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Production of cobalt binding ligands in a *Synechococcus* feature at the Costa Rica upwelling dome

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Abstract

The Costa Rica upwelling dome (CRD; ~8.67°N and 90.6°W) was characterized chemically for cobalt and nickel abundances and speciation and biologically using cyanobacterial abundances and phylogeny. Total dissolved cobalt was 93 pmol L^{-1} at 90 m depth and decreased in surface waters to 45 pmol L^{-1} at 10 m. Cobalt was 40% labile at 90 m but was completely complexed by strong ligands at 10 m. A surface transect out of the dome showed decreasing total dissolved cobalt from 57 pmol L^{-1} to 12 pmol L^{-1}. Detection window studies showed that natural cobalt-ligand complexes have conditional stability constants greater than $10^{16.8}$ and that competition with nickel did not release cobalt bound to organic complexes, consistent with natural cobalt ligands being Co(III) complexes. *Synechococcus* cell densities at the CRD are among the highest reported in nature, varying between 1.2×10^6 and 3.7×10^6 cells ml^{-1}. Phylogenetic analysis using the 16S–23S rDNA internally transcribed spacer showed the majority of clones were related to *Synechococcus* strain MIT S9220, while the remaining subset form a novel group within the marine *Synechococcus* lineage. In a bottle incubation experiment chlorophyll increased with cobalt and iron additions relative to each element alone and the unamended control treatment. Cobalt speciation analysis of incubation experiments revealed large quantities of strong cobalt-ligand complexes in the cobalt addition treatments (401 pmol L^{-1}), whereas cobalt added to a 0.2-μm filtered control remained predominantly labile (387 pmol L^{-1}), demonstrating that the *Synechococcus*-dominated community is a source of strong cobalt ligands.

Processes that influence the production of metal binding ligands in the surface ocean are not well understood. Many of the important biologically used metals (Fe, Cu, Zn, Co, and Cd) have a chemical speciation that is dominated by their complexation with strong organic ligands (e.g., Rue and Bruland 1995; Saito and Moffett 2001; Morel et al. and references therein; Morel et al. 2003). While the chemical structures of these metal-complexing ligands in the natural environment are currently unknown, their destruction by ultraviolet-irradiation provides evidence for their organic composition. Furthermore, conditional stability constants indicate these ligands are very strong, much stronger than most inorganic complexes (i.e., chloro or carbonate complexes). A previous study of iron ligands during the Equatorial Pacific Iron-Ex II enrichment experiment showed rapid production of ligands after the iron fertilization (Rue and Bruland 1997), and methods have been developed to concentrate natural iron ligands from surface waters (Macrellis et al. 2001). Evidence for organic complexation of cobalt in oceanic environments was recently obtained (Ellwood and van den Berg 2001; Saito and Moffett 2001), yet the sources of cobalt ligands in the natural environment have not yet been determined. Culture studies have shown both organically complexed cobalt and inorganic cobalt to be used by the cyanobacterium *Prochlorococcus* (Saito et al. 2002). Furthermore, cobalt is a micronutrient that has been shown to be absolutely required by *Synechococcus* and *Prochlorococcus* (Sunda and Huntsman 1995; Saito et al. 2002). In contrast, eukaryotic algae such as centric diatoms and coccolithophores have been shown to be able to substitute cobalt and cadmium for zinc but do not have an absolute cobalt requirement (Lee and Morel 1995; Sunda and Huntsman 1995; Saito et al. 2002). Determining the source and chemical properties of these natural cobalt ligands is important in understanding the biogeochemistry of cobalt and its influence on phytoplankton communities. The Costa Rica upwelling dome (CRD) provided an ideal environment to study these questions due to the abundant cobalt and high cyanobacterial populations present.

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The Costa Rica upwelling dome is a remarkable environment from both chemical and biological perspectives. Located near 8–11°N and 87–90°W with a diameter of 100 to 900 km, the dome is a shoaling of the thermocline caused by localized cyclonic wind stress curl. The dome forms near the coast in February–March before strengthening offshore between July and November and eventually diminishing by December–January (Hofmann et al. 1981). A characteristic feature of the dome is enhanced primary productivity, as observed by remote sensing Sea-viewing Wide Field-of-view Sensor (SeaWiFS) data (Fiedler 2002). This enhanced primary productivity appears to be a result of physical forcing resulting in upwelling of nutrient-rich waters (McClain et al. 2002), and its effects extend to higher trophic levels (Vecchione 1999). The phytoplankton community was characterized at the dome at the beginning of the seasonal dome formation in March 1981, and the high productivity was dominated by the autotrophic picoplankter *Synechococcus* (Li et al. 1983). That study found cell densities of *Synechococcus* in the dome ranged from 0.5×10^6 to 1.5×10^6 cells ml^{-1}, and *Prochlorococcus* abundances were not measured.

The prevailing view of upwelling regimes is that they are dominated by fast-growing eukaryotic phytoplankton such as diatoms, rather than by cyanobacteria. Yet, in the Costa Rica dome, *Synechococcus* dominate surface waters and are present in concentrations much higher than observed in other open ocean environments (Goericke and Welschmeyer 1993; Campbell et al. 1994; DuRand et al. 2001). This type of enhanced productivity is unusual for the cyanobacteria in marine waters; although cyanobacteria are important and crucial components of the microbial loop of the oligotrophic gyres, cell densities in those regions are an order of magnitude lower than Li et al. observed in the Costa Rica dome. Understanding the factors that lead to high *Synechococcus* abundances here would be useful in understanding their ecology. In the summer of 2000, *Synechococcus* was again observed to be present in extremely high cell densities at the Costa Rica dome. This unique environment made for an ideal natural laboratory to study the interaction between cobalt biogeochemistry and the marine cyanobacteria.

Materials and methods

Sampling and bottle preparation—Seawater was collected using a surface sampler designed for trace metal clean sample collection while in transit. Samples were pumped with a Teflon diaphragm pump into a positive pressure clean area and filtered through 0.4-μm filters into Teflon or polyethylene bottles. All sample bottles were cleaned by overnight soaking in citranox detergent followed by Milli-Q water rinses, overnight soaking in 10% HCl (Baker instra-analyzed) at 60°C, and rinsing and storage in pH 2 Milli-Q water (Baker Ultrex II HCl) until use. Seawater samples were stored at 4°C in darkness until analysis within 2 weeks of collection.

Cobalt speciation: electrochemical instrumentation and reagents—Dissolved cobalt total concentrations and speciation were measured at sea using adsorptive cathodic stripping voltammetry (ACSV) and previously developed methods (Saito and Moffett 2001, 2002). Total dissolved nickel was also measured here using an adapted cobalt analysis protocol. Briefly, a Metrohm 663 hanging mercury drop electrode stand with a Perfluoroalkoxy Teflon (PFA) sample vessel was interfaced with an Eco-chemie μAutolab system and GPES (General Purpose Electrochemical System, Eco Chemie) software. Dimethylglyoxime (DMG) from Aldrich was recrystallized in Milli-Q (Millipore) water in the presence of 10^{-3} ethylenediaminetetraacetic acid (EDTA; Sigma Ultra) to remove impurities, dried, and redissolved in high-performance liquid chromatography (HPLC)-grade methanol. We prepared 1.5 mol L^{-1} sodium nitrite (Fluka Puriss) purified by an overnight equilibration with prepared Chelex-100 beads (BioRad). An N-(2-hydroxyethyl)piperazine-N′-(3-propanesulfonic acid) (EPPS) buffer solution (Fisher) was purified by passing through a column with 3 ml of prepared Chelex-100 beads. For total dissolved cobalt and nickel analyses, 8.50 ml of ultraviolet light irradiated filtered seawater was pipetted into the Teflon sample cup followed by 50 μl of 0.5 mol L^{-1} EPPS, 20 μl 0.1 mol L^{-1} DMG, and 1.5 ml of 1.5 mol L^{-1} sodium nitrite (Saito and Moffett 2002). For cobalt and nickel speciation measurements, 8.5 ml of fresh filtered seawater was pipetted into Teflon equilibration bottles followed by a cobalt nitrate aliquot and 50 μl of 0.01 mol L^{-1} DMG. After overnight equilibration the sample was poured into a clean and preconditioned PFA sample vessel followed by additions of 50 μl of 0.5 mol L^{-1} EPPS and 1.5 ml of 1.5 mol L^{-1} sodium nitrite prior to analysis. Cobalt values were dilution corrected for the nitrite addition. A cobalt blank was measured for each batch of reagents and was subtracted from the total cobalt analyses (~6 pmol L^{-1}). Cobalt speciation measurements consisted of labile cobalt analyses with standard additions of cobalt to DMG-equilibrated samples and detection window studies where DMG concentrations were varied. These labile titrations measure the fraction of cobalt bound to strong organic complexes. The detection window analyses provide estimation of the conditional stability constants of the natural cobalt ligands (*see following*).

The μAutolab protocol involved a 3-min 99.999% N_2 gas purge at 120 kPa, a 90-s deposition time at −0.6 V, a 15-s equilibration period, and a high-speed negative scan from −0.6 to −1.4 V at 10 V s^{-1}. A linear sweep waveform was used on drop size three (0.52 mm^2) and a stirrer speed of five. Cobalt and nickel signals were measured as the peak height from the baseline.

Seawater was prepared for total dissolved cobalt and nickel analysis by irradiation with ultraviolet light for 3.0 ± 0.1 h (Ace Glass) at ambient pH in quartz vials covered with plastic caps, parafilm, and aluminum foil to prevent exposure to dust and losses by evaporation. Previous studies have verified the recovery of nonacidified total cobalt (Vega and van den Berg 1997; Saito and Moffett 2002). Total cobalt concentrations were determined using four to five standard additions of 25 pmol L^{-1} prepared from Fisher certified $Co(NO_3)_2$ stock solutions diluted in pH 2 Milli-Q water and polymethylpentene volumetric flasks. Total nickel concentrations were then determined on the same sample with three standard additions of 1 nmol L^{-1} using a $NiCl_2$ solution prepared in pH 2 Milli-Q water. The PFA sample vessel was rinsed with 10% HCl then pH 3 HCl in Milli-Q water at the

Table 1. Detection windows for K_{CoL}.

DMG (mol L^{-1})	α_{CoHDMG} ($K_{cond}=10^{11.5}$)	Detection window* for K_{CoL}	α_{CoHDMG} ($K_{cond}=10^{12.85}$)	Detection window† for K_{CoL}
10^{-5}	32	$10^{10.8}$ to $10^{12.8}$	708	$10^{12.2}$ to $10^{14.2}$
10^{-4}	3,160	$10^{12.8}$ to $10^{14.8}$	70,800	$10^{14.2}$ to $10^{16.2}$
10^{-3}	3,160,000	$10^{14.8}$ to $10^{16.8}$	7,080,000	$10^{16.2}$ to $10^{18.2}$

* Calculated using K_{cond} of $10^{11.5}$ for $CoHDMG^2$ (Saito and Moffett 2001).
† Calculated using K_{cond} of $10^{12.85}$ for $CoHDMG^2$ (Ellwood and van den Berg 2001).

completion of each titration. The sample vessel was preconditioned with filtered oceanic seawater prior to adding samples.

Detection window theory and experiment—A filtered surface water sample from the Costa Rica upwelling dome station and samples from the bottle incubation experiment were equilibrated with different concentrations of DMG in order to explore the influence of increasing the speciation detection window on natural cobalt ligands. The detection window was adjusted by varying the DMG concentrations: 10^{-5} mol L^{-1}, 10^{-4} mol L^{-1}, and 10^{-3} mol L^{-1} DMG were equilibrated with the seawater sample in Teflon bottles for 20–23 h. The seawater sample had 46.1 ± 5.3 pmol L^{-1} total dissolved cobalt and was determined to be completely complexed to strong organic ligands (no detectable labile cobalt). As with speciation measurements, nitrite and EPPS were added immediately before purging and analysis.

The analytical detection window for strong cobalt ligands is operationally defined as the region in which natural ligands are 90% nonlabile to where they are 90% labile relative to a synthetic competing ligand such as DMG (Zhang et al. 1990). The detection limit can then be expressed mathematically by a comparison of side reaction coefficients (α), where the detectable α_{CoL} is equal to a 10-fold variation relative to α_{CoHDMG_2}:

$$\alpha_{CoL} = K_{CoL}[L] \quad (1)$$

$$\alpha_{CoHDMG_2} = K_{CoHDMG_2}[DMG]^2 \quad (2)$$

The detectable α_{CoL} can then be converted to a range of detectable conditional stability constants (K_{CoL}) by dividing by an estimated ligand concentration [L]. The 10-fold variation relative to α_{CoHDMG_2} is based on the practical range beyond which it is unlikely to determine speciation accurately. The conditional stability constants of $CoHDMG_2$ have been determined for seawater to be $10^{11.5\pm0.3}$ at pH 8.0 and $10^{12.85\pm0.10}$ at pH 8.7 (Zhang et al. 1990; Saito and Moffett 2001). The appropriate range of α_{CoHDMG_2} coefficients can be calculated and used to determine the corresponding analytical detection window for CoL using an estimated 50 pmol L^{-1} concentration of [L] (Table 1). The upper limits of the detection window for conditional stability constants are log K 16.8 and 18.2, for pH 8.0 and 8.7, respectively. The former upper limit is more appropriate since it was calibrated using the same methodology and pH as this study (Saito and Moffett 2001).

Bottle incubations—Iron, cobalt, and combined cobalt and iron enrichments were carried out in triplicate in trace metal clean 1-liter polycarbonate bottles. We added 420 pmol L^{-1} $CoCl_2$ and 0.83 nmol L^{-1} $FeSO_4$ in 0.01 mol L^{-1} HCl in a positive pressure clean environment constructed with laminar flow hoods and plastic sheeting. The acidified Fe(II) stock was used to prevent colloidal formation and/or precipitation that can occur with Fe(III) stocks. Rapid oxidation of Fe(II) to Fe(III) in the seawater incubation bottles was expected. Bottles were filled from a full 50-liter trace metal clean carboy connected to the trace metal clean underway sampler and incubated in an on-deck seawater flow through incubator covered with 35% transmission blue gel (Rosco). After two full days bottles were harvested for chlorophyll and flow cytometry analyses. One of the triplicate bottles from each treatment was returned to the incubator for speciation analysis on day 5. For cobalt speciation analyses each treatment was filtered through acid-washed 0.2-μm syringe filters using all plastic syringes (nonrubber). A control sample consisting of seawater collected through a trace metal clean 0.2-μm filter was incubated under the same environmental conditions.

Flow cytometry—Flow cytometry samples were collected while underway from the trace metal clean surface water sampler. Samples were preserved in 0.125% glutaraldehyde for 10 min in darkness and stored in liquid nitrogen. Samples were thawed at 35°C for 3 min before analysis and run immediately thereafter on a modified FACScan flow cytometer with 0.474-μm fluorescent beads as an internal standard (Dusenberry and Frankel 1994).

Statistical analysis—For the bottle incubation experiment, one-way analysis of variance (ANOVA) on chlorophyll, *Synechococcus* cell abundances, orange fluorescence per cell, and red fluorescence per cell showed all four datasets can reject the null hypothesis that the variation between the experimental treatments is random ($p < 0.01$ for chlorophyll and red fluorescence, and $p < 0.05$ for cell abundance and orange fluorescence datasets). The least significant differences (LSD) were then calculated for each dataset, applying Bonferroni corrections for multiple comparisons (six in these experiments). At the 95% and 99% confidence levels the LSD values are 1.42 and 1.89 for chlorophyll, 521,600 and 696,700 for *Synechococcus* cell abundances, 1.21 and 1.81 for orange fluorescence, and 3.53 and 4.71 for red fluorescence.

DNA isolation, polymerase chain reaction (PCR), and cloning—Biomass was collected on a large volume 0.4-μm cartridge polycarbonate filter using seawater from the trace metal surface water sampling system (8 m depth) during the Costa Rica dome transect. The sample was frozen at −20°C until analysis. For DNA extraction, the cartridge filter was opened and a portion of the filter was minced with a sterile razor blade, added to 1 ml lysis solution (0.5 mol L^{-1} NaCl, 1 mmol L^{-1} EDTA, 10 mmol L^{-1} Tris pH 8.0), and shaken vigorously in a BeadBeater (BioSpec products) for 2 min at maximum speed. To lyse the cells, 0.1 ml of 20 mg ml^{-1} lysozyme was added and the sample was incubated at 37°C

with shaking for 30 min. Next, 0.05 ml 10% sodium dodecyl sulfide and 0.05 ml proteinase K were added and the sample was incubated at 55°C with shaking for 1 h. The sample was extracted twice with 25 : 24 : 1 phenol : chloroform : isoamyl alcohol, and twice with 24 : 1 chloroform : isoamyl alcohol, and then 0.6 ml isopropanol was added. DNA was precipitated at −20°C for 2 h and then spun 10 min in a microcentrifuge, washed with 80% ethanol, dried at room temperature, and resuspended in Tris-EDTA (TE).

Portions of the 16S and 23S rDNAs and the entire internal transcribed spacer (ITS) region were amplified using primers 16S–1247f (CGTACTACAATGCTACGG) and 23S–241r (TTCGCTCGCCRCTACT) (Rocap et al. 2002). Quintuplicate reactions were done in 20 μl volume with final concentrations of reactants as follows: 0.25 mmol L^{-1} deoxynucleotide triphosphate mix (dNTPs), 0.5 μmol L^{-1} each primer, 2.5 μmol L^{-1} $MgCl_2$, and 0.1 U of Taq DNA polymerase. Cycling parameters were 95°C for 4 min followed by 25 cycles of 95°C 1 min, 52°C 1 min, and 72°C 3 min and a final extension at 72°C for 10 min. To reduce the possibility of heteroduplex PCR products in the cloning reaction, a reconditioning PCR was performed using 2 μl of each original reaction as a template (Thompson et al. 2002). Reactant concentrations and cycling parameters were identical to the first PCR, except that only three cycles were performed. After the reconditioning step, the quintuplicate reactions were pooled and excess primers were removed using a Qiaquick PCR purification column (Qiagen). An aliquot was visualized on a 1% agarose gel to ensure that only bands in the 1,100–1,300-bp size range were present.

The pooled purified PCR products were cloned using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Positive colonies, as judged by blue–white color selection, were picked and struck on selective plates to confirm the color phenotype and obtain single colonies, which were picked and grown overnight in Luria-Bertani (LB) broth supplemented with ampicillin. Plasmids were purified from liquid cultures using the Qiaprep Miniprep kit (Qiagen).

Sequencing and phylogenetic analysis—Cloned inserts were sequenced bidirectionally using primers 16S–1247f, 23S–241r, and two primers internal to the ITS (ITS-alaf and ITS-alar) described previously (Rocap et al. 2002). Sequencing reactions were performed with DYEnamic ET dye terminator kit (Amersham Biosciences) according to the manufacturer's instructions and run on a MegaBACE 1000 automated sequencer (Amersham Biosciences). Sequence chromatograms were assembled and base calls manually checked using Sequencher (GeneCodes). Sequences have been deposited in GenBank under accession numbers AY579957–AY579983. Sequences were compiled and aligned manually with ITS sequences of cultured cyanobacteria (Rocap et al. 2002) using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic analyses employed PAUP* version 4.0b10 (Swofford 1999) and 432 positions of the ITS. Distance trees were inferred using minimum evolution as the objective criterion and HKY85 distances. All heuristic searches, including bootstrap analyses (1,000 resamplings), used random addition and tree-bisection reconnection (TBR) branch-swapping methods.

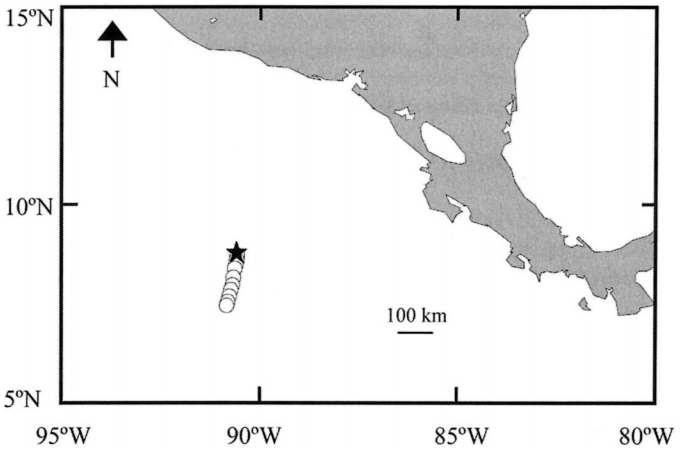

Fig. 1. Location of the Costa Rica upwelling dome (CRD) and transect sampling locations. The location of vertical profile and beginning of surface transect is indicated by a star.

Results and discussion

Cyanobacteria, cobalt, nickel, and nutrient distributions at the Costa Rica dome—A shallow surface profile within the CRD (8.67°N and 90.6°W) and a surface transect south out of the CRD were collected out on 31 August 2000 (Fig. 1). Evidence of the dome was seen in enhanced salinity and decreased temperature at the northern end of the transect (Fig. 2A), consistent with the upwelling of a denser water mass. As previously observed in 1981 (Li et al. 1983), *Synechococcus* abundances in the Costa Rica dome were extremely high with cell densities between 1.2×10^6 to 3.7×10^6 cells ml^{-1} (Fig. 2B). These values are slightly higher than previous *Synechococcus* abundances measured at this site (*see Introduction;* Li et al. 1983). In addition, particulate matter collected on surface seawater filters within the CRD was bright red due to the abundant phycoerythrin in *Synechococcus.* These *Synechococcus* concentrations are significantly higher than maximal cell abundances reported from coastal Peru, the Arabian Sea, and Woods Hole Harbor Massachusetts (8.8×10^5, 1.2×10^5, and 3.5×10^5 cells ml^{-1}, respectively (Waterbury et al. 1979; Sherry and Wood 2001). They are one or more orders of magnitude higher than oceanic environments like the Hawaii ocean time series (HOT) and Bermuda Atlantic time series (BATS) (maximal values of 3.3×10^4 to 5.6×10^4 ml^{-1} for *Synechococcus* at BATS; Goericke and Welschmeyer 1993; Campbell et al. 1994; Durand et al. 2001). *Prochlorococcus* cell numbers, which have not been previously reported for this location, were an order of magnitude lower than that of *Synechococcus* and similar to values observed at HOT and BATS ($\sim 0.5 \times 10^5$ to 2×10^5 cells ml^{-1}). Cell numbers of both *Synechococcus* and *Prochlorococcus* increased at the edge of the dome before decreasing outside, with *Prochlorococcus* reaching extremely high densities of 1.8×10^6 cells ml^{-1} (Fig. 2B). Fluorescence per cell of *Synechococcus* showed a gradual decrease

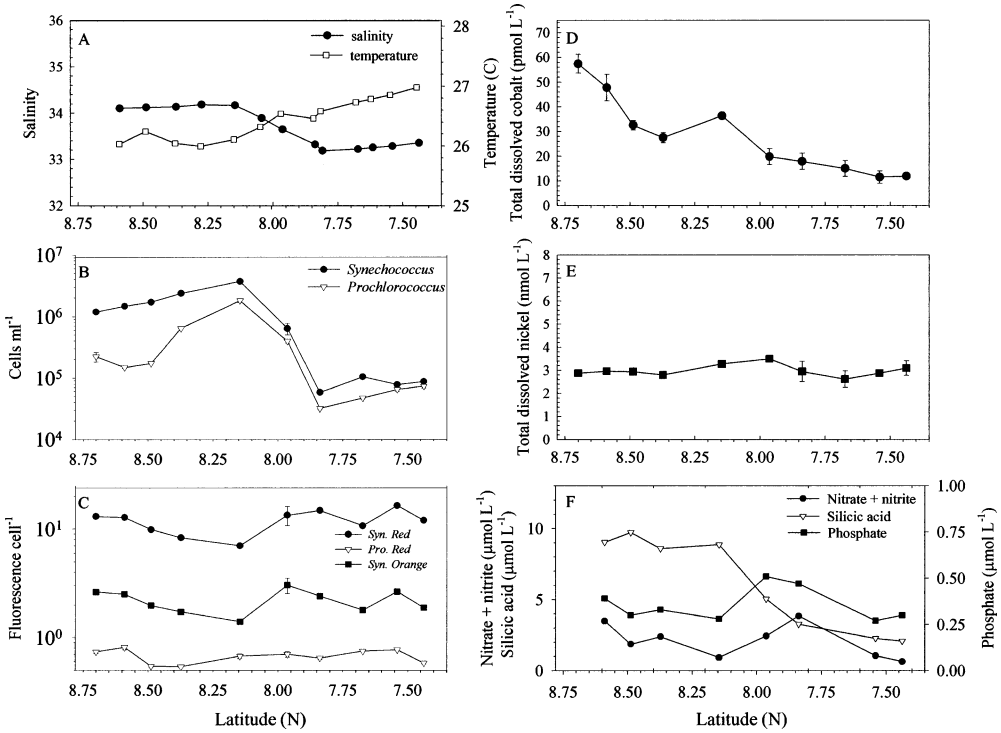

Fig. 2. Chemical and biological characteristics of a surface transect while exiting the CRD. (A) Salinity and temperature, (B) abundances of *Synechococcus* and *Prochlorococcus*, (C) fluorescence per cell, (D) total dissolved cobalt, (E) total dissolved nickel, and (F) phosphate, nitrate + nitrite, and silicic acid. Error bars in panels B–E are often smaller than the symbols.

across the dome and an increase at the boundary and outside, suggestive of nutrient or micronutrient stress within the CRD feature (Fig. 2C).

Three independent lines of evidence indicate that *Synechococcus* was the dominant phytoplankton group in the CRD. First, visual inspection of all surface water filters at the CRD station revealed an unusual pink–red color, characteristic of the phycoerythrin pigments in *Synechococcus.* Second, flow cytometry measurements showed the highest *Synechococcus* cell numbers ever observed in an open ocean environment (this study). Third, calculations of the contribution of carbon by *Synechococcus* to particulate organic carbon (POC) shows them to be a major fraction of the POC: *Synechococcus* contributes between 7.7 and 17.7 μmol L^{-1} C, whereas as small diatoms contribute only 0.94 μmol L^{-1} C. Total POC at the CRD was 27.1 + 2.4 μmol L^{-1} C with 60% of the POC in the 0.7–5.0-μm size class and the remaining 40% in the greater than 5.0-μm size class (Franck et al. 2003). Carbon from *Synechococcus* was calculated by converting cell number (10^6 cells ml^{-1}) to carbon using values (92.4–213 μmol $cell^{-1}$ C) determined in laboratory studies with marine *Synechococcus* strains WH8012 (nonmotile) and WH8103 (motile) (Bertilsson et al. 2003). Carbon in small diatoms was estimated using biogenic silica in the 0.7–5.0-μm size class and converting to carbon using the Si : C ratios measured in two strains of the small pennate diatom *Nitzschia* sp. (0.08, 0.09) (Brzezinski 1985).

Concentrations of total dissolved cobalt were as high as 57 pmol L^{-1} in the surface waters of the dome (Fig. 2D) and decreased to 12 pmol L^{-1} south of the dome. These values are somewhat higher than surface values observed elsewhere. For example, a time series of cobalt in the oligotrophic Sargasso Sea averaged 20 ± 10 pmol L^{-1} between 40 and 47 m throughout 1999 (Saito and Moffett 2002). Total dissolved nickel did not change appreciably throughout the transect (Fig. 2E, mean concentration of 3.0 ± 0.3 nmol L^{-1}), except for a slight increase at the boundary of the CRD. Phosphate and nitrate + nitrite decreased only slightly across CRD region and silicic acid concentrations decreased significantly outside of the dome, suggestive of an increased diatom presence there (Fig. 2F).

A vertical profile within the CRD (8.67°N and 90.6°W) showed a shallow mixed layer of 13 m with 26°C waters, and upwelling of high cobalt from below (Fig. 3A–F). Surface depletion of major nutrients began slightly below the mixed layer (Fig. 3C,D). Surface depletion of cobalt was also observed, with 93 pmol L^{-1} cobalt at 90 m depth decreasing to 45 pmol L^{-1} at 10 m (Fig. 3E). Chemical speciation measurements on these samples showed significant concentrations of labile cobalt being upwelled (40% labile) at 90 m, but in surface waters the only remaining cobalt was bound to strong complexes (Fig. 3F). Together, these data imply rapid biological use of the labile cobalt until its depletion, leaving a residual of organically complexed cobalt in surface waters.

Previously, cultures of *Prochlorococcus* (high light strain MED4) were shown to be capable of using both inorganic Co^{2+} *and* organically complexed cobalt (Saito et al. 2002).

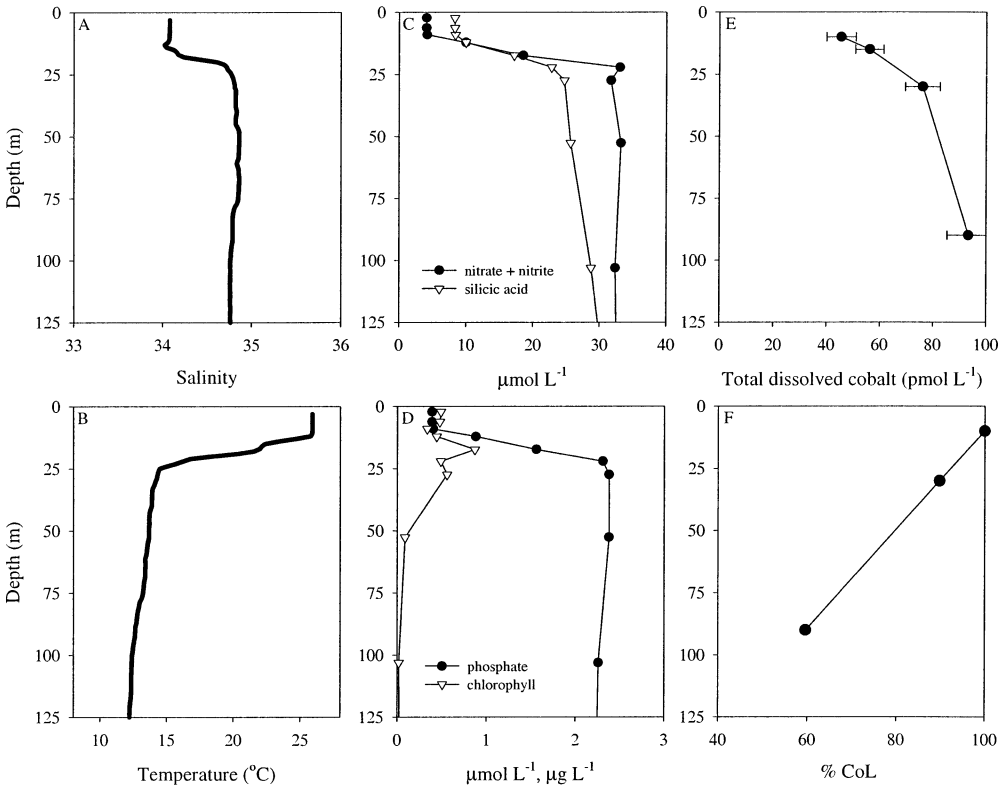

Fig. 3. Vertical profiles of salinity temperature, total dissolved cobalt, cobalt speciation, nitrate + nitrite, phosphate, and chlorophyll at the CRD (northern station on transect).

Those results are consistent with the results found here: a large population of *Synechococcus* was present in surface waters where all cobalt was present as strongly complexed cobalt. In order to persist, the *Synechococcus* population would need to be able to access the organically complexed cobalt reservoir, given the absolute cobalt requirement of *Synechococcus* (Sunda and Huntsman 1995; Saito et al. 2003). The vertical profile showing decreasing labile cobalt suggests this is the preferred chemical form of cobalt and that uptake of strongly complexed cobalt is a secondary mechanism. Similar results were observed in the Peru upwelling region where a large flux of labile cobalt was removed linearly with phosphate on a surface transect, leaving a residual of ~50 pmol L^{-1} strongly complexed cobalt (Saito et al. unpubl. data). These results suggest that cobalt speciation may play an important role on the bioavailability of this metal in the natural environment, where labile cobalt is more easily obtained, but only certain phytoplankton are able to use complexed cobalt. Given the combination of high biomass as a fraction of total POC and the small size of *Synechococcus* (≤ 1 μm) relative to eukaryotic algae, *Synechococcus* also must dominate the cellular surface area present relative to eukaryotic phytoplankton based on scaling of surface area relative to volume. We hypothesize that the abundant *Synechococcus* population in the CRD is both producing cobalt ligands and causing the depletion of labile Co, but since this is a natural assemblage there may be a contribution of other microbial components of the system to cobalt use and transformations.

Cyanobacterial phylogeny—To examine the genetic diversity of the cyanobacterial population in the CRD, portions of the 16S and 23S rDNAs and the entire ITS region between them were amplified. The full-length ITS sequences determined for 27 clones fell into three distinct groups within the marine picoplankton lineage. The majority of the clones (17) are most closely related to *Synechococcus* strain MIT S9220 (Fig. 4) isolated from the Equatorial Pacific (0°, 140°W; Moore et al. 2002). This strain is incapable of growth on nitrate as a sole N source, although it can use nitrite (Moore et al. 2002), raising the possibility that some *Synechococcus* in the CRD may also have this deficiency. The second group includes six clones that form a monophyletic clade (Fig. 4) that does not include any cultured isolate of *Synechococcus* sequenced to date (Rocap et al. 2002) including novel clades recently described from the Red Sea (Fuller et al. 2003). The remaining four clones belong to high light adapted *Prochlorococcus* clade II (data not shown). No clones were found to be members of *Synechococcus* clade II, which has been observed to be dominant in the California current (Toledo and Palenik 2003) and the oligotrophic Red Sea (Fuller et al. 2003).

While *Prochlorococcus* dominates *Synechococcus* numerically in many open ocean environments, a notable exception occurs at BATS in March–April when *Synechococcus* concentrations approach or exceed those of *Prochlorococcus* (DuRand et al. 2001). This is coincident with maximal surface nitrate concentrations in February–March and can be explained by the inability of *Prochlorococcus* to use nitrate

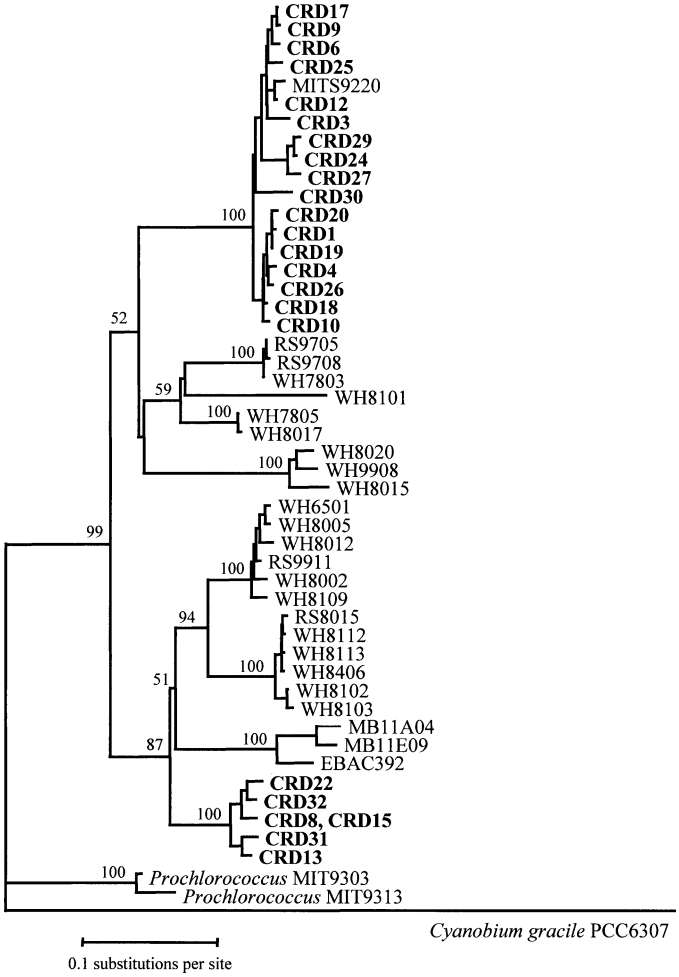

Fig. 4. Phylogenetic tree of marine *Synechococcus* strains and cloned sequences recovered from the Costa Rica dome (shown in bold) inferred from 16S–23S rDNA internally transcribed spacer sequences. Two strains of high B/A *Prochlorococcus* (MIT9303 and MIT9313) and three environmental sequences from Monterey Bay (MB11A04, MB11E09, EBAC392) are also included. The phylogenetic framework was determined using HKY85 distances and minimum evolution as the objective function. Bootstrap values from 1,000 resamplings are listed to the left of each node, with values less than 50 not shown. The tree is rooted with *Cyanobium gracile* PCC 6307.

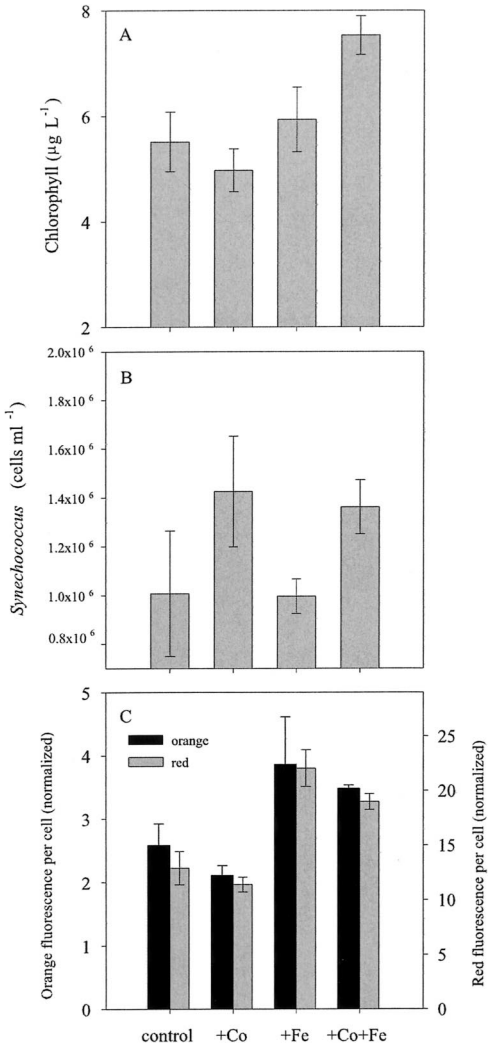

Fig. 5. (A) Bottle incubation chlorophyll results under cobalt, (+Co), iron (+Fe), and combined Co and Fe additions (+Co +Fe), relative to an unamended control. $CoCl_2$ and $FeSO_4$ additions were 420 pmol L^{-1} and 0.83 nmol L^{-1}, respectively. Error bars represent standard deviation of triplicate bottles. (B) *Synechococcus* cell numbers, and (C) *Synechococcus* red and orange fluorescence per cell, normalized to added fluorescent beads. Results reflect 2 d of incubation.

(Waterbury et al. 1986; Moore et al. 2002). Difference in N source use capabilities may also play a role in the dominance of *Synechococcus* at the Costa Rica dome since high concentrations of nitrate are present even in surface waters (Fig. 2F). However, the *Synechococcus* phylogeny presented here shows the majority of clones are most similar to MIT S9220, a strain that cannot use nitrate. Isolation and physiological analysis of *Synechococcus* strains from the Costa Rica dome will be necessary to verify their nitrate use capabilities.

Synechococcus also predominates over *Prochlorococcus* in the mixed layer under stratified conditions in the subtropical north Atlantic. This has been attributed to copper toxicity associated with photodegradation of organic ligands that otherwise would lower copper bioavailability and a higher copper sensitivity in *Prochlorococcus* than *Synechococcus* (Mann et al. 2002). There is no evidence for such a scenario in the Costa Rica dome; no studies to date have shown a similar phenomenon anywhere in the Pacific, where total copper concentrations tend to be lower.

Bottle incubation studies—Bottle incubation studies were conducted to test the hypothesis that this community was a source of natural cobalt ligands. Enrichments of 420 pmol L^{-1} Co and 0.83 nmol L^{-1} Fe alone did not show significant increases in chlorophyll after 48 h, but when added together Co and Fe did show a 35% increase in chlorophyll (Fig. 5A) (significance $p < 0.05$), suggestive of evidence of cobalt and iron colimitation. Flow cytometry analyses (Fig. 5B) showed increases in *Synechococcus* abundances with Co alone and the combined Co and Fe treatments, but not in the Fe-alone

treatment, suggestive of cobalt limitation in *Synechococcus* and Co–Fe colimitation in total chlorophyll ($p < 0.05$). Interestingly, orange and red fluorescence per cell in *Synechococcus* increased with iron treatments but not with cobalt alone (Fig. 5C) (significance $p < 0.05$ for orange fluorescence and $p < 0.01$ for red fluorescence). These results are consistent with laboratory and field studies for cobalt and iron, respectively, where cobalt limitation in *Prochlorococcus* did not influence fluorescence per cell while iron limitation did (Cavender-Bares et al. 1999; Saito 2001). A possible interpretation of these data is that the *Synechococcus* population in the CRD was limited by cobalt since cobalt additions increased cell numbers, yet the *Synechococcus* population was also experiencing iron *stress* since change to iron additions improved fluorescence per cell but not cell numbers. There is currently limited information about the biochemical functions of cobalt in marine phytoplankton. Cobalt is used as the metal center of vitamin B_{12} (cobalamin), as well in a diatom carbonic anhydrase (Lane and Morel 2000; Saito et al. 2002). The concept of colimitation is complex, with at least two possible manifestations: (1) two nutrients with unrelated biochemical functions that are both low enough in concentration to induce limitation of growth simultaneously and (2) two nutrients (e.g., metals) that can substitute for each other biochemically, either within a single enzyme or within two enzymes each designed for a particular metal. The results presented here are suggestive of the first type of colimitation based on the discussion above and the lack of currently known biochemical systems capable of cobalt–iron substitution. Concurrent experiments of a longer duration (6 d) by Franck et al. at the CRD are also suggestive of multiple elements influencing phytoplankton dynamics at the CRD: they observed significant stimulation of total eukaryotic phytoplankton cells by Fe and Zn addition (29-fold) but a much smaller increase with Fe alone (2.5-fold) and no significant effect with Zn alone (Franck et al. 2003). In that study, the larger algal cells were dominated by small pennate diatoms (<5 μm), and no enumeration of cyanobacteria was included.

Speciation measurements on cobalt-amended bottle incubation samples showed that this *Synechococcus*-dominated community appeared to be a significant source of cobalt ligands with 392 pmol L^{-1} and 401 pmol L^{-1} CoL being produced relative to filtered controls in the cobalt-amended and cobalt–iron-amended treatments, respectively (Fig. 6). These concentrations of cobalt ligands produced in the cobalt-enriched bottle experiments are exceptionally high compared with measured water column values. For instance, typical concentrations of cobalt ligands in the Sargasso Sea are in the 5–20 pmol L^{-1} range (Saito and Moffett 2001). In contrast, no production of cobalt ligands was observed in a cobalt-amended control treatment with the microbial community removed by 0.2-μm filtration through a sterile acid-cleaned polycarbonate filter (a small amount of preexisting cobalt remained complexed; Fig. 6). These results show that cobalt-ligand production here was not an abiotic geochemical process but rather required the presence of this cyanobacterial-dominated community to occur. Whether or not these ligands are actively synthesized and secreted (e.g., siderophores or cobalophores; Saito et al. 2002) or produced accidentally upon release from cytoplasms by grazing and/or viral lysis is unknown at this time.

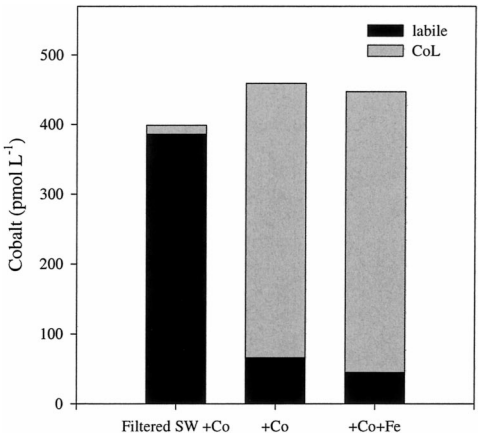

Fig. 6. Cobalt speciation on bottle incubation samples after 5 d. The control treatment represents a filtered seawater control amended with Co and allowed to incubate under the same conditions as the enrichment experiment.

Cobalt binding strength: detection window analysis and nickel substitution studies—Methods to analyze cobalt speciation at the low concentrations found in open ocean environments only became available recently, and hence little is known about the chemical characteristics of natural cobalt ligands (Ellwood and van den Berg 2001; Saito and Moffett 2001). Previously, we estimated a conditional stability constant of $10^{16.3\pm0.9}$ for cobalt ligands in the Sargasso Sea but discussed two alternate interpretations of the data: (1) that the conditional stability constant was a lower limit due to the possibility of a Co(III) redox state or (2) that if cobalt was present in the Co(II) oxidation when bound to the natural ligand(s), there was a significant possibility of nickel competing for this same group of natural ligands (Eq. 3, Saito and Moffett 2001).

$$CoL + Ni^{2+} \Leftrightarrow Co^{2+} + NiL \quad (3)$$

Nickel competition is possible because many synthetic cobalt ligands also have a high affinity for nickel; furthermore, nickel tends to be abundant and labile in seawater, making it available for equilibrium reactions (Achterberg and van den Berg 1997; Saito unpubl. data). Detection window and nickel substitution effects were studied on samples from both the CRD waters and from cobalt-enriched bottle experiments at the CRD. By increasing concentrations of dimethylglyoxime the detection window for cobalt ligands was modulated from $10^{10.8}$ to $10^{16.8}$ (Table 1). Despite sufficient equilibration time (>12 h), these experiments were unable to dissociate significant amounts of cobalt from the cobalt-natural ligands complex by increasing DMG concentrations (Eq. 4, Fig. 7A):

$$CoL + 2HDMG^{-} \Leftrightarrow Co(HDMG)_2 + L^{2-} \quad (4)$$

This experiment was repeated for the cobalt-amended bottle incubation treatments with similar results, demonstrating that the natural ligands at the CRD and those produced by the

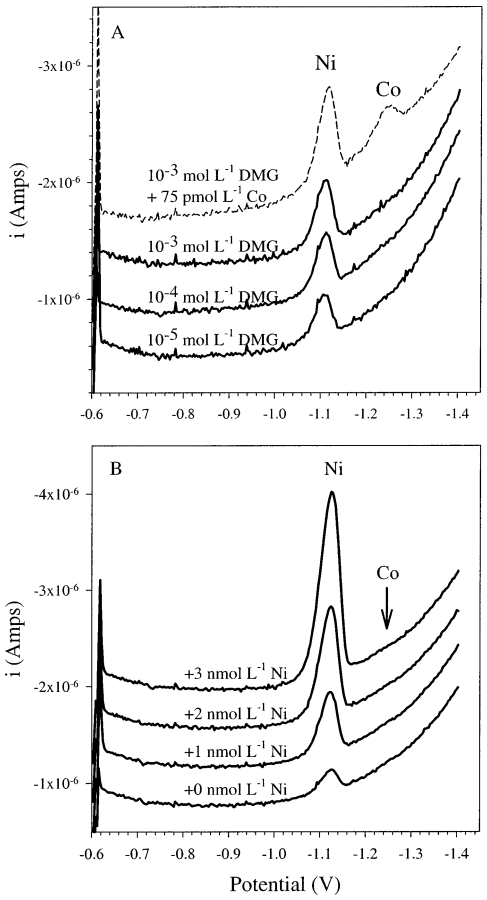

Fig. 7. (A) Detection window results showing strength of cobalt ligands by increasing DMG concentrations (*see Table 1*). The dotted line indicates a cobalt addition to demonstrate where the cobalt peak occurs. Natural cobalt complexes were not observed to dissociate under these conditions. This experiment was repeated with samples from the bottle incubation experiment (Fig. 6) with identical results. (B) Nickel competition experiments are unable to compete for the cobalt site in the natural organic ligands. An arrow is used to demonstrate where the cobalt peak would occur.

Synechococcus-dominated microbial community had a conditional stability constant greater than a log *K* of 16.8. These results are in conflict with previous results by Ellwood and van den Berg showing exchange of CoL with the cobalt ligand nioxime (Ellwood and van den Berg 2001). While nioxime is a slightly stronger ligand for Co than DMG, a much smaller concentration of nioxime relative to DMG was used resulting in similar detection windows (Ellwood and van den Berg 2001). We cannot explain this discrepancy at this time since our detection window extends above their calculated stability constant for CoL. It is possible that this may be due to different chemical properties of CoL in different environments, with weaker CoL complexes present in the North Atlantic/English Channel in their study because of a differing biota. Another possibility is that the synthetic cobalt ligand in their study, nioxime, behaves differently in cobalt speciation experiments.

Cobalt–nickel competition for natural cobalt ligands was also tested using nickel additions that were allowed to equilibrate overnight with the seawater. In all cases the additions were unable to release strongly bound cobalt by competition for natural ligands (Eq. 3; Fig. 7B).

These results show that the ligands in the CRD region and those being produced by the *Synechococcus*-dominated microbial assemblage in bottle incubation experiments are very strong ligands with conditional stability constants greater than $10^{16.8}$ and that nickel cannot compete for the cobalt site in the cobalt-ligand complex. Calculations using thermodynamic stability constants of known strong cobalt and nickel ligands in the National Institute of Standards (NIST) database show that nickel should be able to effectively compete with Co(II) when in higher concentrations than that of cobalt (Martell and Smith 1993; Saito and Moffett 2001). Unless there are other Co(II) ligands that have yet to be characterized and added to the thermodynamic databases, the only means by which nickel and DMG would not be able to effectively compete for either the natural ligand or cobalt, respectively, would be if Co were bound to the complex in a Co(III) redox state. Co(III) is known to be exceptionally inert and forms complexes with exceptionally high stability constants (Shriver et al. 1990; Martell and Smith 1993) for which nickel and DMG would be unlikely to compete. These Ni competition data are consistent with the detection window analysis above, which suggests strong Co(III) ligands. An alternative to this Co(III) explanation is the presence of a colloidal form of Co(II), as has been recently described for Fe speciation (Wu et al. 2001). If Co(II) was physically prevented from approaching equilibrium by being buried within an organic matrix, it likely would also behave inertly.

It is possible that cobalt ligands are in fact cobalamin (vitamin B_{12}) or its degradation products. Cobalamin concentrations, as measured by bioassay, tend to be quite low in oceanic environments (<1 pmol L^{-1}) (Menzel and Spaeth 1962). No measurements have ever been made for B_{12} at the CRD. Cobalt is coordinated in B_{12} by a corrin ring structure and a covalent organometallic bond to a cyanide group at an axial position. While redox activity does occur in B_{12}, it is typically in the Co(III) redox state (Glusker 1995). Previous calculations of the conditional stability constant of cobalt in B_{12} (Ellwood and van den Berg 2001) probably underestimate the actual value since no removal of cobalt from the cobalamin structure was observed.

There are important implications if some or all cobalt ligands in nature are Co(III) rather than Co(II) ligands. In retrospect, such results make sense given that nickel is typically present in nanomolar concentrations of labile form: any labile nickel would likely exchange with Co(II) and release it from natural ligands. Natural Co(III) complexes would be resistant to such competition, allowing the ligands to persist especially in deeper waters where nickel concentrations are high. There are few nickel speciation data available, but they have always been found to be dominated by labile nickel (Achterberg and van den Berg 1997; Saito unpubl. data). This higher predicted stability of natural Co ligand (as Co(III)L) would result in an increased difficulty in releasing cobalt from these complexes prior to their microbial use. A parallel problem occurs for Fe(III), where Fe(III) complexes are much more stable than Fe(II) complexes (Witter et al. 2000). In this case, microbes use iron reduc-

tases to reduce Fe(III) to Fe(II) within the ligand, which then facilitates its exchange with cellular uptake systems. While reduction of cobalt-EDTA has been observed in microbial cultures (Blessing et al. 2001), the enzyme responsible for this reduction process has not yet been elucidated. Furthermore, ''cobalt-reductase'' activity has never been demonstrated in cyanobacterial or algal cultures.

With such a high cobalt conditional stability constant, it is conceivable that cobalt ligands advected into deep waters could persist, effectively protecting cobalt from scavenging for hundreds to thousands of years. Inorganic complexes of Co(II) (e.g., $CoCl^{+}$ and Co^{2+}) are known to be effectively sequestered by manganese oxides via microbial manganese oxidation (Moffett and Ho 1996), as well as nonspecific cation adsorption onto natural organic matter (Brown and Parks 2001), and are presumably significantly more particle reactive than the strong CoL chemical complexes observed here. Hence, the biogenic production of strong Co(III) ligands in surface waters and their transport to deep waters (Saito and Moffett 2001) would help explain the persistence of some total cobalt in deep waters despite the constant flux of particles and associated particle scavenging.

The presence of labile cobalt in upwelled waters of the CRD is intriguing given the measurements of complexed deep-water cobalt in the North Atlantic and North Pacific. A large flux of labile cobalt was recently observed in the Peru upwelling system and was hypothesized to be evidence of release of cobalt from sediments via microbial reduction of manganese oxides (Saito et al. in press) . The high productivity of the Peru upwelling region results in low-oxygen bottom waters that would effectively prevent reoxidation of Mn and Co by association, allowing the cobalt to be carried into surface waters. A similar phenomenon may be occurring here, where cobalt released from sediments near the shelf/slope area is entrained in coastal upwelling. Subsurface isopycnals could then be advected offshore and upwelled in the CRD releasing labile ''new'' cobalt to this environment.

An oceanic Synechococcus bloom?—To the best of our knowledge, the phytoplankton assemblage in the Costa Rica dome has only been studied twice, by Li et al., and during the R/V *Melville* 2000 cruise (Li et al. 1983; Franck et al. 2003; this study). Yet on both visits the CRD was dominated by a *Synechococcus* population with cell numbers more than an order of magnitude higher than other oceanic environments. *Synechococcus,* and unicellular marine cyanobacteria in general, are not typically associated with blooming conditions, but rather with microbial loop ecosystems characterized by tight recycling and regeneration in regions of low chlorophyll due to resource scarcity. Gaining insight to this unique phenomenon of a cyanobacterial bloom in upwelling waters is important in understanding the ecology of these ubiquitous cyanobacteria, as well as improving our understanding of the controls on phytoplankton species composition and resultant effects on the biological carbon pump.

We hypothesize that there may be a unique water column ''chemical signature'' that allows *Synechococcus* instead of larger eukaryotic phytoplankton to bloom. There are several chemical possibilities that can be considered. Major nutrients, including silicic acid, do not appear to be a factor since all are abundant in this upwelling regime (Figs. 2, 3). An iron value of 0.13 nmol L^{-1} was reported for surface waters of the CRD (Franck et al. 2003), yet bottle incubation experiments in both Franck et al. (2003) and this study show only slight iron stimulation effects (2.5-fold increase in eukaryotic phytoplankton after 6 d, or no significant increase in total chlorophyll or *Synechococcus* cell numbers after 2 d, *see above*).

Copper and cadmium are also additional trace elements that could play a role. Both elements are known to be highly toxic to cyanobacteria. Upwelling waters are also known to supply cadmium (Takesue and van Geen 2002). While there are no Cu or Cd data, we suspect that these elements are present in a strongly complexed chemical form in order to allow *Synechococcus* to thrive, given its lower toxicity threshold to these elements relative to eukaryotic algae (Brand et al. 1986; Payne and Price 1999; Saito et al. 2003). Such complexation would also result in a diminishing of the bioavailability of Cd for substitution with Zn nutrition in diatoms. Future studies at this region should test this hypothesis with speciation measurements of these elements.

The chemical attributes of cobalt that we have measured in this study are highly unusual, and yet consistent with what is known about cyanobacteria: the flux of high cobalt into surface waters and its depletion there corresponds with the absolute requirement for cobalt in *Prochlorococcus* and *Synechococcus* (Sunda and Huntsman 1995; Saito et al. 2002). The presence of labile cobalt and complexed cobalt in surface waters corresponds to the capability to use either chemical form in *Prochlorococcus* (and presumably *Synechococcus*) (Saito et al. 2002). The influence of zinc and iron on small pennate diatoms at the CRD (Franck et al. 2003) is also consistent with what is known about diatom requirements for cobalt and zinc: centric diatoms use zinc but are capable of substituting Co and to a lesser extent Cd (Lane and Morel 2000 and references therein; no Co, Zn, and Cd physiological data are available for pennate diatoms). While cobalt is relatively high in concentration at the CRD, it is in complexed form in surface waters and is probably biologically unavailable to diatoms, which are believed to only acquire labile cobalt based on culture experiments (Sunda and Huntsman 1995) and observations of labile cobalt drawdown in the Peru upwelling region where diatoms dominated (Saito et al. in press). While there are no zinc data available for the Costa Rica dome, presumably surface water concentrations are quite low (or highly complexed) in order to cause the stimulation Franck et al. observe.

Thus the chemical signature of the Costa Rica dome may include high Co abundances and ligand production, the complexation of Cd and Cu, and relatively low Fe and Zn concentrations. In this scenario diatoms may be stymied by Zn/Co limitation (with Fe) as shown in this study and other studies (Franck et al. 2003) and are unable to substitute Cd because of complexation. The strong complexation of cobalt lowers its bioavailability to diatoms but enables cyanobacteria to grow, and the high nitrate concentrations favor *Synechococcus* over *Prochlorococcus.* Future work at the Costa Rica dome should include the characterization of the chemical speciation of Cd, Cu, and Zn and the physiological characterization of *Synechococcus* strains in order to better

understand what allows *Synechococcus* to be more competitive than eukaryotic algae in these upwelling waters.

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