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Nitrogenous preference of toxigenic *Pseudo-nitzschia australis* (Bacillariophyceae) from field and laboratory experiments

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Abstract

Field and laboratory experiments were designed to determine the differential growth and toxin response to inorganic and organic nitrogen additions in *Pseudo-nitzschia* spp. Nitrogen enrichments of 50 μM nitrate (KNO_3), 10 μM ammonium (NH_4Cl), 20 μM urea and a control (no addition) were carried out in separate carboys with seawater collected from the mouth of the San Francisco Bay (Bolinas Bay), an area characterized by high concentrations of macronutrients and iron. All treatments showed significant increases in biomass, with chlorophyll *a* peaking on days 4–5 for all treatments except urea, which maintained exponential growth through the termination of the experiment. *Pseudo-nitzschia australis* Frenguelli abundance was 10^3 cells l^{-1} at the start of the experiment and increased by an order of magnitude by day 2. Particulate domoic acid (pDA) was initially low but detectable (0.15 μg l^{-1}), and increased throughout exponential and stationary phases across all treatments. At the termination of the experiment, the urea treatment produced more than double the amount of pDA (9.39 μg l^{-1}) than that produced by the nitrate treatment (4.26 μg l^{-1}) and triple that of the control and ammonium treatments (1.36 μg l^{-1} and 2.64 μg l^{-1}, respectively). The mean specific growth rates, calculated from increases in chlorophyll *a* and from cellular abundance of *P. australis*, were statistically similar across all treatments.

These field results confirmed laboratory experiments conducted with a *P. australis* strain isolated from Monterey Bay, CA (isolate AU221-a) grown in artificial seawater enriched with 50 μM nitrate, 50 μM ammonium or 25 μM of urea as the sole nitrogen source. The exponential growth rate of *P. australis* was significantly slower for cells grown on urea (*ca.* 0.5 day^{-1}) compared to the cells grown on either nitrate or ammonium (*ca.* 0.9 day^{-1}). However the urea-grown cells produced more particulate and dissolved domoic acid (DA) than the ammonium- or nitrate-grown cells. The field and laboratory experiments demonstrate that *P. australis* is able to grow effectively on urea as the primary source of nitrogen and produced more pDA when grown on urea in both natural assemblages and unialgal cultures. These results suggest that the influence of urea from coastal runoff may prove to be more important in the development or maintenance of toxic blooms than previously thought, and that the source of nitrogen may be a determining factor in the relative toxicity of west coast blooms of *P. australis*.

Keywords: Domoic acid; Harmful algae; Nitrogen; *Pseudo-nitzschia australis*; Urea

1. Introduction

The occurrence of harmful algal blooms (HABs) appears to be increasing in both frequency and intensity in recent years (e.g., see reviews by Hallegraeff, 1993;

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Anderson et al., 2002; Glibert et al., 2005a). This is especially evident along the central California coast where amnesic shellfish poisoning (ASP) events caused by toxic blooms of the diatom *Pseudo-nitzschia* spp. have increased dramatically over the last decade (Buck et al., 1992; Scholin et al., 2000; Trainer et al., 2000). The first major ASP event identified in North America occurred in 1987 in eastern Prince Edward Island, Canada, where a toxic bloom of *Pseudo-nitzschia multiseries* Hasle (Bates et al., 1989) resulted in 107 illnesses and the deaths of three people after ingesting contaminated blue mussels (*Mytilus edulis*) (Perl et al., 1990). Blooms of *Pseudo-nitzschia australis* in Monterey Bay, California were first recorded during the fall months of 1989, 1990 and 1991 as well as the spring of 1990 and summer of 1991 (Buck et al., 1992). In September 1991, the first confirmed report of domoic acid poisoning on the U.S. west coast resulted in the mortality of more than 100 brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) (Fritz et al., 1992; Work et al., 1993). In 1998 another bloom, which spanned the central California coast, resulted in the deaths of over 400 sea lions (*Zalophus californianus*) due to consumption of contaminated northern anchovies (*Engraulis mordax*) (Scholin et al., 2000; Trainer et al., 2000).

As a result of these events, a number of laboratory studies on *Pseudo-nitzschia* were initiated, which identified macronutrient limitation as an important factor affecting cellular toxicity (Pan et al., 1996a,b,c; Bates, 1998). Although initial studies focused on macronutrients, iron and copper limitation, as well as elevated copper conditions, have been shown in laboratory studies to induce toxin production in both *P. multiseries* and *P. australis* (Maldonado et al., 2002; Ladizinsky and Smith, 2003; Wells et al., 2005). Domoic acid (DA) has been shown to form chelates with both of these trace metals, potentially increasing the biological availability of iron and/or decreasing the toxicity of copper (Ladizinsky and Smith, 2000; Rue and Bruland, 2001). However, Bates et al. (2000) reported DA levels were 10 times lower in iron-stressed cultures relative to iron-replete cultures of *P. multiseries*.

Substantial variability in growth and toxicity has been shown among different *Pseudo-nitzschia* species as well as isolates of the same species in culture from the same geographical area (Bates, 1998; Kudela et al., 2003a). Macronutrient limitation (silicate and phosphate, but not nitrogen) is known to induce toxin production in *P. multiseries* (Pan et al., 1996a,b; Bates, 1998). Pan et al. (1996a,b) proposed two different stages in the production of DA under silicate limitation. The first occurs during the late exponential phase, characterized by slower growth rates and moderate DA production. The second stage occurs during stationary phase and is distinguished by the depletion of silicate and a significant increase in toxin production per cell (an order of magnitude higher in batch culture). Similar results were found under phosphate limitation. Pan et al. (1996c) showed an increase in the production of domoic acid in *P. multiseries*, in both steady-state continuous culture and batch cultures, when phosphate concentrations were limiting and alkaline phosphatase activity was high. There have been fewer laboratory studies using strains of *P. australis*. Garrison et al. (1992) isolated *P. australis* from the Monterey Bay bloom in 1991, and conducted batch culture experiments with two strains that confirmed DA production by this species with maximum particulate DA concentrations of 37 pg cell^{-1} and 12 pg cell^{-1}.

Despite the many studies on toxin production and nutrient limitation, very few studies have evaluated the nutritional preference of *Pseudo-nitzschia* spp., particularly in terms of nitrogen sources. In laboratory experiments, Bates et al. (1993) grew cultures of *P. multiseries* with varying concentrations of ammonium and nitrogen, ranging from 55 μM to 880 μM, and found that at less than 110 μM N the growth of the cells and the production of DA were the same for nitrate- and ammonium-grown cultures. Hillebrand and Sommer (1996) also evaluated the nitrogenous preference of *P. multiseries* by using nitrate, ammonium and urea in batch cultures. In a batch culture with initial concentrations of 40 μM ammonium, the growth rate increased with increasing concentration of nitrate added to the culture, which the authors interpreted as an alleviation of ammonium inhibition of nitrate uptake, whereas at growth-saturating nitrate concentrations, ammonium additions decreased the growth rate of *P. multiseries*.

While there have been numerous laboratory studies using isolates of *P. multiseries*, this is the first study to examine the nitrogenous nutrition (including urea) of *P. australis*, the predominant DA producer in California waters. In contrast to previous nitrogen-based studies, the field and laboratory experiments reported here were designed to identify differential responses to ammonium, nitrate and urea enrichment using ecologically relevant nitrogen concentrations.

2. Materials and methods

2.1. Field experiment

Nutrient addition experiments were conducted aboard the R/V *Point Sur* during February 2003. Seawater was

collected in the vicinity of the San Francisco Bay (Bolinas Bay, 37°51.30′N, 122°39.13′W). At the time of collection, there was a broad chlorophyll maximum that ranged from 4 m to 14 m depth, a surface mixed layer temperature of 12.5 °C and a practical salinity of 31.5. Ten-liter PVC Niskin bottles (refitted with silicone rubber band springs) mounted on an instrumented rosette were used to collect seawater from 4 m depth. Although water was not collected using strict trace metal clean protocols, reasonable care was taken to reduce the risk of metal contamination. On the same cruise, water collected in the same fashion, but from a high nutrient low chlorophyll (HNLC) coastal region was apparently not contaminated by iron, as evidenced by the lack of enhanced phytoplankton growth under multi-day, deck incubation conditions (data not shown).

Four 9-liter acid-cleaned polycarbonate (Nalgene) carboys were rinsed three times and filled with seawater using multiple Niskin bottles. Carboys were then either unenriched (control) or enriched with nitrogen and placed in a deckboard incubator maintained at the ambient surface temperature (with running seawater) and *ca.* 50% surface irradiance (using neutral density screening). The ambient nitrogen concentration of the seawater collected for the experiment was 6.6 μM nitrate, 1.76 μM ammonium, and 0.9 μM urea. Three separate nutrient treatments were conducted where 42.4 μM nitrate (as KNO_3) was added to the first carboy (total nitrate 49 μM), 10 μM ammonium (as NH_4Cl) was added to the second carboy (total ammonium 11.76 μM) and 20 μM urea was added to the third carboy (total urea 20.9 μM). There was also a control (no addition). Reagent grade stocks and Milli-Q® water were used to prepare all nutrient enrichments. Incubations were maintained for 3 days at sea in a deckboard incubator (mean daily irradiance *ca.* 350 μmol photons m^{-2} s^{-1}), and subsequently transferred to a walk-in environmental chamber (15 °C and *ca.* 100 μmol photons m^{-2} s^{-1} irradiance using "cool-white" fluorescent lamps and a 12:12 light:dark cycle) at the University of California Santa Cruz (due to termination of the cruise) for the remainder of the experiment.

2.1.1. Analytical methods

Samples for chlorophyll *a* were collected daily in triplicate and filtered onto uncombusted glass-fiber filters (Whatman GF/F; nominal pore size 0.7 μm); separate samples for size fractionated chlorophyll *a* were determined using 1-μm and 5-μm polycarbonate filters (Poretics), frozen in liquid nitrogen and processed using the non-acidification method (Welschmeyer, 1994). Macronutrients (nitrate plus nitrite [hereafter referred to as nitrate], silicate and *ortho*-phosphate) were sampled daily, stored frozen, and later analyzed with a Lachat Quick Chem 8000 Flow Injection Analysis system using standard colorimetric techniques (Knepel and Bogren, 2001; Smith and Bogren, 2001a,b). Ammonium and urea samples were collected using 60 ml low-density polyethylene (LDPE) centrifuge tubes (Corning®). Ammonium samples were stored refrigerated after the addition of the phenolic reagent; the addition of the phenolic reagent binds ammonium and eliminates the need to freeze samples (Degobbis, 1973). The remaining reagents were added within 120 h, and the samples were manually analyzed using a spectrophotometer equipped with a 10-cm cell (Solorzano, 1969). Urea samples were frozen at −20 °C, and subsequently thawed ashore at room temperature before manual analysis using the diacetyl monoxime thiosemicarbizide technique (Price and Harrison, 1987), modified to account for a longer time period (72 h) and lower digestion temperature (22 °C). Particulate domoic acid (pDA) samples were filtered onto uncombusted Whatman GF/F filters and processed according to the method of Pocklington et al. (1990), using high performance liquid chromatography with fluorescence detection. At two time points, *P. australis* was identified using large subunit rRNA-targeted fluorescent probes (whole cell) as described by Miller and Scholin (1998). The samples were also probed for *P. multiseries* but this species was not present. Samples (10 ml) from each treatment were collected in triplicate, on days 0 and 2, filtered and immediately preserved in saline ethanol for later microscopic analysis in the laboratory. Because rRNA probes were not available for the remaining sample points, whole-water samples were collected on all days, to be preserved with acidified Lugol's solution; however, after inspecting the samples, it was apparent that 1% non-acidic Lugol's solution was erroneously used. Therefore, it was not possible to use these samples for phytoplankton floristic analysis. Visual examination of the positive whole-cell probe samples (which probes all phytoplankton) provided a record of dominant groups.

2.2. Laboratory experiments

P. australis was isolated from Monterey Bay, California (isolate AU221-a), and grown as batch cultures in filter-sterilized (0.2 μm), nutrient-enriched artificial seawater (modified ESAW; Harrison et al., 1980) at the Romberg Tiburon Center; modifications to this artificial seawater included reducing the nitrogen enrichment to 50 μM nitrate, 50 μM ammonium and 25 μM urea, respectively, for each treatment. Other

modifications are described in detail by Berges et al. (2003). Cultures were maintained at 15 (±0.3) °C with irradiance levels at 100 μmol photons m^{-2} s^{-1} for a 12:12 light:dark cycle. Cell samples were fixed in 1% acidic Lugol's solution and counted using a Palmer-Maloney nanoplankton counter at 100× using a phase contrast microscope (Eclipse E4000, Nikon®). Chlorophyll *a* samples were collected during the late exponential phase; 20 ml of each treatment was filtered onto uncombusted Whatman GF/F filters, extracted in 90% acetone and analyzed as described for the field experiment. Particulate and dissolved DA samples were also taken towards the end (late exponential phase) of the experiment and analyzed using the ELISA method (Garthwaite et al., 2001). For particulate determinations, cells were filtered onto 0.2-μm pore-sized polycarbonate filters (Nuclepore) that were placed in 4-ml vials containing 2-ml deionized water (Millipore). They were cycled through four freeze–thaw sequences using liquid nitrogen and boiling water to liberate the water-soluble DA molecule from the cellular matrix. After the samples were refiltered through a 0.2-μm acrodisc syringe filter, aliquots of the supernatant were analyzed with appropriate dilutions. The 0.2-μm filtrate was collected for measurement of dissolved DA and analyzed directly without dilution. *In vivo* chlorophyll *a* and the ratio of variable (F_v) to maximum (F_m) fluorescence measured during the dark cycle using DCMU (Cullen and Renger, 1979) were determined daily on a Turner Designs 10-AU fluorometer. All experiments were conducted in triplicate for each nitrogen source.

3. Results

3.1. Field experiment

Seawater was collected during a period of minimal prior rainfall, low ambient nutrient levels, and a relatively homogenous water column to 50 m depth. Total biomass (as chlorophyll *a*) in the carboys was initially moderate (4.03 μg l^{-1} on day 0) and increased significantly with the addition of each nitrogen substrate as well as in the control (Fig. 1A). The maximum chlorophyll values were recorded on day 4 for the nitrate addition and the control (29.33 μg l^{-1} and 19.9 μg l^{-1}, respectively) and on day 5 for the ammonium addition (28.48 μg l^{-1}). The urea addition was still in exponential growth on day 7 (30.40 μg l^{-1}) (Fig. 1B). Size fractionated chlorophyll indicated that most of the biomass was greater than 1.0 μm.

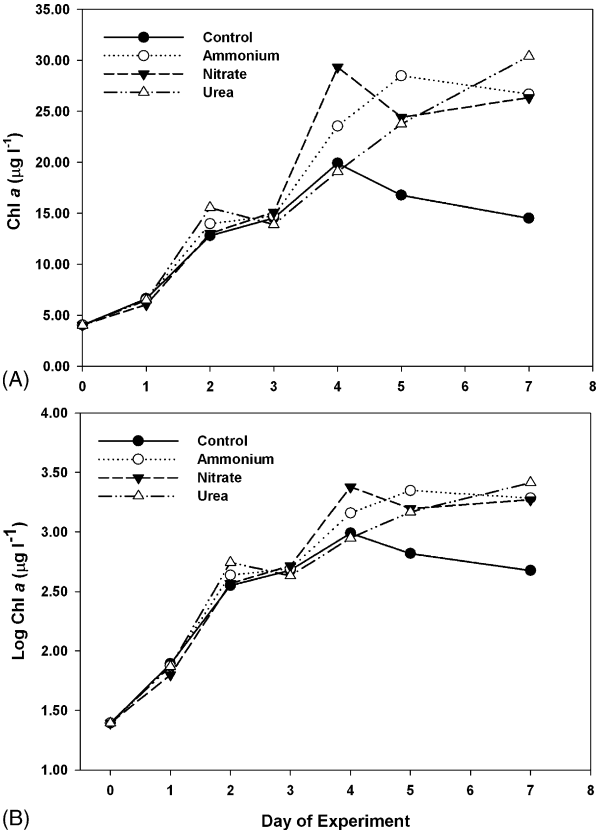

Fig. 1. (A) Total phytoplankton community chlorophyll *a* measured daily from the field experiment and (B) semi-log plot of growth of the same samples for each nitrogen treatment: control (●), ammonium (○), nitrate (▼) and urea (▽). Samples were not taken on day 6 of the experiment. Values are the mean of triplicate samples.

3.1.1. Domoic acid

Initially, particulate domoic acid (pDA) was low but easily detectable, 0.15 μg l^{-1} on day 0 (Fig. 2). However, by day 4 during exponential growth, the pDA of the community increased to the following: nitrate addition, 0.41 μg l^{-1}; ammonium addition (the highest on day 4), 1.50 μg l^{-1}; urea addition, 1.04 μg l^{-1}; the control, 0.46 μg l^{-1}. By the end of the experiment (day 7), the urea treatment produced significantly more pDA (9.39 μg l^{-1}) and was still in exponential growth, while the control was still the lowest at 1.36 μg l^{-1} and it had entered stationary phase. Both the nitrate and ammonium additions were in stationary phase on day 7, when pDA increased to 4.26 μg l^{-1} and 2.64 μg l^{-1}, respectively. Using the days 0 and 2 *P. australis* specific cell counts, per-cell toxicity was 43 pg $cell^{-1}$ on day 0. We do not have simultaneous whole-cell probe data and toxin data for other days; however, using chlorophyll as a proxy, pDA chl^{-1} was 38.7 ng pDA chl^{-1} on day 0. By day 4, the control and nitrate addition decreased to

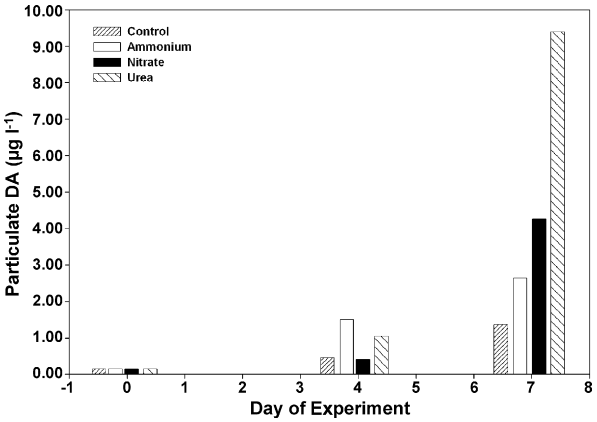

Fig. 2. Particulate domoic acid (μg l^{-1}) sampled on days 0, 4, and 7 of the field experiment for each nitrogen treatment: control (lined bar), ammonium (white bar), nitrate (solid black bar), and urea (hatch bar).

23.5 ng pDA chl^{-1} and 14.0 ng pDA chl^{-1}, respectively, while the ammonium and urea treatments increased to 64.0 ng pDA chl^{-1} and 54.7 ng pDA chl^{-1}. Concentrations increased for all carboys by day 7 to 162.0 ng pDA chl^{-1} in the nitrate addition, 99.3 ng pDA chl^{-1} in the ammonium addition, 309.1 ng pDA chl^{-1} in the urea addition and 94.3 ng pDA chl^{-1} in the control carboy.

3.1.2. Inorganic and organic nitrogen

The ambient nitrogen concentration of the seawater used in the incubation experiments was 6.6 μM nitrate, 1.7 μM ammonium and 0.9 μM urea. An additional 42.4 μM N was added for the nitrate addition treatment, 10 μM N for the ammonium treatment, and 20 μM urea for the urea addition. For the ammonium treatment, the ammonium concentration was half of its initial value by day 2 and was depleted by day 5 (Fig. 3A).

The initial urea concentration did not change significantly in the nitrate (Fig. 3B) ammonium and control treatments (Fig. 3C), but decreased ∼50% by day 5 in the urea treatment (Fig. 3D) which also corresponded to the time point at which nitrate was depleted. At the termination of the experiment there was still 6.6 μM urea in the urea treatment.

Macronutrient depletion rates were calculated from a least-squares linear regression analysis of the exponential growth phase and an analysis of variance (ANOVA) determined from semi-log plots of nutrient concentration versus time (Guillard, 1973). The depletion of total nitrogen in each treatment was highest in the control treatment (Table 1) and lowest in the nitrate treatment. Because of the large amount of nitrate added (relative to the depletion rate) and due to issues associated with the

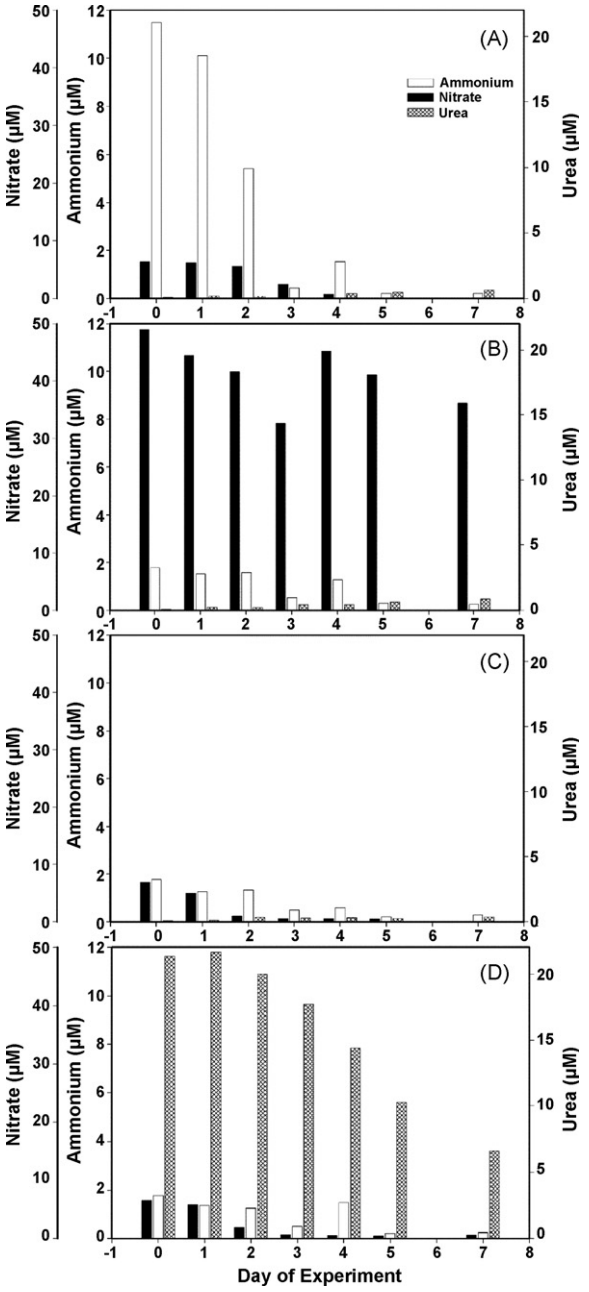

Fig. 3. Nitrate, ammonium and urea concentrations (in μM) measured daily in the field experiment for each nitrogen treatment: (A) ammonium; (B) nitrate; (C) control; (D) urea. Nitrate concentration (solid black bar) on the far left axis, ammonium concentration (white bar) on the left axis and urea concentration (crosshatch bar) on the right axis. Samples were not taken on day 6 of the experiment.

analysis of these high values on the autoanalyzer, the analytical error associated with the estimate of nitrate drawdown precludes accurate estimation of nitrate depletion rates to compare with the drawdown of ammonium and urea. As a result, the depletion rates

Table 1
Net nitrogen depletion rates for all treatments during the field experiment

Treatment	Depletion rates (day^{-1}) from days 0 to 3 (S.E.; n; r^2)											
	Nitrate			Ammonium			Urea			Total nitrogen		
Nitrate		0.13			0.35			−0.47			0.13	
	0.06	4	0.95	0.38	4	0.82	0.31	4	0.92	0.06	4	0.95
Ammonium		0.30			1.47			0.13			0.58	
	0.30	4	0.83	0.88	4	0.88	0.61	4	0.34	0.45	4	0.89
Urea		0.77			0.38			0.06			0.15	
	0.35	4	0.96	0.28	4	0.90	0.05	4	0.89	0.07	4	0.96
Control		0.87			0.36			−0.48			0.62	
	0.36	4	0.96	0.31	4	0.88	0.39	4	0.88	0.16	4	0.98

Standard error (S.E.), number of days included in the rate calculation (n) and r-squared values (r^2) are included in the table.

calculated for the nitrate treatment (total nitrogen and nitrate) do not accurately reflect the utilization of total nitrogen, as evidenced by the growth rates which are statistically indistinguishable across treatments. The highest net nitrate depletion rate (excluding the nitrate treatment) was in the control treatment and the lowest was in the ammonium treatment. For ammonium-based depletion rates, the ammonium treatment had the highest rate of depletion and the nitrate treatment was lowest. The ammonium-based depletion rates for the nitrate, urea and control treatments were not statistically indistinguishable ($p < 0.05$). For urea depletion rates, the highest rates were observed in the ammonium treatment and lowest in the urea treatment, while the nitrate treatment and the control had positive depletion rates for urea, meaning there was an increase in urea with time, probably due to grazers.

3.1.3. Other nutrients

The silicate concentration was initially 23 μM and decreased to 2.7–3.5 μM in the nitrogen additions and to 8.7 μM in the control by the end of the 7-day experiment. Silicate decreased to half of the initial concentration in the nitrate and ammonium treatments by day 3, and in the urea and control treatments, by day 4. The concentration of phosphate was initially 0.8 μM and by day 3 was half of this concentration across all treatments. Phosphate was depleted by day 4 in the nitrogen additions but not until day 7 in the control treatment.

3.1.4. Growth rate

Growth rates for the whole phytoplankton community were calculated from a linear regression analysis of the exponential growth phase and ANOVA determined from semi-log plots of chlorophyll *a* versus time (Guillard, 1973). Growth rates were statistically indistinguishable across all treatments ($p > 0.05$): nitrate 0.48 day^{-1} (calculated over 5 days, days 0–4); ammonium, 0.39 day^{-1} (days 0–5); urea, 0.34 day^{-1} (days 0–5); control, 0.40 day^{-1} (days 0–4). Qualitative indicators of growth of *P. australis* were calculated over 2 days from enumeration of the whole cell probes taken on days 0 and 2. The ammonium treatment was the highest (1.19 day^{-1}), followed by urea (1.16 day^{-1}), then the control (1.08 day^{-1}), and the lowest was the nitrate treatment (0.95 day^{-1}) (Table 2). The growth rates of for *P. australis* were substantially higher than those calculated from the entire assemblage.

3.1.5. Community composition

The phytoplankton assemblage was initially diatom-dominated, with *ca.* 76% centric and 24% pennate

Table 2
Calculated growth rates based on the increase in phytoplankton community chlorophyll *a* (during exponential growth) and *Pseudo-nitzschia australis* cell abundance (days 0–2) during the field experiment off San Francisco Bay

Treatment	Chlorophyll growth rates (day^{-1}) (S.D.; n; r^2)			Growth rate of *P. australis* (day^{-1})
Nitrate		0.48		0.95
	0.14	5	0.97	
Ammonium		0.39		1.19
	0.19	6	0.94	
Urea		0.34		1.16
	0.26	6	0.88	
Control		0.40		1.08
	0.16	5	0.94	

Standard deviation (S.D.), number of days included in the rate calculation (n) and r-squared values (r^2) are included in the table for chlorophyll-based rates.

forms (by number). Dominant genera included *Asterionellopsis*, *Chaetoceros*, and *Thalassiosira* spp.; other genera included *Skeletonema* and *Stephanopyxis* and, to a lesser extent, *Coscinodiscus* and *Eucampia*; dinoflagellates were negligible.

Despite the elevated growth rate for *P. australis* calculated from direct cell counts, there was a reduction from days 0 to 2 in pennate abundance (*ca.* 5–6.5% by number versus *ca.* 91–94% centrics and 0.5–2.1% dinoflagellates).

Although only two time points were available for *P. australis* enumeration (whole cell probe), *P. australis* was present in all samples. *P. australis* cell concentration was initially 3.60×10^3 cells l^{-1} and by day 2 had increased by an order of magnitude in all treatments to the following: nitrate addition, 2.77×10^4 cells l^{-1}; ammonium addition, 4.62×10^4 cell l^{-1}; urea addition, 4.32×10^4 cells l^{-1}; the control, 3.64×10^4 cells l^{-1}. At the start of the experiment, *P. australis* comprised 72% of all pennates and 17% of the whole phytoplankton community. By day 2, *P. australis* accounted for 41% of the pennates in the nitrate treatment, 86% in the ammonium treatment, 55% in the urea treatment and 69% in the control, but only 2.3–5.5% of the entire assemblage.

3.2. Laboratory experiment

3.2.1. Growth rate

Specific growth rates during the exponential growth phase were determined from linear regressions of the natural log of cell abundance versus time (Fig. 4A and B). Comparisons of the mean (±1S.D.) growth rates, using ANOVA (using post-hoc Tukey's test), indicate that the cells maintained on urea grew slower (0.52 ± 0.09 day^{-1}) than the cells grown on either nitrate (0.89 ± 0.08 day^{-1}) or ammonium (0.93 ± 0.001 day^{-1}), which both maintained significantly greater growth rates ($p < 0.01$), but were indistinguishable from each other ($p > 0.05$).

3.2.2. Chlorophyll *a* and cellular fluorescence capacity

Chlorophyll *a* samples were collected during late exponential phase and the chlorophyll *a* per cell was statistically indistinguishable ($p > 0.05$) for the nitrate and ammonium treatments (2.44 ± 0.55 pg cell^{-1} and 2.37 ± 0.48 pg cell^{-1}, respectively). However, the urea treatment exhibited a significantly lower ($p < 0.05$) mean cellular chlorophyll *a* concentration of 0.99 ± 0.43 pg cell^{-1}. The cellular fluorescence capacity for all nitrogen-substrate treatments, determined using DCMU, was indistinguishable from one another during exponential growth; F_v/F_m averaged 0.62 ± 0.05 ($n = 16$). The F_v/F_m values did not decrease during the late exponential phase when samples were collected for chlorophyll *a* and DA.

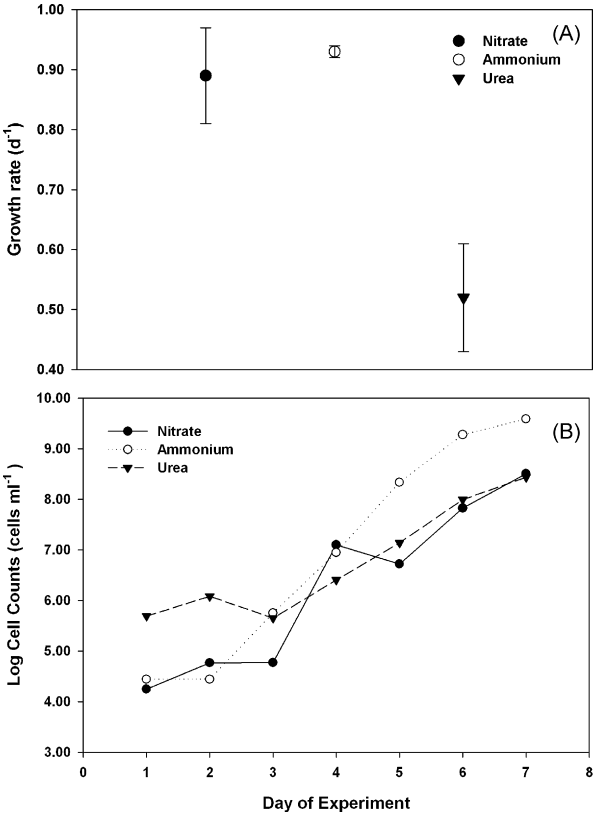

Fig. 4. (A) Growth rates derived from cell count measurements during exponential growth phase; (B) semi-log plot of cell counts measured daily from the laboratory experiment for each nitrogen treatment: (●), nitrate (○), ammonium (▼) and urea. Values are the mean of triplicate samples and error bars on the growth rate plot denote range of replicates.

3.2.3. Domoic acid

Particulate DA was collected during late exponential growth for each culture and normalized to cell abundance; the mean (±1S.D.) pDA per cell for each nitrogen treatment is presented here and graphically (Fig. 5A). The urea treatment had substantially greater pDA per cell, 1.37 ± 0.97 fg cell^{-1}, whereas the nitrate and ammonium treatments were lower, 0.48 ± 0.14 fg cell^{-1} and 0.26 ± 0.098 fg cell^{-1}, respectively. Overall, strain Au221-a was generally less toxic than the natural assemblages, which is consistent with previous laboratory experiments that demonstrate substantial strain-specific variability as well as a gradual loss of toxicity with time (e.g. Villac et al., 1993; Kudela et al., 2003b).

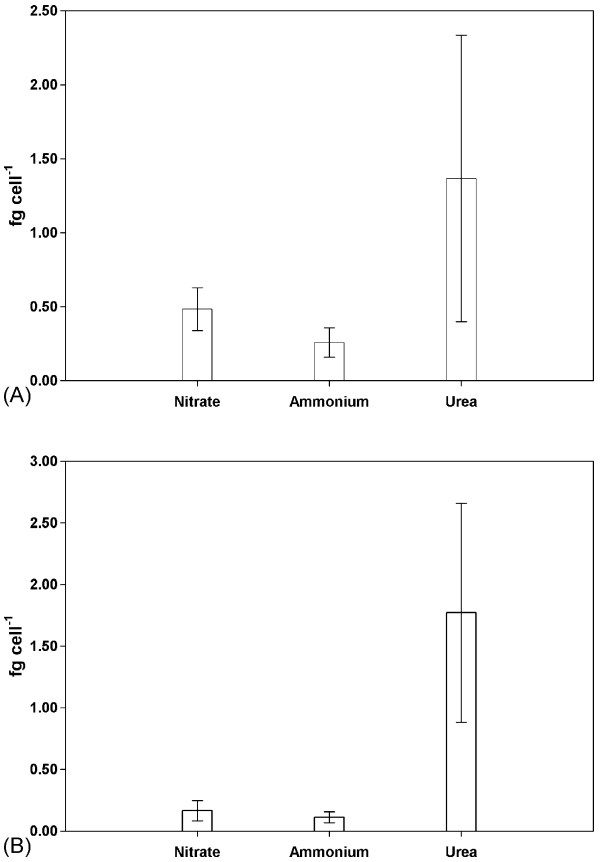

Fig. 5. Particulate (A) and dissolved (B) domoic acid per cell in the laboratory experiment. Values are means ($n = 3$) of domoic acid concentrations normalized to cellular abundance. Error bars represent ±1S.D.

Dissolved domoic acid (dDA), expressed either volumetrically (per ml of culture filtrate) or normalized to cellular abundance (Fig. 5B), was significantly greater ($p < 0.05$) in the urea treatment (5.42 ± 2.24 pg ml^{-1}; 1.77 ± 0.89 fg cell^{-1}), compared to either the nitrate treatment (0.80 ± 0.34 pg ml^{-1}; 0.165 ± 0.082 fg cell^{-1}), or the ammonium treatment (1.51 ± 0.74 pg ml^{-1}; 0.112 ± 0.045 fg cell^{-1}).

4. Discussion

4.1. Field experiment

The central California coast consists of a wide continental shelf characterized by seasonally high concentrations of upwelled macronutrients as well as the micronutrient iron. The supply of fine-grained sediment from river input, combined with upwelling occurring over the shelf, creates an iron-replete region ideal for diatom blooms (Bruland et al., 2001). The phytoplankton assemblage in this region has previously been reported to be largely nitrogen and light limited during the winter (Kudela and Dugdale, 2000; Olivieri and Chavez, 2000). The oceanic conditions experienced during this experiment were typical of the winter season in central California (Wilkerson et al., 2000). Although the elevated ammonium and urea concentrations suggest the influence of San Francisco Bay outflow, Si:N ratios were relatively low (~4:1), substantially lower than reported during high-flow periods (Wilkerson et al., 2000). Consistent with these past observations, biomass (chlorophyll *a*) increased in all the field grow-out treatments, including the control, which suggests that the growth of the phytoplankton community was primarily limited by light availability and not initially limited by nitrogen.

The mean growth rates from our field data during the exponential phase were statistically indistinguishable across all nitrogen-substrate treatments, using either chlorophyll *a* concentrations or the *P. australis* cell abundance. It appears that *P. australis* does not exhibit a strong preference for any particular nitrogen source and can grow equally well on the organic substrate, urea. *P. australis*, initially present at a cell concentration of 10^3 cells l^{-1}, increased by an order of magnitude in all treatments to 10^4 cells l^{-1}, which is considered "bloom" conditions by monitoring agencies. At this cell concentration threshold, increased testing for domoic acid in coastal waters ensues (M. Silver, pers. commun.). This increase in *P. australis* across all treatments suggests that specific conditions such as stratification of the water column (alleviation of light limitation) can increase the growth of *P. australis* when nitrogen is available.

4.1.1. Domoic acid

There is a wide reported range of DA concentrations for central California (Table 3). Buck et al. (1992) reported pDA concentrations in Monterey Bay of 1.1–2.4 μg l^{-1} in October 1991 and 0.1–6.7 μg l^{-1} in November 1991. *P. australis* abundances were (6.5–20) × 10^4 cells l^{-1} and (0.8–67) × 10^4 cells l^{-1}, respectively. Scholin et al. (2000) reported a range of 7.2–31.2 pg DA per *P. australis* cell in Monterey Bay during the 1998 bloom. The calculated pDA in Monterey Bay on 20 May 1998, was 0.36–9.75 μg l^{-1}. Scholin et al. (2000) suggested that the increase in abundance of *P. australis* in the first half of May was a response to increased silicate concentrations, possibly in response to enhanced coastal runoff. El Niño conditions reduced the upwelling intensity signal along the California coast and record levels of rainfall were recorded in 1998 (Trainer

Table 3
Highest concentrations of particulate domoic acid reported in field observations for central California from 1991 to 2003 where *P. australis* was the dominant recorded organism

Location	DA ($\mu g\ l^{-1}$)	Date	Reference
Monterey Bay	1.1–2.4	October 1991	Buck et al. (1992)
Monterey Bay	0.1–6.7	November 1991	Buck et al. (1992)
Monterey Bay	9.75	May 1998	Scholin et al. (2000)
Santa Cruz Wharf	2.5	May 1998	Scholin et al. (2000)
Monterey Bay	0.36	May 1998	Scholin et al. (2000)
Point Lobos	0.18–0.27	June 1998	Trainer et al. (2000)
Morro Bay	1.3–3.8	June 1998	Trainer et al. (2000)
Point Arguello and Point Conception	2.2–7.3	June 1998	Trainer et al. (2000)
Santa Barbara	0.5–1.2	June 1998	Trainer et al. (2000)
Mouth of San Francisco Bay	0.13	June 1998	Trainer et al. (2000)
Above Point Ano Nuevo	0.44	June 1998	Trainer et al. (2000)
Monterey Bay and Point Lobos	0.38	June 1998	Trainer et al. (2000)
Bolinas Bay	0.15–9.39	February 2003	This study

Note that the values from Trainer et al. (2000) are whole water samples (particulate and dissolved domoic acid).

et al., 2000). However, even in May and June during the 1998 bloom, temperature and salinity measurements indicated oceanic conditions. Trainer et al. (2000) suggested upwelling, not enhanced river flow, as the source of nutrients that sustained the bloom. Concentrations of whole water DA on 3–5 June ranged from 1.3 to 3.8 $\mu g\ l^{-1}$ in Morro Bay and 2.2 to 6.3 $\mu g\ l^{-1}$ in Point Conception where *P. australis* was the dominant species, at abundances of 4.9×10^4 cells l^{-1} and 2.3×10^5 cells l^{-1}, respectively. The highest whole water DA concentrations (7.3 $\mu g\ l^{-1}$) were recorded in southern California, at Point Arguello (Trainer et al., 2000).

Results from this experiment (0.15–9.39 μg pDA l^{-1}) fall within the reported range for pDA values, with initial concentrations on the low end, increased concentrations across all treatments by day 4 and the highest levels achieved near the maximum pDA concentrations of 9.75 $\mu g\ l^{-1}$ reported by others (Scholin et al., 2000). All but the urea treatment entered stationary growth phase by the end of the experiment (day 7). The results of the urea treatment are especially significant since it produced the highest pDA on day 7, which was double the amount produced by the nitrate treatment and three times more than that of the control and ammonium treatments. The initial (days 0–2) quantitative indicators of growth of *P. australis* were similar in all of the treatments, and the mean specific growth rates calculated from community chlorophyll concentrations were also statistically indistinguishable from each other. Therefore, the large increase in pDA production cannot be explained simply by higher biomass or lower growth rates in the urea treatment. This suggests that the per-cell production of pDA was substantially greater when grown on urea. Since the cells in the urea treatment were still growing exponentially when pDA was measured, and previous laboratory experiments have shown that the major increase in production of pDA is in stationary phase (at least for *P. multiseries*; Pan et al., 1996a,b; Bates, 1998), one might expect pDA values to be conservative. The implications of these results are that elevated concentrations of urea from anthropogenic sources such as agricultural and urban runoff, or sewage discharge, could be a significant source of nitrogen for toxic bloom development or sustenance of *P. australis*.

4.1.2. Utilization of more than one nitrogen source

The depletion of half of the initial concentration of ammonium and approximately 10% of the concentration of nitrate in the ammonium treatment by day 2, as well as the depletion of both nitrogen sources by day 5, demonstrates simultaneous utilization of more than one nitrogen source. In addition, there was no indication of any inhibitory effects of ammonium on nitrate uptake. Bates et al. (1993) showed that cultures grown at less than 110 μM of nitrate and ammonium had equivalent growth rates and that there was no inhibition of nitrate uptake due to ammonium. As might be expected, ammonium addition stimulated ammonium depletion rates, whereas ammonium depletion rates were similar in the other treatments. Since these experiments utilized mixed phytoplankton assemblages, it is not possible to attribute these nitrogen uptake characteristics to *Pseudo-nitzschia* specifically, but *P. australis* remained the dominant pennate diatom (86%) in the ammonium treatment and accounted for 41% in the nitrate treatment.

In the urea treatment, the depletion of 6 μM of the initial nitrate but only 2 μM of the initial urea by day 2 indicates a slight preference for nitrate over urea. However, the depletion of nitrate by day 5 and the subsequent drawdown of urea indicate the sustained growth capabilities of the assemblage, including *P. australis*, when urea is the sole nitrogen source, particularly since this treatment was still growing exponentially on day 7. The lower depletion rates for urea across all treatments were expected since elevated ammonium concentrations (>1 μM) have been shown to suppress the uptake mechanism for urea in unialgal cultures (e.g. Molloy and Syrett, 1988; Cochlan and Harrison, 1991). In addition, an increase in urea in the carboys can be attributed to grazers. Growth rates were not statistically different across treatments, but based on the growth and nitrogen depletion rates (Tables 1 and 2), there appears to be a slight preference for nitrate and ammonium relative to urea.

Although limitation by other macro- or micronutrients was not directly assessed, ambient silicate and phosphate conditions during the experiment suggest that these nutrients were not limiting (they were not completely depleted for most treatments). Iron limitation and copper toxicity were also not directly addressed; however, initial iron concentrations were elevated, as expected over this shallow shelf region (Bruland, pers. commun.). Copper concentrations can be expected to be similar across nutrient additions, so any changes in growth rate or toxin production are not directly attributable to changes in copper toxicity.

4.2. Laboratory experiment

4.2.1. Growth rates and chlorophyll a

Laboratory experiments indicated that the concentration of chlorophyll *a* per cell was two- to three-fold less in the urea treatment compared to the nitrate and ammonium treatments. The low chlorophyll *a* per cell in the urea treatment could indicate possible nutrient stress, but the cellular fluorescence capacity for each nitrogen treatment did not differ (mean $F_v/F_m = 0.62$, and did not decline with time), suggesting that the physiological status was unaffected by the nitrogen source supporting growth. However, the exponential growth rate (determined using cell abundance) of *P. australis* was significantly slower for cells grown on urea compared to those grown on nitrate and ammonium, which both maintained similar growth rates. These laboratory results demonstrate the capability of this diatom to grow equally well on oxidized and reduced forms of nitrogen, and that *P. australis* is capable of using urea as the sole nitrogen source for growth, albeit at a somewhat slower rate than cultures grown on either nitrate or ammonium.

4.2.2. Domoic acid

The highest level of particulate domoic acid (pDA) per cell occurred in the urea treatment. As with the urea-amended field experiment, cells from the laboratory cultures were harvested in late logarithmic phase for particulate and dissolved domoic acid (dDA) analysis, and it is possible that urea-grown cells could potentially produce higher pDA $cell^{-1}$ once the cells enter stationary phase. In addition, the dDA (normalized to cell abundance) was ~140% of the pDA in the urea treatment whereas in the ammonium and nitrate treatments dDA/pDA only averaged 40–50%. This ratio of dDA/pDA is unusually high for cells harvested in late logarithmic phase. However, there are no other published results at this concentration of ammonium. The results for the laboratory experiment were similar to those of the field experiment in that DA production as a function of nitrogen source was greatest when nitrogen was derived solely from urea, followed by nitrate and ammonium, which were statistically indistinguishable. Assuming that DA production would continue to increase as cells entered stationary phase, and that a considerable fraction of DA is dissolved, our estimates of enhanced toxicity when grown on urea are likely conservative.

5. Conclusions

In California coastal waters, *Pseudo-nitzschia* spp. represent only a minor constituent of the total phytoplankton assemblage during most of the year. Previous authors have suggested a number of potential environmental factors such as coastal runoff (Scholin et al., 2000), metal stress (Rue and Bruland, 2001; Maldonado et al., 2002; Ladizinsky and Smith, 2003; Wells et al., 2005) or macronutrient limitation (Pan et al., 1996a,b,c) that may trigger toxin production in *Pseudo-nitzschia* blooms. In the San Francisco Bay experiment, no conspicuous ''triggers'' of DA production, such as silicate or phosphate limitation, were observed. The phytoplankton assemblage, which included a large proportion of *P. australis*, did not exhibit a preference for any particular nitrogen substrate, and both inorganic and organic nitrogen sources could support the growth of this assemblage, while the unialgal cultures definitively show that *P. australis* can grow on all nitrogen substrates tested. Both the field and laboratory experiments demonstrate

that urea-grown cells were more toxic than cells utilizing either nitrate or ammonium. Given that this diatom blooms during both upwelling and non-upwelling conditions off the west coast of the U.S. (Buck et al., 1992; Fryxell et al., 1997; Trainer et al., 2000), substantial differences in the nitrogenous nutrition of *P. australis* can be expected, and anthropogenic inputs of reduced nitrogen substrates, such as urea, may be more important in harmful algal bloom development than previously thought, as suggested in a recent analysis by Glibert et al. (2005b). We conclude that *P. australis* is capable of using both inorganic and organic nitrogen sources and that nitrogenous source can influence toxin production in this species in central California.

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