BIOGEOCHEMICAL CYCLING OF DOMOIC ACID AND ITS ISOMERS IN THE OCEAN

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ABSTRACT

Domoic acid, a non-protein amino acid, is a neurotoxin produced by species of the marine diatom *Pseudo-nitzschia.* Adsorption of dissolved domoic acid (DA) and its geometrical isomers (iso-DAs D, E, and F) was assessed in filtered seawater solutions containing various types of particles. Adsorption of DA occurred onto natural seawater particles and chromatographic silica. Slight DA adsorption occurred onto estuarine bottom sediment and humic acid. No DA losses were seen in the presence of illite, kaolinite, montmorillonite, or silica sand. Biological degradation accounted for DA losses in seawater without particles. Iso-DAs D, E, and F showed negligible adsorption onto particles, suggesting DA isomers are hydrophilic and not particle reactive. These results suggest that adsorption onto particles is not an important sink for DA or its photochemically produced isomers in natural waters.

Another facet of domoic acid's cycling is its interaction with metals present in natural waters. Results of competitive ligand equilibration/ adsorptive cathodic stripping voltammetric studies (CLE-ACSV) reveal that domoic acid forms a chelate with copper with a conditional stability constant: $K^{cond}_{CuDA,Cu^{2+}} = 6.7 \times 10^{11}\ M^{-1}$. Speciation experiments were also performed to determine binding abilities of DA with 22 nM Zn, 0.5 nM Cd, 0.5 nM Co, and 20 nM Ni in the presence of 5 nM Cu. None of these metals were able to outcompete Cu for DA, suggesting in a natural water matrix Cu is the dominant of these metal chelators.

A second set of experiments designed to determine the metal binding abilities of DA and two of its geometrical isomers, iso-DAs E and F utilized excitation-emission matrix (EEM) fluorescence spectroscopy. EEMs of 0.1 μM DA and 0.1 μM Fe, compared to EEMs of 0.1 μM DA alone, exhibited an increase in fluorescence resulting from the complexation of DA to Fe. Scans repeated with 0.1 μM added Cu also exhibited an increase in fluorescence consistent with

the strong complexation of DA with Cu determined electrochemically. In contrast to DA, its geometrical isomers iso-DAs E and F showed significant quenching of fluorescence upon addition of both iron and copper, indicating that iso-DAs E and F also form complexes with iron and copper. Results of these studies have important ecological implications because they demonstrate that DA and iso-DAs E and F form complexes with both Cu and Fe. The formation of these complexes may affect the toxicity of Cu, the bioavailability of Fe, and the toxicity of DA and its geometrical isomers in natural waters where the blooms occur.

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INTRODUCTION

Little is known about the biogeochemical cycling and fate of marine toxins, particularly that fraction of compounds produced during bloom events not bioaccumulated and transferred through the food chain. This is the case for the marine toxin, domoic acid, a globally dispersed toxin produced by several *Pseudo-nitzschia* diatom species. Domoic acid (Figure 1), a non-protein amino acid, is a potent glutamate agonist (Hampson et al. 1998), and as such is toxic to marine anthropods that graze on diatoms and other phytoplankton (Shaw et al. 1997; Windust, 1992). It has proven to be a threat to public health and some marine life in areas where it is most prominent, particularly North American coastal waters. It was originally discovered in eastern Canada in 1987 as the causative agent in an episode of fatal human poisoning (Wright et al., 1989; Todd, 1993). Domoic acid has been the cause of severe economic losses in the shellfish and crustacean harvesting industry of this area and off the Western coast of North America, and has resulted in the death of many seabirds (Work et al., 1993; Sierra-Beltran et al., 1997) and mammals (Lefebvre et al., 2002; Scholin et al., 2000).

The environmental impact of domoic acid as a toxic compound capable of altering biochemical processes greatly depends upon its residence time and fate in natural waters. Although domoic acid may be degraded by bacterial processes within certain shellfish tissues (e.g., *Mytilus edulis*; Stewart et al., 1998), it has been found in the tissues of a wide variety of marine species, including both pelagic and benthic fish, shellfish, crustaceans, and marine mammals (Lefebvre et al., 2002; Bargu et al., 2003). Many filter-feeding marine species have accumulated domoic acid in their digestive tracts, such as northern anchovies (*Engraulis mordax*), razor clams (*Siliqua patula*), and blue mussels (*Mytilus edulis*) (Quilliam and Wright 1989; Wekell et al. 1994; Altwein et al. 1995; Lefebvre et al. 1999). Some of these species can

be important vectors for DA transfer to organisms at higher trophic levels, especially the northern anchovy (Lefebvre et al. 2001). A toxic bloom of *P. australis* in Monterey Bay, California in 2000 resulted in maximum cell concentrations of greater than 2 x 10^6 cells L^{-1}, and dissolved domoic acid concentrations varying from undetectable to 130 nM (Doucette et al., 2002).

Domoic acid has also accumulated in Portuguese shellfish and greatly affects the shellfish industry. Common cockle (*Cerastoderma edule*) and carpet shell (*Venerupis pullastra*) are two shellfish species that tend to be the most toxic (Vale et al., 2001). Domoic acid and iso-DA D were detected in plankton, shellfish, and fish samples (Vale et. al., 2001). High DA concentrations, reaching 323.1 µg DA/g crab tissue, were reported off the Portuguese coast in 2002 in the swimming crab *Polybius henslowii*. *P. henslowii* is likely an important vector of domoic acid to higher trophic levels such as fish and sea birds in both pelagic and benthic communities (Costa et al., 2003). Towards the end of a *Pseudo-nitzschia* bloom, diatoms containing DA can sink to the seafloor and survive for a short amount of time, allowing benthic communities and nektobenthic species such as cuttlefish to uptake domoic acid (Costa 2005).

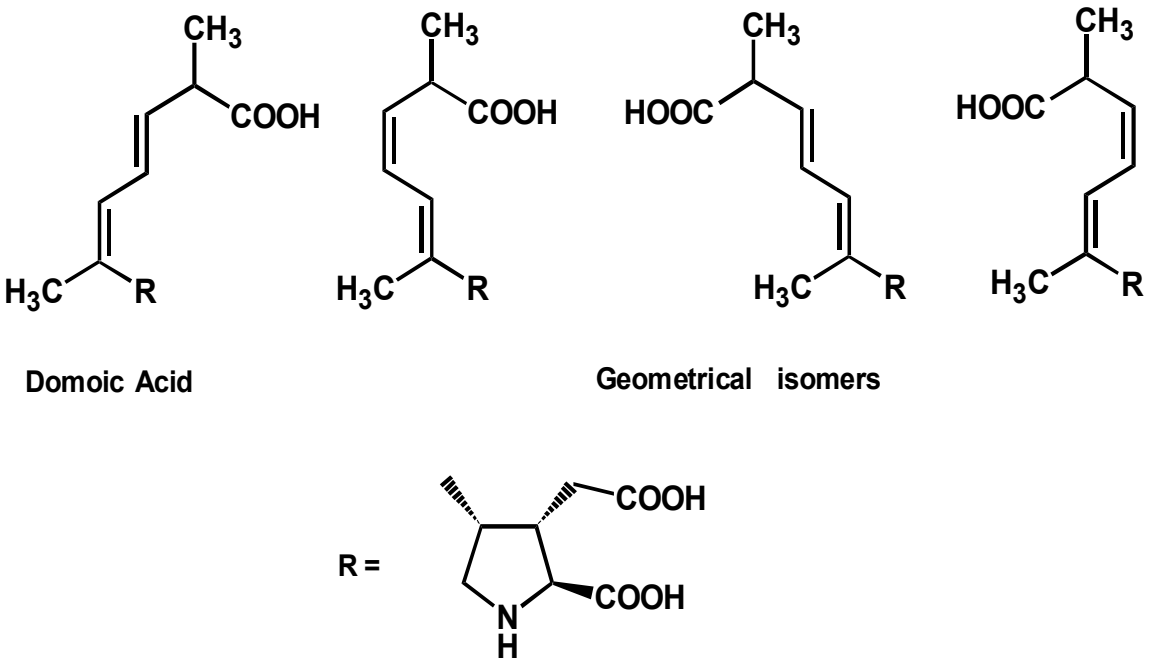


Figure 1. Domoic acid and its geometrical isomers, Iso-domoic acids D, E, and F (Wright et al., 1990).

Virtually nothing is known regarding the biogeochemical cycling and ultimate fate of domoic acid once it is released into the water column. It is known that exposure of domoic acid to sunlight yields a series of three geometrical isomers (Fig. 1), iso-DAs D, E and F (Wright et al., 1990). Domoic acid is most likely degraded through a direct photochemical pathway (Bouillon et al. 2006). Studies have suggested the domoic acid isomers are less potent than domoic acid itself, whereas those isomers with Z configuration at the double bond closest to the ring (like domoic acid) have maximum potency (Hampson, 1992). In addition, exposure of domoic acid to artificial sunlight in seawater for 24 h yielded a group of less polar products believed to be decarboxylated derivatives of domoic acid (Bouillon, unpublished data).

This research focuses on understanding the biogeochemical cycling and fate of domoic acid in the ocean. One aspect of the cycling of domoic acid involves particle interactions. Abiotic and biotic particles can influence the biogeochemical cycling of dissolved algal toxins and other organic compounds in many ways. For example, Satterberg et al, (2003) demonstrated that several common types of clay minerals (chlorite, montmorillonite, and kaolinite) effectively adsorbed a large fraction of soluble intracellular matter produced by four common phytoplankton species. Specifically, the cyanobacterial toxin, microcystin-LR, was efficiently scavenged (up to 81% of total) by clay particles (Morris et al., 2000). Inorganic particles may similarly scavenge domoic acid, leading to its removal to the particulate phase, thereby altering its availability to organisms. Numerous studies report the ability of clay minerals to remove various toxic algal cells, including domoic acid producers, by coagulation (Yu et al., 1995; Zhiming et al., 1998; Sengco et al., 2001). In addition, Hedges and Hare (1987) observed varying degrees of adsorption of 15 protein amino acids onto kaolinite and montmorillonite clay minerals. Thus,

cellular material containing domoic acid may be subjected to removal and possibly biological and chemical degradation processes occurring at particle-water interfaces.

The adsorption of dissolved domoic acid (DA) was previously assessed in aqueous solutions containing various types of particles. The results suggested that DA is relatively hydrophilic and not particle reactive (Lail et al., manuscript submitted). One goal of the research presented here is to evaluate the adsorption behavior of iso-DAs E and F in seawater solutions containing various particles including kaolinite, montmorillonite, illite, and estuarine sediment. The substances chosen represent particle types commonly found in the ocean.

Another facet of the cycling of domoic acid is its interaction with metals present in natural waters. Rue and Bruland (2001) demonstrated that domoic acid is an effective chelator for Cu(II) and Fe(III), both of which are bioactive trace metals of vital importance to a variety of biochemical processes occurring in natural waters. This is not completely unexpected since the carboxyl groups in domoic acid can act as electron donors in the chelation of such metal cations. Trace metal chelation is an important factor controlling the biogeochemical cycling and ultimate fate of domoic acid in natural waters.

Results of Maldonado et al (2002) suggest that domoic acid-producing strains of *Pseudo-nitzschia* may have a competitive advantage over other species of phytoplankton that do not produce this toxin since DA has a strong affinity for Fe and Cu in seawater. Results from these studies indicated that DA production by actively growing *P. multiseries* and *P. australis* is a direct response to bioactive metal stress. For example, Fe complexation by domoic acid may facilitate cellular uptake in Fe-limited waters in which *Pseudo-nitzschia* grows (Hutchins and Bruland, 1998; Maldonado et al., 2002). Some eukaryotic phytoplankton release strong, Fe-specific ligands under Fe-limiting conditions to facilitate Fe uptake (Trick et al., 1983). The rate

of Fe uptake by toxigenic species is apparently increased by DA (Maldonado et al., 2002). Thus it is very likely that cellular release of DA is a controlled process and a response to iron limitation (Wells et al., 2005).

Copper complexation by domoic acid may serve to reduce concentrations of free hydrated Cu(II), which is widely recognized as a toxic form of Cu for marine phytoplankton (Sunda and Guillard, 1976; Brand et al., 1986; Moffett and Brand, 1996). Certain marine species, such as the cyanobacteria *Synechococcus*, are sensitive to free Cu(II) levels as low as 10^{-11} M (Brand et al., 1986). Moffett and Brand (1996) demonstrated that *Synechococcus* produces a strong ligand under Cu-stressed conditions in coastal waters, presumably as a detoxifying mechanism. In a similar manner, DA release may be an effective strategy for toxic strains of *Pseudo-nitzschia* to deal with Cu toxicity (Maldonado et al., 2002).

The observation of production of domoic acid photoisomers under UV radiation (254 nm) (Wright and Quilliam, 1995) raises several questions regarding the environmental cycling and fate of domoic acid. For example, are the photoisomers, iso-DAs E and F, particle-reactive? Do isomers of DA complex Fe(III) and Cu(II)? Can other trace metals such as Zn(II), Cd(II), or Ni(II) outcompete Cu(II) to complex with DA?

METHODS

Reagents and Standards

DA (95%) used for adsorption experiments and to synthesize its geometrical isomers was obtained from MP Biomedical, LCC (Aurora, OH). Fluorenylmethoxycarbonyl (FMOC, 99.0%) was obtained from Fluka Chemical (Buchs, Switzerland). Acetonitrile (HPLC grade) was purchased from Burdick & Jackson (Muskegon, MI). Acetonitrile was pre-filtered through a 0.2 μm GTTP filter membrane (Millipore®). Reagent grade boric acid and fine granular silica sand

were obtained from Fisher Scientific Company (Fair Lawn, NJ). Ethyl acetate, 230-400 mesh chromatographic silica gel, and technical grade humic acid were acquired from Sigma-Aldrich Co. (St-Louis, MA). Ethyl alcohol was supplied from AAPER Alcohol and Chemical Co (Shelbyville, KY). Trifluoroacetic acid (TFA, 99.5%) was obtained from Alfa Aesar (Ward Hill, MA). Kaolinite (Ward's API #9), Na-montmorillonite, illite, and dried surface sediment originated from Mesa Alta, NM, Clay Spur, WY, Silver Hill, MT, and upper Delaware Bay, respectively. All aqueous solutions were prepared with deionized water (DIW) supplied from a Millipore water purification system (Millipore Corp., Bedford, MA) and have a resistance greater than 18 MΩ cm^{-1}.

Solutions for adsorption and metal binding studies were prepared using both DIW and coastal seawater. Natural seawater samples (WBSW) were collected in 10-L fluorinated polyethylene (FLPE) carboys from Wrightsville Beach, North Carolina (34.2°N; 77.8°W). Seawater samples were filtered through a Meissner® Stylux 0.2-μm (500-cm^2) filter capsule pre-rinsed with seawater. Prior to sample collection, filter capsules were pre-cleaned with acidic solution (pH 2 Fisher Trace Metal grade HCl) followed by DIW. Seawater samples were stored in the dark at 5°C.

All bottles used to collect water or hold treatments were rinsed with DIW three times and stored in 10% HCl for at least two days. All glassware was rinsed with DIW three times, rinsed with methanol, and rinsed twice with DIW before use. High-density polyethylene (HPLE) and fluorinated high-density polyethylene (FHDPE) bottles were used to hold samples.

Production and purification of DA geometrical isomers

Iso-DAs D, E, and F were produced photochemically following procedures adapted from Wright and colleagues (Wright et al., 1990; Walter et al., 1994). A solution of 321 μmol L^{-1} DA

in methanol was irradiated in a quartz chamber with UV radiation from a 1000 W mercury lamp for approximately 12 min. Individual geometrical isomers of DA were separated from the mixture by semi-preparative HPLC. The HPLC system was a Hewlett-Packard Model 1100 Series. A C_{18} reversed phase column (25 cm × 10.0 mm; 10 μm; Vydac 201TP column) was used for chromatography, and elution at room temperature with the mobile phase 8% acetonitrile/92% DIW at 5 ml min^{-1}. Effluent from the column was continuously monitored with a UV detector at 242 nm. Each peak corresponding to a fraction was collected manually based on visual inspection of the chromatogram. After pooling all fractions containing the same isomer, the solvent was evaporated to dryness under reduced pressure and re-dissolved into an aqueous solution of acetonitrile (10%).

Quantification of DA, Iso-DAs

The concentrations of dissolved DA and iso-DAs were determined using a modified version of the fluorenylmethyoxycarbonyl-high performance liquid chromatography (FMOC-HPLC) method described by Pocklington et al. (1990). This method is based on fluorometric detection after a pre-column reaction to produce fluorescent derivatives separable by HPLC. The fluorenylmethyl chloroformate (FMOC-Cl) reagent solution was prepared according to the method described by Pocklington et al. (1990). Approximately 1.0 g of 9-fluorenylmethyl chloroformate (Lancaster) was weighed into a 250 mL volumetric flask and made to volume with 0.2 μm filtered acetonitrile. The solution was then placed into 2 mL glass vials, purged with nitrogen for ~25 s, capped with Teflon-lined caps, and placed in a desiccator at -20ºC. Vials were removed from the freezer as needed, and any unused portion was discarded.

A 1 M borate buffer solution was prepared by dissolving 6.18 g of orthoboric acid in 95 mL DIW. The pH of the solution was adjusted to 6.2 with 5 M sodium hydroxide. The volume

of buffer was then brought to 100 mL with DIW. The borate buffer was filtered through a 0.2 μm filter on a weekly basis in order to remove particles that may have precipitated out of solution.

Domoic acid and iso-domoic acid samples were derivatized by combining 400 μL of sample, 40 μL of borate buffer, and 500 μL of FMOC-Cl in a 3.5 mL glass test tube. The sample was shaken with a vortex mixer (Vortex Genie) for 45 s. Ethyl acetate (1 mL) was added to the sample mixture to extract excess FMOC-Cl reagent, after which the sample was vortex-mixed a second time for 45 s, and the top organic layer removed via a disposable Pasteur pipette. An additional aliquot of ethyl acetate (1 mL) was added to the sample and vortex-mixed for another 20 s followed by removal of the organic layer. This was repeated for a third and final time, after which the bottom layer was removed with a Pasteur pipette and placed in a HPLC vial containing a glass micro-insert. Once derivatized, all samples were refrigerated until analyzed by HPLC. Storage experiments done by Pocklington et al. (1990) indicate derivatized solutions are stable for at least one week in the light at room temperature. Domoic acid samples were stored in the dark at 4°C for no more than 1-2 weeks before being analyzed.

Size fractionation of particles

Montmorillonite and kaolinite particles were size fractionated to obtain a particle size less than 5 μm according to Jackson (1973). Clean 600 mL beakers were marked to a height of 1 cm from the bottom of the beaker, and again at a height of 6 cm from the bottom of the beaker. Clay suspensions were poured into the beakers and DIW was added to the 6 cm line. The suspension was thoroughly stirred with a glass stirring rod to disperse the clays. When the suspension became still, time was started. At a temperature of ~23˚C, 35.5 min were allowed to pass until the portion between the 1 and 5 cm marks is decanted into a clean 1 L FHDPE bottle. The clay

portions > 5 μm were discarded. This time allows any particles larger than 5 μm to settle to the bottom of the beaker. This process was repeated several times until the suspensions became relatively clear.

Several grams of reagent grade NaCl (99+%, Acros) were added to the decanted portions of the suspensions to enhance flocculation of the clay. The bottles were left overnight and the clean supernatant poured off and discarded. The flocculated clays were rinsed with DIW three times before use, utilizing a centrifuge to accelerate settling between rinses (2510 rpm, 25˚C, Marathon 22 KBR, Fisher Scientific).

H_2O_2 Treatment

Size-fractionated clays were treated with H_2O_2 to remove organics (Jackson 1973). The clays were poured into clean 600 mL beakers, using ~2 mL DIW to rinse the clay out of the centrifuge tubes. Five mL 30% H_2O_2 was added and stirred for several minutes. The beakers were placed on a hot plate with the heat set at 2.5. Another 5 mL 30% H_2O_2 was added after scrubbing the beaker with a rubber policeman. After stirring for five min, an additional 10 mL 30% H_2O_2 was added, a watch glass was placed on top of the beaker, and it was allowed to digest for 2.25 h.

After digesting, the clays were returned to centrifuge tubes with ~15 mL DIW added. They were then centrifuged and the water decanted. This process was repeated 3 times. Next 95% MeOH was added to the 40 mL line of the tube, it was centrifuged, and the top layer was decanted.

Freeze-drying

Clays were subsequently freeze-dried using a Freezone 4.5 benchtop model freeze-dry system (Labconco). Before freeze-drying, the clays were shell-freezed by placing in an ice bath

at about -70˚C that was prepared with dry ice and acetone. The bath needed to be viscous but with dry ice still present. The clays, contained in polyethylene cups, were rotated in the ice bath to freeze the clays in a thin layer on the inside surface of the vessel. Once shell-freezing was completed and the freeze-dryer had a sufficiently low vacuum (~100 x 10^3 Mbar), the freeze-drying process was initiated.

The polyethylene cup was quickly placed into a glass ampoule, the rubber adapter was placed on top of the ampoule, the ampoule was connected to the freeze-dryer, and the vacuum opened. Additional ampoules were added to the freeze-dryer once the vacuum was back down to approximately 100 x 10^3 Mbar. Ampoules were left on the freeze-dryer for approximately 24 h or until samples were dry (the sample was dry if the ampoule was no longer cold to the touch or defrosting). Once taken off the freeze-dryer, the clays were kept in a desiccator at room temperature until needed.

High Performance Liquid Chromatography

A Hewlett-Packard series 1100 high performance liquid chromatograph (HPLC) was used to analyze the domoic acid samples. The HPLC was outfitted with a variable volume (1-25 μL) injector and auto sampler, heated column compartment, and Agilent Technologies data system. It was coupled to an external Shimadzu RF-551 PC spectrofluorometric detector set at an excitation wavelength of 264 nm and emission wavelength of 313 nm. A 25 cm x 4.6 mm I.D. reverse phase C_{18} column packed with 5 μm particles (Vydac 201TP column) maintained at a temperature of 55ºC was used to separate the domoic acid derivatives. The mobile phase consisted of aqueous acetonitrile with 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 mL min^{-1}. Gradient elution was programmed from 30% acetonitrile with 0.1% TFA to 70% over 15 min, followed by an increase to 100% acetonitrile with 0.1% TFA over the next 2 min, which

was maintained for 7 min before cycling back to 30% acetonitrile with 0.1% TFA. The total analysis time was 35 min, and the injection volume was 10 μL.

A 2.9 μM stock solution of domoic acid standard was prepared by diluting the standard reference material in DIW followed by dark storage at 8ºC. Domoic acid calibration solutions were prepared by diluting the standard stock solution in DIW to final concentrations of 10, 25, 50, 100, and 150 nM. The calibration solutions were then derivatized with FMOC-Cl and stored in the dark at 8ºC until analyzed on the HPLC. A new calibration curve (Figure 2) was created for each set of samples run on the HPLC. Standards of 100 nM were run between several domoic acid samples in order to calibrate for instrument drift. Blank samples (sample matrix minus domoic acid) contained non-detectable levels of domoic acid. The detection limit, defined as a signal to noise ratio of 3, was 5 nmol L^{-1} ± 1% (rsd) at typical seawater concentrations. Reproducibility, based on triplicate analyses, was 1.8% rsd. The certified calibration solutions of DA (99.5 mg L^{-1}) were purchased through the National Research Council of Canada Reference Materials Program. Concentrations of DA isomers were determined assuming the signal response of isomers was equal to that of DA (Pocklington et al., 1990).

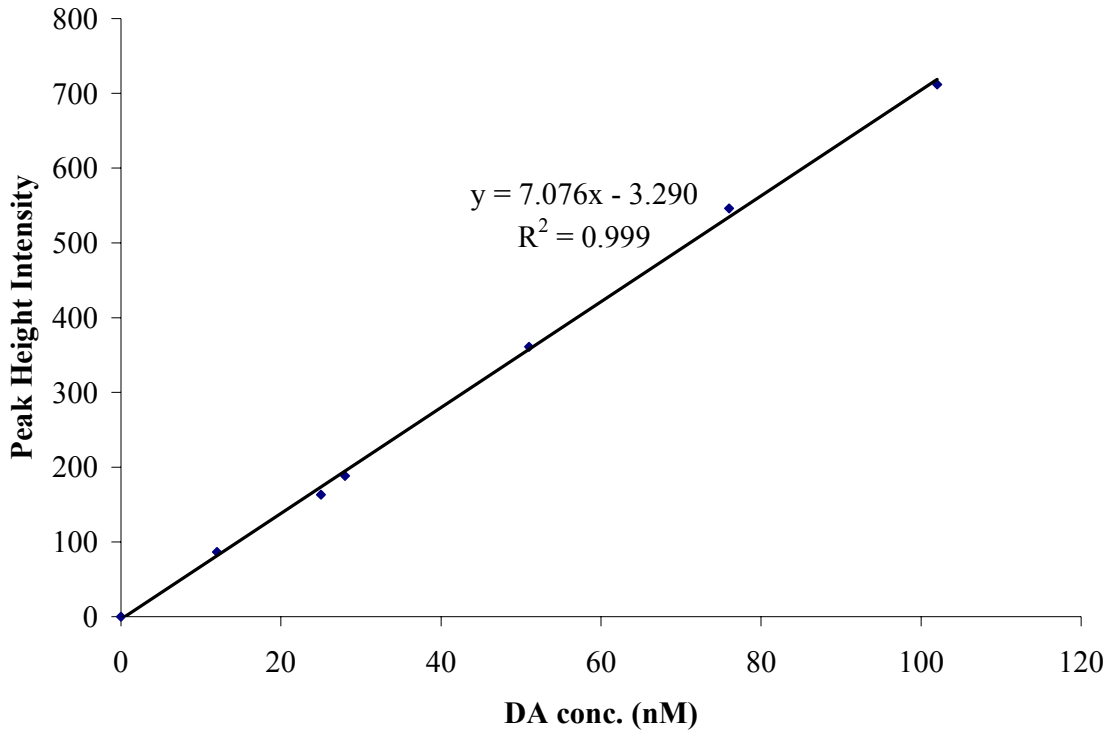


Figure 2. Peak height as a function of added domoic acid (nM) in a typical HPLC calibration curve.

Adsorption experiments

A series of experiments were carried out to examine the adsorption behavior of iso-DAs E and F onto various types of particles in seawater solutions. Iso-DA D was not evaluated due to lack of availability. The particles used in this series of experiments were kaolinite, montmorillonite, illite, and Delaware Bay sediment. Kaolinite and montmorillonite clays were size fractionated, treated with hydrogen peroxide and freeze-dried. The <5 μm fractions were used for the adsorption experiment. A particle free solution prepared with filtered WBSW and 100 nM DA served as a control. Another experiment was also performed using unfiltered WBSW containing ambient natural particles. Particles were added to a final concentration of ~500 mg L^{-1}, to WBSW solutions (pH ~7.9) containing 73, 79, and 125 nmol L^{-1} of iso-DAs D, E, and F, respectively.

The adsorption experiments were carried out in duplicate using 50-mL fluorinated high density polyethylene (FHDPE) centrifuge tubes. The centrifuge tubes were placed on a platform shaker at 300 rpm (Bigger Bill, Thermolyne). Aliquots (1 mL) were taken at 2 different time points (0 and 24 h). Aliquots were filtered immediately after collection using a 0.2 μm cellulose acetate filter (HPLC certified, Gelman Sciences). Experiments were conducted at room temperature, in the dark. Concentrations of dissolved iso-DAs were determined using a modified version of the FMOC-HPLC method described previously.

Pre-treatment of seawater

For metal binding experiments only, seawater collected from Wrightsville Beach was filtered through a Meissner® Stylux 0.2-μm (500-cm^2) filter capsule pre-rinsed with seawater and UV-irradiated (1200 W mercury vapor lamp; Ace Glass) for 3.5 h to destroy natural organic material.

Metal binding experiments, Series 1

The ability of Cu to complex DA was evaluated using a competitive ligand equilibration / adsorptive cathodic stripping voltammetric (CLE-ACSV) technique adapted from Rue and Bruland (2001). Experiments by Rue and Bruland (2001) were repeated to ensure agreement with their results. UV-irradiated seawater was buffered with 6.5 mM HEPPS and spiked with 5 nM Cu. Aliquots (5 mL) of the buffered solution were delivered into FEP-Teflon cups. The cups were spiked with DA concentrations ranging from 0 μM to 1.3 μM. The solutions equilibrated for 300 min, when 2.5 μM salicylaldoxime (SA) was spiked into the cups. After 15 min of equilibration, the solutions were analyzed. A Bioanalytical Systems model CV50W potentiostat connected to a model CGME mercury drop electrode was utilized for these experiments. Conditional stability constants were determined from these experiments.

In order to see if other metals found in natural waters would outcompete Cu for DA binding sites, a second series of experiments was conducted. The experiments were carried out to determine the binding abilities of domoic acid with Zn, Cd, Co, and Ni in the presence of Cu. The CLE-ACSV method involved establishment of a competitive equilibrium between Cu, one of the four aforementioned metals, domoic acid, and a competing ligand, salicylaldoxime (SA).

To avoid contamination of samples, all equipment used for trace metal analyses, including bottles, stir bars, and sampling and analytical implements, were rigorously acid-washed. All sample manipulations and analyses were performed under class 100 laminar flow hoods within a clean room.

An aliquot of UV-irradiated Wrightsville Beach seawater (UVWBSW) was buffered with 6.5 mM HEPPS, and spiked with Cu to a concentration of 5 nM. Subsamples (5 mL) were distributed from the buffered solution into FEP-Teflon cups. Half the cups were spiked with

either Ni, Zn, Cd, or Co. For experiments involving Ni, 20 nM was added; for Zn, 22 nM; for Cd, 0.5 nM; and for Co, 0.5 nM was added. The concentrations were chosen based on ten times the concentration typically found in nature. Next, 652 nM DA, ten times the concentration of toxin usually found in blooms, was added to all cups. The cups were then allowed to equilibrate for 300 min.

After equilibration, 2.5 μM of the competing ligand, SA, was added to each cup and allowed to equilibrate for 15 min. The sample was purged and stirred for 240 s with ultrapure N_2 (bubbled through DI water to minimize sample evaporation) to eliminate oxygen, which causes a background signal that interferes with the analysis. The $Cu(SA)_2$ complexes were deposited at a hanging mercury drop electrode on the CGME, and then a negative-going potential scan was applied. The reduction current from the Cu bound to the added competing ligand SA was then measured (in the presence and absence of the competing metal) as a function of potential using differential pulse voltammetry in stirred samples (300 rpm) with 5-minute adsorption periods at a deposition potential of -0.05 V.

Metal binding experiments, Series 2

Additional studies were designed to determine the binding of metals to DA and its isomers. Solutions were examined by excitation-emission matrix (EEM) fluorescence spectroscopy. Excitation and emission spectra (EEM) can be employed to gain insight into complexation of iso-DAs E and F with metals. One major fluorescence peak, the T peak, was used to describe domoic acid fluorescence. Domoic acid fluorescence occurred in this region, around excitation 260 nm and emission 305 nm. The T peak indicates the presence of protein-like substances (Coble 1996). DA and its isomers occurred in the region referred to as the T peak (Fig. 3).

Fluorescence quenching is a method which allows assessment of the complexing ability of metals such as Cu in natural waters (Boussemart et al. 1989). Most systems, upon complexation of a metal, exhibit complexation-induced fluorescence quenching (CHEQ), although a few molecular systems are known to exhibit an enhancement of fluorescence upon complexation of a metal. Copper (II) and mercury (II) typically show quenching upon complexation but complexation of Cu (II) or Hg (II) has also been known to result in fluorescence enhancement (Hennrich et al., 2001). Studies have been done where formation of a chromium (VI) complex produced quenching of fluorescence in some cases and enhancement in others (Xiang, 2007). Based on this information, a change in fluorescence upon addition of iron to domoic acid was seawater indicative of domoic acid binding to iron.

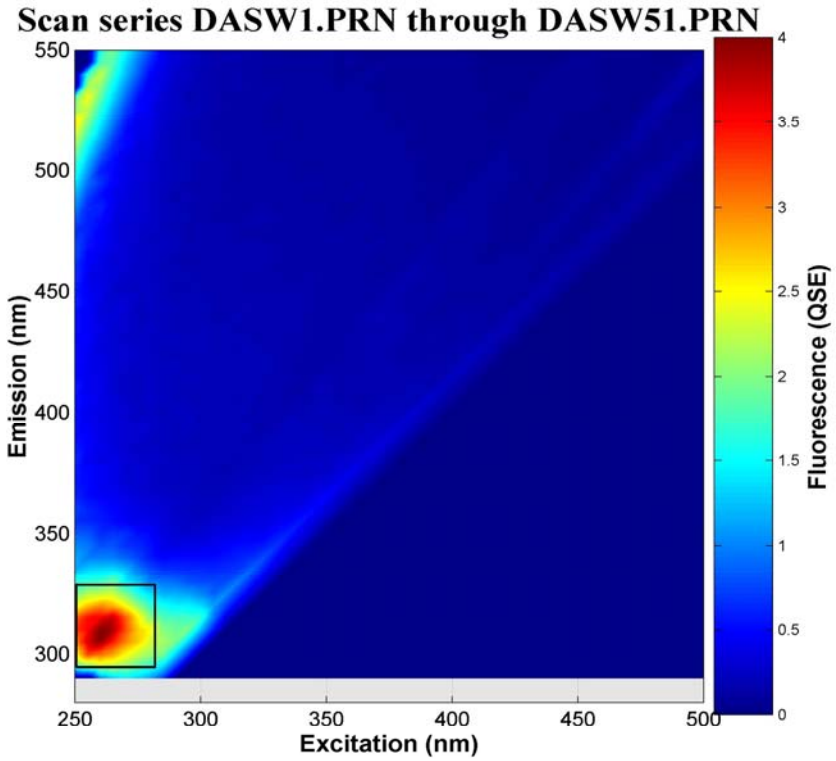


Figure 3. EEM spectra of domoic acid in seawater. The boxed portion shows where domoic acid and its isomers fluoresce.

Samples for fluorescence were warmed to room temperature prior to analysis. Deionized water was used in the reference cell. An aliquot of UV-irradiated Wrightsville Beach seawater (UVWBSW) was spiked with 0.1 μm Fe and 0.1 μm DA (DAFeSW). A second aliquot of UVWBSW was spiked with 0.1 μm DA(DASW). Two solutions composed of 0.1 μm Fe and either 0.1 μm iso-DA E or iso-DA F (IEFeSW and IFFeSW, respectively). Iso-DA E was added to UVWBSW, with a final concentration of 0.1 μm (IESW). Iso-DA F was added to UVWBSW, with a final concentration of 0.1 μm (IFSW).

Excitation-emission matrix (EEM) fluorescence properties were determined on a Jobin Yvon SPEX Fluoromax-3 scanning fluorometer equipped with a 150 W Xe arc lamp and a R928P detector. The instrument is configured to collect the signal in ratio mode with dark offset using 5 nm bandpasses on both the excitation and emission monochromators. The EEMs were created by concatenating emission spectra measured every 5 nm from 250 to 500 nm at 51 separate excitation wavelengths (Del Castillo et al. 1999). Scans are corrected for instrument configuration using factory supplied correction factors, which are determined essentially as described in Method 1 of Coble et al. (1993).

Post processing of scans was performed using FLToolbox 1.91 developed by Wade Sheldon (University of Georgia) for MATLAB® (Release 11). The software eliminates Rayleigh and Raman scattering peaks by excising portions (± 10-15 nm FW) of each scan centered on the respective scatter peak. The excised data is replaced using three-dimensional interpolation of the remaining data according to the Delaunay triangulation method and constraining the interpolation such that all nonexcised data is retained. Following removal of scatter peaks, data were normalized to a daily-determined water Raman intensity (275ex /

303em, 5 nm bandpasses) and converted to Raman normalized quinine sulfate equivalents (QSE) in ppb (Coble et al. 1998).

To detect a change in fluorescence upon addition of iron to domoic acid, scans without added iron were compared to scans with added iron. A scan of 0.1 μm DA in UVWBSW were compared to a scan of 0.1 μm Fe and 0.1 μm DA in UVWBSW. Scans including iso-DA E or iso-DA F were compared in the same manner. A change in fluorescence was indicative of either DA, iso-DA E, or iso-DA F complexing to iron, depending on the scan.

RESULTS

Adsorption experiments

A series of experiments were conducted to evaluate the adsorption behavior of iso-DAs E and F in seawater solutions containing various particles including kaolinite, montmorillonite, illite, and Delaware Bay sediment. Particles were added, to yield a final concentration of ~500 mg L^{-1}, to WBSW solutions (pH ~7.9) containing 73, 79, and 125 nmol L^{-1} of iso-DAs D, E, and F, respectively. Another experiment was also performed using unfiltered WBSW containing ambient particles. Aliquots (1 mL) were taken at 2 different time points (0 and 24 h), and immediately filtered after collection. Concentrations of dissolved iso-DAs (Figure 1) were determined using a modified version of the fluorenylmethyoxycarbonyl-high performance liquid chromatography (FMOC-HPLC) method described by Pocklington et al. (1990).

In all treatments, the loss of DA and iso-DAs adsorbed onto particles was negligible (Table 1). Previous experiments assessing DA particle reactivity showed minimal net adsorption onto a variety of particles in filtered seawater (Lail et al., manuscript submitted). Other previous experiments using organic-free kaolinite in DIW demonstrated that ~70% DA adsorption onto

kaolinite. These experiments imply that major ions in seawater neutralize electrostatic attractions between particles and DA isomers (Lail et al., manuscript submitted). This implies that DA and iso-DAs are relatively hydrophilic, not particle reactive, and that this is not a significant loss mechanism in natural waters.

Table 1. Adsorption of ISO solutions in seawater with and without added particles.
ISO = 73, 79, and 125 nM of iso-DAs D, E, and F, respectively. WBSW = Wrightsville Beach seawater (salinity ~34). DA = 100nM domoic acid. a. Mesa Alta, N.M., Ward's API #9. b. Na-montmorillonite, Clay Spur, WY. c. Dried surface sediment from upper Delaware Bay. d. Illite, Silver Hill, MT. Montmorillonite and kaolinite were size-fractionated.

Solution composition	**% DA lost relative to initial concentration**		**% iso-DA-D lost relative to initial concentration**		**% iso-DA-E lost relative to initial concentration**		**% iso-DA-F lost relative to initial concentration**	
	0 h	**24 h**	**0 h**	**24 h**	**0 h**	**24 h**	**0 h**	**24 h**
filtered WBSW + 100 nM DA	0	0	0	5	0	6	0	1
unfiltered WBSW + ISO (natural particles)	0	0	0	8	0	6	0	1
filtered WBSW + ISO + ~0.500g/L kaolinite[a]	0	0	0	0	0	0	0	0
filtered WBSW + ISO + ~0.500g/L montmorillonite[b]	0	0	0	6	0	6	0	6
filtered WBSW + ISO + ~0.494g/L Delaware bay sediment[c]	0	0	0	0	0	0	0	0
filtered WBSW + ISO + ~0.494g/L illite[d]	0	0	0	0	0	0	0	0

Metal binding experiments, Series 1

A series of experiments was conducted to determine the binding of DA and its isomers to a series of metals based on the approach used by Rue and Bruland (2001). Experiments by Rue and Bruland (2001) were repeated to ensure agreement of my data with their results. The speciation of Cu was determined using competitive ligand equilibration adsorptive cathodic stripping voltammetry (CLE-ACSV), where a titration is carried out in which increasing amounts of domoic acid are added to the sample while salicylaldoxime (SA) and the metal have constant concentrations. The peak showing on the voltammogram results from the electroactive $Cu(SA)_2$ complex. As more domoic acid was added, domoic acid competed to bind with Cu, thus forming less of the $Cu(SA)_2$ complex. The addition of more domoic acid contributed to a progressively smaller peak size on the voltammogram (Fig. 4).

Domoic acid formed a chelate with Cu with a conditional stability constant of: $K^{cond}_{CuDA,Cu^{2+}} = 10^{11}$ M^{-1}. This value correlates closely with the value determined by Rue and Bruland (2001) of $K^{cond}_{CuDA,Cu^{2+}} = 10^{10.3\pm0.2}$ M^{-1}. This conditional stability constant was calculated (Eq. 1) according to Rue and Bruland (2001).

$$K^{cond}_{CuDA,Cu^{2+}} = \frac{X((B^{cond}_{Cu(SA)_2,Cu^{2+}})[SA]^2)}{([DA]-X[DA])} \quad (1)$$

In the calculation, X is a ratio of the reduction current obtained in the presence of domoic acid to that obtained when no domoic acid was present. The conditional stability constant with respect to Cu and SA is $B^{cond}_{Cu(SA)_2,Cu^{2+}}$, [DA] is the concentration of domoic acid, and [SA] is the concentration of the electroactive ligand salicylaldoxime (SA).

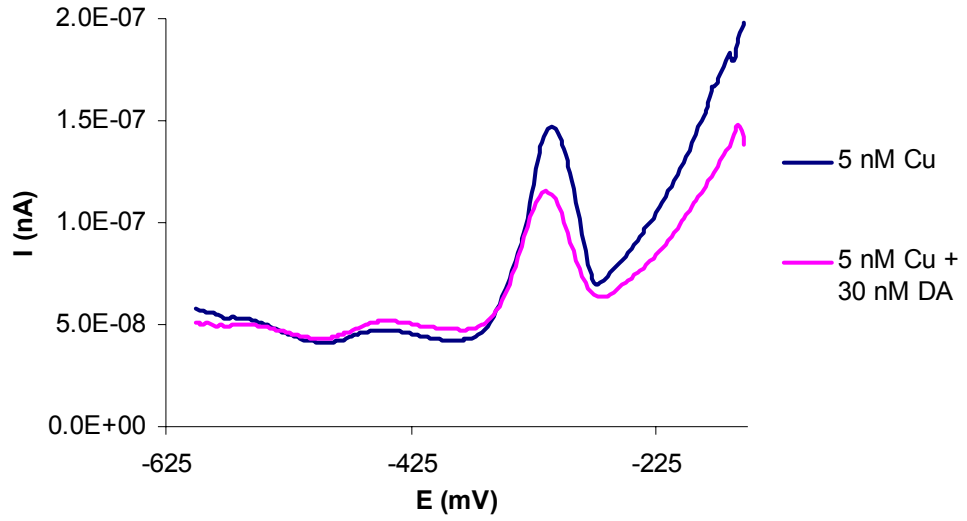


Figure 4. Plot of Cu titration with and without added domoic acid.

A second series of experiments was conducted in order to determine if other metals found in natural waters would outcompete Cu for DA binding sites. Experiments were carried out to determine binding abilities of domoic acid with Zn, Cd, Co, and Ni in the presence of 5 nM Cu. The CSV-CLE method involved establishment of a competitive equilibrium between Cu, one of the four aforementioned metals, domoic acid, and the competing ligand, salicylaldoxime (SA).

There was no statistical difference (t_{test}) in the signals (nA) resulting from the binding of 5 nM Cu and 652 nM DA solutions to those with either 20 nM Ni, 22 nM Zn, 0.5 nM Cd, or 0.5 nM Co added to the matrix. Triplicate measurements were made. This suggests that Ni, Zn, Cd, and Co could not outcompete Cu for binding sites with domoic acid, indicating that Cu is the dominant chelator in this series of metals.

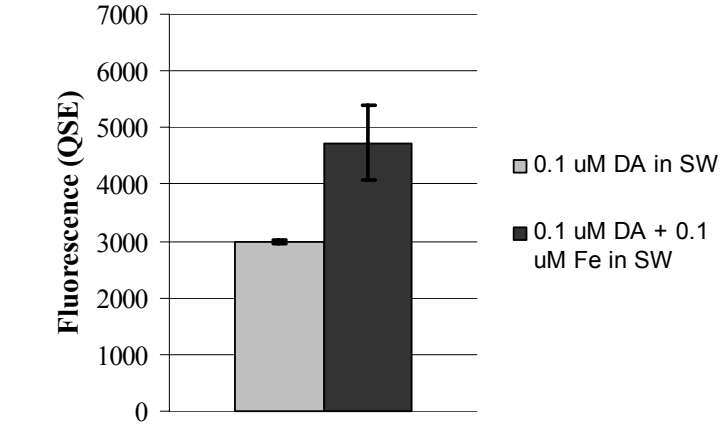
Metal binding experiments, Series 2

Electrochemistry experiments could not be performed on iso-DAs E and F due to some unknown interaction between the isomers and SA. Iso-DA D was not studied at all due to lack of availability. Additional studies were designed using excitation-emission matrix (EEM) fluorescence spectroscopy to determine the binding of metals to DA and its isomers. Excitation and emission spectra can be employed to gain insight into complexation of iso-DAs E and F with metals by comparison of spectra in the presence and absence of metals. One major fluorescence peak, in the region around excitation 260 nm and emission 305 nm, was used to describe domoic acid fluorescence. A change in fluorescence of DA or its isomer upon addition of Fe or Cu confirmed complexation of the toxin to the added metal.

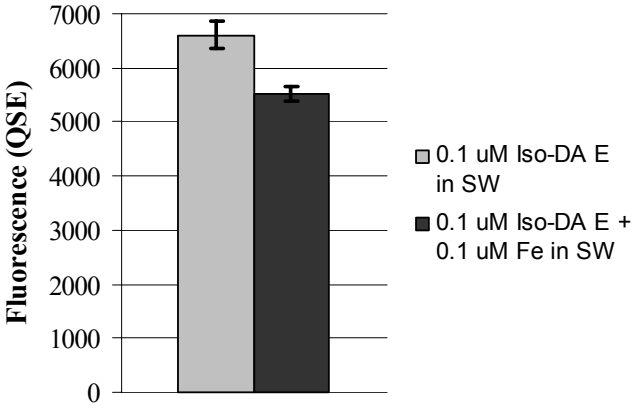
The integrated fluorescence of the DA and Fe mixture exhibited an enhancement of fluorescence when compared to EEMs of DA alone (Fig 5a). Scans were repeated with added Cu as well, with enhanced fluorescence in the presence of Cu relative to DA with no added metal

(Fig. 6a). Scans of each solution were repeated three times. Iso-DAs E and F showed quenching of fluorescence upon addition of both iron or copper, indicating that iso-DAs E and F also form complexes with iron and copper (Figs. 5b-c, 6b-c). Iso-DAs E and F showed higher initial fluorescence than DA. The conformational structure of the isomers is different than domoic acid, so they fluoresce differently. Domoic acid shows approximately the same enhancement of fluorescence when either iron or copper is added. Upon addition of either iron or copper, iso-DAs E and F both show a quenching of about 1000 integrated fluorescence units.

Fluorescence quenching is a method which allows assessment of complexing capacities in natural waters (Boussemart 1989). Most systems, upon complexation of a metal, exhibit complexation-induced fluorescence quenching (CHEQ), although a few molecular systems are known to exhibit an enhancement of fluorescence upon complexation of a metal. Copper (II) and mercury (II) typically show quenching upon complexation but complexation of Cu (II) or Hg (II) has also been known to result in fluorescence enhancement (Hennrich et al., 2001). Studies have been done where binding of chromium (VI) to a fluorescent probe produced quenching of fluorescence in some cases and enhancement in others (Xiang, 2007). It is not known why one ligand will quench fluorescence and another will enhance fluorescence, but it is likely to due rigidity of the structures and their ability to bind a metal ion. Iso-DAs E and F have different conformational structures than domoic acid and could have different metal binding abilities.



a.



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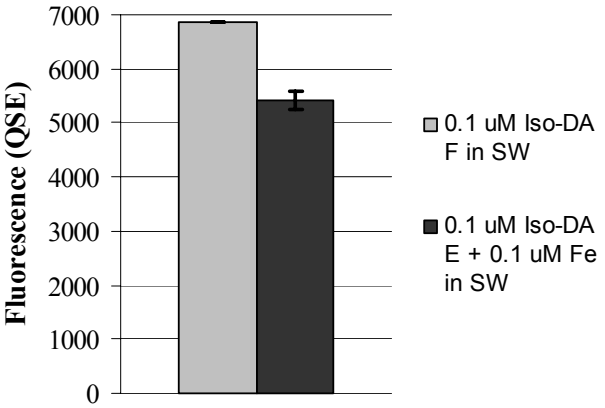
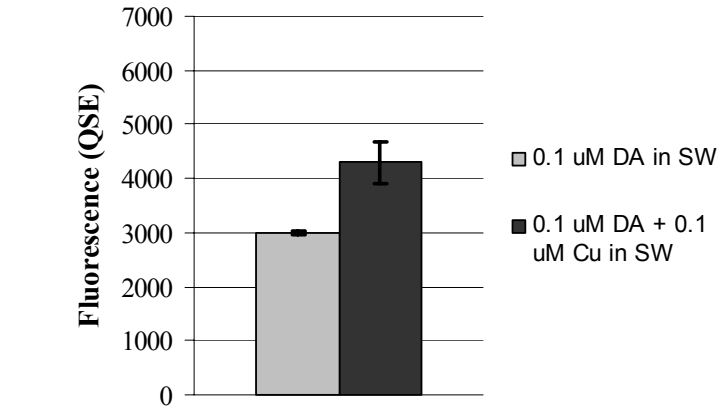
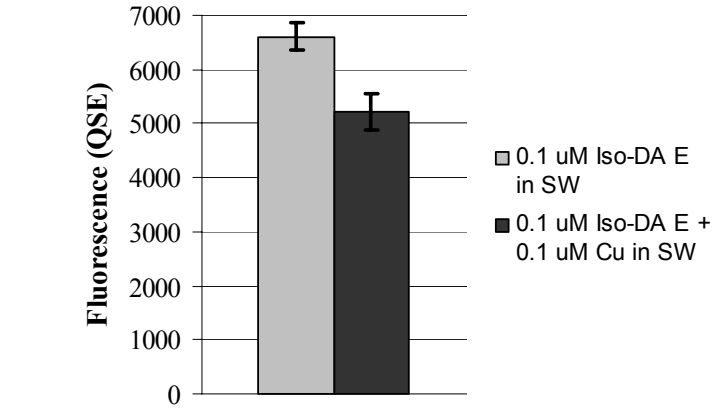


c.

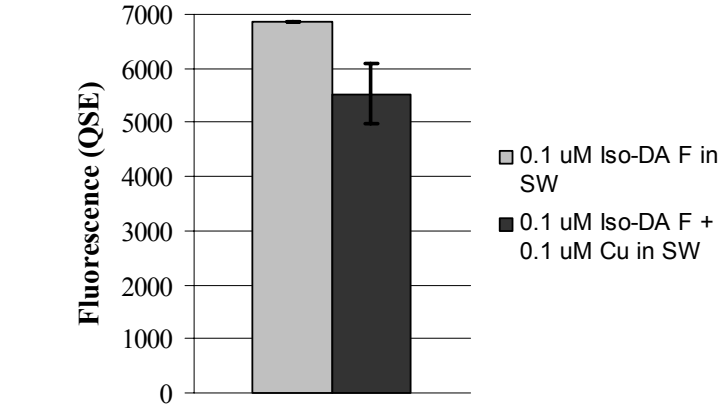
Figure 5 a-c. Integrated fluorescence intensity- Iron. a. dasw = 0.1 µM DA DA in UV-irradiated seawater; dafesw = 0.1 µM DA and 0. 1 µM Fe in UV-irradiated seawater. b. iesw = 0.1 µM iso-DA E in UV-irradiated seawater; iefesw = 0.1 µM iso-DA E and 0. 1 µM Fe in UV-irradiated seawater. c. . ifsw = 0.1 µM iso-DA F in UV-irradiated seawater; iffesw = 0.1 µM iso-DA F and 0. 1 µM Fe in UV-irradiated seawater.



a.



b.



c.

Figure 6. a-c. Integrated fluorescence intensity- Copper. a. dasw = 0.1 µM DA DA in UV-irradiated seawater; dacusw = 0.1 µM DA and 0. 1 µM Cu in UV-irradiated seawater. b. iesw = 0.1 µM iso-DA E in UV-irradiated seawater; iecusw = 0.1 µM iso-DA E and 0. 1 µM Cu in UV-irradiated seawater. c. . ifsw = 0.1 µM Iso-DA F in UV-irradiated seawater; ifcusw = 0.1 µM iso-DA F and 0. 1 µM Cu in UV-irradiated seawater.

DISCUSSION

Adsorption experiments

All treatments showed little or no loss of iso-DA D, E, and F in the presence of particles suspended in filtered WBSW. Controls showed approximately the same adsorption as did montmorillonite and unfiltered WBSW, indicating that removal of these geometrical isomers by adsorption onto particles in the water column is insignificant. This is similar to what has already been shown for DA in seawater (Lail et al., manuscript submitted).

In seawater, high concentrations of cations and anions are present that neutralize both negative and positive sites on clay minerals and other types of particles used in these experiments. Furthermore, most particles in seawater are coated with organic matter (Hunter, 1979), which would further retard electrostatic adsorption of iso-DAs D, E, and F onto their surfaces. Observations of negligible adsorption of iso-DAs D, E, and F are consistent with these observations. In addition, DA is quite hydrophilic, as indicated by its low octanol-water partition coefficient (K_{ow} = 0.0037; Falk et al, 1991). Consequently, DA would tend to remain dissolved in the aqueous phase rather than adsorb onto organically-coated particles in seawater. The isomers of DA are similarly dominated by carboxylic acid groups and are therefore hydrophilic, resulting in no net adsorption onto organically coated particles. These results suggest that removal of DA and its isomers by particles is not a significant sink of these toxins in natural waters.

Metal binding experiments, Series 1

Results of competitive ligand equilibration/ adsorptive cathodic stripping voltammetric studies (CLE-ACSV) reveal that domoic acid forms a strong chelate with copper with a conditional stability constant: $K^{cond}_{CuDA,Cu^{2+}} = 10^{11}$ M^{-1}. This correlates closely with Rue and

Bruland's value: $K^{cond}_{CuDA,Cu^{2+}} = 10^{10.3\pm0.2}$ M^{-1} (2001). Speciation experiments were also performed to determine binding abilities of DA with 22 nM Zn, 0.5 nM Cd, 0.5 nM Co, and 20 nM Ni in the presence of 5 nM Cu. After comparing solutions containing 5 nM Cu and 652 nM DA to those with either 20 nM Ni, 22 nM Zn, 0.5 nM Cd, or 0.5 nM Co added to the matrix, no statistical difference was observed. This suggests no systematic competition from the added metal to complex with DA in the presence of Cu. None of these metals could outcompete Cu for DA, suggesting in a natural water matrix that Cu is the dominant of these metal chelators. Rue and Bruland (2001) found the following stability constant of the Fe(III) -DA complex: $K^{cond}_{FeDA,Fe^{3+}} = 10^{18.7\pm0.5}$ M^{-1}, indicating that this is also a stable complex, and very likely another dominant metal chelator in seawater.

Metal binding experiments, Series 2

A second set of experiments designed to determine the metal binding abilities of DA and two of its geometrical isomers, iso-DAs E and F utilized excitation-emission matrix (EEM) fluorescence spectroscopy. EEMs of 0.1 μM DA and 0.1 μM Fe, compared to EEMs of 0.1 μM DA alone, exhibited enhanced fluorescence resulting from the complexation of DA to Fe. Scans repeated with 0.1 μM added Cu also exhibited an increase in fluorescence consistent with the strong complexation of DA with Cu determined electrochemically. In contrast to DA, its geometrical isomers iso-DAs E and F showed significant quenching of fluorescence upon addition of both iron and copper, indicating that iso-DAs E and F also form complexes with iron and copper. Results of these studies have important ecological implications because they demonstrate that DA and iso-DAs E and F form complexes with both Cu and Fe which could impact both the toxicity of DA as well as the cycling of the metals.

Other factors will ultimately influence the ability of the isomers to have an effect in the ocean. Since domoic acid is known to be photodegraded, forming iso-DAs D, E, and F, the amount of sunlight present will determine the amount of isomers produced. Dispersal of a *Pseudo-nitzschia* bloom spreads out domoic acid and its isomers, diluting their concentrations. Mixing of seawater also dilutes the concentrations of domoic acid and its isomers. Further studies are needed to determine how important DA isomers would be in the natural ocean environment.

CONCLUSIONS

Results from the adsorption study imply that adsorption onto suspended particles represent a minor sink of DA and its geometrical isomers in the ocean. The highly hydrophilic nature of DA and its isomers suggests that these species will remain dissolved in the water column until they are photodegraded or taken up biologically. Previous work supports these results, with domoic acid showing little adsorption onto particles (Lail et al., unpublished).

The hydrophilicity of DA and its isomers and consequent lack of particle reactivity suggests that DA and its isomers are not likely to accumulate in the sea surface microlayer, which is enriched in hydrophobic organic material (Carlson, 1983). Recently it was demonstrated that photodegradation is an important mechanism for DA removal in natural waters (Bouillon et al. unpublished data). The results presented here suggest that photolysis will likely occur in bulk seawater and not be directly altered by particle-water interactions. Since DA has been found in relatively high concentrations in a variety of benthic organisms (Goldberg, 2003), these results suggest the dominant vector for transport of DA and its isomers to bottom

sediments must be biological. Field experiments are needed to assess the relative importance of DA sinks in toxigenic blooms in the coastal ocean.

Results from the electrochemical metal binding study demonstrate that a stable complex is formed between domoic acid and copper, with a conditional stability constant: $K^{cond}_{CuDA,Cu^{2+}} = 10^{11}$ M^{-1}. Other metals present in natural waters, such as Zn, Cd, Co, and Ni, did not outcompete Cu for binding sites on domoic acid. These findings support Rue and Bruland's proposal (2001) of DA production by *Pseudo-nitzschia* as a detoxification mechanism. Since Cu outcompetes other metals in natural waters, DA can act to detoxify Cu for these organisms. These diatoms may also release DA to acquire an essential nutrient, iron. Rue and Bruland (2001) found the following stability constant of Fe-DA complex: $K^{cond}_{FeDA,Fe^{3+}} = 10^{18.7\pm0.5}$ M^{-1}, indicating that this is also a stable complex, and very likely another dominant metal chelator in natural waters.

The second set of metal binding experiments demonstrated that both domoic acid and its geometrical isomers iso-DAs E and F form complexes with iron and copper. This has important ecological implications because formation of isomer-Cu complexes may affect the toxicity and bioavailability of Cu. Domoic acid is produced and subsequently photodegraded in surface waters, leaving the possibility that domoic acid isomers could also serve to detoxify Cu. It is possible that other ligands present in natural waters could outcompete DA isomers to complex Cu. These issues still need to be addressed, as conditional stability constants of these isomer-Cu complexes are unknown.

It is possible that DA isomers could serve another role in the acquisition of iron for *Pseudo-nitzschia* species since domoic acid is known to chelate iron. This would affect the bioavailability of iron, with increasing iron availability to the diatoms, and less availability of iron to other marine life. The speciation and bioavailability of iron are affected by formation of

isomer-Fe complexes. The formation of these complexes may affect the toxicity and bioavailability of Cu, the bioavailability of Fe, and the toxicity of DA and its geometrical isomers in natural waters where the blooms occur.

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