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Vitamin B_{12} and iron colimitation of phytoplankton growth in the Ross Sea

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Abstract

Primary production in the Ross Sea, one of the most productive areas in the Southern Ocean, has previously been shown to be seasonally limited by iron. In two of three bottle incubation experiments conducted in the austral summer, significantly higher chlorophyll *a* (Chl *a*) concentrations were measured upon the addition of iron and B_{12}, relative to iron additions alone. Initial bacterial abundances were significantly lower in the two experiments that showed phytoplankton stimulation upon addition of B_{12} and iron relative to the experiment that did not show this stimulation. This is consistent with the hypothesis that the bacteria and archaea in the upper water column are an important source of B_{12} to marine phytoplankton. The addition of iron alone increased the growth of *Phaeocystis antarctica* relative to diatoms, whereas in an experiment where iron and B_{12} stimulated total phytoplankton growth, the diatom *Pseudonitzschia subcurvata* went from comprising approximately 70% of the phytoplankton community to over 90%. Cobalt additions, with and without iron, did not alter Chl *a* biomass relative to controls and iron additions alone in the Ross Sea. Iron and vitamin B_{12} plus iron treatments caused reductions in the DMSP (dimethyl sulfoniopropionate) : Chl *a* ratio relative to the control and B_{12} treatments, consistent with the notion of an antioxidant function for DMSP. These results demonstrate the importance of a vitamin to phytoplankton growth and community composition in the marine environment.

The nutritional controls on marine phytoplankton growth have important implications for the regulation of the global carbon cycle. Nitrogen and iron are thought to be the dominant controllers of phytoplankton growth in the oceans, and hence the discovery of a vitamin such as B_{12} having an influence on marine primary productivity would be a finding of significance. The limited information about the biogeochemical cycle of this vitamin suggests that it may be in limiting quantities in seawater. B_{12} is a biologically produced cobalt-containing organometallic molecule, and only select bacteria and archaea possess the capability for B_{12} biosynthesis. As a result, all eukaryotic organisms, from eukaryotic phytoplankton to humans, must either acquire B_{12} from the environment or possess an alternate biochemistry that does not require the vitamin. Removal of B_{12} from the water column has never been directly quantified but likely includes photodegradation

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(Carlucci et al. 1969; Saito and Noble unpubl. data), phytoplankton and bacterial uptake, export in sinking biogenic material, and physical transport (Karl 2002). The biogeochemical cycle of vitamin B_{12} must also be inextricably tied to that of cobalt, given the cobalt metal center inside the corrin ring of B_{12}. Both of these substances (B_{12} and cobalt) are found in vanishingly low concentrations in seawater. The few measurements of vitamin B_{12} in seawater reveal extremely low concentrations, in the subpicomolar range, in oceanic regions and higher concentrations in a heavily populated coastal region (Menzel and Spaeth 1962; Okbamichael and Sañudo-Wilhelmy 2004; Sañudo-Wilhelmy et al. 2006). Moreover, B_{12} concentrations have been found to vary seasonally, with a maximum in winter followed by a decline during the spring bloom in the Gulf of Maine and the Sargasso Sea (Menzel and Spaeth 1962; Swift 1981).

Early workers demonstrated a B_{12} requirement in many marine algae and hypothesized that B_{12} could influence marine primary production and phytoplankton species composition (Droop 1957; Guillard and Cassie 1963; Swift 1981). A recent literature review of 326 algal species found more than half to be B_{12} auxotrophs (Croft et al. 2005), similar to earlier estimates that 70% of phytoplankton species require the vitamin (Swift 1981). The enzyme methionine synthase is believed to be responsible for this B_{12} requirement in phytoplankton, where B_{12}-requiring phytoplankton have the B_{12}-dependent methionine synthase (MetH), while nonrequirers have a B_{12}-independent methionine synthase (MetE) (Rodionov et al. 2003; Croft et al. 2005). This enzyme catalyzes the last step in the synthesis of the amino acid methionine. The variation in methionine synthase isoforms is a likely mechanism for the hypothesized influence of vitamin B_{12} concentrations on phytoplankton species composition in the ocean.

The sources of vitamin B_{12} to marine phytoplankton in the natural environment are only beginning to be understood. The arrival of whole genome sequencing suggests distinct niches in the surface ocean. For example, the need for an exogenous source of B_{12} in eukaryotic phytoplankton is evident in the first marine eukaryotic phytoplankton genome, *Thalassiosira pseudonana*: it lacks the vast majority of the B_{12} biosynthesis pathway (Armbrust et al. 2004) and contains the B_{12}-requiring metH for methionine synthesis (Croft et al. 2005), consistent with the culture studies described above. In contrast, the B_{12} biosynthesis pathway is found in many, though not all, bacteria and archaea (Rodionov et al. 2003 and references therein), including all of the currently available genomes of marine cyanobacteria (oxygenic photoautotrophs) such as the globally abundant *Prochlorococcus* and *Synechococcus* (Partensky et al. 1999; Palenik et al. 2003). Interestingly, the genome of the marine heterotrophic bacterium *Pelagibacter ubique* (a cultured isolate from the highly abundant SAR11 clade) lacks the B_{12} biosynthetic pathway as well as that of several other vitamins (Giovannoni et al. 2005), suggesting that this microbe is dependent on an external dissolved supply of vitamins. Early work suggested that marine heterotrophic bacteria could supply phytoplankton with enough B_{12} for growth in culture experiments (Haines and Guillard 1974). More recently, a laboratory study has shown that a eukaryotic phytoplankter (*Porphyridium purpureum*) can acquire vitamin B_{12} through a close bitrophic symbiotic relationship with cell-surface-associated heterotrophic bacterial populations (Croft et al. 2005). Together this information suggests at least two possible sources of B_{12} to eukaryotic marine phytoplankton in the natural environment: uptake of dissolved B_{12} and acquisition of B_{12} through cell-surface symbioses. We hypothesize that in the former scenario, dissolved B_{12} is released through grazing and viral lysis of bacteria and archaea as part of the microbial loop (Azam 1998 and references therein), and this flux may be important in regions where the cyanobacteria are a major component of the ecosystem.

It has been hypothesized that methionine biosynthesis could be involved in controlling the rate of dimethyl sulfoniopropionate (DMSP) production by some phytoplankton (Gröne and Kirst 1992). Because of the role of vitamin B_{12} in methionine synthesis, it is possible that there may be a connection between B_{12} and the cycling of the DMSP in the surface ocean. DMSP serves as the precursor to dimethyl sulfoxide and other atmospherically and climatically important chemical species (Charlson et al. 1987). DMSP is believed to be produced by phytoplankton for several biochemical roles, including as an osmolyte, a cryoprotectant (Stefels 2000), and as an antioxidant (Sunda et al. 2002).

In this study we present experimental data from the Ross Sea, which harbors one of the most extensive phytoplankton blooms in the Southern Ocean (Smith and Nelson 1985) and hence is believed to play a significant role in the global carbon cycle (Arrigo et al. 1999). The phytoplankton community in the Ross Sea is dominated by the colonial haptophyte *Phaeocystis antarctica* and a variety of diatoms such as *Pseudonitzschia subcurvata* (Arrigo et al. 1999). The phytoplankton population varies seasonally (Smith et al. 2000), with *P. antarctica* typically blooming in the spring and early summer, followed by an increase in diatom growth in the later summer (Arrigo et al. 1999 and references therein; Leventer and Dunbar 1996; Smith et al. 2000). Primary production in the Ross Sea has been shown to be controlled by the availability of iron as a micronutrient as well as physical factors such as irradiance (Martin et al. 1990 and references therein; Sedwick et al. 2000; Coale et al. 2003). Alternative micronutrients, such as zinc, have not demonstrated any influence on phytoplankton growth in the Ross Sea (Coale et al. 2003), likely due to relatively high concentrations of these metals near the photic zone. The Ross Sea is a region of biogeochemical significance because of its particularly efficient carbon export (Buesseler et al. 2001), and the high rate of biological production of DMSP by phytoplankton, notably *P. antarctica*. The efficient export of biogenic material in this region (DiTullio et al. 2000; Buesseler et al. 2001) also suggests that incorporated micronutrients such as vitamin B_{12} are being exported rather than recycled within the ecosystem.

The low heterotrophic bacterial production rates (Ducklow and Carlson 1992; Ducklow 2000; Ducklow et al. 2001 and references therein), low grazing rates (Caron et al.

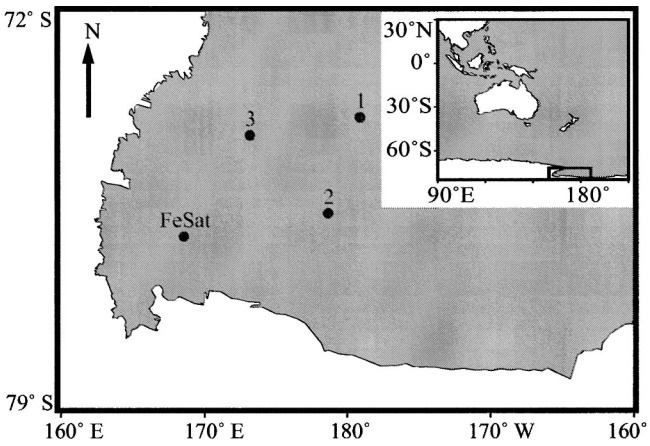

Fig. 1. Location of incubation experiments in the Ross Sea on the Antarctic continental shelf. Incubation 1 was carried out at 74°26′S, 179°23′W; incubation 2 at 76°00′S, 178°66′E; incubation 3 at 74°60′S, 173°20′E; and the iron saturation curve experiment (FeSat) at 076°39′S, 168°58′E.

2000), and absence of cyanobacterial populations in the Ross Sea (Walker and Marchant 1989; Caron et al. 2000; Marchant 2005 and references therein) suggest that this region may lack the potential sources of B_{12} to the marine environment that are common in subtropical and tropical oceanic environments. In addition, the higher intensity of ultraviolet irradiation in the Southern Ocean due to the ozone hole in the austral spring (Cruzen 1992) could conceivably increase photodegradation of the vitamin relative to other areas of the ocean. Hence, the Ross Sea is an ideal location to study the influence of vitamin B_{12} on primary productivity and phytoplankton community structure. In this manuscript, we present experiments demonstrating the colimitation of the Ross Sea by iron and vitamin B_{12} during the austral summer.

Materials and methods

Study area and water collection—All experiments were conducted in the Ross Sea on the CORSACS 1 (Controls on Ross Sea Algal Community Structure) cruise, in the austral summer of 2005 (NBP0601). Experiment 1 was started on 27 December 2005 at 74°26′S, 179°23′W. Experiment 2 was started on 08 January 2006 at 76°00′S, 178°67′E. Experiment 3 was started 16 January 2006 at 74°60′S 173°20′E (Fig. 1). For all experiments, water was collected from 5–8 m depth using a trace-metal-clean Teflon pumping system. Water was dispensed into a 50-liter trace-metal-clean mixing carboy and then into detergent- and acid-washed (0.1% citranox for 48 h, 10% HCl for 7 d, clean pH 2 water rinsed) 1.1- or 4.5-liter polycarbonate bottles. Incubation bottles were filled in a positive-pressure trace-metal-clean environment constructed with laminar flow hoods and plastic sheeting to avoid trace metal contamination.

Shipboard incubations—In general, bottle incubations with additions of 1 or 2 nmol L^{-1} iron, 500 pmol L^{-1} added cobalt, and 100 pmol L^{-1} added vitamin B_{12} were started from three locations within the Ross Sea and carried out for 7–9 d. The length of the experiments was determined by the extent of nutrient depletion in each experiment using shipboard analyses in near-real time. Treatment concentrations were later corrected to account for exact bottle volume. Experiment 1 consisted of six treatments (control, Fe, B_{12}, B_{12}Fe, Co, and CoFe). Duplicate treatments were prepared in 1.1-liter bottles and single treatments were prepared in 4.5-liter bottles at concentrations of 0.9 nmol L^{-1} added iron (Fe), 90 pmol L^{-1} added vitamin B_{12} (B_{12}), 450 pmol L^{-1} added cobalt (Co), 450 pmol L^{-1} added cobalt and 0.9 nmol L^{-1} added iron (CoFe), or 90 pmol L^{-1} B_{12} and 0.9 nmol L^{-1} iron (B_{12}Fe). Experiment 2 consisted of six treatments (control, Fe, B_{12}, B_{12}Fe, Co, and CoFe). Triplicate treatments were prepared in 1.1-liter bottles and single treatments were prepared in 4.5-liter bottles at concentrations of 1.8 nmol L^{-1} added iron (Fe), 90 pmol L^{-1} added vitamin B_{12} (B_{12}), 450 pmol L^{-1} added cobalt (Co), 450 pmol L^{-1} added cobalt and 1.8 nmol L^{-1} added iron (CoFe), or 90 pmol L^{-1} B_{12} and 1.8 nmol L^{-1} iron (B_{12}Fe). Experiment 3 consisted of four treatments (control, Fe, B_{12}, and B_{12}Fe). Triplicate treatments in 1.1-liter bottles were prepared in concentrations of 1.8 nmol L^{-1} added iron (Fe), 90 pmol L^{-1} added vitamin B_{12} (B_{12}), or 90 pmol L^{-1} B_{12} and 1.8 nmol L^{-1} iron (B_{12}Fe). When nitrate levels in any of the treatments dropped below approximately 5 μmol L^{-1}, the experiment was ended.

All three experiments were tightly capped and placed in deckboard flow-through incubators at ~20% ambient light, shielded with neutral density screening. Ambient temperature was maintained by a constant flow of surface seawater through the incubators. In all cases, iron was added as $FeCl_3$ (Fluka) in pH 2 (SeaStar HCl) MilliQ water. Cobalt was added as $CoCl_2$ (Fluka) in pH 2 (SeaStar HCl) MilliQ water. Vitamin B_{12} (Sigma, plant cell culture tested cyanocobalamin, 99%) was added as a solution in Milli-Q water, cleaned for trace metals by running through a column with 2–3 mL of prepared Chelex-100 beads (BioRad) (Price et al. 1988/1989). Student's unpaired *t*-tests were used to establish significant difference between treatments; degrees of freedom = 4 in all analyses and *p* values are presented with each data set.

Nutrient analysis—Nutrients, including N+N, nitrite, phosphate, and silicic acid, were measured in the incubation experiments approximately every 60 h on 0.2-μm-filtered samples from each bottle. Analysis was performed at sea using a Lachat QuickChem Autoanalyzer. Minimum detectable levels were 0.02 μmol L^{-1} for phosphate, 0.16 μmol L^{-1} for N+N, 0.03 μmol L^{-1} for nitrite, and 0.18 μmol L^{-1} for silicic acid.

Biomass analysis—Total chlorophyll *a* (Chl *a*) was measured approximately every 60 h using the nonacidified fluorometric method of JGOFS (Joint Global Ocean Flux Study), with a Turner Designs TD700 fluorometer (Whatman GF/F filtered). Bacteria and archaea were enumerated using a previously published method involving DAPI (4′-6-diamidino-2-phenylindole) staining (Porter and Feig,

1980). Diatoms were identified by transmitted light microscopy at ×400 and ×1000 magnification on gridded mixed cellulose ester membrane filters with a 0.45 μm pore size. Phytoplankton were enumerated by 400-individual counts, using standard epifluoresence microscope techniques at ×1000 magnification on 0.2 μm pore size polycarbonate membrane filters. All epifluorescence slides were filtered from 10-mL samples to facilitate visual comparison.

In the iron treatments in incubation 1, atypically high *P. antarctica* populations combined with generally high phytoplankton populations made phytoplankton cell enumeration by our method problematic. These atypical samples were found to have heterogeneous cell distribution across the slide, with diatoms adhering to numerous irregularly dispersed *Phaeocystis* colonies, resulting in large standard deviations for cell counts, but low standard deviations for community composition. In addition, the extremely dense cell concentrations in incubation 1 reduced the count area necessary for enumeration and identification of 400 cells. Given that the *P. antarctica* component increases relative to diatoms only in the Fe addition treatment, that *P. antarctica* colonies greatly increased sample heterogeneity, and that high cellular densities resulted in fewer fields being counted to obtain species composition, we infer that our random field counting method underestimated true cell concentrations in these samples.

Total iron measurements—Total dissolved Fe concentrations were measured using adsorptive cathodic stripping voltammetry (ACSV) based on the method described by Rue and Bruland (1995). Reagents were prepared as follows: A 5 mmol L^{-1} salicylaldoxime (SA: Aldrich, ≥98%) solution was prepared in quartz-distilled methanol (Q-MeOH) and stored in the refrigerator. A final concentration of 25 μmol L^{-1} SA was used for total dissolved Fe measurements. A 1.5 mol L^{-1} borate buffer was prepared as previously described (Ellwood and Van Den Berg 2000). Fe standards were prepared from dilution of a 1,000 parts per million atomic adsorption standard with pH 1.7 quartz-distilled hydrochloric acid (Q-HCl).

The voltammetric system consisted of Princeton Applied Research (PAR) 303A interfaced with a computer-controlled μAutolabII potentiostat/galvanostat (Eco Chemie). The working electrode was a "large" mercury drop (2.8 mm^2), the reference electrode was Ag : saturated AgCl, saturated KCl, and the counterelectrode was a platinum wire. During ACSV analyses, all samples were contained in fluorinated ethylene propylene–Teflon voltammetric cell cups, and stirred with a PTFE (polytetraflrethylane) – Teflon-coated stirring bar driven by a PAR magnetic stirrer (model 305).

Filtered samples were acidified to pH 1.7 with 4 mL L^{-1} Q-HCl. Samples were microwaved 2 × 15 s at 1,100 W to release dissolved Fe from ambient organic ligands (Bruland et al. 2005), neutralized once with cool 1 mol L^{-1} Q-NH_4OH, and buffered to pH 8.2 with the borate buffer. Once buffered, Fe and SA additions were made and following ACSV analysis Fe concentrations were determined from a linear regression of the standard addition curve. The detection limit for the ACSV method is 0.02 nmol L^{-1}, calculated from three times the standard deviation of a 0.05 nmol L^{-1} Fe addition, as no peak is observed in either Milli-Q or ultraviolet (UV)-oxidized seawater (from which trace metals and metal-chelating organic ligands are removed from seawater [Donat and Bruland 1988]) at deposition times of up to 600 s. Deposition times for sample analyses here were between 10 and 300 s, depending on ambient Fe and ligand concentrations.

Cobalt total concentration and speciation measurements—Total cobalt and cobalt speciation analyses were performed by ACSV using a Metrohm 663 hanging mercury drop electrode and Eco-Chemie μAutolab III as described previously (Saito and Moffett 2002; Saito et al. 2005). Briefly, cobalt total measurements were made by first UV-irradiating the seawater for 1 h to destroy the strong organic ligands that bind cobalt. The seawater (9.25 mL) was then analyzed with 0.2 mmol L^{-1} dimethylglyoxime, 0.113 mol L^{-1} sodium nitrite, and 2.5 mmol L^{-1} *N*-(2-hydroxyethy)piperazine-*N*-(3-propanesulfonic acid), as cobalt ligand, catalyst, and buffer respectively. Cobalt speciation was measured similarly but without UV irradiation and with overnight equilibration with the dimethylglyoxime ligand. Cobalt was then measured using 25 pmol L^{-1} standard additions, deposition for 90 s at −0.6 V, and linear sweep stripping from −0.6 V to −1.4 V at 10 V s^{-1}.

Iron blank in vitamin stock—Total iron in the vitamin B_{12} stock was measured using the above technique through dilution into seawater with a known total iron concentration 7 d after it was first used to prepare incubation 1. After use in incubation 1, the B_{12} stock had been used outside a clean area for experiments not described here. At that time, it was found to contain 76 pmol L^{-1} total iron for every 100 pmol L^{-1} vitamin B_{12}. The vitamin stock was then treated with Chelex-100 and the iron concentration was subsequently found to be 29 pmol L^{-1} for every 100 pmol L^{-1} vitamin B_{12}. This stock was then used in incubations 2 and 3. Since the stock had been carefully treated with Chelex-100 and not used outside a clean area before its use with incubation 1, we assume the initial B_{12} solution had an iron concentration of 29 pmol L^{-1} for every 100 pmol L^{-1} vitamin B_{12} when used in all three incubations, although the 76 pmol L^{-1} concentration would not alter the interpretation of experiments presented here. The small amounts of iron added with the B_{12} solution only increased the iron in the iron-B_{12} treatments marginally (from 0.89 nmol L^{-1} to 0.92 nmol L^{-1} iron (Fe) and (B_{12}Fe) iron concentrations in experiment 1, and from 1.81 nmol L^{-1} to 1.84 nmol L^{-1} (Fe) and (B_{12}Fe) iron concentrations in experiments 2 and 3).

Iron saturation curve experiment—An iron saturation curve was constructed (after Hutchins et al. 2002) to demonstrate that this small iron addition with the B_{12} stock could not account for additional phytoplankton growth.

Chl *a* net specific growth rate as a function of total dissolved iron concentration (nmol L^{-1}) was calculated at 76°39′S, 168°58′E on 18 January 2006 where total ambient dissolved iron concentration was 0.09 nmol L^{-1}. This experiment was performed at similar geographical location to the incubation studies (Fig. 1) and with similar initial nutrient profile and Chl *a* concentrations (Table 1). Water was collected with a 10-liter Go-Flo bottle (General Oceanics) from 10 m depth. This water was dispensed, using trace-metal-clean techniques, into 60-mL polycarbonate bottles, trace-metal-cleaned as described above. Duplicate unamended controls and duplicates of 0.2 nmol L^{-1}, 0.45 nmol L^{-1}, 0.9 nmol L^{-1}, and 2.5 nmol L^{-1} added iron were placed in a sealed plastic bag in deckboard flow-through incubators for 7 d at ~20% ambient light, shielded with neutral density screening. At the end of the 7-d incubation period, 50 mL of each bottle were used to measure total Chl *a*. Chl *a* net specific growth rate (μ) was calculated using standard growth rate equations. The relation between iron concentration and Chl *a* net specific growth rate was assumed to follow the Michaelis–Menten equation and the data were fit to this equation using a nonlinear regression.

DMSP measurements—Samples for DMSP were collected following the small-volume gravity filtration procedure of Kiene and Slezak (2006). In a cold room held at 0°C, a small aliquot (≤20 mL) of each sample was gravity-filtered through a Whatman GF/F filter, recollected, and acidified with 100 μL of 50% sulfuric acid for the determination of dissolved DMSP. A second unfiltered aliquot of sample was acidified with 100 μL of 50% sulfuric acid for the measurement of total DMSP. Particulate DMSP was calculated as the difference between the total and dissolved DMSP fractions. All DMSP samples were base-hydrolyzed in strong alkali (>1 mol L^{-1} sodium hydroxide; [White 1982]) and analyzed for dimethyl sulfide (DMS) using a cryogenic purge and trap system coupled to either a Hewlett-Packard 6890 or 5890 Series II gas chromatograph fitted with flame photometric detector (DiTullio and Smith 1995).

Vitamin B_{12} uptake—^{57}Co-labeled cyanocobalamin was used to measure the rate of vitamin B_{12} uptake by the community at 30 m depth at 74°40′S, 168°52′E on 20 January 2006. Uptake by the greater-than-2-μm-size fraction and greater-than-0.2-μm-size fraction was measured.

Radiolabeled vitamin B_{12} (^{57}Co B_{12}) was isolated from Rubratope57 pills (Radiopharmacy). The gelatin capsule coating was removed from the pill and the remaining sponge, laden with ^{57}Co B_{12}, was placed in 5 mL of pH 2.5 Milli-Q water (HCl) and mixed until the sponge was pulverized. The mixture was left to stand protected from light at 4°C for 24 h. The liquid was decanted to remove any large remaining pieces of sponge. The pH of the solution was raised to 7 with NaOH and was cleaned for inorganic ^{57}Co and other trace metals by running through a column with 2–3 mL of Chelex-100 beads (BioRad) and

Table 1. Initial physical, chemical, and biological conditions at each location where an experiment was started. <MDL is less than minimum detectable limit. N.M. is parameter not measured. Where applicable, ±1 SD is indicated.

	Latitude	Longitude	NO_3 (μmol L^{-1})	PO_4 (μmol L^{-1})	SiO_2 (μmol L^{-1})	NH_4 (μmol L^{-1})	Total Fe (nmol L^{-1})	Total Co (pmol L^{-1})	Labile Co (pmol L^{-1})	Chl *a* (μg L^{-1})	Bacteria and archaea (cells mL^{-1})	Phytoplankton (cells mL^{-1})
Incubation 1	074°26′S	179°23′W	19.89	1.36	63.64	0.17	0.31	31.0	30.1	4.319	$6.47\times10^4\pm7.2\times10^3$	1.4×10^4
Incubation 2	076°00′S	178°67′E	20.00	1.33	62.00	0.12	0.11	38.8	8.4	1.489	$9.86\times10^5\pm1.2\times10^4$	$0.4\times10^4\pm0.1\times10^4$
Incubation 3	074°60′S	173°20′E	22.98	1.63	62.00	0.49	0.13	51.0	21.0	0.886	$1.76\times10^5\pm3.0\times10^4$	0.2×10^4
Saturation experiment	076°39′S	168°58′E	13.37	1.49	74.75	<MDL	0.09	N.M.	N.M.	1.470	N.M.	N.M.

filtered through a 0.2-μm sterile filter to remove any remaining sponge particles. The concentration of ^{57}Co B_{12} in this stock was measured by gamma detection and normalized to $^{57}CoCl_2$ standards using Canberra Germanium Gamma detector.

Six identical unfiltered seawater samples were taken from 30 m depth using a trace-metal-clean Go-Flo bottle (General Oceanics) and dispensed into six acid- and detergent-cleaned 125-mL polycarbonate bottles. Immediately after dispensing the water (within 1 h of collection), approximately 0.09 pmol L^{-1} ^{57}Co B_{12} and 43 pmol L^{-1} unlabeled vitamin B_{12} were added to each and the bottles were placed in a deckboard incubator. Exact concentrations were later calculated for individual replicates on the basis of slight variations in volume associated with each bottle. After 24 h, the bottle incubations were measured for volume and filtered at 48 kPa, three replicates through a 2-μm polycarbonate filter membrane and three through 0.2-μm polycarbonate filter membrane. The filters were rinsed with 1–2 mL of 0.4-μm-filtered seawater each. The filter was centered and placed in a tight-lid petri dish (Fisher Scientific) and sealed with Parafilm.

^{57}Co radioactivity on each filter was determined using a Canberra Germanium Gamma detector. Counts per minute at 122 keV were corrected for decay and normalized to percentage uptake per day, calculated by dividing the activity on each filter by the total activity added. Since natural concentrations of vitamin B_{12} in pristine environments are believed to be subpicomolar (Menzel and Spaeth 1962; Swift 1981; Okbamichael and Sañudo-Wilhelmy 2004) and ^{57}Co-labeled B_{12} was also added in subpicomolar concentrations, the total concentration of B_{12} should be equivalent to the amount of unlabeled B_{12} added in these experiments, approximately 43 pmol L^{-1}. Using the percentage uptake of labeled B_{12} and this total concentration, total vitamin B_{12} uptake per day was calculated for each filter size. The 0.2-μm filter was considered total community uptake. The 2-μm filter represented uptake from the >2-μm-size fraction, whereas the 0.2-μm filter minus the 2-μm filter represented the 0.2-μm- to 2-μm-size fraction of the community.

Results

Three bottle incubation experiments were conducted examining the influence of iron, vitamin B_{12}, and cobalt in the Ross Sea (locations shown in Fig. 1, with initial conditions described in Table 1). Nutrients, including N+N, nitrite, phosphate, and silicic acid, as well as the micronutrients cobalt and iron, were measured over the course of these incubations. Phytoplankton growth and community composition was measured by Chl *a* fluorescence and epifluorescence microscopy. Bacterial and archaeal abundance was analyzed by microscopy, and DMSP was measured over the course of the experiments. In all cases, iron addition yielded increased phytoplankton growth. In two of three cases, vitamin B_{12} addition along with iron resulted in greater stimulation of phytoplankton growth than iron alone, and in no case did vitamin B_{12} alone result in significant stimulation. Where bacterial and archaeal abundance was highest, the least stimulation upon B_{12} addition with iron was seen; where it was lowest, the greatest stimulation was seen.

B_{12} enrichment bottle incubation results—In all three bottle incubation experiments, iron additions caused a significant ($p < 0.01$) increase in phytoplankton growth relative to unamended controls, shown by maximum Chl *a* concentration and nutrient consumption, as is consistent with previous studies in the Ross Sea (Martin et al. 1990; Sedwick and DiTullio 1997; Sedwick et al. 2000). Trends in phytoplankton cell concentrations agreed with those in Chl *a* concentration except in incubation 1, where enumeration of cells in the iron treatment was complicated by significant *Phaeocystis* colony formation (see Methods, Biomass analysis, and Table 2).

In two of the three incubation experiments (experiments 1 and 3), combined B_{12} and iron amendments, hereafter referred to as $B_{12}Fe$, resulted in a significant increase in phytoplankton growth above that seen in the iron treatments, as evidenced by maximum Chl *a* concentration ($p < 0.01$; final time point results summarized in Fig. 2), macronutrient consumption (Fig. 3A–H), and phytoplankton cell concentration (Table 2). This $B_{12}Fe$ stimulation was not observed in one of the three experiments (experiment 2; Figs. 2, 3; Table 2). Additions of vitamin B_{12} alone did not result in a significant ($p > 0.05$) stimulation of Chl *a* relative to the unamended control in all three experiments. These results suggest that the Ross Sea polynya in late austral summer is limited by iron, and variably colimited by iron and the B_{12} vitamin.

Changes in community composition, nutrient depletion, and DMSP production—The Fe-B_{12} colimitation effect observed in the Ross Sea is evident in all of the relevant biological measurements from experiments 1 and 3. There were statistically significant increases in Chl *a* concentrations, nearly doubling in experiment 1 and over 20% higher in experiment 3 relative to iron treatments (Figs. 2, 3A–D, I–L). This increase is associated primarily with an increase in the diatom species *P. subcurvata*, which increased from 73% of the population to over 92% in experiment 1, and from 59% to 74% in experiment 3 (Table 3). In contrast, in these experiments (1 and 3) the *P. antarctica* component of the community decreased in all treatments except the iron-only addition where it increased from 14% to 34% in incubation 3 and remained at 28% in incubation 1 (Table 2). These results indicate that combined $B_{12}Fe$ additions can influence the growth rates of phytoplankton as well as alter the phytoplankton species composition relative to iron-only additions and unamended controls.

The diatom community in incubation experiment 2, where no $B_{12}Fe$ stimulation was observed, was substantially different from that in incubations 1 and 3. Notably, *Fragilariopsis cylindrus* and *Chaetoceros* spp. comprised a much greater fraction of the diatom community than in incubations 1 and 3 (Table 3). This may have been due to variable inputs from pack ice, as these diatoms are known to form a large component of Antarctic pack ice

Table 2. Community composition (shown in relative abundance of diatoms and *Phaeocystis* with 1 SD where applicable) displayed with bacterial and phytoplankton abundances in the initial conditions (t_0) and at the final time points (t_F) (160, 223, and 180 h respectively) in the unamended (Control) treatment, added vitamin B_{12} (B_{12}), added cobalt (Co), added iron (Fe), added iron and cobalt (CoFe), and added iron and vitamin B_{12} (B_{12}Fe) from incubation experiments 1, 2, and 3. N.M. is parameter not measured.

	Phaeocystis antarctica: Percentage of total community	Total diatoms: Percentage of total community	Phytoplankton cells mL^{-1}	Bacterial and archaeal cells mL^{-1}
Incubation 1				
t_0	27.8±5.2%	72.3±5.5%	1.4×10^4	$6.47\times10^4\pm7.2\times10^3$
Control (t_F)	18.2±3.2%	81.8±4.2%	$7.45\times10^4\pm2.5\times10^4$*	$1.44\times10^5\pm1.6\times10^4$
B_{12} (t_F)	15.6±4.9%	84.4±4.3%	$5.86\times10^4\pm4.0\times10^4$*	$1.76\times10^5\pm1.3\times10^4$
Co (t_F)	N.M.	N.M.	N.M.	$1.36\times10^5\pm1.3\times10^4$
Fe (t_F)	28.7±4.8%	71.2±6.1%	$7.08\times10^4\pm1.3\times10^4$*	$3.47\times10^5\pm3.5\times10^4$
CoFe (t_F)	N.M.	N.M.	N.M.	$3.78\times10^5\pm3.2\times10^4$
B_{12}Fe (t_F)	5.48±1.3%	94.5±1.5%	$18.0\times10^4\pm2.9\times10^4$	$2.64\times10^5\pm4.7\times10^4$
Incubation 2				
t_0	35.9±4.3%	64.1±8.2%	$0.4\times10^4\pm0.1\times10^4$	$9.86\times10^5\pm1.2\times10^4$
Control (t_F)	16.1±4.4%	84.3±7.9%	$1.30\times10^4\pm0.2\times10^4$	$1.17\times10^6\pm1.5\times10^5$
B_{12} (t_F)	17.9±3.2%	82.1±5.8%	$1.39\times10^4\pm0.1\times10^4$	$1.21\times10^6\pm1.7\times10^5$
Co (t_F)	N.M.	N.M.	N.M.	$1.41\times10^6\pm1.6\times10^5$
Fe (t_F)	13.0±2.5%	87.0±7.9%	$3.45\times10^4\pm0.9\times10^4$	$1.17\times10^6\pm8.8\times10^4$
CoFe (t_F)	N.M.	N.M.	N.M.	$1.09\times10^6\pm9.0\times10^4$
B_{12}Fe (t_F)	17.9±0.7%	82.1±3.6%	$3.35\times10^4\pm0.05\times10^4$	$1.11\times10^6\pm1.5\times10^5$
Incubation 3				
t_0	13.6%	86.4%	0.2×10^4	$1.76\times10^5\pm3.0\times10^4$
Control (t_F)	14.13±6.1%	85.9±9.6%	$3.31\times10^4\pm0.1\times10^4$	$2.90\times10^5\pm2.4\times10^4$
B_{12} (t_F)	12.7±1.9%	87.2±3.0%	$3.50\times10^4\pm0.5\times10^4$	$3.02\times10^5\pm1.0\times10^4$
Fe (t_F)	24.3±4.6%	75.7±3.8%	$7.32\times10^4\pm0.6\times10^4$	$3.09\times10^5\pm3.3\times10^4$
B_{12}Fe (t_F)	13.3±5.0%	86.7±5.4%	$8.94\times10^4\pm1.1\times10^4$	$3.96\times10^5\pm1.2\times10^4$

* Approximate representation only; high *Phaeocystis* abundance prevented accurate cell counts by our method (see Methods, Biomass analysis).

communities (Leventer and Dunbar 1996 and references therein; Arrigo et al. 2003). Communities in open water near melting sea ice have been shown to mirror the assemblages in sea ice (Leventer and Dunbar 1996). This substantially different community yielded a very different response to the incubation amendments. Iron addition alone did not yield a relative increase in *Phaeocystis* populations as seen in incubations 1 and 3, but rather favored an increase in *P. subcurvata* and *F. cylindrus* populations relative to the control. Most strikingly, there was no increase in phytoplankton growth in the iron and B_{12} treatment relative to the iron treatment alone. This result cannot likely be explained by a difference in community structure only, as the species that responded to B_{12}Fe supplementation most (*P. subcurvata*) in incubations 1 and 3 were still present in the incubation 2 community, but did not yield the same response. An alternative explanation for the geographical variability in B_{12} effects is the relative abundances of bacteria and archaea as a source of the vitamin (see Bacterial section below).

The depletion of seawater nutrients is also indicative of phytoplankton species composition changes and changes in growth parameters. In the B_{12}Fe-supplemented incubations in experiments 1 and 3, nutrient drawdown was enhanced over the control and B_{12}-alone additions (Fig. 3). Notably, the silicic acid drawdown by B_{12} and iron treatments was significantly more ($p < 0.01$) in incubations 1 and 3 relative to iron treatments. Diatoms are the major users of silicic acid in the Ross Sea, and hence this result is consistent with the measurements of increasing *P. subcurvata* described above. The consumption of the micronutrients cobalt and iron were strongly influenced by iron additions but did not show differences between the B_{12}Fe additions and iron-only treatments (Table 4). Iron drawdown in all three incubation experiments was substantial; for example, in experiment 1 iron-supplemented treatments were depleted to 0.09 and 0.08 nmol L^{-1}, whereas the control and B_{12} additions were depleted to 0.13 and 0.18 nmol L^{-1}. Cobalt drawdown was also substantial in the treatments where iron was added, with an undetectable level (close to the 3 pmol L^{-1} detection limit) of labile cobalt present in the Fe and B_{12}Fe treatments (Table 4).

DMSP is produced by phytoplankton such as *Phaeocystis* for several biochemical roles including as an osmolyte, a cryoprotectant (Stefels 2000), and as an antioxidant (Sunda et al. 2002). Our incubation experiments are consistent with previous observations of DMSP cycling where nutrient stress by iron and carbon dioxide have been shown to induce DMSP production against the resulting oxidative stress experienced by the cell (Sunda et al. 2002), and iron stress has been shown to result in increased DMSP production in *P. antarctica* (Stefels and Leeuwe 1998). In our experiments, total DMSP (dissolved and particulate) increased as a result of iron additions (both Fe and B_{12}Fe, Fig. 4 and Table 5). When normalized to Chl *a* $DMSP_P$ (particulate DMSP) decreased, likely indicating alleviation of micronutrient (iron) limitation-induced oxidative stress.

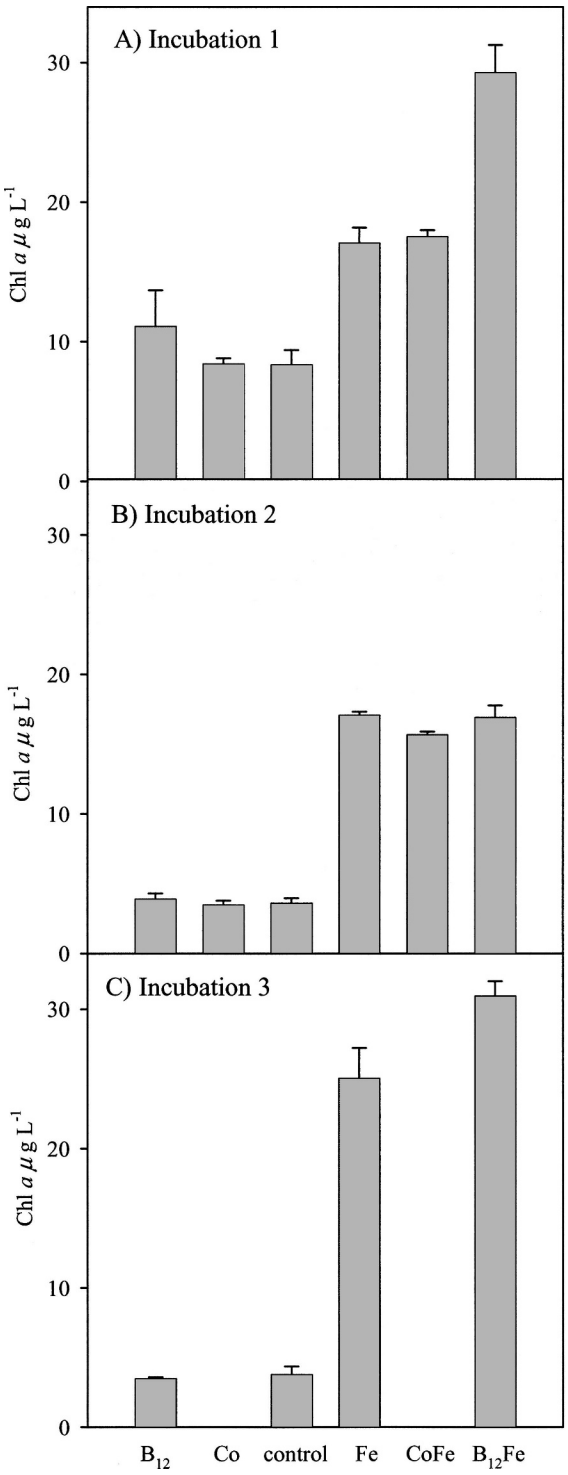

Fig. 2. Total Chl *a* concentrations in the unamended (control) treatment, added vitamin B_{12} (B_{12}), added cobalt (Co), added iron (Fe), added iron and cobalt (CoFe), and added iron and vitamin B_{12} (B_{12}Fe) from (A) incubation experiment 1 after 137 h of incubation, (B) incubation experiment 2 after 223 h, and (C) incubation experiment 3 after 180 h. Different incubation times were adopted to maximize the length of each experiment while preventing major nutrients (nitrate, phosphate, silicic acid) from becoming limiting. Values shown are means of triplicate treatments in 1.1- or 4.5-liter bottles (experiment 1) or triplicate treatments in 1.1-liter bottles (experiments 2 and 3) with error bars representing 1 SD. In all incubations, the addition of iron resulted in significantly more Chl *a* (t = 9.9, 54, 16; $p = 6 \times 10^{-4}$, 7×10^{-7}, 8×10^{-5} respectively, t-test). In incubations 1 and 3, there is a significant difference between Chl *a* in the B_{12}Fe treatment versus the Fe treatment: t = 9.3, 4 .2; $p = 7 \times 10^{-4}$, 0.01. B_{12} treatments did not show any significant stimulation relative to the control in any of the three incubation experiments: t = 1.7, 0.9, 0.8; p = 0.2, 0.4, 0.5.

Of the major phytoplankton species in the Ross Sea, *Phaeocystis* is believed to be an important producer of DMSP (DiTullio and Smith 1995). Methionine is hypothesized to control the rate of DMSP production by some phytoplankton (Gröne and Kirst 1992), and as described above, vitamin B_{12} has been implicated in methionine production and utilization. When $DMSP_P$ production is normalized to estimated *Phaeocystis* cellular densities, it tends to be higher in B_{12} additions (with and without added iron) than in Fe-only treatments in all incubation experiments (data not shown), despite no obvious influence of B_{12} when $DMSP_P$ is normalized to Chl *a*, with the possible exception of incubation 2 (Fig. 4). One possible explanation for these observations is that B_{12} is influencing methionine biosynthesis in *Phaeocystis*, and methionine availability is in turn influencing DMSP production rates (Gröne and Kirst 1992). As a result, higher B_{12} abundances in seawater could potentially lead to increased DMSP production. This creates an interesting dichotomy of competing mechanisms where iron additions decrease DMSP production via a supposed reduction of oxidative stress, but alleviation from B_{12} limitation would result in subsequent recovery of methionine biosynthesis and allow increases in DMSP production. The possibility of these competing mechanisms in DMSP production should be investigated through future laboratory experiments.

Bacterial and archaeal abundances—Variation in bacterial and archaeal abundance (hereafter referred to as bacterial abundance for simplicity) is consistent with the geographical variability we observe in Fe-B_{12} colimitation in the Ross Sea where the degree of B_{12} stimulation is negatively related to bacterial abundance. Initial bacterial abundances were much higher in experiment 2 (986,000 ± 12,300 cells mL^{-1}) where no stimulatory B_{12} effect was observed, relative to initial abundances in experiments 1 and 3 (64,700 ± 7,180 and 176,000 ± 29,900 cells mL^{-1} respectively) where B_{12} stimulation of growth was observed (see Fig. 5, Tables 1, 2). In addition, B_{12}Fe treatment stimulated more growth in experiment 1, where bacterial numbers were lowest, than in experiment 3, where numbers were slightly higher (Fig. 5). This is consistent with the idea that bacteria provide vitamin B_{12} to phytoplankton: in experiment 2 there seemed to be enough bacteria to supply an abundance of the vitamin, whereas in experiments 1 and 3, the increase in phytoplankton biomass upon iron addition likely exhausted the vitamin B_{12} naturally available until it became limiting. In fact, the initial abundance

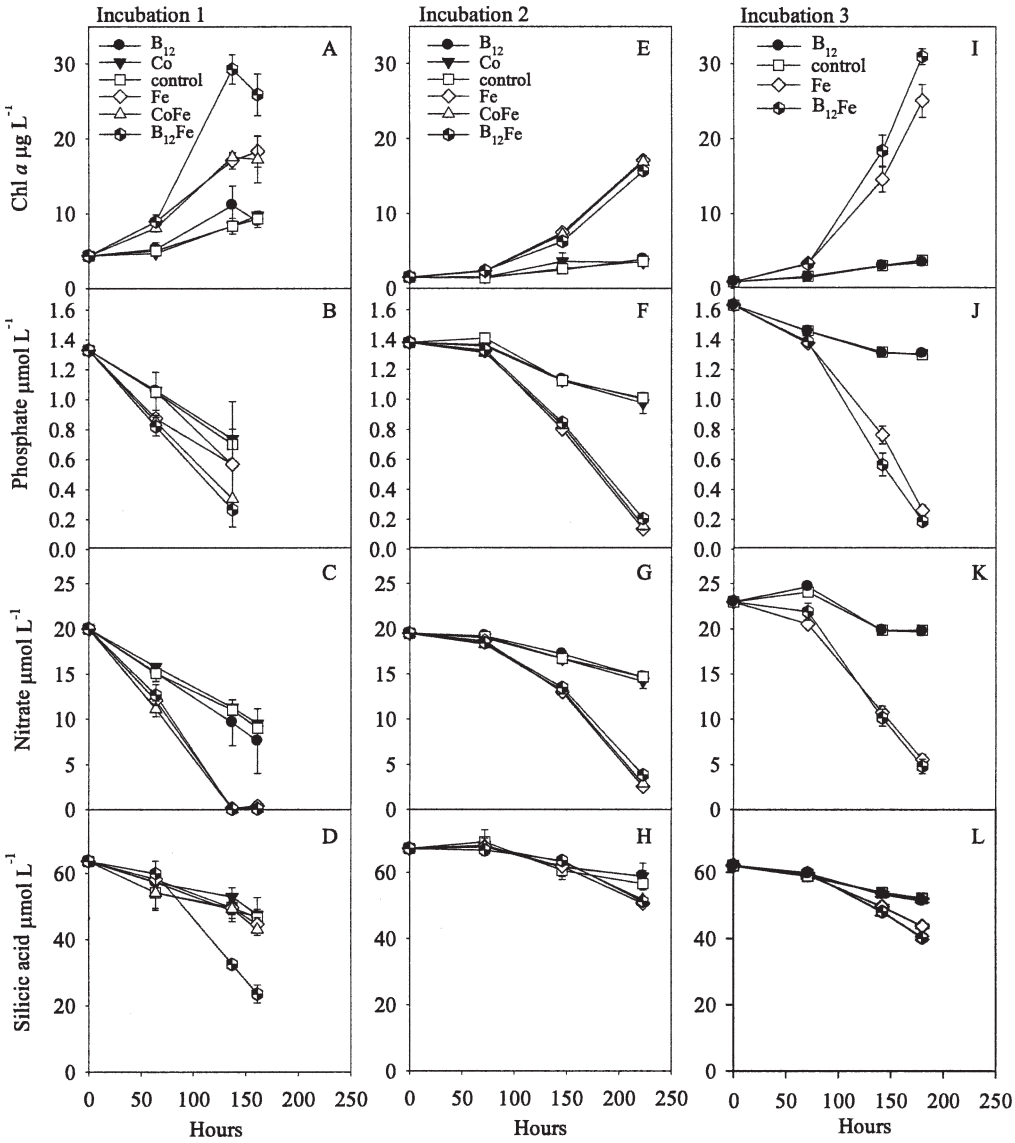

Fig. 3. (A–D): Total chloropyll *a* (Chl *a*), phosphate, dissolved nitrate, and silicic acid over time for incubation 1. Concentrations of these variables are shown for the unamended (control) treatment, 90 pmol L^{-1} added vitamin B_{12} (B_{12}), 450 pmol L^{-1} added cobalt (Co), 0.9 nmol L^{-1} added iron (Fe), 450 pmol L^{-1} added cobalt and 0.9 nmol L^{-1} added iron (CoFe), and 0.90 nmol L^{-1} added iron and 90 pmol L^{-1} vitamin B_{12} ($B_{12}Fe$) from incubation 1. Values shown are means of triplicate treatments (1.1- and 4.5-liter bottles) with error bars representing standard deviations. Values for phosphate at 161 h were omitted because of an observed systematic error in this analysis on the day these values were measured. (E–H) Total Chl *a*, phosphate, dissolved nitrate, and silicic acid over time for incubation 2. Concentrations of these variables are shown for the unamended (control) treatment, 90 pmol L^{-1} added vitamin B_{12} (B_{12}), 450 pmol L^{-1} added cobalt (Co), 1.8 nmol L^{-1} added iron (Fe), 450 pmol L^{-1} added cobalt and 1.8 nmol L^{-1} added iron (CoFe), and 1.8 nmol L^{-1} added iron and 90 pmol L^{-1} vitamin B_{12} ($B_{12}Fe$). Values shown are means of triplicate treatments (1.1-liter bottles) with error bars representing standard deviations. (I–L) Total Chl *a*, phosphate, dissolved nitrate, and silicic acid over time for incubation 3. Concentrations of these variables are shown for the Fe, B_{12}, control, and $B_{12}Fe$ treatments in the same concentrations as incubation 2. Values shown are means of triplicate treatments (1.1-liter bottles) with error bars representing 1 SD.

in incubation 2 was in line with the highest values observed in the Ross Sea (Ducklow et al. 2001 and references therein). Bacterial production of B_{12} has been shown to be an effective source of vitamin B_{12} to phytoplankton cultures (Haines and Guillard 1974; Croft et al. 2005).

Size-fractionated uptake experiments from the Ross Sea—Experiments were performed to discern what size fraction of the community in the Ross Sea was responsible for the majority of the vitamin B_{12} uptake in this study area and confirm the ability of phytoplankton to take up dissolved vitamin B_{12}. Size-fractionated vitamin B_{12} uptake in the surface waters as measured by addition of 43 pmol L^{-1} cyanocobalamin and trace amounts of ^{57}Co-labeled cyanocobalamin showed that 2.08 ± 0.05 pmol L^{-1} d^{-1} B_{12} was utilized by the >2-μm-size fraction, while the smaller (0.2–2 μm) size fraction utilized 0.88 ± 0.27 pmol L^{-1} d^{-1} B_{12}. Since this smaller-size fraction is comprised mostly of

Table 3. Relative abundance of diatoms in total community in the initial conditions (t_0) and at the final time point (t_F) (160, 223, and 180 h respectively) in the unamended (Control) treatment, added vitamin B_{12} (B_{12}), added iron (Fe), and added iron and vitamin B_{12} (B_{12}Fe) from incubation experiments 1, 2, and 3. Rare indicates where the indicated diatom comprised <0.5% of the total community. N.M. is parameter not measured.

	Pseudonitzchia subcurvata	*Fragilariopsis cylindrus*	*F. curta*	*Chaetoceros* spp.*	*Pseudonitzschia* sp.	*Fragiliaropsis* sp.	*Corethron pennatum*
Incubation 1							
t_0	Dominated	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
Control (t_F)	77.2%±3.2%	3.86%±2.7%	Rare	0.62%±0.7%	0	0	Rare
B_{12} (t_F)	79.9%±4.1%	3.95%±1.2%	0	Rare	Rare	0	0
Fe (t_F)	66.3%±5.6%	3.34%±1.1%	0	1.57%±1.9%	0	0	Rare
B_{12}Fe (t_F)	92.7%±1.1%	1.10%±0.8%	0	0.72%±0.7%	0	0	0
Incubation 2							
t_0	15.6%±2.5%	11.3%±0.5%	1.1%±1.1%	34.2%±7.7%	0.60%±0.5%	0	Rare
Control (t_F)	14.9%±2.3%	35.3%±3.2%	0.67%±0.7%	29.4%±6.7%	0.95%±0.7%	0.89%±1.3%	Rare
B_{12} (t_F)	14.1%±2.1%	30.9%±2.7%	0.56%±0.5%	33.3%±4.5%	0.88%±0.9%	0	Rare
Fe (t_F)	26.5%±3.3%	36.4%±1.1%	1.01%±0.5%	21.0%±6.9%	Rare	0	Rare
B_{12}Fe (t_F)	18.7%±2.3%	43.7%±0.6%	0.85%±0.3%	16.1%±2.2%	2.17%±1.4%	0	0
Incubation 3							
t_0	58.90%	12.70%	1.15%	10.37%	Rare	0	0.92%
Control (t_F)	76.9%±9.2 %	6.63%±2.3%	0.73%±0.9%	0.81%±0.4%	Rare	0	Rare
B_{12} (t_F)	79.0%±0.8%	7.37%±2.8%	0	Rare	Rare	0	Rare
Fe (t_F)	62.4%±3.2%	9.7%±1.6%	0	2.61%±1.1%	Rare	0	0
B_{12}Fe (t_F)	73.8%±4.7%	10.2%±2.2%	Rare	2.12%±1.6%	0	0	Rare

	Nitzschia stellata	*Plagiotropis gaussii*	*Trichotoxon reinboldii*	*Eucampia antarctica*	*Asteromphalus parvulus*	Other centrics	Other pennates
Incubation 1							
t_0	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
Control (t_F)	0	0	0	0	0	0	0
B_{12} (t_F)	0	0	0	0	0	0	0
Fe (t_F)	0	0	0	0	0	0	0
B_{12}Fe (t_F)	0	0	0	0	0	0	0
Incubation 2							
t_0	Rare	0	Rare	0	Rare	Rare	Rare
Control (t_F)	Rare	Rare	Rare	Rare	Rare	0	0.62%±0.7%
B_{12} (t_F)	Rare	0.80%±0.1%	Rare	0	Rare	Rare	0.87%±0.8%
Fe (t_F)	Rare	Rare	Rare	0	0	Rare	Rare
B_{12}Fe (t_F)	Rare	Rare	Rare	Rare	Rare	0	Rare
Incubation 3							
t_0	Rare	0	0	0	0	0.69%	0.92%
Control (t_F)	0	0	0	0	0	0	Rare
B_{12} (t_F)	0	0	0	0	Rare	0	Rare
Fe (t_F)	0	0	0	0	Rare	Rare	Rare
B_{12}Fe (t_F)	Rare	0	0	0	0	0	0

* Identified under light microscopy as *C. criophilum*, *C. dichaeta*, and small specimens similar to *C. neglectus*.

Table 4. Metal (cobalt and iron) concentrations at final time points of incubation experiments 1, 2, and 3 (160, 223, and 180 h respectively) in the unamended (Control) treatment, added vitamin B_{12} (B_{12}), added cobalt (Co), added iron (Fe), added iron and cobalt (CoFe), and added iron and vitamin B_{12} (B_{12}Fe). N.M. indicates no measurement made; ND indicates none detected. Measurements were made on one replicate bottle only except the incubation 3 cobalt determinations, which were measured in all three replicates. Values presented are averages ± 1 SD (where available).

	Total Fe (nmol L^{-1})	Total Co (pmol L^{-1})	Labile Co (pmol L^{-1})
Incubation 1			
Cont.	0.13	26	6
B_{12}	0.18	88	7
Co	N.M.	104	50
Fe	0.08	35	ND
CoFe	0.08	97	34
B_{12}Fe	0.09	99	ND
Incubation 2			
Cont.	N.M.	35	11
B_{12}	N.M.	95	8
Co	N.M.	530	186
Fe	N.M.	30	5
CoFe	N.M.	203	50
B_{12}Fe	N.M.	101	7
Incubation 3			
Cont.	0.08	32±3	3±2
B_{12}	0.08	101±22	2±2
Fe	N.M.	31± 9	ND
B_{12}Fe	0.03	58±12	2±1

bacteria and archaea along with some picoeukaryotes, and on the basis of the microscopically determined phytoplankton community profiles in this study, picoeukaryotes are not abundant; these data indicate that approximately one-third of the vitamin B_{12} uptake under these conditions can be attributed to bacteria and archaea, which are believed to be responsible for vitamin B_{12} production in the ecosystem as well. The larger-size fraction, comprised of eukaryotic phytoplankton including diatoms and *Phaeocystis* (colonial and free-living cells), takes up about two-thirds of the B_{12} consumed. These data suggest that the microbial community of the Ross Sea can take up dissolved vitamin B_{12}, implying a vitamin cycling pattern within the microbial loop there, with recycling of dissolved B_{12} and export by sinking eukaryotic phytoplankton.

Consideration of alternative explanations for phytoplankton stimulation—The possibility that a vitamin could substantially influence phytoplankton growth and community composition in the marine environment is a novel and exciting finding. To confirm these results, we analytically and experimentally verified that the B_{12} stimulation effects described above were not due to iron contamination (see Materials and methods for complete description). Quantifying the small iron blank associated with the added B_{12}, and then comparing it to an iron saturation growth curve (Fig. 6) demonstrates that the marginal increase in iron

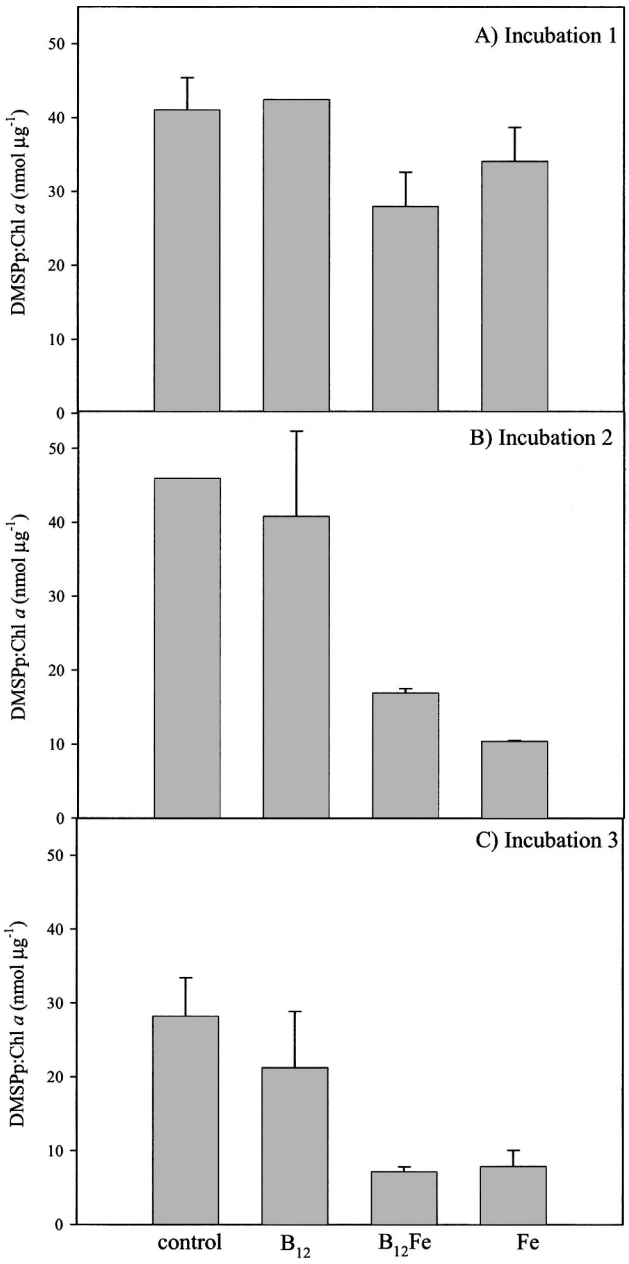

Fig. 4. Particulate DMSP production (nmol μg^{-1} Chl *a*) in the unamended (control) treatment, added vitamin B_{12} (B_{12}), added iron and vitamin B_{12} (B_{12}Fe), and added iron (Fe), from (A) incubation experiment 1 after 160 h of incubation, from (B) incubation experiment 2 after 223 h, and (C) incubation experiment 3 after 180 h. Values shown are means of single to triplicate treatments in 1.1- or 4.5-liter bottles (experiment 1) or single to triplicate treatments in 1.1-liter bottles (experiments 2 and 3) with error bars representing 1 SD. Particulate DMSP production at the start of incubation 2 was 52.7 ± 4.1 nmol μg^{-1} Chl *a* and was 68.8 ± 9.6 nmol μg^{-1} Chl *a* in incubation 3.

in the B_{12}Fe treatments relative to Fe-only treatments cannot account for the stimulation observed in the B_{12}Fe treatments of experiments 1 and 3: the small iron increase associated with the blank in the B_{12} solution would have resulted in changes to total iron concentrations only in the saturating portion of the apparent growth curve, where

Table 5. DMS (dimethyl sulfide) and DMSP (β-dimethyl sulfoniopropionate) in the initial conditions (t_0) and at the final time points (t_F) (160, 223, and 180 h respectively) in the unamended (Control) treatment, added vitamin B_{12} (B_{12}), added iron (Fe), and added iron and vitamin B_{12} (B_{12}Fe) from incubation experiments 1, 2, and 3. $DMSP_T$ is total DMSP; $DMSP_p$ is particulate (>0.2 μm) DMSP, and $DMSP_d$ is dissolved (<0.2 μmol L^{-1}) DMSP. Values are averages of single to triplicate treatments shown with 1 SD. N.M. indicates not measured.

	DMS (nmol L^{-1})	$DMSP_t$ (nmol L^{-1})	$DMSP_d$ (nmol L^{-1})	$DMSP_p$ (nmol L^{-1})
Incubation 1				
t_0	N.M.	77.1±3.1	N.M.	N.M.
t_F Control	N.M.	427.2±14	45.2±43	382±39
t_F B_{12}	N.M.	540±130	36.1±13	445±130
t_F Fe	N.M.	641±71	22.3±6.4	618±68
t_F B_{12}Fe	N.M.	747±144	23.7±4.1	722±140
Incubation 2				
t_0	10.7±6.5	98.9±1.7	20.6±1.8	78.2±0.1
t_F Control	5.08±3.0	198±9.0	5.37	182
t_F B_{12}	3.57±0.6	165±27	8.75±1.3	156±25
t_F Fe	6.22±2.6	189±1.0	12.2±6.0	177±5.1
t_F B_{12}Fe	4.45±0.7	264±18	9.27±2.9	255±16
Incubation 3				
t_0	14.7±2.1	95.9±1.0	35.1±5.4	60.8±6.4
t_F Control	17.2±7.6	120±8.5	16.7±7.6	104±12
t_F B_{12}	15.4±3.8	111±9.6	38.0±13	73.3±22.5
t_F Fe	14.6±3.9	223±24	30.0±20	193±40
t_F B_{12}Fe	9.23±6.1	240±21	19.3±2.1	220±19

increases in iron are not expected to significantly improve growth rates. By this logic, the iron blank in the B_{12} stock cannot account for any increase in phytoplankton growth.

The increase in phytoplankton growth in the B_{12} and iron addition also does not appear to be the result of B_{12} being used as a source of nitrogen. Vitamin B_{12} contains 14 atoms of nitrogen, thus the 90 pmol L^{-1} addition of B_{12} to these incubations translated into a 1.3 nmol L^{-1} addition of B_{12}-associated nitrogen. This was between 0.005% and 0.01% of the nitrogen found as nitrate at the start of each of these experiments and thus an insignificant addition.

Discussion

Our bottle incubation experiments demonstrate that vitamin B_{12} and iron colimit phytoplankton growth in the Ross Sea during the austral summer. Given that neither iron contamination nor the contribution of nitrogen from within the B_{12} molecule explain the results shown here, we hypothesize that the B_{12}Fe colimitation observed resulted from depletion of naturally present levels of dissolved vitamin B_{12} upon Fe fertilization. When vitamin B_{12} was added with iron, concentrations of the vitamin were sufficient to prevent vitamin limitation in the iron-caused phytoplankton bloom. This Fe-B_{12} colimitation effect was not seen in our second of three incubation experiments, suggesting that there was a difference in the chemistry or biology of this site relative to the other two experimental

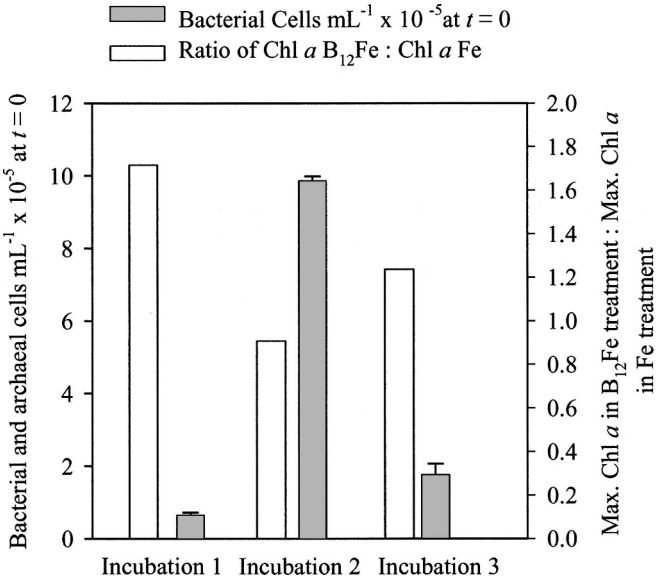

Fig. 5. Bacterial and archaeal counts at initiation of each experiment (gray) shown with the ratio of the maximum Chl *a* concentration in the vitamin B_{12} and iron addition to the maximum Chl *a* concentration in the iron-only addition (white). A ratio of 1 would mean that vitamin B_{12} added with iron yielded no change from the iron addition alone. A ratio of 2 would mean that the addition of B_{12} with iron doubled the Chl *a* yield above iron alone. This demonstrates that when bacterial and archaeal abundances were lowest in the initial conditions, vitamin B_{12} additions made the greatest difference in Chl *a* yield.

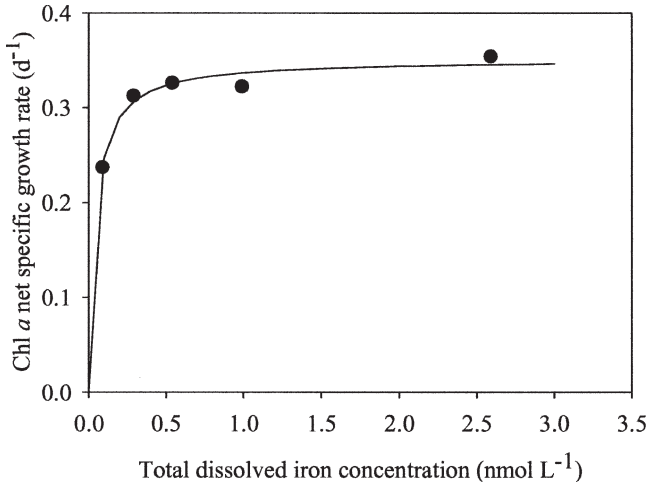

Fig. 6. Chl *a* net specific growth rate as a function of total dissolved iron concentration (iron added plus naturally occurring). The curve was fit with an r^2 value of 0.95 using the Droop equation. μ_m corresponded to a net specific growth rate of 0.35 per day and K_m corresponded to an iron concentration of 42 pmol L^{-1}. Duplicate measurements of total Chl *a* used to create this graph varied by less than 10%. Concentrations of 0.89 and 0.92 nmol L^{-1} iron ([Fe] and [B_{12}Fe] iron concentrations in experiment 1) and 1.81 and 1.84 ([Fe] and [B_{12}Fe] iron concentrations in experiments 2 and 3) occur only in the saturating portion of the curve. This suggests that the small iron blank associated with the B_{12} additions in the (B_{12}Fe) treatments could not account for the increase in Chl *a* observed.

stations. We hypothesize that this difference was caused by variation in the bacteria and archaeoplankton community within the three study sites.

Colimitation versus secondary limitation—We should note that this colimitation may also be described by the term "secondary limitation" where B_{12} is limiting once iron is replete. Moreover, since iron and B_{12} are micronutrients with independent biological functions, this would be a type I colimitation scenario, relative to type II colimitation in which biochemical substitution occurs (e.g., cobalt–zinc substitution), and type III colimitation between two interdependent nutrients (e.g., zinc-dependent carbon acquisition) as recently described (Saito, Goepfert, and Ritt unpubl.).

Potential sources of vitamin B_{12} in the Ross Sea—Although the sources of B_{12} to the Ross Sea have yet to be characterized, we can describe the potential components of a B_{12} biogeochemical cycle on the basis of phytoplankton culture studies, genomic information, and results presented here. As mentioned above, only the members of bacterial and archaeal domains are known to be capable of biosynthesizing vitamin B_{12}, and hence eukaryotic phytoplankton with a B_{12} requirement must acquire this vitamin from external sources. We hypothesize that given their abundance in tropical and subtropical marine environments (Partensky et al. 1999), the marine cyanobacteria are likely a major source of B_{12} to the marine environment, yet these microbes are virtually nonexistent in the Southern Ocean and Ross Sea (Caron et al. 2000; Marchant 2005). As a result, the remaining possible sources of B_{12} to the Ross Sea are either the bacteria and archaea living in the water column, or physical advection of B_{12} from other water masses or sedimentary environments. The bacterial production rates in the Ross Sea are among the lowest measured anywhere in the oceans, contributing only 5.5 mg m^{-2} d^{-1} C, or only 4% of phytoplankton production (Carlson et al. 1998)—much lower than the 25–30% found in other marine environments (Ducklow 2000; Ducklow and Carlson 1992). The specific reasons for these lower bacterial production rates are unknown, but are likely related to organic matter limitation (Ducklow 2000). Given these lower bacterial production rates in the Ross Sea and Southern Ocean, phytoplankton growth in this region could be especially prone to vitamin B_{12} limitation.

There is limited information about the bacterial diversity of the Ross Sea and the vitamin B_{12} production capabilities of that diversity. If bacteria in the water column of the Ross Sea are the major source of vitamin B_{12} (and other vitamins), they would have to be significantly different from *Pelagibacter ubique*, which is known to be lacking the B_{12} biosynthetic pathway and is representative of the SAR11 clade that numerically dominates clone libraries from the tropical and subtropical regions. 16S rDNA sequence analysis has revealed members of the *Pseudoalteromonas, Phychrobacter, Roseobacter, Paracoccus, Arthrobacter, Rhodococcus, Janibacter,* and *Planococcus* genera in McMurdo Sound of the Ross Sea (Michaud et al. 2004), at least some of which have members with vitamin B_{12} biosynthesis capabilities (Rodionov et al. 2003). The sequenced genome (Medigue et al. 2005) of the Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125 reveals that it has the genetic machinery to take up B_{12} and possible B_{12} degradation products, as it has the gene *btuB*, which can take up the vitamin as well as cobinamides and other corrinoids (Rodionov et al 2003). It also possesses the genes needed to complete the last few steps in vitamin B_{12} synthesis only (*cobS, cobU*) but cannot make the molecule outright. We hypothesize that without a cyanobacterial foundation to the high-latitude microbial food web, a number of bacteria and archaea would have retained their B_{12} biosynthesis capabilities in contrast to *P. ubique* of the SAR11 clade.

B_{12} requirements of phytoplankton from the Ross Sea—Cultivated phytoplankton strains related to those found in the Ross Sea have been shown to have a vitamin B_{12} requirement. Of the 55 diatoms reviewed by Croft et al. (2005), 35 (65%) required vitamin B_{12} for growth, including 5 of the 13 *Nitzschia* (closely related to *Pseudonitzschia*) strains surveyed. There is currently no direct evidence for a B_{12} requirement for *P. subcurvata* (the strain shown to comprise a very large portion of the community upon B_{12}Fe addition, Table 3). When cultured in the laboratory, its culture media contains added B_{12}. Our incubation results suggest that *P. subcurvata* likely requires vitamin B_{12} in the Ross Sea.

A vitamin B_{12} requirement for *Phaeocystis globosa* has been reported (Peperzak et al. 2000), and our experiments demonstrate slightly improved growth rates and yields of *P. antarctica* when grown in culture with B_{12} amendments (data not shown), although these experiments were conducted with nonaxenic cultures that may have B_{12} contributions from the co-occurring heterotrophic bacteria. If *P. antarctica* does require vitamin B_{12} in the field, the fact that diatom growth increased more than *Phaeocystis* growth upon vitamin and iron addition in our Ross Sea experiments indicates that *P. antarctica* likely has an alternative source of vitamin B_{12} that diatoms cannot access. This source could possibly be a close association with heterotrophic bacteria, similar to the symbiosis observed in the laboratory experiments using *Porphyridium purpureum* and heterotrophic bacteria (Croft et al. 2005). Putt et al. (1994) observed 2–11-fold increase in concentration of bacteria around *Phaeocystis* sp. colonies over ambient bacterial concentrations in McMurdo Sound of the Ross Sea. This close bacterial association could provide *Phaeocystis* with sufficient concentrations of the B_{12} and explain how the vitamin addition spurred diatom growth over *Phaeocystis* growth. This scenario also suggests that diatoms rely on dissolved vitamin B_{12} released through the microbial loop while *Phaeocystis* may acquire their vitamins through direct interaction with heterotrophic bacteria. These two distinct means of acquiring B_{12}, uptake of dissolved B_{12} or symbiosis with heterotrophic bacteria, could result in unique niches for phytoplankton species.

In the Ross Sea, *P. antarctica* dominates in the spring and early summer; a bacterioplankton bloom follows (Ducklow et al. 2001), perhaps due to the decay of the *Phaeocystis* bloom. Diatom species such as *P. subcurvata* (Arrigo et al. 1999) and *Fragilariopsis curta* (Leventer and

Dunbar 1996) dominate after the *Phaeocystis* bloom. Our results suggest that this bacterioplankton bloom could supply the vitamin B_{12} needed by diatoms and may be involved in the phytoplankton community shift observed seasonally in the Ross Sea.

Implications for the biogeochemical cycling of B_{12}, cobalt, and carbon—Our observations that iron and the B_{12} vitamin colimit phytoplankton growth in the Ross Sea suggest that B_{12} sources are limited in this region. In the Ross Sea, the microbial food web is lacking in cyanobacteria and has low bacterial production rates, two presumed major sources of B_{12} in the ocean. The combination of this microbial profile, a high export rate of biologically produced material, and the increased UV irradiation found seasonally in this region could together cause this region to become B_{12} limited. This is a significantly different picture of B_{12} cycling from what must exist in most of the world's oceans, where cyanobacteria are often a significant or major component of the phytoplankton community and where heterotrophic bacteria are more abundant and productive. A large fraction of the dissolved cobalt in the Ross Sea was found to be in a labile form, meaning it was not bound to strong organic ligands (Table 1). This labile cobalt is operationally defined as exchangeable with added strong competitor ligands and is believed to be the more bioavailable fraction (Saito and Moffett 2001; Saito et al. 2005 and references therein). These findings are consistent with results from incubation experiments 1 and 2 in which the cobalt addition did not yield any significant phytoplankton growth over the unamended control, and the combined cobalt and iron addition yielded no significant phytoplankton growth over the iron treatment alone. Because B_{12} contains a cobalt atom, this indicates that the stimulatory effect of B_{12} is not attributable to the cobalt atom being extracted and used for other metabolic functions.

These results form a picture of cobalt and vitamin B_{12} cocycling within the Ross Sea. It appears that here, cobalt is abundant in a bioavailable form, and that the amount of available cobalt does not limit the amount of B_{12} produced and cycled within the microbial food web. It is also possible that vitamin B_{12} or its degradation products comprise a significant if not numerically dominant portion of the organically bound cobalt in this study area as suggested for some tropical regions (Saito et al. 2005), particularly since in some areas of the Ross Sea (Table 1) less than 1 pmol L^{-1} of cobalt was found to be organically complexed.

The colimitation of phytoplankton growth by iron and B_{12} in the Ross Sea illustrates the potential importance of this vitamin to marine primary productivity and carbon cycling. We have shown that the addition of B_{12} and iron together can both increase phytoplankton growth and modulate phytoplankton community composition, compared to the addition of iron alone. Community composition in the Ross Sea was previously shown to be an important factor in determining the rate of carbon export (Arrigo et al. 1999). This study also suggests an important mechanism by which bacterial and archaeal populations within the microbial loop can affect carbon fixation and export. Our results also suggest that different modes of vitamin B_{12} acquisition, whether through uptake of dissolved B_{12} or through a symbiosis with cell-surface-associated bacteria, could have significant effects for phytoplankton and confer ecological advantages. This study highlights vitamin B_{12} as a biogeochemically relevant micronutrient and suggests that it can influence the cycling of carbon in the marine environment.

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