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The effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*

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Abstract

Toxigenic pennate diatoms of the genus *Pseudo-nitzschia* produce domoic acid (DA), the neurotoxin linked to amnesic shellfish poisoning. We investigated how Fe and Cu affect growth and DA production by *P. multiseries* and *P. australis* clones isolated from Monterey Bay, California. Growth rates of these species could be inhibited by both Fe limitation (pFe 20.5, $\mu = 50\%\ \mu_{max}$) and Cu toxicity (pCu 10.5, $\mu = 30$–$50\%\ \mu_{max}$). The rate of DA production during the exponential phase was a significant inverse function of cellular growth rates ($P < 0.02$). Voltammetric measurements of dissolved DA-equivalents in short-term experiments indicated that the cellular production of DA increased from ~5 amol DA cell^{-1} h^{-1} under optimal growth conditions to ~40 amol DA cell^{-1} h^{-1} under Fe-deficient conditions and ~105 amol DA cell^{-1} h^{-1} under Cu-stressed conditions. The DA was released to the medium under metal stress conditions, with intracellular DA concentrations decreasing relative to nonstressed cells. Fe uptake rates by *P. multiseries* were slow compared to other marine diatoms in the absence of dissolved DA but were enhanced threefold ($P < 0.03$) by adding DA to the medium. DA addition also partially alleviated toxic Cu conditions. Our findings suggest that DA production during exponential growth of these two toxigenic *Pseudo-nitzschia* species is directly induced by Fe-deficient or Cu stress conditions and that 95% of this DA is actively released into the medium. Changing trace metal conditions in coastal waters therefore may have a profound effect upon intracellular DA concentrations and thereby influence the toxic effect of these harmful bloom events.

Fewer than two dozen of the more than 5,000 known marine phytoplankton species produce powerful toxins that affect food webs (Taylor 1990), although blooms of these toxic species appear to be increasing worldwide (e.g., Smayda 1992; Anderson 1995). Until recently, only planktonic dinoflagellates were recognized to produce algal toxins. However, a deadly outbreak of the diatom *Pseudo-nitzschia multiseries* (formerly *Nitzschia pungens* f. *multiseries*) occurred in 1987 in bays of Prince Edward Island, Canada. The resultant syndrome became known as amnesic shellfish poisoning (ASP) because of the permanent memory loss experienced by survivors (Perl et al. 1990; Todd 1993). This unique epidemiology was traced to a neurotoxin identified as domoic acid (Subba Rao et al. 1988; Bates et al. 1989). Since then, other toxic blooms of the genus *Pseudo-nitzschia* have been recorded on both east and west coasts of North America (Buck et al. 1992; Fritz et al. 1992; Garrison et al. 1992). Ascertaining the distribution of these toxigenic diatoms has been difficult because *Pseudo-nitzschia* spp. often have been identified only to the genus level, and few of these species are known to produce domoic acid (DA) (Bates 2000). Furthermore, toxigenic species sometimes bloom without producing significant amounts of toxin (Villac et al. 1993; Bates et al. 1997), so records of toxic events alone are not useful for establishing their distribution.

Domoic acid is found both intracellularly and in the dissolved phase in cultures of *Pseudo-nitzschia* spp. (Bates et al. 1991 and others). It is a secondary metabolite, apparently formed by the condensation of an activated citric acid cycle derivative (probably glutamate arising from α-ketoglutarate) with geranyl (probably as geranyl pyrophosphate) (Douglas et al. 1992). The result is a water-soluble amino acid that mimics the excitatory neurotransmitter L-glutamic acid and binds irreversibly to glutamate receptor sites, causing destructive neuronal depolarization (Debonnel et al. 1989) and permanent short-term memory loss in mammals (Perl et al. 1990; Todd 1993). However, the physiological role of domoic acid in algal metabolism has remained entirely obscure.

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The relationship between production of DA by *Pseudo-nitzschia* spp. and environmental conditions also is unclear (Buck et al. 1992; Garrison et al. 1992; Lange et al. 1994; Taylor et al. 1994). Initial batch culture experiments suggested that cellular accumulation of DA began only at the onset of stationary phase (Bates et al. 1989, 1991, 1993). However, more recent findings show that DA production also occurs during late exponential phase, but at a slower rate (Garrison et al. 1992; Pan et al. 1996*a*; Garrison unpubl. data). Chemostat studies demonstrate that cellular accumulation of DA by *Pseudo-nitzschia* spp. is negatively correlated with growth rate (Pan et al. 1996*b,c*; Garrison unpubl. data) and that intracellular DA content is enhanced by either phosphorus or silicate limitation (Bates et al. 1991; Pan et al. 1996*b,c*; Garrison unpubl. data). To date, laboratory studies have focused mainly on the potential linkages between macronutrient limitation and DA production.

Rue and Bruland (2001) noted the similar architecture of mugenic acid, a phytosiderophore (Takagi et al. 1984), and domoic acid, a small, tricarboxylic acid and nitrogen-containing molecule. Phytosiderophores are analogous to bacterial siderophores in that they are low–molecular weight chelators released under Fe stress to enhance Fe uptake. Although siderophores are highly specific Fe chelators, they also bind Cu sufficiently well to alleviate Cu toxicity in algal cultures (McKnight and Morel 1979, 1980; Clarke et al. 1987). Rue and Bruland (2001) have measured the conditional stability constants for DA complexation of Fe and Cu in seawater and suggest that DA released to solution during *Pseudo-nitzschia* blooms could potentially alter the chemical speciation of these metals in seawater. The production and release of DA thus might be related to bioactive metal nutrition.

This study examines the relationship between trace metal availability and DA production by toxigenic *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis.* Because metal availability is extremely difficult to regulate in enriched seawater media, we modified the chemically well-defined synthetic seawater medium Aquil (Price et al. 1989) to culture these two toxigenic *Pseudo-nitzschia* species under precise and reproducible trace metal regimes. The Fe requirements and Cu tolerances of these species were determined by measuring growth rates under Fe-sufficient, Fe-limiting, and Cu-stressed conditions. We then examined whether Fe limitation or Cu toxicity affected the cellular production of DA and the extracellular release of DA-like ligands by these species during their log phase of growth. Experiments were performed to investigate the physiological role of DA in these toxigenic *Pseudo-nitzschia* species. A nontoxic *Pseudo-nitzschia pungens* strain was used as a control in some experiments.

Materials and methods

Study organisms—The two toxigenic species of *Pseudo-nitzschia* (*P. multiseries* and *P. australis*) were isolated from Monterey Bay, California, during the spring/summer of 1998 by M. Hughes. The nontoxigenic strain of *Pseudo-nitzschia pungens* used in our experiments was kindly donated by P. Miller (Monterey Bay Aquarium Research Institute).

Table 1. Trace metal enrichments in our synthetic seawater, a modification and combination of the f/2 medium (Guillard and Hargraves 1993) and the Aquil (Price et al. 1989) nutrient amendments. Macronutrient additions to this medium were 100 μM NO_3^-, 10 μM PO_4^-, and 50 μM Si. Trace metal additions for the three conditions tested are listed. The Fe-sufficient medium was our basal medium. The Fe-limiting or Cu-stressed medium was made by modifying only the concentration of either Fe or Cu, respectively. The concentration of pM was calculated for nonilluminated medium using the chemical equilibrium program MINEQL+ (Schecher and McAvoy 1998).

Element	Concentration (μM)	pM $-\log[M^+]$
Fe-sufficient		
EDTA	11.7	
Fe	0.15	18.6
Cu	0.01	12.9
Mn	0.9	6.5
Zn	0.08	9.8
Co	0.05	9.8
Ni	0.01	13.1
V	0.01	—
Cr	0.001	—
Mo	0.09	—
Se	0.01	—
Fe-limited		
Fe	0.0017	20.5
Cu-stressed		
Cu	1.8	10.5

Media preparation—Stock cultures of these isolates were grown and maintained in sterilized filtered seawater using f/2 medium enrichments of 2.16 μM ethylenediaminetetraacetic acid (EDTA), 0.216 μM Fe, 0.073 μM Mn, 500 μM NO_3^-, 40 μM PO_4^{3-}, and 50 μM SiO_3^{2-} (Guillard and Hargraves 1993). For the culture experiments, isolates were transferred to and acclimated in synthetic seawater media (Table 1).

Our synthetic seawater medium was a modified and combined version of the f/2 (Guillard and Hargraves 1993) and the Aquil media (Price et al. 1989), containing 100 μM NO_3^-, 10 μM PO_4^{3-}, and 50 μM SiO_3^{2-} (Table 1). Synthetic ocean water was prepared and purified of trace metals using Chelex 100 ion exchange resin (Bio-Rad Laboratories) according to the procedure of Price et al. (1989). Media were sterilized by microwaving in acid-washed Teflon bottles (Keller et al. 1988) and enriched with filter-sterilized (0.2 μm Acrodisc) EDTA–trace metal and vitamin (B12, thiamine, and biotin) solutions. All trace metals (Cu, Zn, Mn, Co, Se, Mo, V, Cr, and Ni) except Fe were added according to the f/2 medium additions (Guillard and Hargraves 1993) and were buffered with 10 μM EDTA (Table 1). The free ion activities of Cu ($10^{-12.9}$), Zn ($10^{-9.79}$), Mn ($10^{-6.5}$), Ni ($10^{-13.1}$), and Co ($10^{-9.76}$) were calculated using the chemical equilibrium program MINEQL+ (Schecher and McAvoy 1998). Premixed FeEDTA (1 : 1.05) was added separately to create Fe-sufficient or Fe-limiting media. The concentration of Fe was adjusted to achieve free ferric ion activities of $10^{-18.6}$ (pFe = $-\log[Fe^{3+}]$ = 18.6, [Fe] = 0.15 μM), and

$10^{-20.5}$ (pFe = $-\log[Fe^{3+}]$ = 20.5, [Fe] = 1.7 nM) for the Fe-sufficient and Fe-deficient media, respectively. To induce Cu stress, the Fe-sufficient medium was enriched with 1.8 μM Cu to give a free cupric ion activity of $10^{-10.5}$ (pCu = $-\log[Cu^{2+}]$ = 10.5). Two additional growth media were prepared by enriching this Cu stress medium with either 1.8 or 0.18 μM DA (Sigma) to test the effect of DA additions on the growth of Cu-stressed *Pseudo-nitzschia* sp.

All media were allowed to chemically equilibrate overnight in the dark before use and were stored in sterile, acid-washed polycarbonate bottles. Bottles and tubes used in our experiments were acid washed (soaked for at least 24 h in 10% HCl solution), followed by repetitive rinses with Milli-Q H_2O (Millipore). To minimize trace-metal contamination, all media manipulations were performed in a Class-100 laminar flow, metal-free hood. Sterile techniques were used to the fullest extent possible with trace-metal clean protocols to minimize bacterial contamination; cultures were not axenic but bacteria were sparse. Independent experiments have demonstrated that marine bacteria are not a source of DA in our cultures (Garrison et al. unpubl. data). Phytoplankton were grown in acid-washed, 28-ml polycarbonate tubes at 12°C under a continuous, saturating photon flux density of 130 μE m^{-2} s^{-1}, and cultures were gently mixed each day.

Growth rates—All cultures were maintained in constant exponential growth using semicontinuous batch culture techniques described by Brand et al. (1981). Biomass (cells ml^{-1}) was determined daily. Culture samples were preserved with Lugol's solution, and cell density was measured in duplicate by light microscope counting using a Palmer–Maloney chamber. Specific growth rates (d^{-1}) were determined from linear regressions of ln cells ml^{-1} versus time. All of the rates reported here are *acclimated* growth rates. Acclimation was judged to be complete when the growth rates of successive transfers varied by less than 10% (Brand et al. 1981). Statistical analysis was performed on all the acclimated growth rates.

Estimates of cell volume, surface area, and cellular C—Cell volumes were calculated by assuming that these pennate diatoms have the shape of a rectangular box with double trapezoidal prisms: V = (A × B × C × D) + [(1 − D) × A × (B + 0.5)/2 × C], where A is the length of the valve, B is the width of the valve in valve view, C is the depth of valve in girdle view, and D = 0.8 is the ratio of the linear part of the cell length to the total cell length. The cellular dimensions (A, B, C, and D) were determined for at least 100 cells using an inverted light microscope. Cellular surface area calculations were simplified by assuming that the cells were rectangular boxes (Area = 2[AB + BC + AC]), given that the trapezoidal prisms accounted for only 10% of the total surface area. The cell volume and surface area of *P. multiseries* were estimated to be 1,270 ± 100 μm^3 and 1,510 ± 180 μm^2, respectively. *P. australis* had a cell volume of 4,750 ± 300 μm^3 and a cell surface area of 1,166 ± 193 μm^2. *Pseudo-nitzschia pungens* had a cell volume of 705 ± 98 μm^3 and a surface area of 814 ± 55 μm^2. Fe uptake rates were normalized to cell surface area before comparison among the different phytoplankton species. Estimates of cellular carbon contents of *Pseudo-nitzschia* spp. were determined from cell volumes using the modified Strathmann equations for diatoms [log(C, pg) = 0.76 × log(cell volume, μm^3) − 0.352, Eppley et al. 1970]. The calculated cellular C contents of *P. multiseries* and *P. pungens* are 8.45 and 5.41 pmol C $cell^{-1}$, respectively.

Measuring Fe : C ratios—The cellular quotas of Fe and C (μmol Fe $cell^{-1}$, mol C $cell^{-1}$, and μmol Fe : mol C) for Fe-limited *P. multiseries* and Fe-sufficient *P. multiseries* and *P. pungens* were determined simultaneously using $^{55}FeCl_3$ (specific activity 30 mCi mg^{-1}, NEN) and $H^{14}CO_3^-$ (0.595 mCi mg^{-1}, NEN) (Tortell et al. 1996). After acclimation of the cultures to a specific set of growth conditions, inocula were transferred in triplicate to fresh media in which 100 or 2% of the total Fe was added as the ^{55}Fe-radiotracer solution (pFe 20.5 and pFe 18.6, respectively). The media also were spiked with 25 μCi L^{-1} of $H^{14}CO_3^-$. To ensure complete labeling of cellular Fe, eight or more divisions were allowed before harvesting. During midexponential growth, samples were filtered onto 5-μm pore-sized polycarbonate Poretics filters and rinsed for 3 min with Ti(III) EDTA-citrate reagent (Hudson and Morel 1989). Activities of ^{14}C and ^{55}Fe (disintegrations per minute, dpm) were determined by dual-label liquid scintillation counting (Beckman model LKB) after correcting for quenching and decay. Total cellular Fe and C were calculated using the dpm, the specific activities of ^{55}Fe and ^{14}C in the media, and cell densities (cells ml^{-1}).

Domoic acid production—Acclimated cultures of *Pseudo-nitzschia* spp. were grown under Fe-limiting, Fe-sufficient, or Cu-stressed conditions in our modified medium. During midexponential growth, cultures of *Pseudo-nitzschia* spp. (2 L, ~1,000–3,000 cells ml^{-1}) were harvested by gravity onto acid-washed, 5-μm pore-sized polycarbonate Poretics filters. Before running dry, the filters were gently flushed with sterile synthetic seawater (without trace metal, EDTA, or vitamin additions). Cells on the filter were then immediately resuspended in 500 ml of sterile synthetic seawater (without trace metal, EDTA, or vitamin additions) and left under culturing light and temperature conditions. A sample for cell counts was taken from this culture immediately after resuspension. After 12 h, cell counts were compared with the initial densities to ensure that cell growth remained in log phase during this 12-h period. The cell suspension then was immediately refiltered onto acid-washed, 0.4-μm pore-sized polycarbonate Poretics filters using the trace-metal clean filtration apparatus described by Phinney and Bruland (1994). Cellular DA concentrations were determined from analysis of the filters using the fluorenylmethyoxycarbonyl–high-performance liquid chromatography (FMOC-HPLC) method (Pocklington et al. 1990).

Concentrations of dissolved DA in the filtrate of these experiments were below the analytical detection limit of the FMOC method. Instead, competitive ligand equilibration/adsorptive cathodic stripping voltammetry (CLE-CSV) was used to measure the concentration and conditional stability constants of dissolved Fe and Cu complexing ligands (L) in the filtrate (Rue and Bruland 2001). Briefly, the electrochemically active ligand salicylaldoxime is added to the sample

to compete for metals with the ambient organic ligands (L). The ligand mixture then is titrated with increasing additions of Fe or Cu while the fraction of metal bound to salicylaldoxime is measured voltammetrically. Scatchard and Ruzic transformations of these titration data are used to resolve the concentration and conditional stability constants of the ligand class(es) (L_n) present in the sample (*see* Rue and Bruland 1995). The single ligand class detected in the filtrate of the *Pseudo-nitzschia* spp. cultures had conditional stability constants for Fe and Cu that were analytically indistinguishable from those of a purified DA standard (DACS-1C, NRC Certified Reference Materials Program), indicating that L in our samples was indeed dissolved DA (Rue and Bruland 2001). This expectation was strengthened by independent analyses using a DA-specific competitive enzyme-linked immunosorbent assay (cELISA, Garthwaite et al. 1996; *see Results*).

Cellular release rates of DA were estimated from voltammetrically determined dissolved DA-equivalent ligand concentrations, the measured cell densities, and the time the cells were resuspended in the sterile synthetic seawater (without trace metals). Intracellular DA production rates (mol DA $cell^{-1}$ d^{-1}) were calculated from the particulate (cellular) DA concentrations on the filters (mol DA $cell^{-1}$, determined with FMOC-HPLC) after 12 h, and growth rates (d^{-1}). We assumed steady state conditions for these calculations given that the cells were in log phase growth during all experiments.

Short-term Fe uptake experiments—Short-term (6 h) Fe uptake rates of acclimated, Fe-limited (pFe 20.5) or Fe-sufficient (pFe 18.6) *P. multiseries* were measured in the dark using $^{55}FeCl_3$ (0.55 mM in 0.005 M HCl, pH 2.3, specific activity 30 mCi mg^{-1}, NEN, Life Science Products). Fe uptake rates were compared in sterile synthetic seawater (without trace metal or vitamin additions) enriched with FeEDTA (1–10 nM Fe : 10 μM EDTA) or both FeEDTA (10 μM) and FeDA (1–10 nM Fe : 2 μM DA, Sigma). In addition, we measured the uptake rates of Fe bound to the fungal siderophore desferrioxamine B (FeDFB, 10 nM Fe : 100 nM DFB) in the presence or absence of DA (1–10 nM Fe : 2 μM DA). For the uptake experiments with EDTA and DFB, ^{55}Fe was pre-equilibrated with the chelator for 2 h before adding it to the culture media (Maldonado and Price 2001). In the DA competition experiments, ^{55}Fe was pre-equilibrated with DA for >2 h before adding it to the stock containing either EDTA or DFB. After subsequent addition of the ^{55}Fe-chelator stocks to sterile synthetic seawater, the medium was allowed to chemically equilibrate overnight before initiating the uptake experiment. The medium pH (8.1) did not change with metal-chelator additions or during the course of the uptake experiments.

Acclimated cultures of *P. multiseries* were harvested during midexponential growth by gravity onto acid-washed, 5-μm pore-sized polycarbonate filters, and the cells were immediately resuspended into the ^{55}Fe uptake media described above. Uptake was measured in duplicate from each flask every 1–1.5 h over 6 h by filtering cells onto 5-μm pore-sized polycarbonate filters and rinsing them with the Ti(III) EDTA-citrate reagent described by Hudson and Morel (1989) to desorb Fe bound to extracellular surfaces. ^{55}Fe activities (dpm) on the filters were determined by liquid scintillation counting (Beckman model LKB) after correcting for quenching and decay. Total cellular Fe was calculated from the measured dpm, the specific activity of ^{55}Fe in the media, and cell densities (cells ml^{-1}). ^{55}Fe uptake rates were calculated from the slope of the least-squares linear regression between Fe quota (Fe $cell^{-1}$) and time. The experimental controls for the uptake experiments were glutaraldehyde-killed cells (2% glutaraldehyde fixed for 1 h). Iron uptake in these controls was less than 5% of that measured for living cells.

Table 2. Growth rates (d^{-1}) of *P. multiseries* and *P. australis* grown in Fe-sufficient (pFe 18.6), Fe-limited (pFe 20.5) or Cu-toxic (pCu 10.5) synthetic seawater and in enriched filtered seawater medium (mean ± SD). The number of replicate cultures are given in parentheses. $pM = -\log[M^+]$.

Medium	Growth rate (d^{-1})	
	P. multiseries	*P. australis*
Enriched filtered seawater	1.5±0.49(7)	1.0±0.3(6)
Synthetic seawater		
Fe-sufficient (pFe 18.6, pCu 13.5)	1.0±0.17(30)	0.8±0.24(22)
Fe-limited (pFe 20.5, pCu 13.5)	0.50±0.14(22)*	0.4±0.15(5)*
Cu-stressed (pFe 18.6, pCu 10.5)	0.51±0.14(6)*	0.26±0.03(2)*

* Growth rates differed significantly from that in the Fe-sufficient treatment (*t*-test, $P < 0.05$).

Results

Growth media—Our initial efforts focused on experimenting with different formulations of the f/2-Aquil synthetic medium in order to optimize it for growth of *Pseudo-nitzschia* spp. We varied the additions of trace metals, EDTA, and Si and compared the division rates achieved by *Pseudo-nitzschia* spp. in these synthetic media to those in parallel enriched filtered seawater media (Table 2, *see Discussion*). With respect to the f/2 medium (Guillard and Hargraves 1993), we decreased the concentrations of Fe (100-fold), silicate (twofold) and phosphate (threefold) (Table 1). In comparison to Aquil (Price et al. 1989), our medium has (1) half the silicate addition and 10-fold lower EDTA levels; (2) at least 10-fold higher concentrations of Mn, Cu, Zn, and Co; (3) eightfold lower Fe concentrations relative to EDTA; and (4) amendments of V, Cr, and Ni (Table 1). The recipe presented in Table 1 yielded the best growth response *Pseudo-nitzschia* spp. and high biomass at the end of exponential growth. Growth rates of *P. australis* and *P. multiseries* in the Fe-sufficient modified f/2-Aquil medium ranged from 0.8 to 1.0 d^{-1} and were between 66 and 80% of those achieved in enriched natural filtered seawater (Table 2). This basal recipe (Table 1) was used in all subsequent experiments.

Growth rates—Fe and Cu stress—In general, *P. multiseries* achieved faster growth rates than *P. australis* in metal-

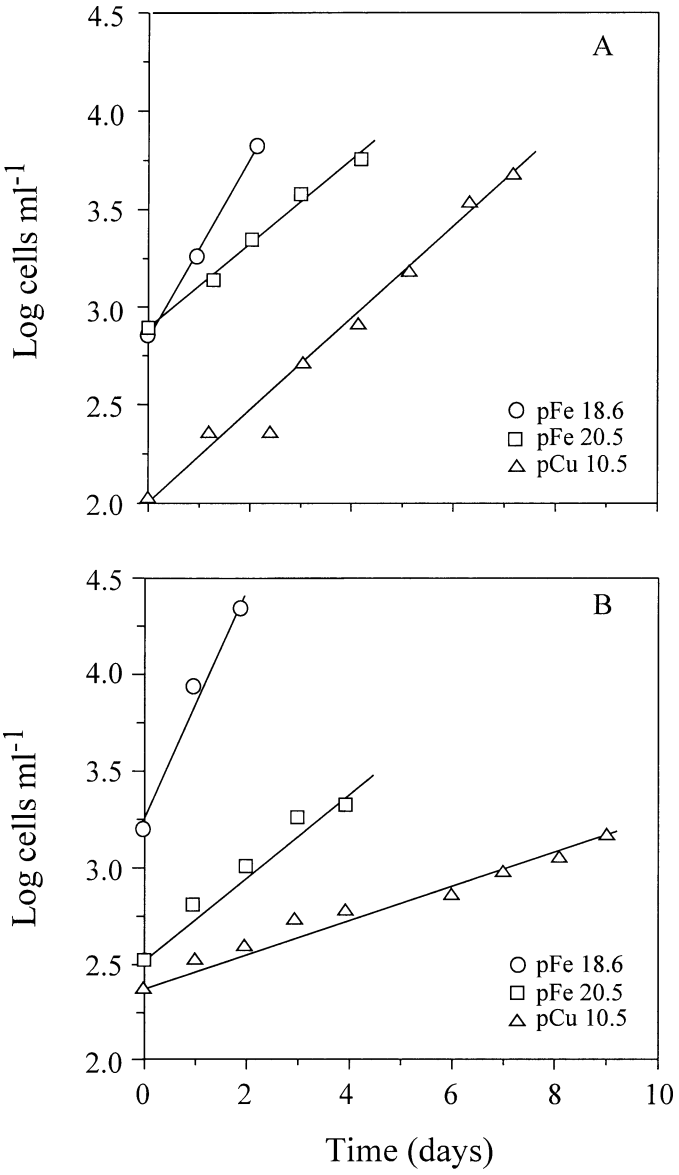

Fig. 1. Cell densities (cells ml^{-1}) as a function of time (days) for cultures of (A) *P. multiseries* and (B) *P. australis* grown in Fe-sufficient (pFe 18.6), Fe-limiting (pFe 20.5) or high-Cu (pCu 10.5) synthetic seawater medium (*see Table 1*).

sufficient media (Table 2). Growth rates of both species were reduced by 50% under Fe-deficient conditions (pFe 20.5) (Table 2, Fig. 1). Increasing the Cu concentration in the medium (or lowering pCu from 12.9 to 10.5) significantly depressed (30–50%) growth rates of both species; however, *P. australis* was slightly more sensitive to Cu toxicity than was *P. multiseries* (Table 2). In separate experiments, adding DA to high-Cu media reduced Cu toxicity for *P. multiseries* by up to 20% (pCu 10.5: no DA, $\mu = 0.45 \pm 0.17$ d^{-1}, $n = 3$; with 180 nM DA added, $\mu = 0.56 \pm 0.06$ d^{-1}, $n = 3$; and with 1.8 μM DA, $\mu = 0.51 \pm 0.1$ d^{-1}, $n = 3$).

Fe : C ratios—The Fe : C ratios of *P. multiseries* and *P. pungens* grown in Fe-sufficient medium (pFe 18.5) were very similar, averaging 140 ± 5.8 ($n = 3$) and 148 ($n = 1$) μmol Fe : mol C, respectively. Under Fe-deficient conditions (pFe 20.5), the Fe : C ratio of *P. multiseries* decreased to 13 ± 3.2 μmol Fe : mol C ($n = 3$), corresponding to a 7.3× lower cellular Fe quota (0.129 ± 0.004 vs. 0.937 ± 0.005 × 10^{-15} mol Fe $cell^{-1}$, respectively). Iron limitation had little effect on the cell volume of *P. multiseries* (1,259 ± 129 vs. 1,283 ± 91 μm^3 for Fe-limited and Fe-replete cells, respectively) or the calculated cellular C concentrations (7.6 ± 1.6 vs. 5.2 ± 0.8 fmol C fl^{-1}, respectively).

Table 3. Methods comparison of dissolved DA concentrations determined in cultures of *P. multiseries* as a function of Fe and Cu stress. Replicate subsamples of the culture media were analyzed by the DA-specific cELISA immunoassay method (DA) and by the DA-equivalent ligand (DA-E) determinations of CLE-ACSV (Rue and Bruland 2001). Neither method yielded detectable levels of DA in cultures of *Pseudo-nitzschia pungens*, a clone in which no intracellular DA has been detectable using the FMOC method. (ND, not detected). The terms [Fe′] and [Cu′] refer to the concentration of the sum of inorganic metal species.

Culture conditions	cELISA immunoassay dissolved DA (nM)	CLE-ACSV dissolved DA-E (nM)
P. multiseries		
Fe-sufficient (pFe 18.5, [Fe′] = 10 nM) (pCu 13.5, [Cu′] = 0.5 pM)	ND	0.7±0.2
Fe-limited (pFe 20.5, [Fe′] = 0.03 nM)	8.0±2.5	5.8±0.1
Cu-stressed (pCu 10.5, [Cu′] = 0.5 nM)	7.9±1.8	10.9±0.5
P. pungens		
Fe-limited (pFe 20.5, [Fe′] = 0.03 nM)	ND	ND

Cellular domoic acid production—Both Fe-sufficient and Fe-limited (μ = 80% μ_{max}) cultures of *P. australis* and *P. multiseries* produced a dissolved Fe-binding chelator having a conditional stability constant that was analytically identical to a certified DA standard ($K_{FeDA,Fe(III)'} = 10^{8.7\pm0.5}$ M^{-1}; Rue and Bruland 2001). By the courtesy of I. Garthwaite (AgResearch, New Zealand), this ligand also was analyzed in a subset of experiments using a newly developed cELISA that is molecularly specific for DA (Garthwaite et al. 1996). Parallel measurements on three independent filtrate samples yielded analytically indistinguishable values for DA (cELISA) and DA-equivalent (CLE-CSV) ligand concentrations (Table 3). As a further check, the comparison was repeated with *P. pungens*, a non-DA producer, and no detectable concentrations of DA (cELISA) or DA-equivalent (CLE-CSV) ligand were found under Fe-limited growth conditions.

Domoic acid production by *P. multiseries* and *P. australis* was measured in the cellular and dissolved fractions as a function of Fe stress. Intracellular DA concentrations and production (accumulation) rates were two times higher for

Table 4. Concentrations and production rates of domoic acid (dissolved and intracellular) in *Pseudo-nitzschia multiseries* cultures grown under Fe-sufficient (pFe 18.6), Fe-limited (pFe 20.5) or toxic Cu (pCu 10.5) conditions. (pM = $-\log[M^+]$, amol = 10^{-18} mol, DA molecular weight = 311.3 g mol^{-1}).

Dissolved [DA] nM (μg L^{-1})	Dissolved DA production rate amol $cell^{-1}h^{-1}$ (fg $cell^{-1}h^{-1}$)	Particulate [DA] amol $cell^{-1}$ (fg $cell^{-1}$)	Particulate DA production rate amol $cell^{-1}$ h^{-1} (fg $cell^{-1}h^{-1}$)
Fe-sufficient			
0.5(0.16)	5.1(1.6)	67.7(21.1)	1.94(0.61)
0.84(0.26)	5.2(1.6)	—	—
Fe-limited			
5.8(1.8)	49.9(15.5)	34 (10.6)	0.78(0.24)
5.9(1.8)	28.2(8.8)		
7.6(2.4)*	41 (12.6)	—	—
Cu-stressed			
>17(5.3)	>163(50.7)	—	—
10.9(3.4)	81.6(25.5)		
11.6(3.6)	69.3(21.7)		

* *Pseudo-nitzschia australis.*

Fe-sufficient than Fe-deficient cells (Table 4). Dissolved DA-equivalent ligand concentrations showed the reverse trend, with concentrations being, on average, an order of magnitude higher under Fe-deficient (6.43 ± 1.0 nM, $n = 3$) than Fe-sufficient conditions (0.67 ± 0.2 nM, $n = 2$) despite similar cell densities (Table 4). Both *P. multiseries* and *P. australis* responded similarly to Fe-limitation, with *P. australis* producing 7.6 nM and *P. multiseries* 5.85 ± 0.07 nM of dissolved DA-equivalents ($n = 3$). The combined rate of intracellular DA production and extracellular release was ~10× faster for Fe-deficient than Fe-sufficient cells (Table 4).

The effect of Cu stress on DA production and release by *P. multiseries* appeared to be even greater than that measured for Fe stress. As with Fe, the conditional stability constant of Cu complexation by the released ligand was analytically indistinguishable from that determined for the certified DA standard ($K_{CuDA,Cu(II)'} = 10^{9.0\pm0.2}$ M^{-1}; Rue and Bruland 2001). Unfortunately, the particulate DA samples from the Cu experiments were lost during analysis, so we are unable to calculate intracellular DA production rates under Cu stress. Nonetheless, release of DA-equivalent Cu-binding ligands was approximately three times higher under Cu stress (105 ± 51 amol DA $cell^{-1}$ h^{-1}, $n = 3$) than under Fe deficiency (39.7 ± 11 amol DA $cell^{-1}$ h^{-1}, $n = 3$), and up to 20× higher than for Fe-replete conditions (5.2 ± 0.1 amol DA $cell^{-1}$ h^{-1}, $n = 2$).

Fe uptake rates—To measure the effect of DA on Fe uptake, cells were preconditioned in Fe-limiting (pFe 20.5) or Fe-sufficient media (pFe 18.5) and then resuspended in synthetic seawater enriched with different concentrations of FeEDTA or a FeEDTA/FeDA mixture. Fe uptake rates increased linearly with increasing Fe additions in both the EDTA (mol Fe $cell^{-1}$ = 0.036 + 0.047 [Fe] nM, $r^2 = 0.95$) and EDTA/DA treatments (mol Fe $cell^{-1}$ = 0.134 + 0.143 [Fe] nM, $r^2 = 0.84$) (Fig. 2). However, uptake rates in the FeEDTA/FeDA treatment were ~3× higher than that with FeEDTA alone (paired *t*-test: $t = 2.07$, $P = 0.03$, df=10; Table 5, Fig. 2), as illustrated by the higher regression slope for the DA treatment. DA also significantly enhanced Fe acquisition by a *P. pungens* clone that has not been observed to produce DA (Table 5).

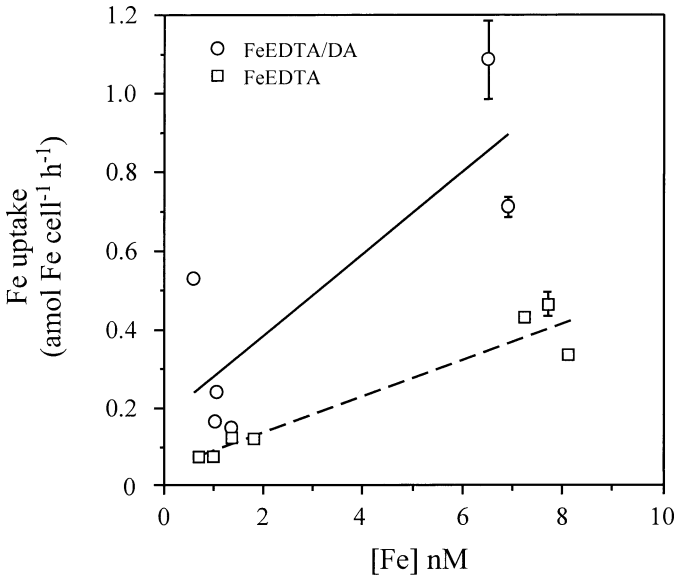

Fig. 2. Short-term Fe uptake rates (amol ^{55}Fe $cell^{-1}$ h^{-1}) of Fe-limited (pFe 20.5, 1.7 nM dissolved Fe) and Fe-sufficient (pFe 18.6, 0.15 μM dissolved Fe) *Pseudo-nitzschia multiseries* (mean ± SD of duplicate analyses, data from Table 4). Rates were determined in the dark at subsaturating Fe concentrations in the presence of 10 μM EDTA (FeEDTA) or 10 μM EDTA and 2 μM DA (FeEDTA/DA). The Fe concentration reported is the measured dissolved ^{55}Fe concentration in the Fe uptake media at the start of the experiment. The plotted lines were obtained by least-squares regression (*see Results*).

In contrast, addition of DFB to *P. multiseries* cultures yielded Fe uptake rates that were indistinguishable from killed controls (0.016 ± 0.002 amol Fe $cell^{-1}$ h^{-1}, $n = 1$). This low Fe uptake was not measurably improved by adding DA to the medium (0.016 ± 0.002 vs. 0.013 ± 0.003 amol Fe $cell^{-1}$ h^{-1}, $n = 1$).

All Fe uptake experiments were performed in the dark to avoid complications from photolysis of the Fe ligand complexes. To investigate the possible energy requirement for Fe uptake by *P. multiseries*, we compared Fe uptake in the presence or absence of light with and without 10 nM DCMU (dichlorophenyldimethyl urea) added. Fe uptake rates from an FeEDTA/FeDA mixture (0.65 nM Fe : 10 μM EDTA : 2 μM DA) were considerably faster in the light than those measured the dark (3.55 ± 0.25 vs. 0.53 ± 0.03 amol Fe $cell^{-1}$ h^{-1}, respectively). However, low-level additions of DCMU, a photosynthetic inhibitor, did not significantly affect short-term Fe uptake rates in either the light (2.96 ± 0.4 vs. 3.55 ± 0.25 amol Fe $cell^{-1}$ h^{-1}) or the dark (0.36 ± 0.02 vs. 0.53 ± 0.03 amol Fe $cell^{-1}$ h^{-1}; with and without DCMU, respectively) (mean ± range of duplicates).

Table 5. Short-term Fe uptake rates (amol^{55} Fe cell^{-1}h^{-1}) of Fe-limited (pFe 20.5, 1.7 nM dissolved Fe) and Fe-sufficient (pFe 18.6, 0.15 μM dissolved Fe) *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia pungens* (mean ± SD of duplicate analyses). Rates were determined in the dark at subsaturating Fe concentrations in the presence of 10 μM EDTA (FeEDTA) or 10 μM EDTA and 2 μM DA (FeEDTA/DA) (amol = 10^{-18} mol, DA molecular weight = 311.3 g mol^{-1}). The Fe concentration reported is the measured dissolved ^{55}Fe concentration in the Fe uptake media at the start of the experiment.

Dissolved [Fe] (nM)		Cell density (cell ml^{-1})		Fe uptake rate [amol cell^{-1} h^{-1} × 10^{-21} ± S.D. (mol Fe μm^{-2} h^{-1})]	
FeEDTA/DA	Fe-EDTA	FeEDTA/DA	FeEDTA	FeEDTA/DA	FeEDTA
Fe-sufficient					
1.1	1.8	5,220	6,215	0.163±0.01(0.106)	0.121±0.004(0.079)
6.5	8.1	4,110	3,605	1.086±0.1(0.709)	0.326±0.018(0.212)
1.0*	1.0	4,510	3,890	0.236±0.009(0.29)	0.071±0.004(0.087)
Fe-limited					
0.6	0.7	645	875	0.529(0.47)	0.079(0.07)
1.4	1.3	5,930	7,073	0.148±0.01(0.098)	0.123±0.01(0.082)
6.9	7.7	2,280	4,755	0.711±0.024(0.472)	0.463±0.03(0.308)
7.3		5,315			0.427(0.284)

* *Pseudo-nitzschia pungens.*

Discussion

Synthetic seawater and growth rates—Evaluating the role that metals might play in the production of DA by toxigenic *Pseudo-nitzschia* spp. is extremely difficult using enriched seawater media because trace-metal chemistry (and thus metal availability) cannot be precisely or reproducibly controlled. Our synthetic medium, a modification and combination of the Guillard f/2 and the Aquil nutrient amendments, allows *P. multiseries* and *P. australis* to grow at ~75% of the maximum division rates achieved with enriched natural seawater. By manipulating Fe and Cu additions to this medium, we are able to induce Fe limitation (μ = 50% μ_{max}) and Cu toxicity (μ = 30–50% μ_{max}) to the cells during exponential growth (Fig. 1, Table 2). However, although we can successfully grow and maintain these organisms for extended periods of time in our synthetic seawater medium, maximal cell division rates slow or cease altogether after ~6 months. This response also occurs with amended natural seawater media and highlights the challenge in rearing these toxigenic species. Nonetheless, the synthetic medium described here provides an adequate starting point for critically assessing the effects of trace metal stress on the physiology and toxin production of *Pseudo-nitzschia* spp.

The response of these toxigenic diatoms to metal stress was notably different from that of other marine phytoplankton. The relative growth rates of *P. multiseries* and *P. australis* under Fe-deficient conditions ([Fe′] = 25–30 pM; mean μ/μ_{max} = 0.5) were faster than other coastal species at similar Fe levels (mean μ/μ_{max} = 0.40 at [Fe′] ≈ 25 pM, Sunda and Huntsman 1995; mean μ/μ_{max} = 0.20, [Fe′] ≈ 25–30 pM, Maldonado and Price 1996). In contrast, these *Pseudo-nitzschia* species were more sensitive to Cu toxicity (pCu = 10.5) than other coastal diatoms (μ/μ_{max} = 0.3 for *P. australis* and μ/μ_{max} = 0.5 for *P. multiseries* vs. μ/μ_{max} = 0.65–0.9, Sunda and Hunstman 1995). Even so, both *P. multiseries* and *P. australis* were more Cu tolerant than *Synechococcus* spp. (μ/μ_{max} = 0.01, Brand et al. 1986). Given that DA has a high affinity for both Fe and Cu in seawater (Rue and Bruland 2001), it is conceivable that DA release might impart some competitive advantage to these cells.

Domoic acid concentrations and production rates—P. multiseries had relatively low intracellular DA concentrations during middle to late exponential growth, in accordance with previous findings (Bates et al. 1991; Pan et al. 1996*a*,*b*,*c*). These values (0.034–0.068 fmol DA cell^{-1}) lie within the lower range of intracellular DA concentrations reported (0.022 fmol DA cell^{-1} for *Pseudo-nitzschia pseudodelicatissima* to 119 fmol DA cell^{-1} in cultures of *P. australis*, Subba Rao et al. 1988; Bates et al. 1989, 1991, 1993; Martin et al. 1990; Buck et al. 1992; Garrison et al. 1992; Pan et al. 1996*a*,*b*,*c*; Scholin et al. 2000). By comparison, dissolved DA-equivalent ligand concentrations were much higher in these actively growing cultures (≥0.5 nM, Table 4). It is important to recognize that CLE-ACSV does not provide structural information about the ligands measured. However, the analytically identical conditional stability constants of the released ligand and certified DA standard for Fe and Cu, the close agreement between voltammetric and DA-specific cELISA determinations on a subset of samples (Table 3), and the absence of any measurable DA by either method in cultures of a non-DA–producing diatom combine to offer reasonable certainty that the released ligand measured by voltammetry was DA. Nonetheless, to clarify the discussion, we denote ''DA'' for FMOC-derived domoic acid values and ''DA-E'' for domoic acid–equivalent values determined by voltammetric analysis.

P. multiseries and *P. australis* showed a low-level release of DA-E (~5 amol cell^{-1} h^{-1}) under optimal growth con-

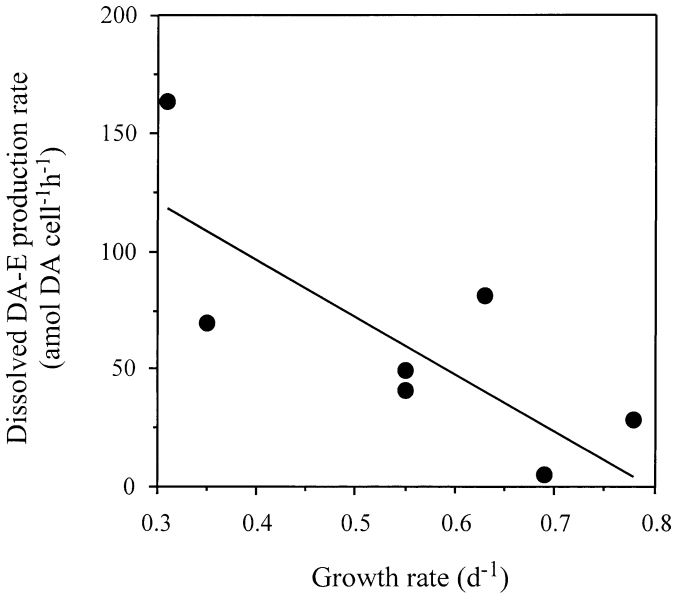

Fig. 3. Dissolved DA production rates (amol cell^{-1} h^{-1}) as a function of absolute growth rates (d^{-1}) for cultures of *Pseudo-nitzschia* spp. grown in Fe-sufficient (pFe 18.6), Fe-limiting (pFe 20.5), or high-Cu (pCu 10.5) synthetic seawater medium. Production measurements were made during midexponential phase. [$y = 193.23 - 242.3x$, where y = DA production rate (amol DA cell^{-1} h^{-1}) and x = growth rate (d^{-1}), $r^2 = 0.6$; ANOVA $F_{2,5} = 9.54$, $P = 0.025$]. (amol = 10^{-18} mol). Data from Table 4, *P. multiseries* ($n = 7$) and for *P. australis* ($n = 1$).

ditions, but this increased ~10-fold (~40 amol cell^{-1} h^{-1}) when cells grew under Fe-deficient conditions and by ~20-fold for *P. multiseries* (~105 amol cell^{-1} h^{-1}) with increased Cu toxicity (Table 4). The dissolved DA-E concentrations measured were far too great to result from simple transport of intracellular DA reserves but, instead, indicate the rapid production (and release) of new DA molecules by the growing cells. These production rates are 6–25× higher than those reported for Si- or P-limited *P. multiseries* at equivalent growth rates (Pan et al. 1996*b*,*c*). Using the measured DA-E production rates and the intracellular DA pool, it is estimated that the average residence time of DA molecules in the cell is ~13 h under Fe-sufficient conditions and only ~40 min under Fe-deficient conditions. Production rates of dissolved DA-E were a significant inverse function of growth rates (Fig. 3). Similar relationships between total DA production rates and growth rates have been found for Si- and P-limited continuous cultures of *P. multiseries* (Pan et al. 1996*b*,*c*).

The striking difference between trace metal and macronutrient effects on DA production in batch cultures of *Pseudo-nitzschia* spp. is the partitioning between intracellular DA and that released into the medium. Under P- or Si-limitation, *P. multiseries* begins to significantly accumulate intracellular DA only during mid- to late–stationary phase (S. Bates pers. comm.). Similarly, DA release to the medium becomes significant only during mid- to late–stationary phase (after ~15 d, Bates et al. 1991; Pan et al. 1996*a*,*c*; Bates et al. 2000), presumably because of increased leakage of the cell membrane. Our findings indicate for the first time that toxigenic *Pseudo-nitzschia* spp. produce DA during exponential growth in response to Fe-deficient or Cu-toxic conditions and that 95% of the total DA produced is released into the medium. Because DA is a small charged molecule, and thus very hydrophilic, this release would require active transport of the molecule across the intact cell membrane.

It is extremely unlikely that the DA-E release measured in our experiments is an artifact resulting from cell rupture during filtration or resuspension. First, the low pressure (<2.5 psi) filtration would have minimized shear stress on the diatoms; second, twice the number of cells recovered would have had to rupture *after* rinsing and resuspension in the 500 ml of sterile media to account for the dissolved DA-E concentration measured (~4×10^6 cells × 67 amol DA cell^{-1} × 2 ≈ 0.5 nmol DA L^{-1}; Table 4). Thus, our findings indicate that the production and release of DA by the cells is an active physiological response to trace metal stress.

The active release of strong metal-complexing organic ligands by phytoplankton has been observed previously. Siderophore production has been measured for a few marine heterotrophic bacteria (Trick 1989; Reid et al. 1993; Granger and Price 1999) and prokaryotic phytoplankton (Wilhelm and Trick 1994; Trick and Wilhelm 1995). A few eukaryotic phytoplankton have been found to release strong, Fe-specific organic ligands under Fe-limiting conditions that have hydroxamate (siderophore-like) functionalities, apparently to facilitate Fe acquisition (Trick et al. 1983). On the other hand, Cu stress causes both prokaryotic and eukaryotic phytoplankton to release Cu-complexing organic ligands (McKnight and Morel 1979) having conditional stability constants very similar to the Cu-binding ligands in seawater (McKnight and Morel 1980; Croot et al. 2000). Our results demonstrate that *P. multiseries* and *P. australis* also release an organic metal-complexing ligand under metal stress. Unlike other phytochelators, this ligand also has fatal neurological effects in vertebrates, likely because of the glutamic acid functionality of the metal complexing site.

Bates et al. (2000) also showed that *P. multiseries* had lower intracellular DA concentrations when experiencing Fe stress during stationary phase, but they found that dissolved DA concentrations increased faster in Fe-replete than Fe-deficient cultures during mid- to late-stationary phase (i.e., the opposite trend shown in our short-term growth experiments). The cause for this difference is not clear, although it may be linked to differential effects of Fe stress over the very different time scales of the two studies. For example, Fe limitation curtails the ability of phytoplankton to assimilate nitrate (e.g., Price et al. 1991). As the Fe-deficient cultures entered stationary phase, the cells then likely also experienced N stress, and N limitation has been shown to inhibit the production of DA (Bates et al. 1993). The coupling of Fe availability and DA production/release indicated here might then be constrained to conditions where these toxigenic species are experiencing Fe *stress* rather than severe Fe *limitation.*

Domoic acid effect on Fe uptake—If DA release under Fe limitation is a physiological response to increase Fe acquisition, then addition of DA to the medium should enhance

Fe uptake. Indeed, our results show that Fe uptake rates were three times faster ($P = 0.03$) in the presence of DA than with EDTA alone. But is DA serving the role of siderophore for these toxigenic diatoms? The evidence would suggest not. First, the conditional stability constant of DA for Fe is low ($K_{FeDA,Fe(III)'} = 10^{8.7\pm0.5}$ M^{-1}, Rue and Bruland 2001) compared to that of siderophore molecules in seawater ($\sim 10^{10.7}$–$10^{16.5}$ M^{-1}, Rue and Bruland 1995). Second, organisms utilizing siderophores typically display significantly (20–30×) faster Fe uptake rates under Fe-deficient conditions compared to Fe-sufficient conditions, when siderophores are not utilized (Braun et al. 1998; Granger and Price 1999; Maldonado and Price 2001). That result was not observed in this study, but DA clearly increases the rate of Fe uptake by these toxigenic species. Rather than out-competing the ligand classes for Fe in seawater (which it might do at high dissolved DA concentrations, Rue and Bruland 2001), DA might still facilitate Fe uptake by increasing the rate of Fe exchange among ligands in solution and at the cell surface (Albrecht-Gary and Crumbliss 1998). DA enhancement of Fe lability might explain why the Fe uptake rate of *P. pungens*, a nonproducer of DA, increased when DA was added to the medium.

The ability of *Pseudo-nitzschia* spp. to augment their Fe acquisition could be critical for their success in coastal waters. Although the Fe use efficiency for Fe-stressed *P. multiseries* [(1/13 μmol Fe (mol C)$^{-1}$) × 0.5 d^{-1} = 0.4 × 10^5 mol C (mol Fe)$^{-1}$ d^{-1}] is within the range reported for other Fe-stressed coastal phytoplankton (0.3–1.11 × 10^5 mol C (mol Fe)$^{-1}$ d^{-1}, Maldonado and Price 1996), their short-term, surface-normalized Fe uptake rates were much slower. For example, the mean surface-normalized Fe uptake rate for *P. multiseries* at 25 pM Fe′ was 0.27 ± 0.18 × 10^{-21} mol Fe μm^{-2} h^{-1} ($n = 4$) in the presence of DA, whereas surface area normalized uptake rates of Fe-limited *T. oceanica* and *T. weissflogii* are about five times faster at equivalent [Fe′] (1.41 × 10^{-21} mol μm^{-2} h^{-1}, Maldonado and Price 2000; 1.6 × 10^{-21} mol μm^{-2} h^{-1}, Harrison and Morel 1986; Hudson and Morel 1990). This inefficiency in Fe uptake presumably should have a significant influence on the growth of these toxigenic diatoms in coastal waters.

In fact, the short-term Fe uptake rates measured in the dark with DA additions were below the calculated steady-state cellular Fe demand (e.g., $\rho Fe = Q_{Fe} \times \mu$ = 13 μmol Fe (mol C)$^{-1}$ × 0.5 d^{-1} × 8.45 pmol C cell^{-1} = 2.3 amol Fe cell^{-1} h^{-1} vs. the 0.2–1.1 amol Fe cell^{-1} h^{-1} measured). However, Fe uptake rates in the light were similar to the steady-state Fe uptake requirement calculated. As with other marine diatoms (Anderson and Morel 1982; Hudson and Morel 1990), Fe uptake by *P. multiseries* does not appear to be a large energy-requiring process, as suggested by the absence of a low-level DCMU effect on Fe uptake. Thus, higher rates of Fe uptake in the light (3.55 ± 0.25 vs. 0.53 ± 0.03 amol Fe cell^{-1} h^{-1}) likely are related to increases in [Fe′] resulting from photoreduction of FeEDTA (and perhaps FeDA). Given their comparatively poor ability to acquire Fe, photochemical processes therefore may be particularly important for these toxigenic species to flourish in coastal waters.

Domoic acid production rates by Cu-stressed cells—*Synechococcus* spp. are particularly sensitive to Cu toxicity (Brand et al. 1986) and release strong Cu-chelating ligands ($\log K_{Cu}^{cond} > 13$) to lower cupric ion activities to nontoxic levels (Moffett and Brand 1996). The *Pseudo-nitzschia* species studied here were less sensitive to Cu than *Synechococcus* but were substantially more fastidious with respect to Cu than other coastal diatoms (Sunda and Huntsman 1995). Cu stress caused *P. multiseries* to increase DA-E release by 20-fold, and addition of DA to Cu-stressed cultures improved cell growth (data not shown). It is conceivable then that DA release is an effective strategy for *P. multiseries* to deal with Cu toxicity. Other diatoms also release Cu-complexing ligands under stress conditions, generating dissolved ligand concentrations similar to the DA-E release measured here (McKnight and Morel 1979; Croot et al. 2000). However, the conditional stability constant for Cu complexation by those ligands are one to two orders of magnitude higher than for DA ($K_{CuDA,Cu(II)'} = 10^{9.0\pm0.2}$ M^{-1}, Rue and Bruland 2001), which might help explain why other coastal diatoms are more Cu tolerant.

Effect of domoic acid release in the environment—Rue and Bruland (2001) combine the dissolved DA-E production rates determined here with the measured affinity of DA for Fe and Cu to suggest that DA could alter the chemical speciation of Fe and Cu in coastal waters. Based on thermodynamics, they calculated that 100 nM dissolved DA would compete with in situ Fe-complexing ligands to bind 25% of the total dissolved Fe pool (Rue and Bruland 2001). Given the upper range of DA-E production rates measured (Table 4, Fig. 3), it is conceivable that DA concentrations of this magnitude might be achieved during intense *Pseudo-nitzschia* blooms (0.8 μM DA = 163 amol DA cell^{-1} h^{-1} × 24 h d^{-1} × 30,000 cells ml^{-1} × 1,000 ml L^{-1} × 7 d). Unfortunately, there are no dissolved DA data from the 1998 *Pseudo-nitzschia* outbreak along the California coast to evaluate this possibility.

Based on our steady-state Fe uptake rate estimates, we calculate that *P. multiseries* and *P. australis* can fulfill their minimal Fe quota without DA release if the steady-state [Fe′] is ≥20–25 pM (2.3 × 10^{-18} mol Fe cell^{-1} h^{-1} = 13 μmol Fe (mol C)$^{-1}$ × 8.5 pmol C cell^{-1} × 0.5 d^{-1} × d/24 h). As a bloom develops and [Fe′] is driven below ~20 pM toward ~7 pM Fe′, the cells would be able to maintain rapid growth by releasing DA to the surrounding seawater at the rates measured here. If the bloom continued to grow, drawing [Fe′] below ~7 pM, cell division would slow due to Fe limitation despite DA release. The calculated equilibrium [Fe′] in recently upwelled waters north of Monterey Bay are >25 pM, based on measured concentrations and conditional stability constants of Fe-complexing organic ligands (Rue and Bruland unpubl. data). Aging of these recently upwelled waters, with the resultant intense diatom growth, forces [Fe′] to <1 pM, although steady-state [Fe′] should be slightly higher during daylight hours because of photochemical cycling (Rue and Bruland 1995). Thus, [Fe′] in coastal waters fall within the range shown here to cause the increased production and extracellular release of DA-E by these two *Pseudo-nitzschia* species.

The case for Cu is less clear. Production of 10 nM dissolved DA would lower free cupric ion [Cu^{2+}] activities in surface waters 100-fold (Rue and Bruland 2001)—enough to convert even high Cu^{2+} activities in some coastal waters (e.g., $\sim 10^{-9.6}$ M in Narragansett Bay, Kozelka and Bruland 1997) to nontoxic levels for these *Pseudo-nitzschia* species. However, in most coastal waters, the pCu lies within the apparent range of tolerance for these species, so Fe stress would appear to be a more likely stimulant for DA release than Cu toxicity. Nonetheless, changes in pCu presumably could affect the production and release of DA in coastal waters influenced by high metal inputs.

Synthesis—Domoic acid has a high affinity for Fe and Cu in seawater (Rue and Bruland 2001), and the findings here indicate for the first time that DA production by actively growing toxigenic *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis* is a function of bioactive metal stress. Our results suggest that the metal : DA relationship is causative rather than an indirect outcome of a dysfunctional metabolic system. Enhanced rates of DA-E release from exponentially growing cells under metal-deficient (Fe) and -toxic (Cu) conditions would require active transport of the hydrophilic molecule across the cell membrane, adding further evidence that production of DA-E is a direct response to metal stress. Although *P. multiseries* is less efficient at obtaining Fe compared to other marine diatoms, Fe acquisition improves markedly when DA is added to the medium, indicating that one physiological role for DA in these cells may be to alleviate Fe limitation. Moreover, the intracellular turnover rates of DA change from a few hours to a few minutes once cells encounter Fe stress conditions, indicating a tight coupling between cellular metabolism and metal availability outside the cell. Rates of DA-E production and release by *P. multiseries* were substantially higher under Cu stress than Fe stress in our experiments, but this may be because the degree of stress was greater with Cu than Fe ($\mu/\mu_{max} = 0.3$–0.5 for Cu-stressed vs. $\mu/\mu_{max} = 0.5$ for Fe-stressed). Increasing the dissolved DA concentration also partially alleviated Cu toxicity, suggesting a second possible physiological role for DA. Under conditions of severe Fe or Cu stress, DA production rates could be as high as $\sim$150 amol DA $cell^{-1}$ h^{-1} (Fig. 3). It is our opinion then, that the toxic neurological effect DA expressed in mammals is a misdirected result of the metal-binding characteristic of the glutamate functional moiety.

Extensive work (Bates et al. 1989, 1991, 1993, 1997; Pan et al. 1996*a*, 1966*b*, 1996*c*) has shown that *P. multiseries* begins to strongly accumulate intracellular DA when forced into Si- or P-induced senescence. One interpretation of these findings is that P or Si metabolism may be directly involved with DA function within the cell, contrary to the findings presented here. We suggest that combining these apparently disparate observations might provide some predictive insight to when *Pseudo-nitzschia* blooms will become toxic in nature.

It is clear that low-level DA production occurs during optimal growth conditions (Table 4), but these intracellular DA concentrations remain low. However, as the results of Pan, Bates, and colleagues show, there is a sharp increase in intracellular DA accumulation once cell division begins to slow and the cells enter stationary phase; slower cell growth is more conducive to DA accumulation (Pan et al. 1998). The key implication of our findings is that intracellular DA accumulation would not occur if cells were experiencing metal stress simultaneously with Si or P stress. In that case, our results suggest that active transport of DA across the cell membrane would rapidly deplete intracellular DA pools, and that the majority of the subsequent enhanced DA production would be targeted for release outside the cell.

Given that DA production is affected by both Fe and Cu, which metal is more likely to affect the toxicity of cells in coastal waters? Because blooms will not develop under toxic Cu conditions, the effects of Cu on DA production likely would occur primarily when cell densities are low. On the other hand, there is recent evidence that Fe stress is more common during bloom development in coastal seawaters than previously recognized (Hutchins et al. 1998), raising the likelihood that Fe may well influence cell toxicity when it is most important from a public and marine ecosystem health perspective—namely, when cell densities are high.

We suggest that a direct coupling between Fe availability and DA *release* by the cell is responsible for the widely variable intracellular (particulate) DA levels observed for *Pseudo-nitzschia* blooms in coastal waters (Villac et al. 1993; Trainer et al. 2000). In this scenario, intensely toxic blooms develop only when complete macronutrient (P or Si) draw-down occurs before Fe availability falls below the physiological requirements of these toxigenic species. Conversely, marginally toxic or nontoxic blooms result when cell growth drives the system into Fe limitation before complete Si draw-down has occurred. Our results suggest then that changing Fe inputs related to runoff and sediment resuspension and the character of upwelling zones may be critical parameters controlling the outcome of bloom toxicity. Furthermore, because Fe is essential for nitrate uptake and assimilation (Price et al. 1991), toxigenic *Pseudo-nitzschia* spp. should have significantly lower Fe requirements if grown primarily on ammonium. One implication of our findings then is that localized regions with high anthropogenic inputs of reduced N, such as near domestic sewage outfalls or extensive finfish aquaculture facilities, might be susceptible to experiencing particularly toxic blooms compared to other coastal waters.

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