JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Borate as a Synergistic Anion for *Marinobacter algicola* Ferric Binding Protein, FbpA: A Role for Boron in Iron Transport in Marine Life

Aruna J. Weerasinghe,[†] Shady A. Amin,[‡] Ryan A. Barker,[‡] Thaer Othman,[‡] Ariel N. Romano,[‡] Claire J. Parker Siburt,[†] Jerrell Tisnado,[‡] Lisa A. Lambert,[§] Tom Huxford,[‡] Carl J. Carrano,*[,‡] and Alvin L. Crumbliss*[,†]

[†]Department of Chemistry, Duke University, Durham, North Carolina, 27708-0346, United States
[‡]Department of Chemistry and Biochemistry, San Diego State University, San Diego, California 92182-1030, United States
[§]Department of Biology, Chatham University, Pittsburgh, Pennsylvania 15232, United States

Supporting Information

ABSTRACT: Boron in the ocean is generally considered a nonbiological element due to its relatively high concentration (0.4 mM) and depth independent concentration profile. Here we report an unexpected role for boron in the iron transport system of the marine bacterium *Marinobacter algicola*. Proteome analysis under varying boron concentrations revealed that the periplasmic ferric binding protein (Mb-FbpA) was among the proteins whose expression was most affected, strongly implicating the involvement of boron in iron utilization. Here we show that boron facilitates Fe^{3+} sequestration by Mb-FbpA at pH 8 (oceanic pH) by acting as a synergistic anion ($B(OH)_4^{1-}$). Fe^{3+} sequestration does not occur at pH 6.5 where boric acid ($B(OH)_3$; $pK_a = 8.55$) is the predominant species. Borate anion is also shown to bind to apo-Mb-FbpA with mM affinity at pH 8, consistent with the biological relevance implied from boron's oceanic concentration (0.4 mM). Borate is among those synergistic anions tested which support the strongest Fe^{3+} binding to Mb-FbpA, where the range of anion dependent affinity constants is log K'_{eff} = 21−22. Since the pK_a of boric acid (8.55) lies near the pH of ocean water, changes in oceanic pH, as a consequence of fluctuations in atmospheric CO_2, may perturb iron uptake in many marine heterotrophic bacteria due to a decrease in oceanic borate anion concentration.

Boron is a required trace element for many organisms, especially plants, although it can be toxic at higher concentrations.[1−3] While the terrestrial environment is typically boron poor, the concentration of this element in the world's oceans is relatively high (0.4 mM).[4] That it exhibits a conservative (not depth dependent), non-nutrient like profile, indicates that it is seldom, if ever, a growth limiting micronutrient. However, the fact that virtually all of the few natural products known to contain boron come from the marine environment suggests that marine organisms have developed homeostatic mechanisms for boron that have not only allowed them to adapt to the presence of high boron concentrations in their environment but also make use of its abundance for a variety of biological purposes.[5]

Marinobacter algicola was selected as a model marine bacterium to study the effect of boron on bacterial iron transport due to the fact that this bacterium produces vibrioferrin, a siderophore known to bind boron as well as iron.[6] We have found that boron affects the expression level of some genes and proteins associated with iron transport in *Marinobacter algicola*.[7] Under iron limited conditions the expression of the periplasmic ferric binding protein Mb-FbpA was down-regulated greater than 10-fold in the presence of boron.[7] Surprisingly, the other iron uptake genes that were tested showed upregulated expression in the presence of low iron and high boron conditions.[7] Although sensitive to iron deficiency in the absence of boron, no classical iron responsive element, i.e., Fur-box sequence, has been found for Mb-FbpA.[8]

The amino acid sequence of Mb-FbpA shows that it is related to a family of periplasmic binding proteins (PBP) that bind Fe^{3+} directly (Figure S4). Some homologues of Mb-FbpA have been shown to sequester naked Fe^{3+} in the presence of a synergistic anion and transport it across the periplasm into the cyptoplasm as part of an ABC transport system in Gram-negative bacteria (Figure 1).[9] These classical FbpAs are members of the transferrin superfamily, which, like human serum transferrin, transport Fe^{3+}, interact with membrane receptors, and require an exogenous or synergistic anion for tight iron sequestration.[9]

A fundamental question is: how can boron affect the expression level of Mb-FbpA? One possibility is that boron binds directly to the Mb-FbpA protein in some fashion that affects iron transport efficiency, which in turn indirectly affects the expression of the protein through an iron-sensing non-Fur-dependent mechanism. In this study, we test the hypothesis that boron directly interacts with the active site of the periplasmic iron transport protein Mb-FbpA by serving as an effective synergistic anion. Our model for Fe^{3+} sequestration by Mb-FbpA using a synergistic borate anion is shown in Figure 2 and is structurally similar to the homologous *Mannheimia hemolytica* protein.[10] Based on sequence alignments shown on the phylogenetic tree in Figure S4, the Mb-FbpA Fe^{3+} binding cleft is composed of three tyrosine residues (Tyr141, 197, and 198) all from the C-domain. This is in contrast to FbpA from

Received: June 28, 2013
Published: September 12, 2013

   dx.doi.org/10.1021/ja406609s | *J. Am. Chem. Soc.* 2013, 135, 14504−14507

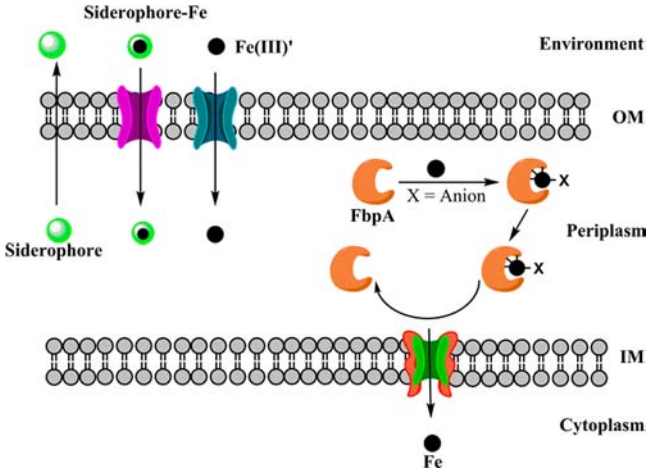

Figure 1. Schematic representation of FbpA mediated iron uptake in Gram-negative marine bacteria. Iron is transported across the outer membrane from a variety of iron sources including siderophore-Fe complexes and Fe(III)′ (soluble inorganic ferric ion). In the periplasm, iron is shuttled by a PBP. One PBP, Mb-FbpA, chelates Fe^{3+} in the presence of a synergistic anion X. Iron is transported across the inner membrane via an ABC transport complex.

the terrestrial pathogen *Neisseria gonorrheae* (Ng-FbpA) where the Fe^{3+} is sequestered between the C and N domains by Tyr195/Tyr196 and Glu57/His9, respectively.[11] This structural difference provides a more open iron binding site for Mb-FbpA. The higher extinction coefficients of all Mb-FeFbpA-X (X = anion) assemblies compared to Ng-FeFbpA-X and the blue-shifted ligand to metal charge transfer (LMCT) absorption maxima are consistent with three tyrosines being bound.[12] The model in Figure 2 also shows a likely synergistic anion binding site for Mb-FbpA at Arg10, Gln11, and Arg101. The modeled structure in Figure 2 and the sequence alignment data in Figure S4 illustrate the plausibility of Fe^{3+} sequestration by Mb-FbpA in the presence of a synergistic borate anion.

To test the efficacy of the hypothesis summarized by Figure 2, we set about to characterize the interactions of boron and Fe^{3+} with Mb-FbpA at pH 8 (oceanic pH) and 6.5. We also characterized Fe^{3+} sequestration by Mb-FbpA in the presence of relevant nonboron-containing anions since FbpAs as a subclass of the transferrin superfamily are characterized by their synergistic anion promiscuity.[9]

UV-difference spectroscopy confirms a binding event with aqueous boric acid and apo-Mb-FbpA at pH 8.0 (reaction 1), and Figure S1 shows an excellent fit of the data to a single site binding model (eq S1) with K_d = 1.5 ± 1.0 mM.

$$\text{Apo}-\text{FbpA}-\text{B(OH)}_x \leftrightarrows \text{Apo}-\text{FbpA} + \text{B(OH)}_x \qquad (1)$$

A similar experiment at pH 6.5 did not detect any binding with apo-Mb-FbpA. Given that the pK_a of boric acid at a background aqueous ionic strength comparable to that of seawater is 8.55 (reaction 2),[13] a binding event at pH 8.0 and

$$\text{B(OH)}_3 + \text{H}_2\text{O} \leftrightarrows \text{B(OH)}_4^- + \text{H}^+ \quad \text{p}K_a = 8.55 \qquad (2)$$

not at 6.5 is consistent with borate anion ($B(OH)_4^{1-}$) and not boric acid binding to apo-Mb-FbpA, presumably at the site shown in Figure 2. In addition, we ruled out the possibility of binding polyborate anions to Mb-FbpA as these species are only formed at high boron concentrations (>0.025 M) between pH 6 and pH 11.[14]

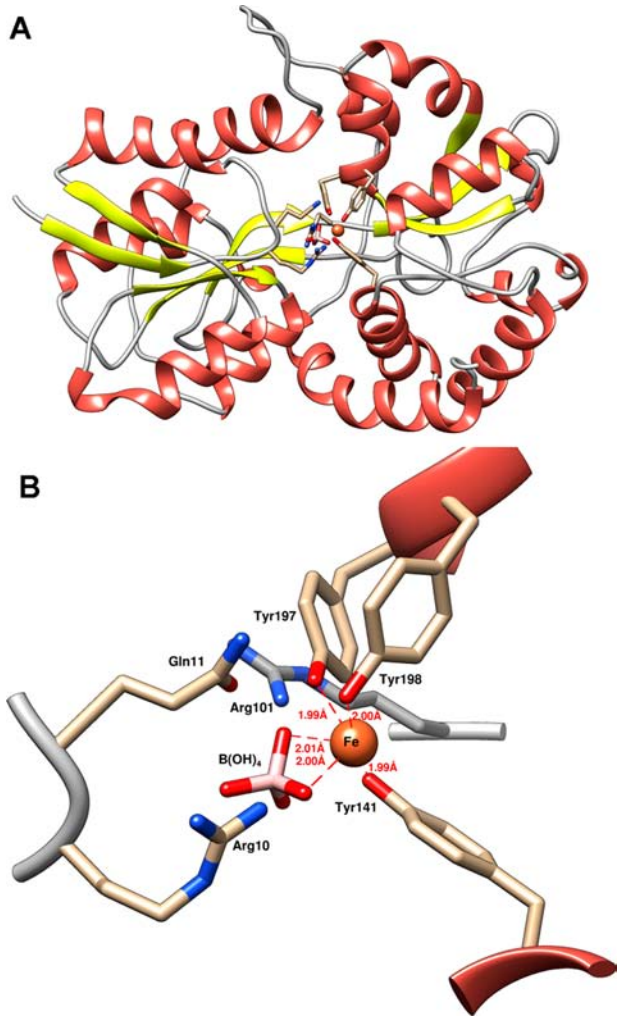

Figure 2. Model of *M. algicola* DG893 ferric binding protein with bound Fe^{3+} and $B(OH)_4^{1-}$. (A) Full model colored according to secondary structure. N-domain is on the left. (B) Close-up of proposed Fe^{3+} binding site (Tyrs 141, 197, 198) with bidentate $B(OH)_4^{1-}$ bound at anion binding site (Arg10, Gln11, Arg101). Metal binding distances shown in red. H atoms not shown for clarity. Model was created using I-Tasser based on the Genbank sequence: ZP_01892821 and further refined using Molprobity and Chimera. ERRAT model score: 97.068 (Figure S3). Bidentate borate binding is analogous to that found in Mh-FeFbpA-CO_3.[10]

Interestingly, apo-Ng-FbpA (Figure S2) showed a much lower binding affinity for borate (K_d = 37 ± 28 mM at pH 8) compared to apo-Mb-FbpA, confirming a greater affinity of Mb-FbpA for borate. This is consistent with borate binding to Mb-FbpA in the marine environment, where boron concentrations are high and not binding to Ng-FbpA in terrestrial environments with typically very low boron concentration.

We find that Mb-FbpA sequesters Fe^{3+} in the presence of aqueous boron at pH 8.0 as evidenced by the appearance of a new LMCT band at 419 nm (Figure 3; Table 1). When the Mb-FeFbpA-$B(OH)_4$ prepared (Supporting Information, SI) at pH 8.0 was dialyzed against pH 6.5 buffer, the charge transfer band at 419 nm disappeared indicating a loss of bound Fe^{3+}. In addition, we were unable to load Mb-FbpA with Fe^{3+} in the presence of aqueous boron at pH 6.5 (SI). Our observation that Mb-FbpA will load Fe^{3+} in the presence of aqueous boron at pH 8.0, where the speciation distribution includes a significant

dx.doi.org/10.1021/ja406609s | J. Am. Chem. Soc. 2013, 135, 14504−14507

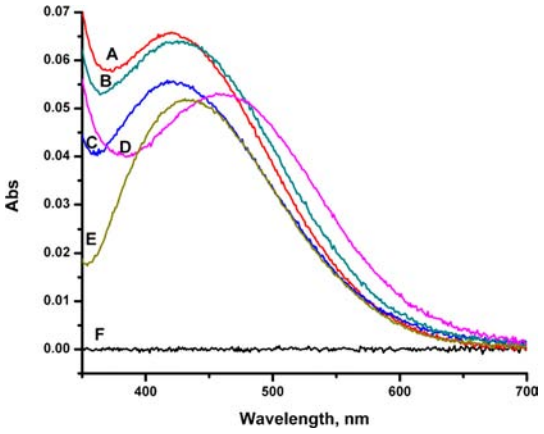

Figure 3. UV−vis spectra of Mb-FeFbpA-X (X = $B(OH)_4^{1-}$, citrate, NTA, carbonate, and sulfate) in 50 mM HEPES, 100 mM NaCl at pH 8.0. (A) Mb-FeFbpA-borate (17.5×10^{-6} M); (B) Mb-FeFbpA-sulfate (19.7×10^{-6} M); (C) Mb-FeFbpA-carbonate (13.5×10^{-6} M); (D) Mb-FeFbpA-NTA (15.5×10^{-6} M); (E) Mb-FeFbpA-citrate (14.0×10^{-6} M); and (F) buffer baseline.

Table 1. Biophysical Characterization of Mb-FeFbpA-X (X = Citrate, Borate, Carbonate, NTA, and Sulfate) at pH 8.0

anion	λ_{max}, nm[a]	ε, $M^{-1}cm^{-1}$[b]	K'_{eff}, M^{-1}[c]
borate	419	3741	$6.0 \pm 3.1 \times 10^{22}$
carbonate	423	4103	$2.4 \pm 0.4 \times 10^{22}$
citrate	430	3668	$2.5 \pm 1.6 \times 10^{22}$
NTA	463	3394	$2.0 \pm 0.6 \times 10^{21}$
sulfate	428	3233	-

[a]Absorbance maximum of Mb-FeFbpA-X in the visible region in 50 mM HEPES, 100 mM NaCl at pH 8.0. [b]Molar absorptivity of the absorbance band maximum at pH 8.0 in 50 mM HEPES, 100 mM NaCl. [c]Effective stability constants corresponding to reaction 3 at pH 8.0 in 50 mM HEPES, 100 mM NaCl (T = 25 °C). Each value reported is an average of two independent determinations at 1:4.5 to 1:2 Mb-FeFbpA-X:EDTA ratios.

fraction of $B(OH)_4^{1-}$ (28%) but will not load Fe^{3+} at pH 6.5 where very little $B(OH)_4^{1-}$ (0.9%) is present, suggests that it is the borate anion ($B(OH)_4^{1-}$) and not boric acid that is acting as the synergistic anion as shown in Figure 2. This interpretation is further supported by the fact that we have confirmed that the Mb-FbpA protein can function as an Fe^{3+} sequestering protein at pH 6.5 by showing that Mb-FeFbpA-NTA (SI) exhibits a stable characteristic charge transfer band λ_{max} = 463 nm (Figure 3; Table 1) at pH 8.0 and 6.5. These observations are consistent with the model presented in Figure 2 where $B(OH)_4^{1-}$ is bound at the anion binding site and the first coordination shell of Fe^{3+}. This is further supported by the fact that borate anion has previously been shown to coordinate to Fe^{3+} in aqueous solution.[15]

We find that Mb-FbpA behaves as a classical FbpA and a member of the transferrin superfamily. That is, we have found no evidence for Fe^{3+} sequestration by Mb-FbpA in the absence of a suitable synergistic anion, and the protein appears to be promiscuous in its requirement for specific anions, as observed previously for Ng-FbpA.[12,16−19] Ternary protein complexes (Mb-FbpA-Fe-anion) were prepared as described in SI. The anion modulates the spectral properties of these ternary complexes in the visible region of the spectrum (Figure 3), suggesting Fe^{3+} first coordination shell interaction as observed for Ng-FbpA.[12,16−19]

The affinity of Mb-FbpA for Fe^{3+} in the presence of various anions (X = $B(OH)_4^{1-}$, citrate, NTA, and carbonate) was determined as described in SI. Table 1 summarizes the biophysical characteristics of Mb-FeFbpA-X prepared at pH 8.0, including stability constants for equilibrium reaction 3 calculated using EDTA competition experiments (reaction 4).

$$\text{FbpA}-\text{X} + \text{Fe}^{3+}_{aq} \leftrightarrows \text{FeFbpA}-\text{X} \quad (3)$$

$$\text{FeFbpA}-\text{X} + \text{EDTA} \leftrightarrows \text{FbpA}-\text{X} + \text{FeEDTA} \quad (4)$$

M. algicola FbpA generally showed a high affinity for Fe^{3+} with a variety of synergistic anions (log K'_{eff} = 21−22; Table 1), which is comparable to that of human transferrin and greater than that observed for the terrestrial Ng-FbpA.[12] Importantly, borate as a synergistic anion is among those anions forming the most stable (K'_{eff}) holo protein complex with Mb-FbpA. This observation is consistent with the hypothesis that borate plays a synergistic anion role critical to *in vivo* iron transport in *M. algicola*. Other synergistic anions for this study were selected based on their relative concentration in the marine environment and their biological significance. The dominant anion in the marine environment of *M. algicola* is chloride, but other anions, such as sulfate and borate, are also present in significant quantities (Table S2).[4,20,21] Of the ambient anions, chloride does not function as a synergistic anion for Mb-FbpA at any reasonable concentration. Sulfate, the second most abundant anion in the ocean and likely one of the key anions in the periplasm of *M. algicola* due to its ready diffusion through porins in the outer membrane, can serve as a synergistic anion for Mb-FbpA (Table 1). However, it forms a very weak ternary complex that dissociates in the absence of excess sulfate, precluding the quantitative determination of its binding constant, which in any event is very low. Citrate is a biologically important anion, while NTA is considered as a nonbiological anion but is included as a model amine carboxylate anion in iron loading experiments.

Since the pK_a of boric acid lies near the pH of seawater, any change in the pH of the latter will have a major effect on the boric acid/borate equilibrium. Since $B(OH)_4^{1-}$ has been shown by our work to act as an effective synergistic anion for the sequestration of Fe^{3+} by Mb-FbpA, variations in the $B(OH)_4^{1-}$ concentration have the potential of altering periplasmic iron transport thermodynamics in Gram-negative bacteria that utilize Mb-FbpA homologues. Variations in atmospheric CO_2 concentrations are proposed to influence oceanic pH.[22] Results presented here suggest such an environmental change could impact iron transport and the survival of *M. algicola*. In addition, *M. algicola* has been identified as an "algal-associated" species which enhances iron availability to associated phytoplankton.[23,24] Consequently, this report represents an initial assessment of the impact of boron and the role that ocean acidification may play not only in iron transport by marine bacteria but also on these associated primary producers.

In conclusion, we have shown that although boron has a nonnutrient oceanic concentration profile, it may play an important role in iron transport. Mb-FbpA binds iron as effectively in the presence of borate as any other synergistic anion tested. Direct binding of Mb-FbpA with borate as a synergistic anion may lead to the regulation of this crucial protein in iron transport. The biological relevance of this study was further confirmed by the fact that the affinity of aqueous boron toward apo-Mb-FbpA (K_d = 1.5 mM) is comparable to the boron concentration found in the ocean. Since borate anion is likely the most important

dx.doi.org/10.1021/ja406609s | J. Am. Chem. Soc. 2013, 135, 14504−14507

boron species involved in the uptake of iron by bacteria compared to neutral boric acid, a decrease in ocean borate concentration, could have significant impact on marine life.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, K_d and Fe^{3+} binding constant calculations, and phylogenetic tree. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

Alvin.Crumbliss@duke.edu
carrano@sciences.sdsu.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by NOAA grant nos. NA04OAR4170038 and NA08OAR4170669, California Sea Grant College Program Project numbers R/CZ-198 and R/CONT-205 and NSF grants CHE-0809466 and CHE-0924313. A.N.R. and J.T. were supported by the National Institutes of Health SDSU MBRS grant no. 2R25GM058906. We thank S. Banerjee, R. T. Kreulen, W. Nielsen and G. A. Wray, Duke University, for experimental assistance and helpful discussion.

■ REFERENCES

(1) *Boron in Plant and Animal Nutrition*, Goldbach, H. E.; Rerkasem, B.; Wimmer, M. A.; Brown, P. H.; Thellier, M.; Bell. R. W., Eds.; Kluwer Academic/Plenum Publishers: New York, 2002.
(2) Lewin, J. *J. Phycol.* **1966**, *2*, 160−163.
(3) Bonilla, I.; Garcia-Gonzalez, M.; Mateo, P. *Plant Physiol.* **1990**, *94*, 1554−1560.
(4) Uppstrom, L. R. *Deep-Sea Res.* **1974**, *21*, 161−162.
(5) Carrano, C. J.; Schellenberg, S.; Amin, S. A.; Green, D. H.; Küpper, F. C. *Mar. Biotechnol.* **2009**, *11*, 431−440.
(6) Amin, S. A.; Küpper, F. C.; Green, D. H.; Harris, W. R.; Carrano, C. J. *J. Am. Chem. Soc.* **2007**, *129*, 478−479.
(7) Romano, A.; Amin, S. A.; Trimble, L.; Hobusch, A. R.; Schroeder, K. J.; Harnett, A. D.; Crumbliss, A. L.; Carrano, C. J. *Metallomics* **2013**, *5*, 1025−1030.
(8) Amin, S. A.; Green, D. H.; Waheeb, D. A.; Gardes, A.; Carrano, C. J. *BioMetals* **2012**, *25*, 135−147.
(9) Parker Siburt, C. J.; Mietzner, T. A.; Crumbliss, A. L. *Biochim. Biophys. Acta, Gen. Subj.* **2012**, *1820*, 379−392.
(10) Shouldice, S. R.; Skene, R. J.; Dougan, D. R.; Snell, G.; McRee, D. E.; A. B. Schryvers, A. B.; Tari, L. W. *J. Bact.* **2004**, *186*, 3903−3910.
(11) Bruns, C. M.; Nowalk, A. J.; Arvail, A. S.; McTigue, M. A.; Vaughan, K. G.; Mietzner, T. A.; McRee, D. E. *Nat. Struct. Biol.* **1997**, *4*, 919−924.
(12) Dhungana, S.; Taboy, C. H.; Anderson, D. S.; Vaughan, K. G.; Aisen, P.; Mietzner, T. A.; Crumbliss, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3659−3664.
(13) Millero, F. J. *Chemical Oceanography*, 2nd ed.; CRC Press: Boca Raton, FL, 1996.
(14) Duffin, A. M.; Schwartz, C. P.; England, A. H.; Uejio, J. S.; Prendergast, D.; Saykally, R. J. *J. Chem. Phys.* **2011**, *134*, 154503.
(15) Elrod, J. A.; Kester, D. R. *J. Sol. Chem.* **1980**, *9*, 885−894.
(16) Heymann, J. J.; Weaver, K. D.; Mietzner, T. A.; Crumbliss, A. L. *J. Am. Chem. Soc.* **2007**, *129*, 9704−9712.
(17) Taboy, C. H.; Vaughan, K. G.; Mietzner, T. A.; Aisen, P.; Crumbliss, A. L. *J. Biol. Chem.* **2001**, *276*, 2719−2724.
(18) Roulhac, P. L.; Powell, K. D.; Dhungana, S.; Weaver, K. D.; Mietzner, T. A.; Crumbliss, A. L.; Fitzgerald, M. C. *Biochemistry* **2004**, *43*, 15767−15774.
(19) Guo, M.; Harvey, I.; Yang, W.; Coghill, L.; Campopiano, D. J.; Parkinson, J. A.; MacGillivray, R. T. A.; Harris, W. A.; Sadler, P. J. *J. Biol. Chem.* **2003**, *278*, 2490−2502.
(20) Hutchinson, G. E. *Ecol. Monogr.* **1947**, *17*, 299−307.
(21) Morris, A. W.; Riley, J. P. *Deep-Sea Res.* **1966**, *13*, 699−705.
(22) Caldeira, K.; Wickett, M. *Nature* **2003**, *425*, 365.
(23) Amin, S. A.; Green, D. H.; Hart, M. C.; Küpper, F. C.; Sunda, W. G.; Carrano, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 17071−17076.
(24) Gärdes, A.; Triana, C.; Amin, S. A.; Green, D. H.; Romano, A.; Trimble, L.; Carrano, C. J. *BioMetals* **2013**, *26*, 507−516.