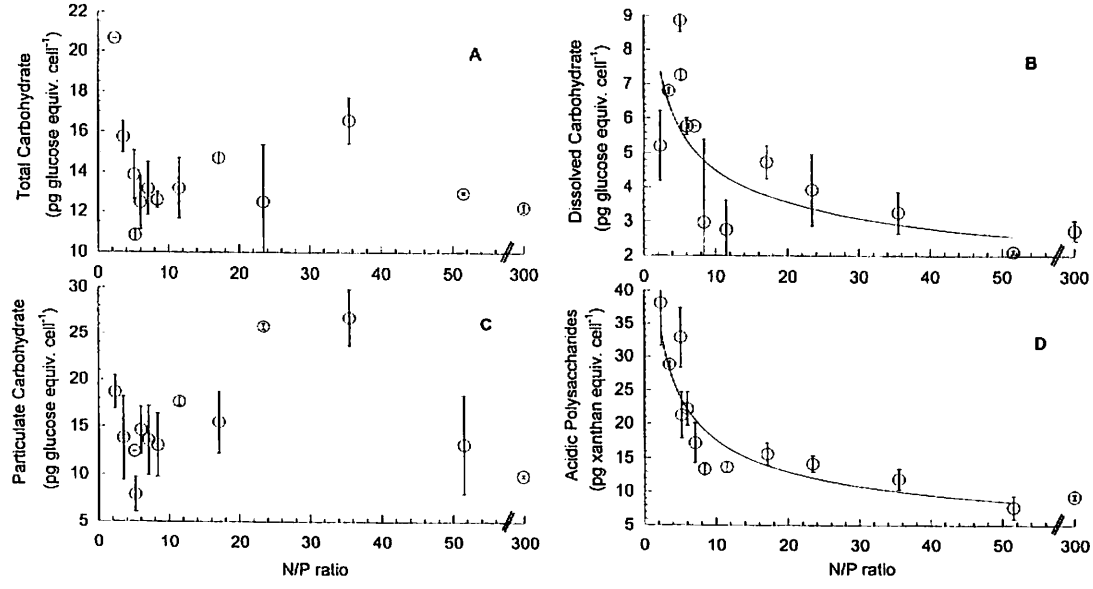

Fig. 4. Total (A), dissolved (B) and particulate carbohydrates (C) and mucus acidic polysaccharides (D) plotted against media N : P ratio for *A. lagunensis* growing in a continuous culture with medium gradually shifted from low N : P to high N : P ratio (see text for details). Error bars are the standard deviations.

extracellular polysaccharide at high rates long after the production of cellular carbohydrate had ceased for *C. affinis* under phosphate limitation. *A. lagunensis* in our present study may have displayed the same physiological characteristics under nitrogen limitation.

An alternative explanation for the disagreement between the measurement of total carbohydrates and alcian blue stainable acidic polysaccharides mucus could be that the composition of the EPS changed under different nutrient stress. Nutrient status profoundly affects the amount and composition of the exudates. Severe N- and P- limitation favors the release of carbohydrates that do not contain these elements (Myklestad 1995). However, the change of EPS composition can not wholly explain the discrepancy in the two measurements, because carbohydrates (mostly polysaccharides) usually comprise 80–90% of the total extracellular release (Myklestad 1995). Lower concentrations of dissolved carbohydrates for *A. lagunensis* in high N : P medium also rule out the possibility of increased release of monosaccharides at P-limitation (Fig. 4b).

It has been demonstrated that *A. lagunensis* is well adapted to the phosphate-limited environment in the Laguna Madre by its extremely low cellular phosphorus requirement and a potential ability to use organic phosphorus (Villareal et al. 1998, Liu et al. 2001). It is worth to point out that *A. lagunensis* growing in N : P gradient experiment might not be under P-limitation during the most of time because even in the highest media N : P ratio (301) at the end of the experiment its N : P cell quota (149) was just reaching its N : P critical ratio, a ratio of N : P cell quota at the transition from N-limitation to P-limitation at a given growth rate. The estimated N : P critical ratio for *A. lagunensis* growing at 0.34 d^{-1} is about 140, which is very high compare to other phytoplankton species with known N : P critical ratio (Liu et al., 2001). Nonetheless, our finding that *A. lagunensis* EPS release was low under severe phosphate-limited condition, as opposed to the increased EPS release under nitrogen limitation, support the conclusion that *A. lagunensis* may be well adapted to the phosphate-limited environment.

Previous study has shown that high *A. lagunensis* densities occurred when surface water N : P ratio was high (Rhudy et al. 1999), suggesting its preference to grow in low phosphate environment (Liu et al. 2001). Since the N : P ratio in the surface water of the Laguna Madre is less than 16 during the most time of the year (Rhudy et al. 1999), *A. lagunensis* may have been under N stress. Because enhanced EPS production protects *A. lagunensis* from grazers (Liu and Buskey 2000, Bersano et al. 2002), our finding in this study that *A. lagunensis* increases its EPS production under nitrogen limitation may help to solve the mystery of uninterrupted bloom of *A. lagunensis* in the Laguna Madre during last decade.

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