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Domoic acid: The synergy of iron, copper, and the toxicity of diatoms

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Abstract

Diatom blooms generated by the alleviation of iron limitation in high nitrate–low chlorophyll (HNLC) regions of the oceans often are composed of pennate diatoms of the genus *Pseudo-nitzschia*, many of which periodically produce the potent neurotoxin domoic acid. We show that toxigenic diatoms have an inducible high-affinity iron uptake capability that enables them to grow efficiently on iron complexed by strong organic ligands in seawater. This low-iron adaptive strategy requires copper and domoic acid, a copper chelator whose production increases sharply when both iron and copper are limiting. Addition of either domoic acid or copper to seawater improves the growth of *Pseudo-nitzschia* spp. on strongly complexed iron during deck incubation experiments with natural phytoplankton. Our findings indicate that domoic acid is a functional component of the unusual high-affinity iron acquisition system of these organisms. This system may help explain why *Pseudo-nitzschia* spp. are persistent seed populations in oceanic HNLC regions, as well as in some neritic regions. Our findings also indicate that in the absence of an adequate copper supply, iron-limited natural populations of *Pseudo-nitzschia* will become increasingly toxic.

The discovery that iron can limit phytoplankton growth in offshore high nitrate–low chlorophyll (HNLC) regimes (Coale 1991; Tsuda et al. 2003) and in some nearshore upwelling waters (Hutchins et al. 1998) has spawned detailed studies into the iron acquisition strategies of marine phytoplankton. Although low dissolved iron concentrations contribute greatly to iron limitation in ocean waters, the presence of very strong, iron-specific organic complexing ligands (Rue and Bruland 1995) can substantially reduce iron availability to larger eukaryotic phytoplankton (Wells 1999). Thus, the presence of persistent, viable populations of coastal-type pennate diatoms of the genus *Pseudo-nitzschia* in offshore HNLC waters (e.g., Harrison et al. 1999) is puzzling, given their expected inability to access strongly complexed iron resources. One possibility is that these organisms have evolved either novel or more efficient strategies for iron acquisition than have other neritic-type phytoplankton.

Iron uptake by eukaryotic phytoplankton initially was modeled as being proportional to the free ferric ion activity (e.g., Brand et al. 1983), in the same manner as that shown for the uptake of other bioactive metals (e.g., Sunda and Huntsman 1998). These models stem from the early work of Anderson and Morel (1982), showing the dependence of iron uptake on free ferric ion activities, and are fundamentally linked to the concentration of kinetically labile, soluble ferric hydrolysis species (Hudson and Morel 1990). However, this scenario proved to be overly simplistic for natural systems, in which ambient concentrations of inorganic iron species are extremely low as a result of complexation by at

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least two classes of strong organic chelators (Rue and Bruland 1995). One conceptual framework that has emerged recently is that the stronger class of complexing ligands comprises siderophores (Wilhelm and Trick 1994), molecules released by microbes to chelate iron and facilitate its cellular uptake by specific siderophore transport proteins on the outer membranes (Neilands 1995). The ''weaker'' class of ligands, which generally are found in higher concentrations, are attributed to intracellular metabolic constituents (and their partial breakdown products) released from cells by leakage, grazing, and viral lysis. As a first approximation, iron bound to the stronger class of ligands appears to be more available to prokaryotic phytoplankton, while the comparatively weaker ligand class better supports eukaryotic phytoplankton (Hutchins et al. 1999). While some eukaryotic phytoplankton appear to reduce strong Fe(III) chelates at the cell surface (Maldonado and Price 2001), the resultant iron uptake rates often are slow relative to rates of Fe(III) uptake (e.g., Wells and Trick 2004). The question, then, is whether pennate diatoms of the genus *Pseudo-nitzschia* have developed more efficient strategies for accessing organically complexed Fe(III).

All *Pseudo-nitzschia* spp. examined to date with highly sensitive, enzyme-linked immunosorbent assay (ELISA)–based analytical methods have been found, at one time or another, to produce the toxin domoic acid (DA). However, the factors regulating toxin production are not known, and toxin concentrations in a given species can vary from high to undetectable in both laboratory cultures (e.g., Pan et al. 1996; Maldonado et al. 2002) as well as in natural waters (e.g., Trainer et al. 2000, 2002). Early research indicated that growth limitation by silicon (Si) and phosphorus (P) would increase cell toxicity after prolonged senescence (Bates et al. 1991; Pan et al. 1996). Subsequent findings have shown that cell toxicity during exponential growth is stimulated by low iron availability, resulting in the release of DA to solution, which, in turn, increases rates of iron uptake (Maldonado et al. 2002). These findings indicate that DA is involved in enhancing iron acquisition by *Pseudo-nitzschia* species.

In the present study, we examine the growth responses of the toxigenic diatoms *Pseudo-nitzschia multiseries*, *Pseudo-nitzschia australis*, and *Pseudo-nitzschia fraudulenta* under iron stress relative to that of the nontoxic centric diatom *Thalassiosira weissflogii*. Based on our earlier findings (Maldonado et al. 2002), we hypothesized that these growth responses are linked to DA production and release, and that iron limitation is the primary trigger for toxin production. The present results demonstrate that these toxigenic diatoms have an unusual ability to adapt to iron limitation in synthetic growth medium and that there is a synergistic link between toxin production and limitation by iron and copper. These observations are supported by field incubation studies conducted in the *Pseudo-nitzschia*–rich waters of the Juan de Fuca eddy located off the coasts of Washington (U.S.A.) and British Columbia (Canada). Our findings indicate a novel functional role for DA that might explain the persistence of *Pseudo-nitzschia* spp. in offshore HNLC waters and may provide a framework for predicting when coastal blooms of *Pseudo-nitzschia* spp. become toxic.

Materials and methods

Laboratory culture experiments were performed using a basal synthetic medium consisting of a modified and combined version of f/2 (Guillard and Hargraves 1993) and Aquil media (Price et al. 1989). The basal salt medium and individual nutrient stocks were pumped ($\sim$2 mL min^{-1}) through an ion-exchange column (Chelex 100, Biorad) containing resin prepared according to Price et al. (1989). Macronutrients were added at initial concentrations of 300 μmol L^{-1} NO_3^-, 15 μmol L^{-1} PO_4^{3-}, and 50 μmol L^{-1} $Si(OH)_4$. Greater concentrations of silicate had a detrimental effect on cell growth, and this level was sufficient to ensure that cells did not encounter silicate limitation over the $\leq$5-d experimental durations (final $Si(OH)_4 \geq 7$ μmol L^{-1}). Media batches 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 (B_{12}, thiamine, and biotin) solutions. The trace metals zinc (Zn), manganese (Mn), cobalt (Co), selenium (Se), molybdenum (Mo), vanadium (V), and chromium (Cr) were added as in our previous studies (Maldonado et al. 2002); nickel (Ni) additions were reduced to 1×10^{-13} mol L^{-1} because this lower concentration was found to improve cell growth rates (Wells unpubl. data). Metals were buffered with 11.7 μmol L^{-1} EDTA and the free ion activities calculated using the chemical equilibrium program MINEQL+ (Schecher and McAvoy 1998). Iron (Fe) and copper (Cu) stock solutions were added separately to achieve the desired conditions. Reagent grade DA (Sigma) used in the experiments was dissolved in deionized water and treated ($\geq$24 h) in batch with clean Chelex 100 deionizing resin (Biorad) to remove metal contamination. All bottles and apparatus were acid cleaned, and sample manipulations were conducted within a laminar-flow hood (HEPA, class 100) using trace-metal clean techniques (Maldonado et al. 2002).

The three *Pseudo-nitzschia* clones used in these experiments were isolated from Monterey Bay (*P. australis*, 17 April 2001; *P. multiseries*, 11 April 2001; and *P. fraudulenta*, 11 April 2001). Phytoplankton were reared in semicontinuous batch cultures, in which inocula of exponentially growing cells were transferred to new media every 4–5 d, thereby ensuring that cells remained in continuous exponential growth through the experiment. Single cultures were used for each treatment, with successive transfers under the same conditions providing a measure of precision for the growth rate determinations. Specific growth rates were determined from linear regressions of the natural log of in vivo fluorescence versus time, after confirming a proportional relationship between fluorescence and cell concentration during exponential growth (Guillard 1973). Cells were grown under iron availabilities ranging from pFe = 18.6 to 21.4 (pFe = $-\log[Fe^{3+}]$), while copper was varied from pCu = 14.8 to 18.8 (pCu = $-\log[Cu^{2+}]$). These conditions were calculated using the chemical equilibrium program MINEQL+ (Schecher and McAvoy 1998) for additions of 2–150 nmol L^{-1} Fe and 1–1,000 pmol L^{-1} Cu (for EDTA = 11.7 μmol L^{-1}). Past analyses of the basal medium have shown contaminant levels of Fe and Cu to be <50 pmol

L^{-1}; however, actual free ion activities in these experiments likely were somewhat higher than calculated because of light effects on Fe-EDTA complexes (Sunda and Huntsman 2003) and trace copper contamination in the medium. Any possible differences would be small and would not substantially alter the findings because the gradient of metal availability is more relevant here than the arbitrarily chosen specific metal activities. Cell cultures were transferred 5 to 14 times in semicontinuous mode depending on the experiment. After transferring clones, the remaining culture medium was harvested periodically for analysis of dissolved and particulate DA.

Three separate laboratory culture experiments were performed. The first was designed to ascertain the growth response of *P. multiseries, P. australis,* and *P. fraudulenta* to decreasing inorganic iron concentrations and to contrast these responses to that of the well-characterized centric diatom *T. weissflogii* (obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Science, Boothbay Harbor, Maine). In the second experiment we tested whether the successful adaptation of these toxigenic diatoms to iron stress was influenced by copper availability. The third experiment examined how addition of dissolved DA to the medium affected cell growth under low copper conditions.

Deckboard incubation experiments were conducted during September 2003 in the Juan de Fuca eddy off British Columbia and Washington State—a cold-water gyre identified as a region frequently containing high concentrations of DA and *Pseudo-nitzschia* cells (Trainer et al. 2000, 2002). Seawater was pumped from 5m depth using an all-plastic, trace-metal clean system into a 50-liter acid-cleaned polyethylene carboy, mixed thoroughly, and dispersed into acid-cleaned and rinsed 10-liter polycarbonate carboys. Treatments were prepared as described in the text, and the carboys (duplicate treatments and controls) placed within a clear Plexiglas deck incubator maintained at sea-surface temperature and 50% surface irradiance. The carboys remained sealed until the end of the experiment but were subsampled daily by compressed air overpressure, as described in Coale (1991). Treatment and control samples were filtered (Poretics, 5.0 μm) over the 4-d experiment for determination of cellular DA and chlorophyll *a,* and subsamples were collected for quantification of cell abundance and species composition.

The DA concentrations in field samples were determined using the receptor binding assay (Trainer et al. 2000). A more sensitive direct ELISA method was used in the laboratory studies through collaboration with N. Towers and I. Garthwaite (AgResearch, Hamilton, New Zealand). A similar analysis kit now is available from Biosense. The analytical detection limit, defined as three times the standard deviation of the lowest samples, was ~20 fg mL^{-1} DA, while the working range extended up to ~5 ng mL^{-1} DA (Wells et al. 2002). For particulate determinations, cells were filtered onto 25-mm GF/F filters (Whatman) and the filters placed in vials containing 2-mL deionized water (Millipore). They were cycled through four freeze–thaw sequences using liquid nitrogen and a hot water bath to liberate the water-soluble DA molecule from the cellular matrix. After mixing, the samples were syringe filtered through 0.2-μm acrodisc filters and the supernatant diluted to a ratio of 1 : 60 with deionized water to minimize interferences (Wells et al. 2002). Dissolved DA in the culture media was syringe filtered using a 0.2-μm acrodisc filter and then analyzed directly without dilution. Chlorophyll *a* concentrations were determined by in vitro fluorometry (Parsons et al. 1984) and phytoplankton enumeration by phase-contrast microscopy; *Pseudo-nitzschia* species identification was confirmed by scanning electron microscopy.

Results

The growth responses of *T. weissflogii, P. multiseries, P. australis,* and *P. fraudulenta* as a function of pFe are shown in Fig. 1. After four successive transfers to new media, *T. weissflogii* achieved a maximum growth rate of ~0.7 d^{-1}, and a stable pattern appeared whereby growth rates decreased with decreasing free metal ion activity (Fig. 1A). In contrast, the growth rates of the *Pseudo-nitzschia* spp. did not achieve a prolonged steady state. After four successive transfers, their maximum growth rates (~0.8 d^{-1}) decreased monotonically between pFe 18.5 to 20.4 but remained elevated in the lower free Fe^{3+} treatments, approaching even optimal growth at pFe 21.4 (Fig. 1B–D; closed circles). Continuing the successive transfers led to full acclimation of all three *Pseudo-nitzschia* species to the iron stress conditions, indicated by maximal growth rates across the range of EDTA-buffered free ferric ion activities (Fig. 1B–D; open circles).

The acclimation rate of these organisms to iron stress appeared to be fastest when iron stress was greatest. Growth rates in the lowest pFe treatment (21.4) remained high, if not optimal from the beginning; however, cells apparently did not acclimate as rapidly to the intermediate levels of iron stress (as indicated by the lower growth rates at pFe ≈ 20–20.5; Fig. 1B–D). Nonetheless, after expression of maximal iron stress during transfers 5 and 6, the recovery of growth rates was rapid (≤4 transfers) and complete (Fig. 1B–D). In short, it was not possible to fully starve these toxigenic diatoms for iron under these culture conditions, in direct contrast to the centric diatom *T. weissflogii.*

The DA per cell decreased with increasing iron stress during the early stages (transfer 3) of *P. multiseries* acclimation (Fig. 2A). However, the reverse trend—DA per cell increasing with increasing iron stress—was measured after *P. multiseries* had acclimated fully to iron stress (transfer 12) (Fig. 2B). The same trends occurred with *P. australis* and *P. fraudulenta*; however, DA per cell was much lower and the differences were less dramatic (data not shown). The intracellular : dissolved partitioning of DA also changed as the cells adapted to iron limitation (Fig. 3). A higher proportion of DA was found in the medium during the early stages of acclimation than in later stages of the experiment. Differences between treatments showed the relative (and absolute) dissolved DA concentrations to be greatest when iron availability was low, before cells had acclimated to the iron stress. The partitioning between dissolved (D) and particulate (P) DA became much more uniform (average D : P = 0.3 ± 0.1) across the range of iron-limited conditions after

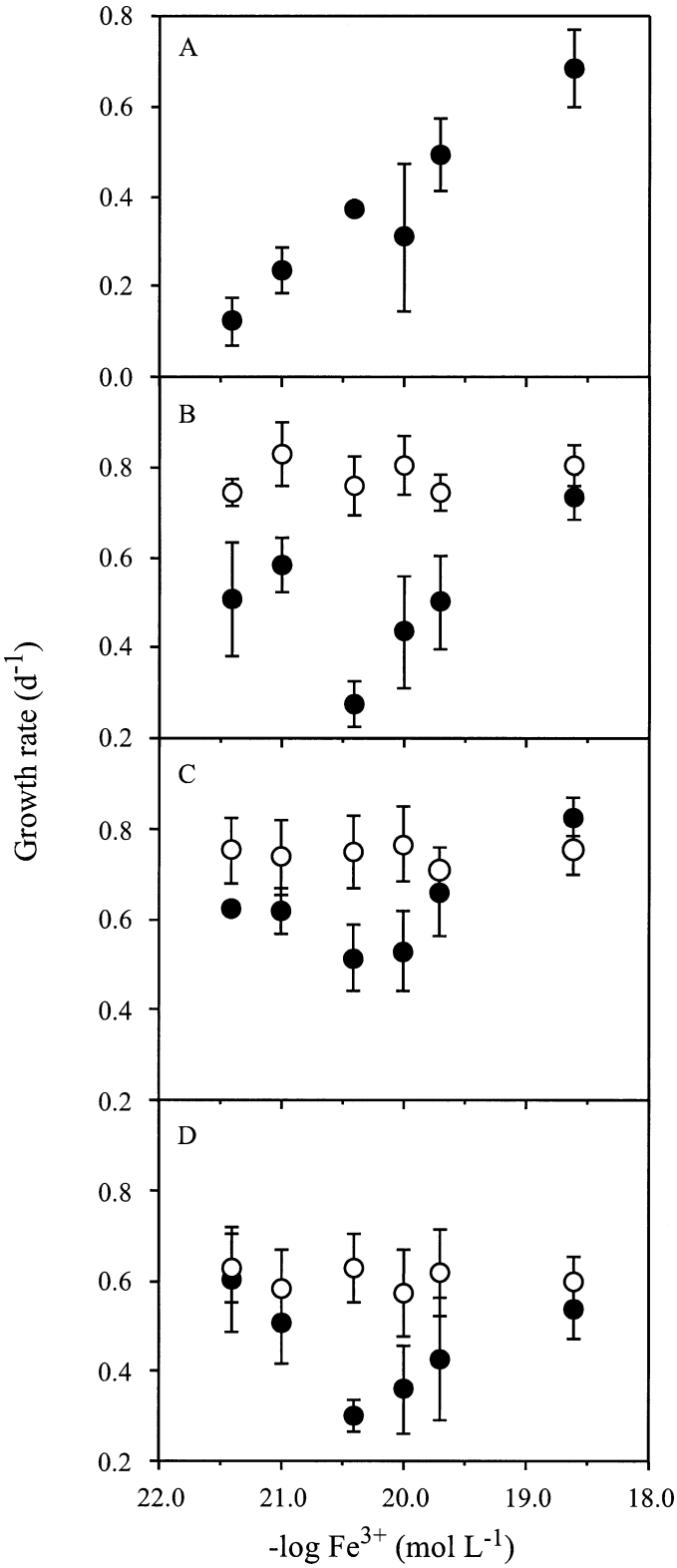

Fig. 1. (A) Average cellular growth rates of the nontoxic diatom *T. weissflogii*, (B) toxigenic diatoms *P. australis*, (C) *P. multiseries*, and (D) *P. fraudulenta* as a function of free ferric ion concentrations. Acclimated growth rates during the early stages of successive semicontinuous batch cultures (transfers 5 and 6) are depicted as closed circles, while open circles show later stages (transfers 9–13) of the experiment. Error bars represent the range of transfers 5 and 6 and the SD of successive transfers (9–13).

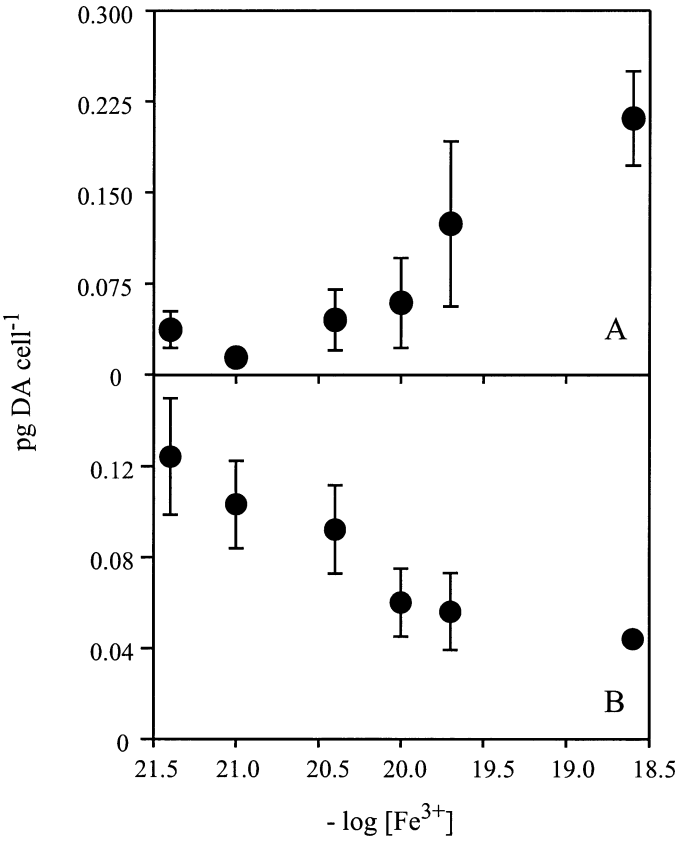

Fig. 2. Differences in DA per cell determined in exponentially growing cultures of *P. multiseries* as a function of $-\log[Fe^{3+}]$; (A) during early acclimation (transfer 3) and (B) after full acclimation to iron stress conditions (transfer 12). Data points are the mean ± 1 SD of ≥10 replicate measurements from the individual culture vessels.

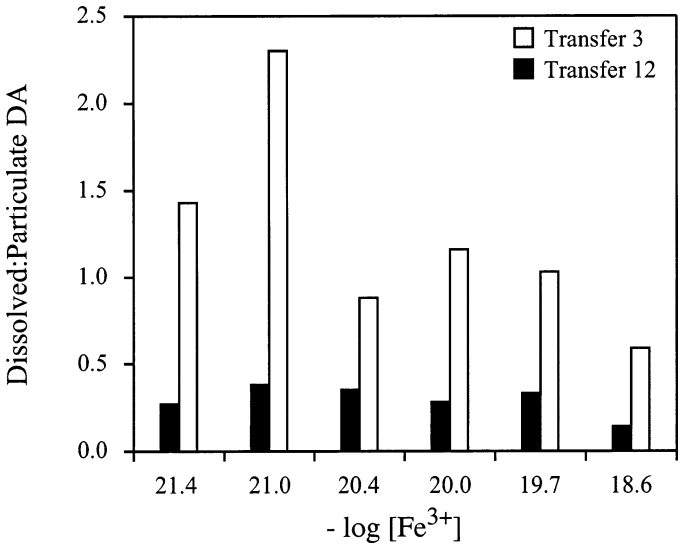

Fig. 3. Ratios of total dissolved : total particulate DA in the *P. multiseries* cultures shown in Fig. 2A and B as a function of pFe.

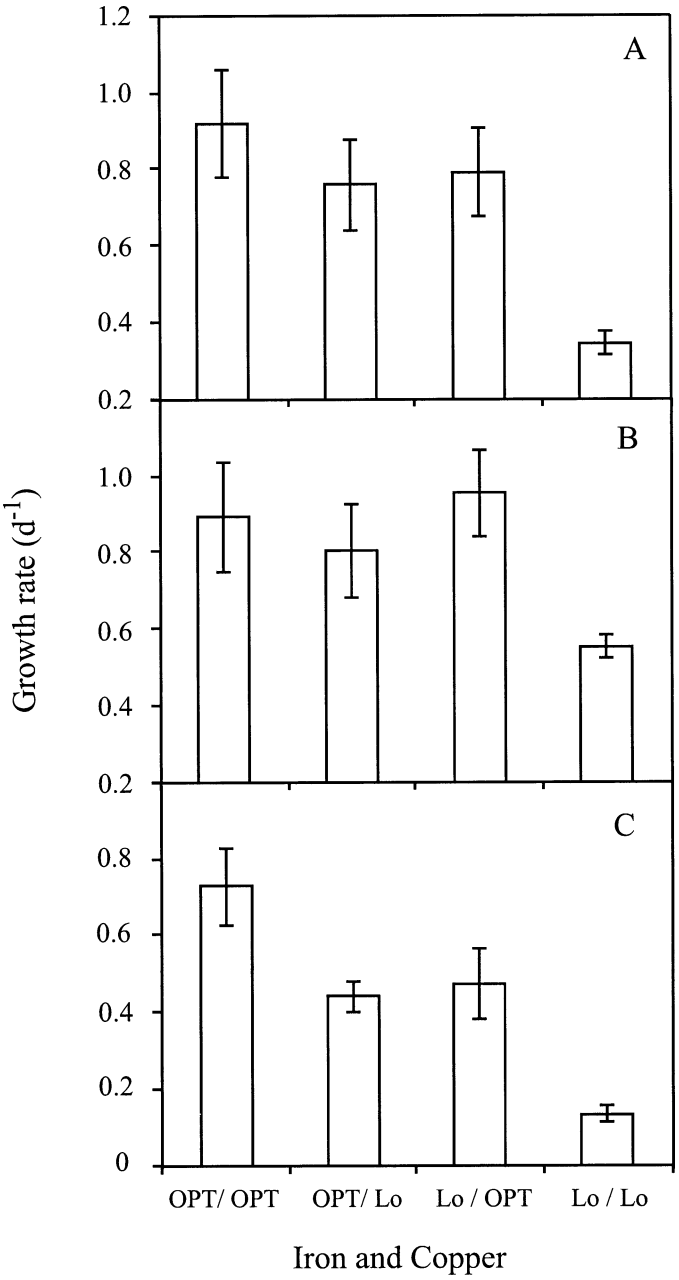

Fig. 4. The synergistic effect of iron and copper on the cellular growth rates of (A) *P. australis*, (B) *P. multiseries*, and (C) *P. fraudulenta* in semicontinuous batch cultures after short-term equilibration (generally the mean of transfers 5 and 6). Concentrations for iron were: $-\log Fe^{3+} = 10^{-18.6}$ mol L^{-1} (optimal, OPT) and 10^{-21} mol L^{-1} (low, Lo). Concentrations for copper were: $-\log Cu^{2+} = 10^{-13}$ mol L^{-1} (OPT) and 10^{-17} mol L^{-1} (LO). Error bars show the range for transfers 5 and 6.

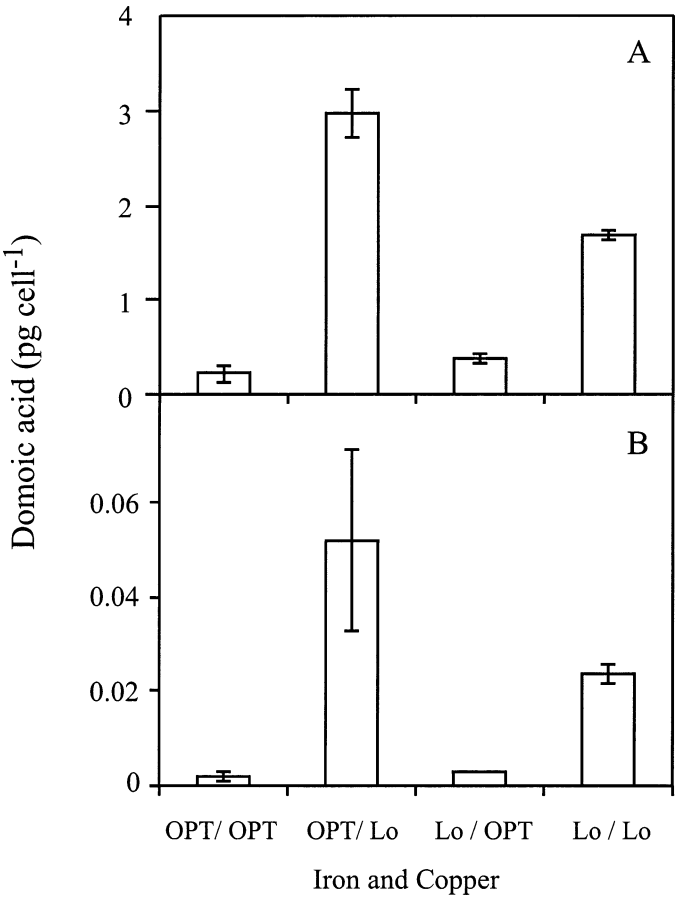

Fig. 5. (A) Concentrations of cellular and (B) dissolved DA produced by *P. australis* in the growth experiment depicted in Fig. 4. Error bars are the standard deviation of replicate analyses (n = 12).

cells had fully acclimated to the various degrees of iron stress. Because cells were kept in exponential growth phase during these experiments, this DA partitioning likely resulted from cellular control rather than the inadvertent leakage of DA through damaged cell membranes.

The unusual growth response of these toxigenic diatoms under iron stress led us to hypothesize that the cells were acquiring iron directly from the EDTA-Fe complexes in the media and that this ability was linked to the bioavailability of copper (*see Discussion*). We tested this hypothesis by contrasting the growth rates under control conditions (optimal iron, pFe 18.6, and copper, pCu 13) with three treatments: low iron/optimal copper (pFe 21, pCu 13); optimal iron/low copper (pFe 18.6, pCu 17); and low iron/low copper (pFe 21, pCu 17) (Fig. 4). After short-term equilibration (four to six transfers), the growth rates of *P. multiseries* and *P. australis* were indistinguishable from optimal rates (~0.8 d^{-1}) under either low iron or low copper, but these rates decreased by 40–70% when both metals were deficient (Fig. 4A,B). *P. fraudulenta* showed a similar decrease in growth (80%) relative to the control cultures under combined iron and copper limitation. *P. fraudulenta* had not fully acclimated to iron stress in this experiment (40% lower growth rates) and appeared to also be sensitive to copper limitation (Fig. 4C) (*but see following*).

Copper deficiencies had a dramatic effect on cellular production of DA. While cell toxin levels doubled in *P. australis* under iron stress, copper deficiency increased DA per cell by 20-fold (Fig. 5A). When normalized to cell abundance, dissolved DA concentrations showed a similar pattern to the DA per-cell levels in these cultures, with low copper

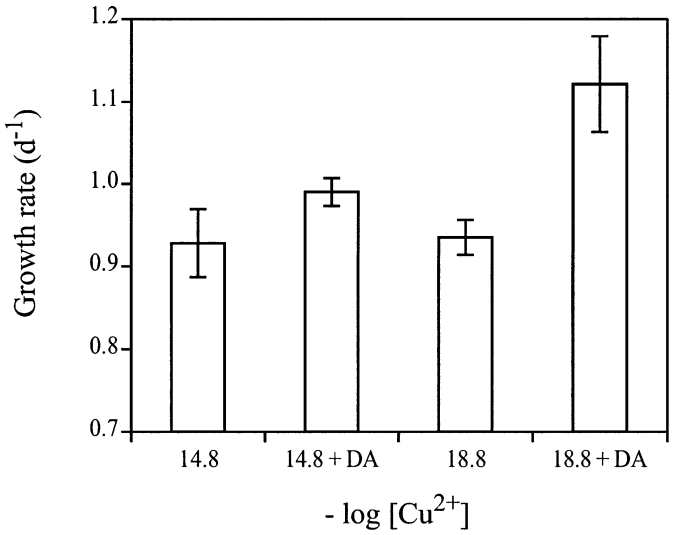

Fig. 6. Effect of DA addition on the growth rates of *P. fraudulenta* at optimal (pCu = 14.9) and low (pCu = 18.8) copper conditions. Data are the mean growth rates for successive transfers 5–9 in the EDTA-buffered medium. Error bars are ±1 SD.

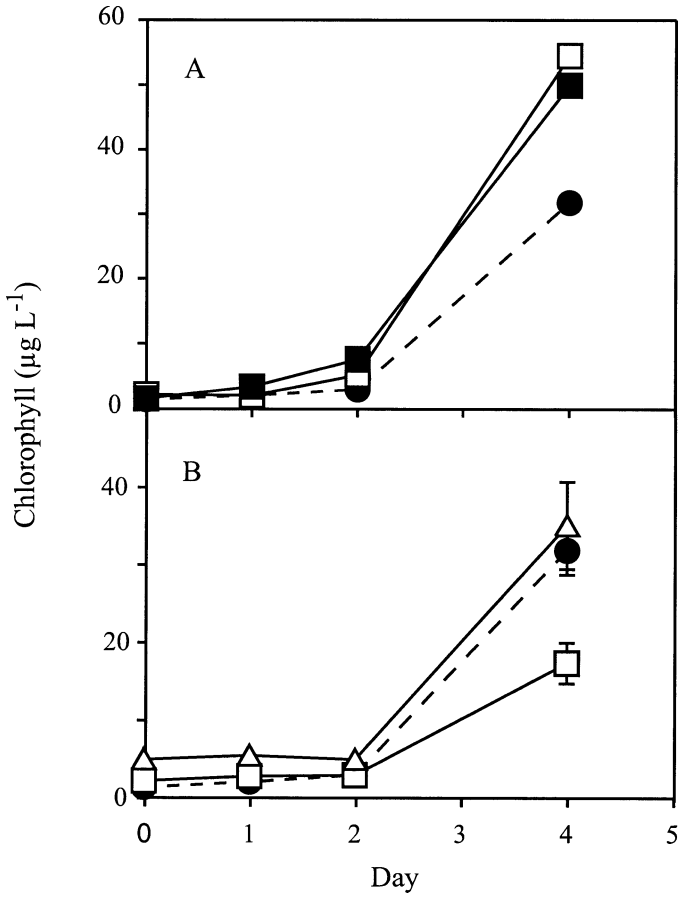

Fig. 7. (A) Growth response of the predominantly (80–100%) *Pseudo-nitzschia* phytoplankton assemblage in the Juan de Fuca eddy to amendments with iron (10 nmol L^{-1}—open squares) and dissolved DA (100 nmol L^{-1}—filled squares) compared to controls with no enrichment (filled circles). (B) A parallel incubation experiment showing phytoplankton biomass (chlorophyll *a*) as a function of time reveals cell growth inhibition with addition of ferrichrome (100 nmol L^{-1}—open squares) relative to the control (filled circles). Reversal of this iron deficiency is accomplished by copper amendment (2 nmol L^{-1}—open triangles). Error bars show the range for duplicate carboys.

conditions stimulating a much higher release of DA (Fig. 5B). Analogous relationships were observed for *P. multiseries* and *P. fraudulenta,* although cellular levels were lower and in some cases more variable at the time during which these experiments were conducted (data not shown).

To determine whether DA release to solution would generate any physiological benefit for the cells, *P. fraudulenta* was grown under favorable copper (pCu = 14.8) and copper-limiting (pCu = 18.8) conditions with and without the addition of 100 nmol L^{-1} dissolved DA. This level of DA is similar to the highest values measured off Monterey Bay, California (120 nmol L^{-1}, G. Doucette, NOAA Marine Biotoxins Program, Charleston, North Carolina, pers. comm.). All other metals were adjusted to their optimal free ion activities. The addition of dissolved DA improved growth rates slightly (7%) in the copper-favorable medium, relative to the control, but greatly improved growth rates (20%) of cells grown in the low pCu medium (Fig. 6). Addition of dissolved DA also improved algal growth rate by 20% for cells experiencing copper toxicity (pCu = 12.8) (data not shown).

The apparently strong relationship observed in laboratory experiments between iron, copper, and DA production and release by these toxigenic diatoms led us to test this linkage further with field incubation studies in the Juan de Fuca (Tully) eddy, off the mouth of the Strait of Juan de Fuca between British Columbia and Washington (Trainer et al. 2002). These waters contained up to 4×10^5 cells L^{-1} of *Pseudo-nitzschia delicatissima*/cf. *pseudodelicatissima* as well as *P. australis* and *Pseudo-nitzschia heimii,* and particulate DA concentrations of generally ~0.1–3.0 nmol L^{-1} (but up to 14 nmol L^{-1} at one site; Trainer et al. unpubl. data). Iron additions (10 nmol L^{-1}) significantly enhanced growth in these natural population cultures that comprised primarily (80–100%) *Pseudo-nitzschia* spp., demonstrating that these cells were experiencing iron stress (Fig. 7A). This finding was surprising because dissolved iron concentrations in these waters were >0.2 nmol L^{-1}; a level normally sufficient to support rapid growth of pennate diatoms (e.g., Wells 2003). The implication was that iron bioavailability was diminished by the complexation of iron with strong organic ligands (Wells et al. unpubl. data). Iron limitation in these natural assemblage cultures also was alleviated by the addition of 100 nmol L^{-1} dissolved DA (Fig. 7A). These dissolved DA additions also yielded the highest short-term (6-h) ^{59}Fe uptake rates of any treatment (Wells et al. unpubl. data), demonstrating a strong linkage between DA and iron acquisition in these iron-stressed cells.

Iron availability was significantly reduced by the addition of the nonmarine siderophore ferrichrome (100 nmol L^{-1}), evidenced by decreased phytoplankton biomass (Fig. 7B) and *Pseudo-nitzschia* cell counts (not shown). Ferrichrome addition also greatly decreased iron uptake rates (>80%) relative to the control (Wells et al. unpubl. data). However,

Table 1. Concentrations of *Pseudo-nitzschia* spp. and calculated domoic acid (DA) per cell from day 2 of the Juan de Fuca eddy deck incubation experiments described in Fig. 7, before macronutrients became depleted. Precision of the cell counts is ±7%. The mean and range for DA determinations in the duplicate treatment bottles are shown.

Treatment	*Pseudo-nitzschia* concentrations ($\times 10^5$ cells L^{-1})	Cellular DA (pg DA $cell^{-1}$)
Control	4.07±0.29	0.76±0.02
Iron added	4.53±0.32	0.60±0.01
Ferrichrome added	2.69±0.19	2.61±0.57
Ferrichrome+Cu	13.4±0.94	0.96±0.02
DA	5.69±0.40	0.70±0.11

the addition of copper (2 nmol L^{-1}) to a subset of the ferrichrome treatments ameliorated this inhibition of growth, so that final cell yields were indistinguishable from that achieved in control cultures (Fig. 7B).

Total cellular DA and direct counts of *Pseudo-nitzschia* cell abundance were used to calculate DA per cell on day 2 of the experiment, when cells still were in exponential growth and well before macronutrients were depleted (Cochlan et al. unpubl. data). Up to four *Pseudo-nitzschia* species may have been present in these samples, and not all may have been producing DA. But a first-order assumption of a uniform distribution of toxin among these species yielded cellular DA values from 0.6 to 2.6 pg DA $cell^{-1}$ (Table 1). The addition of iron decreased toxin levels by ~20% (0.76 to 0.60 pg DA $cell^{-1}$), while the addition of dissolved DA had little effect. Cellular toxin content increased three- to fourfold in the ferrichrome treatment (2.6 pg DA $cell^{-1}$), but this stimulation was not evident when the ferrichrome-treated cultures were supplemented with copper (2 nmol L^{-1}). These findings indicate that without sufficient iron or copper, natural assemblages of *Pseudo-nitzschia* will become more toxic.

Discussion

The progressive decrease in *T. weissflogii* growth rates with decreasing free ferric ion activity is the characteristic growth response reported for coastal diatoms and other marine eukaryotic phytoplankton (e.g., Sunda and Huntsman 1995). The growth response of toxigenic diatoms studied here was dramatically different and demonstrates that these organisms can adapt efficiently to iron stress. This adaptation apparently is not due to unusual reduction in cellular Fe : C requirements (Maldonado et al. 2002) but instead appears to represent an enhanced iron uptake capability in all three of these toxigenic *Pseudo-nitzschia* species. Given that the total dissolved iron concentrations were reasonably high in all of these media (e.g., pFe 21.4 = 2 nmol L^{-1} total Fe), it appears that these organisms circumvented iron limitation by stripping iron directly from the Fe-EDTA complexes, a capability that eukaryotic phytoplankton are not normally recognized to have (Hudson and Morel 1990).

The initial V-shaped growth response shown here as a function of inorganic iron availability is characteristic for many prokaryotic organisms and represents the induction of high-affinity iron uptake systems to augment normal iron acquisition pathways (Trick and Wilhelm 1995). Efficient high-affinity iron uptake capabilities have not been reported for eukaryotic phytoplankton, so it is noteworthy that all three of the toxigenic diatoms examined here displayed this unusual capability, contrasting sharply with other coastal phytoplankton (Sunda and Huntsman 1995). We speculate that this efficient high-affinity uptake system might functionally separate toxigenic from nontoxic diatoms and may help explain the persistence of *Pseudo-nitzschia* spp. in HNLC waters and in some coastal regions.

The relationship between cellular DA levels and the degree of iron stress changes from an inverse to a direct relationship with increasing acclimation time. The DA content per cell decreased with increasing iron stress during the early stages of the experiment (Fig. 2). This finding is consistent with our previous work showing that toxigenic diatoms initially respond to iron limitation by rapidly releasing DA to the surrounding medium (Maldonado et al. 2002), thereby lowering the cellular toxin load. However, this pattern reversed as cells acclimated (Fig. 2), so that cell toxicity increased with increasing iron ''stress'' (i.e., decreasing free inorganic Fe, though cell growth rates returned to optimal; Fig. 1B–D). Combined, these findings indicate there is a greater metabolic demand for DA as inorganic iron availability decreases and that the metabolic role of DA lies outside the cell. In other words, it appears that DA production in the iron-replete (pFe = 18.6) cells used in the initial transfer was insufficient to meet the extracellular need for DA, so that the enhanced release caused depletion of the intracellular DA reservoir. But as cells acclimated to the lower availability of inorganic iron (i.e., low pFe), DA production appeared to increase to match the elevated extracellular requirements. Evidence for this shift is the change in dissolved : particulate DA ratios from >1 (DA release > DA production) at low pFe before acclimation to a uniform value of ~0.3 after acclimation (Fig. 3). If this pattern indeed reflects a cell-induced homeostasis between intracellular and extracellular DA, then the relative partitioning of cellular and dissolved DA in natural waters may signal the degree to which the cells have acclimated to iron stress.

It is not likely that the cellular release of DA measured in these experiments is an uncontrolled process, even though DA is a low-molecular-weight compound. In all cases, cells remained in exponential growth, so membrane integrity should have remained high. The evolution of a uniform dissolved : particulate partitioning of DA during acclimation to iron stress also strongly indicates that cells regulate DA release. This enhanced release appears to directly benefit cells under iron limiting conditions. Adding dissolved DA to the naturally iron-stressed waters of the Juan de Fuca eddy enhanced growth of the mainly (80–100%) *Pseudo-nitzschia* assemblage (Fig. 7A) and increased short-term ^{59}Fe uptake rates (Wells et al. unpubl. data), as has been reported previously for laboratory cultures of *Pseudo-nitzschia* spp. (Maldonado et al. 2002). But it remains unclear what role, if any, DA serves in the high-affinity iron uptake system of the toxigenic diatoms shown here.

High-affinity iron uptake systems of bacteria often involve the production and release of siderophores—low-molecular-weight compounds having a high and specific affinity for iron. These molecules serve as competitive tools among bacteria and against eukaryotic organisms, whereby iron complexed to a given siderophore becomes available primarily to only the organism that produced it via cell surface transporters or reaction centers. The chemical speciation of dissolved iron in seawater is overwhelmingly controlled (>99%) by Fe(III)-complexing organic ligands of unknown origin having high binding affinities ($K^{\text{cond}}_{\text{FeL}_2,\text{Fe}'} \cong 10^{11}$–$10^{12}$ L mol^{-1}) (Rue and Bruland 1995). Siderophores obtained from cultures of marine bacteria have conditional binding constants near the upper range of marine ligands ($\sim 10^{12}$ L mol^{-1}) (Barbeau et al. 2003), indicating that siderophore-based iron uptake systems may be used widely by marine prokaryotes. But although DA can complex iron in seawater, it seems an unlikely candidate for a eukaryotic ''siderophore'' because its conditional Fe(III)-binding constant ($K^{\text{cond}}_{\text{FeDA},\text{Fe}'} \cong 10^{8.7}$ L mol^{-1}) (Rue and Bruland 2001) is too low to compete effectively with the ambient organic ligands, other than at very high DA concentrations ($\sim$100 nmol L^{-1}) (Rue and Bruland 2001). Such high levels are found rarely during natural blooms of *Pseudo-nitzschia* (Trainer et al. unpubl. data), although they have been observed on occasion (G. Doucette, NOAA Marine Biotoxin Program pers. comm.). So if DA indeed facilitates iron uptake in natural waters, as appears to be the case for the Juan de Fuca eddy assemblages, it likely does so via an indirect process.

The common yeast *Saccharomyces cerevisiae* has evolved to utilize iron bound to bacterial siderophores even though it is not known to produce these molecules (Dancis et al. 1994; Crichton and Pierre 2001). Fe(III)-siderophore complexes diffusing to *S. cerevisiae* cell surfaces are reduced by surface-bound ferrireductases, thus liberating Fe(II) from the chelates (Crichton and Pierre 2001). Although yeast have low-affinity Fe(II) transporters, most of the transient free Fe(II) produced under iron stress is quickly reoxidized by membrane-associated multi-copper oxidases before it diffuses away (Dancis et al. 1994). These oxidases, in turn, are linked closely with high-affinity Fe(III) transporters, enabling the sequential oxidation and uptake of iron (Fig. 8). This rapid redox cycling on the outer membrane enables *S. cerevisiae* to pirate iron from siderophore complexes otherwise destined for its prokaryotic neighbors. Of particular note here is that this high-affinity uptake system requires an adequate copper supply.

Copper also is essential for the adaptation of *P. multiseries, P. australis,* and *P. fraudulenta* to iron stress. Growth of *P. multiseries* and *P. australis* remained optimal under either iron or (low) copper stress, but decreased by 40–70% when both metals were deficient (Fig. 4A,B). These results demonstrate a synergism between iron and copper that would be consistent with the well-described high-affinity iron uptake system of *S. cerevisiae,* and indicates that copper is a key factor facilitating the success of toxigenic diatoms in iron-deficient waters. *P. fraudulenta* showed a similar negative impact (80%) from simultaneous iron and copper deficiency, but its growth rates also decreased by $\sim$40% under either low iron or low copper in this experimental series

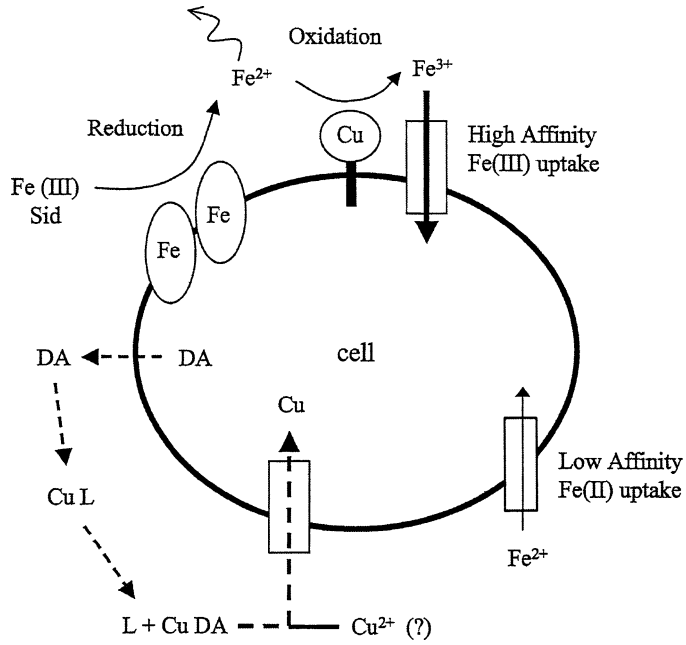

Fig. 8. Conceptual model of the high-affinity iron uptake system found in *S. cerevisiae,* comprising a membrane-bound, iron-containing iron reductase, a multi-copper iron oxidase, and high-affinity Fe(III) transporter. In this sequence, organically bound Fe(III) is reduced, releasing Fe(II) from the complex. This Fe(II) may have a lifetime of minutes to hours depending on temperature, so before diffusion transports Fe(II) away for the cell it is oxidized enzymatically, with the resulting Fe(III) being bound and transported into the cell. Copper uptake in this case is shown modulated by the release of DA; a primary hypothesis evolved from the findings presented here. Modified from Crichton and Pierre (2001).

(Fig. 4C). The high-affinity iron uptake system of *P. fraudulenta* thus appears to be less efficient than that noted in either *P. multiseries* or *P. australis,* although a longer acclimation time in this case may have eliminated these differences. *P. fraudulenta* also produced by far the lowest levels of DA of the three species examined.

In addition to iron, DA also complexes copper in seawater (Rue and Bruland 2001), and although DA production doubles under iron stress (Fig. 5A), it increases by an order of magnitude under copper limiting conditions (Fig. 5A). Similar increases in DA production occur under toxic copper conditions and are presumed to reduce copper toxicity in *P. multiseries* and *P. australis* (Maldonado et al. 2002; Ladizinsky 2003). Furthermore, these laboratory results have been reinforced by recent field observations in which strong correlations between elevated (toxic) Cu and DA per cell have been established (Ladizinsky 2003).

The sharp enhancement of DA production caused by copper limitation shown here indicates that while DA production is linked to iron stress, it is more closely tied with copper supply to the cell. Additions of DA to laboratory strains grown in low copper medium (pCu 18.8) increased cell growth rates by 20% (0.9 $\pm$ 0.04 d^{-1} to 1.1 $\pm$ 0.06 d^{-1}) (Fig. 6), indicating that dissolved DA facilitates copper acquisition by the cell. How might DA enhance copper acquisition by these diatoms in natural seawater?

Copper uptake by marine phytoplankton has been shown in laboratory cultures to be dependent on the free cupric ion (Cu^{2+}) activity (Moffett and Brand 1995). However, as seen for iron, most copper in seawater is complexed by organic ligands (Moffett and Brand 1995) released at least in part by marine cyanobacteria, which are particularly sensitive to copper toxicity (Moffett and Brand 1995). This copper complexation also reduces copper availability to eukaryotic phytoplankton and that, in turn, could restrict the induction of (copper-reliant) high-affinity iron uptake systems. However, DA released to seawater could effectively compete for Cu(II) relative to the weaker class of copper-binding organic ligands measured in nearshore seawaters [log $K^{\text{cond}}_{\text{CuL}_2,\text{Cu}'} \cong 9.0$–9.5 L mol^{-1} (Donat et al. 1994) vs. log $K^{\text{cond}}_{\text{CuDA},\text{Cu}'} \cong 9.0$–9.6 L mol^{-1} (Rue and Bruland 2001)]. Ladizinsky (2003) reported a slightly higher conditional constant for DA (log $K^{\text{cond}}_{\text{CuDA},\text{Cu}'} \cong 10.7$, corrected for $\alpha_{Cu} = 1.3$), but this does not take into account the unknown side reaction coefficient for DA (α_{DA}) in seawater. Other marine eukaryotic phytoplankton have been shown to release copper complexing ligands having similar conditional constants for copper (Croot et al. 2000), but, as for *Synechococcus*, this release has been only suggested to decrease copper toxicity. We hypothesize that *Pseudo-nitzschia* spp. actively release DA under iron stress to facilitate copper acquisition, thereby allowing induction of their high-affinity transport system to access iron bound to strong ligands (Fig. 8).

Compelling field evidence for a physiological role of DA as a facilitator for iron uptake comes from deck incubation studies conducted in the *Pseudo-nitzschia*–rich waters of the Juan de Fuca (Tully) eddy. Iron enrichment resulted in increased growth of the primarily *Pseudo-nitzschia* assemblage during multi-day incubation experiments, showing that these cells were experiencing iron stress in situ (Fig. 7A). The addition of DA to these samples caused similar increases in growth (Fig. 7A) and yielded the highest iron uptake rates of any treatment (Wells et al. unpubl. data).

In contrast, adding the nonmarine siderophore ferrichrome to these waters significantly decreased iron availability to *Pseudo-nitzschia*, evidenced by the dramatic reduction in biomass (Fig. 7B) and by a sharp decline in iron uptake rates (>80%) compared to control cultures (data not shown). This result is consistent with findings that eukaryotic cells in general have difficulty accessing strong Fe-ligand complexes (Hutchins et al. 1999). However, small amendments of copper (2 nmol L^{-1}) enabled *Pseudo-nitzschia* growth to recover in these siderophore treatments. Given that there is little functional substitution of copper for iron in cellular metabolism, these findings support the hypothesis that toxigenic diatoms can efficiently access siderophore-bound iron, provided the availability of copper is adequate.

The DA per cell in the natural assemblage cultures decreased upon iron enrichment but increased several-fold when iron availability was lowered by ferrichrome addition (Table 1). The same result was observed in our laboratory experiments (Figs. 2, 5). However, cellular DA content decreased in the ferrichrome treatment if a low concentration (2 nmol L^{-1}) of copper was added (Table 1). This observation is consistent with our hypothesized scenario (Fig. 8), whereby *Pseudo-nitzschia* would not need to produce as much DA under iron stress when copper was already readily available. The implication is that in the absence of adequate copper availability, iron limited natural populations of *Pseudo-nitzschia* will become more toxic.

The laboratory growth experiments here lack an ideal control, namely a nontoxic species of *Pseudo-nitzschia*. Although it is generally accepted that there are a number of nontoxic species of *Pseudo-nitzschia*, our experience indicates that this view may not be correct. The *P. fraudulenta* strain used here was chosen initially because the strain was not believed to produce DA. However, analytical sensitivity of the ELISA method used here is considerably better than previous methods, and DA production was detected, albeit in much lower quantities than either *P. multiseries* and *P. australis*. Based on our finding, and on the fact that at least one species of the related *Nitzschia* genus also produces DA (Kotaki et al. 2000), we believe it is reasonable to anticipate that all *Pseudo-nitzschia* species produce DA to some degree, though not all will produce enough to generate toxicity at higher trophic levels under all conditions.

Our previous work has indicated linkages between iron, copper, and the release of DA by toxigenic diatoms (Maldonado et al. 2002). The findings here indicate that this linkage is synergistic and can be modeled similarly to the copper-dependent, high-affinity iron acquisition system of *S. cerevisiae*. This high-affinity iron uptake system has been identified in the green alga *Chlamydomonas* (La Fontaine et al. 2002), and genetic homologues for these proteins are found in the genome of the marine diatom *Thalassiosira pseudonana* (Armbrust et al. 2004). In this scenario, decreasing pools of available iron, stemming from low dissolved iron concentrations and the presence of strong iron-complexing ligands, induce a high-affinity iron uptake system in toxigenic diatoms that, in turn, increases their copper requirements. DA appears to enhance copper uptake in laboratory cultures of *Pseudo-nitzschia* and should function similarly in coastal waters. But the success of this strategy would depend upon the concentration and conditional stability constants of the ambient copper-complexing ligands in seawater. It is notable, then, that species of the marine prokaryote genus *Synechoccoccus* abundant in HNLC waters releases not only siderophores to facilitate iron uptake (Wilhelm and Trick 1994), but also very strong copper-complexing organic ligands (Moffett and Brand 1995) that would challenge weaker ligands, like DA, for copper. We speculate that very high conditional stability constants of the copper-binding compounds released by some marine prokaryotes ($K^{\text{cond}}_{\text{CuL}_1,\text{Cu}'} \cong 10^{13}$ L mol^{-1}) (Moffett and Brand 1995) may function primarily to limit the piracy of their siderophore-bound iron by eukaryotic competitors. If true, then gaining a predictive understanding of the factors regulating iron availability to marine phytoplankton, and of their effects on global climate change, will require an understanding of the marine biogeochemical cycles of both iron and copper.

References

ANDERSON, M. A., AND F. M. M. MOREL. 1982. The influence of aqueous iron chemistry on the uptake of iron by the coastal diatom *Thalassiosira weissflogii*. Limnol. Oceanogr. **27:** 789–813.

ARMBRUST, E. V., AND OTHERS. 2004. The genome of the diatom *Thalassiosira pseudonana*: Ecology, evolution, and metabolism. Science **306:** 79–86.

BARBEAU, K., E. L. RUE, C. G. TRICK, K. W. BRULAND, AND A. BUTLER. 2003. Photochemical reactivity of siderophores produced by marine heterotrophic bacteria and cyanobacteria by characteristic Fe(III) binding groups. Limnol. Oceanogr. **48:** 1069–1073.

BATES, S. S., A. S. W. DEFREITAS, J. E. MILLEY, R. POCKLINGTON, M. A. QUILLIAM, J. C. SMITH, AND J. WORMS. 1991. Controls on domoic acid production by the diatom *Nitzschia pungens f. multiseries* in culture: Nutrients and irradiance. Can. J. Fish. Res. Aquat. Sci. **48:** 1136–1144.

BRAND, L. E., W. G. SUNDA, AND R. R. L. GUILLARD. 1983. Limitation of marine phytoplankton reproductive rates by zinc, manganese, and iron. Limnol. Oceanogr. **28:** 1182–1198.

COALE, K. H. 1991. Effects of iron, manganese, copper, and zinc enrichments on productivity and biomass in the subarctic Pacific. Limnol. Oceanogr. **36:** 1851–1864.

CRICHTON, R. R., AND J.-L. PIERRE. 2001. Old iron, young copper: From Mars to Venus. BioMetals **14:** 99–112.

CROOT, P. L., J. W. MOFFETT, AND L. E. BRAND. 2000. Production of extracellular Cu complexing ligands by eucarotyic phytoplankton in response to Cu stress. Limnol. Oceanogr. **45:** 619–627.

DANCIS, A., D. S. YUAN, AND D. HALLE. 1994. Molecular characterization of a copper transport protein in S. cerevisiae: An unexpected role for copper in iron transport. Cell **76:** 393–402.

DONAT, J. R., K. A. LAO, AND K. W. BRULAND. 1994. Speciation of dissolved copper and nickel in south San Francisco Bay—A multi-method approach. Anal. Chim. Acta **284:** 547–571.

GUILLARD, R. R. L. 1973. Division rates, p. 290–311. *In* J. R. Stein [ed.], Handbook of phycological methods—Culture methods and growth measurements. Cambridge Univ. Press.

———, AND P. E. HARGRAVES. 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia **32:** 234–236.

HARRISON, P. J., P. W. BOYD, D. E. VARELA, S. TAKEDA, A. SHIOMOTO, AND T. ODATE. 1999. Comparison of factors controlling phytoplankton productivity in the NE and NW subarctic Pacific gyres. Prog. Oceanogr. **43:** 205–234.

HUDSON, R. J. M., AND F. M. M. MOREL. 1990. Iron transport in marine phytoplankton: Kinetics of cellular and medium coordination reactions. Limnol. Oceanogr. **35:** 1002–1020.

HUTCHINS, D. A., G. R. DITULLIO, Y. ZHANG, AND K. W. BRULAND. 1998. An iron limitation mosaic in the California upwelling regime. Limnol. Oceanogr. **43:** 1037–1054.

———, A. E. WITTER, A. BUTLER, AND G. W. LUTHER. 1999. Competition among marine phytoplankton for different chelated iron species. Nature **400:** 858–860.

KELLER, M. D., W. K. BELLOWS, AND R. R. L. GUILLARD. 1988. Microwave treatment for sterilization of phytoplankton culture media. J. Exp. Mar. Biol. Ecol. **117:** 279–283.

KOTAKI, Y., AND OTHERS. 2000. Domoic acid production in Nitzschia isolated from a shrimp-culture pond in Do Son, Vietnam. J. Phycol. **36:** 1057–1060.

LADIZINSKY, N. L. 2003. The influence of dissolved copper on the production of domoic acid by *Pseudo-nitzschia* species in Monterey Bay, California: Laboratory experiments and field observations. M.Sc. thesis, California State University, Monterey Bay.

LA FONTAINE, S., AND OTHERS. 2002. Copper-dependent iron assimilation pathway in the model photosynthetic eukaryote *Chlamydomonas reinhardtii*. Eukaryotic Cell **1:** 736–757.

MALDONADO, M. T., M. HUGHES, E. RUE, AND M. L. WELLS. 2002. The effect of Fe and Cu on the growth and domoic acid production of *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis.* Limnol. Oceanogr. **47:** 515–526.

———, AND N. M. PRICE. 2001. Reduction and transport of organically bound iron by *Thalassiosira oceanica* (Bacillariophyceae). J. Phycol. **37:** 298–309.

MOFFETT, J. W., AND L. E. BRAND. 1995. Production of strong, extracellular Cu chelators by marine cyanobacteria in response to Cu stress. Limnol. Oceanogr. **41:** 388–395.

NEILANDS, J. B. 1995. Siderophores—structure and function of microbial iron transport compounds. J. Biol. Chem. **270:** 26723–26726.

PAN, Y., D. V. S. RAO, AND K. H. MANN. 1996. Changes in domoic acid production and cellular chemical composition of the toxigenic diatom *Pseudo-nitzschia multiseries* under phosphate limitation. J. Phycol. **32:** 371–381.

PARSONS, T. R., Y. MAITA, AND C. LALLI. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon.

PRICE, N. M., G. I. HARRISON, J. G. HERING, R. J. HUDSON, M. V. N. PASCALE, G. PALENIK, AND F. M. M. MOREL. 1989. Preparation and chemistry of the artificial algal culture medium aquil. Biol. Oceanogr. **6:** 443–462.

RUE, E., AND K. W. BRULAND. 2001. Domoic acid binds iron and copper: A possible role for the toxin produced by the marine diatom *Pseudo-nitzschia.* Mar. Chem. **76:** 127–134.

———, AND ———. 1995. Complexation of Fe(III) by natural organic ligands in the central North Pacific as determined by a new competitive ligand equilibration adsorptive cathodic stripping voltammetric method. Mar. Chem. **50:** 117–138.

SCHECHER, W. D., AND D. C. MCAVOY. 1998. MINEQL+: A chemical equilibrium modeling system, Version 4 for Windows, Users Manual. Environmental Research Software.

SUNDA, W. G., AND S. A. HUNTSMAN. 1995. Iron uptake and growth limitation in oceanic and coastal phytoplankton. Mar. Chem. **50:** 189–206.

———. 1998. Interactions among Cu^{2+}, Zn^{2+}, and Mn^{2+} in controlling cellular Mn, Zn and growth rate in the coastal algal *Chlamydomonas.* Limnol. Oceanogr. **43:** 1055–1064.

———. 2003. Effect of pH, light, and temperature on Fe-EDTA chelation and Fe hydrolysis in seawater. Mar. Chem. **84:** 35–47.

TRAINER, V. L., AND OTHERS. 2000. Domoic acid production near California coastal upwelling zones, June 1998. Limnol. Oceanogr. **45:** 1818–1833.

———, B. M. HICKEY, AND R. A. HORNER. 2002. Biological and physical dynamics of domoic acid production off the Washington Coast. Limnol. Oceanogr. **47:** 1438–1446.

TRICK, C. G., AND S. W. WILHELM. 1995. Physiological changes in the coastal marine cyanobacterium *Synechococcus* sp. PCC7002 exposed to low ferric ion levels. Mar. Chem. **50:** 207–217.

TSUDA, A., AND OTHERS. 2003. A mesoscale iron enrichment in the Western Subarctic Pacific induces a large centric diatom bloom. Science **300:** 958–961.

WELLS, M. L. 1999. Manipulating iron availability in nearshore waters. Limnol. Oceanogr. **44:** 1002–1008.

———. 2003. The level of iron enrichment required to initiate diatoms blooms in HNLC waters. Mar. Chem. **82:** 101–114.

———, I. GARTHWAITE, M. P. HUGHES, K. ROSS, M. BRIGGS, AND N. R. TOWERS. 2002. Evaluation of cELISA and an analytical tool for measuring sub-nanomolar concentrations of domoic acid in phytoplankton and seawater. Tenth International Conference on Harmful Algae.

———, AND C. G. TRICK. 2004. Controlling iron availability to phytoplankton in iron-replete coastal waters. Mar. Chem. **86:** 1–13.

WILHELM, S., AND C. G. TRICK. 1994. Iron-limited growth of cyanobacteria: Multiple siderophore production is a common response. Limnol. Oceanogr. **39:** 1979–1984.

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