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Iron limitation of a springtime bacterial and phytoplankton community in the Ross Sea: implications for vitamin B₁₂ nutrition

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The Ross Sea is home to some of the largest phytoplankton blooms in the Southern Ocean. Primary production in this system has previously been shown to be iron limited in the summer and periodically iron and vitamin B₁₂ colimited. In this study, we examined trace metal limitation of biological activity in the Ross Sea in the austral spring and considered possible implications for vitamin B₁₂ nutrition. Bottle incubation experiments demonstrated that iron limited phytoplankton growth in the austral spring while B₁₂, cobalt, and zinc did not. This is the first demonstration of iron limitation in a *Phaeocystis antarctica*-dominated, early season Ross Sea phytoplankton community. The lack of B₁₂ limitation in this location is consistent with previous Ross Sea studies in the austral summer, wherein vitamin additions did not stimulate *P. antarctica* growth and B₁₂ was limiting only when bacterial abundance was low. Bottle incubation experiments and a bacterial regrowth experiment also revealed that iron addition directly enhanced bacterial growth. B₁₂ uptake measurements in natural water samples and in an iron fertilized bottle incubation demonstrated that bacteria serve not only as a source for vitamin B₁₂, but also as a significant sink, and that iron additions enhanced B₁₂ uptake rates in phytoplankton but not bacteria. Additionally, vitamin uptake rates did not become saturated upon the addition of up to 95 pM B₁₂. A rapid B₁₂ uptake rate was observed after 13 min, which then decreased to a slower constant uptake rate over the next 52 h. Results from this study highlight the importance of iron availability in limiting early season Ross Sea phytoplankton growth and suggest that rates of vitamin B₁₂ production and consumption may be impacted by iron availability.

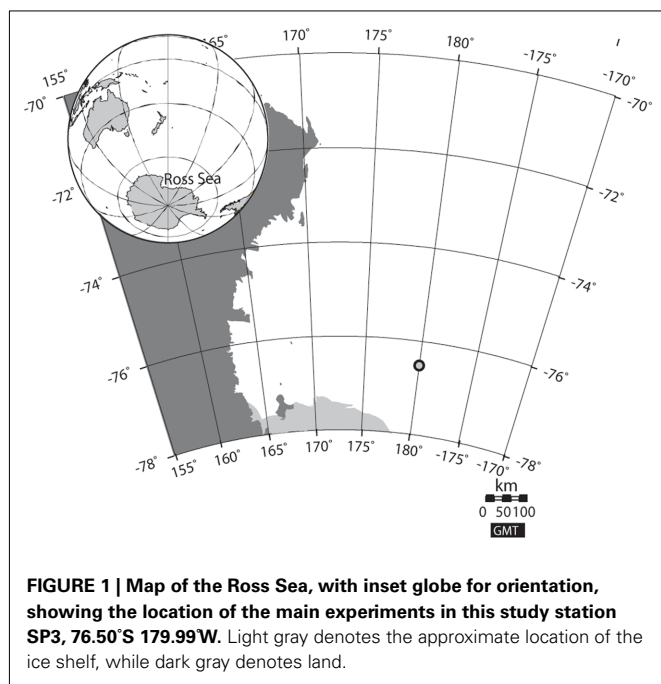
Keywords: iron limitation, vitamin B₁₂, Ross Sea, colimitation, bacteria, phytoplankton, iron fertilization

INTRODUCTION

The Ross Sea of the Southern Ocean (**Figure 1**) hosts massive phytoplankton blooms (Smith and Nelson, 1985) and high rates of dimethylsulfoniopropionate (DMSP) production (DiTullio and Smith, 1995). As a result, this and similar Antarctic coastal ecosystems are considered to have a significant impact on global biogeochemical cycles (Arrigo et al., 1999, 2008). The phytoplankton community in the Ross Sea is typically dominated by the colonial haptophyte *Phaeocystis antarctica* (DiTullio and Smith, 1996) and pennate diatoms such as *Fragilariopsis* and *Pseudonitzschia* spp. (Leventer and Dunbar, 1996; Arrigo et al., 1999; Armand et al., 2005). Because of the significantly higher C:P and N:P ratios of *P. antarctica* compared to diatom communities, the relative abundances of *P. antarctica* and diatoms in the Ross Sea may control carbon uptake and export in the region (Arrigo et al., 1999). These community dynamics are thought to be controlled in part

by irradiance, with diatoms dominating when mixed layers are shallow (high irradiance, associated with sea ice meltwater), and *P. antarctica* dominating when mixed layers are deep (low irradiance; DiTullio and Smith, 1996; Arrigo et al., 1999).

Iron has been shown to exert a controlling influence on primary productivity in the Ross Sea, particularly in the austral summer months (Martin et al., 1990; Sedwick and DiTullio, 1997; Sedwick et al., 2000; Bertrand et al., 2007). Iron availability is also thought to impact Ross Sea phytoplankton community composition, as iron additions have in some cases caused substantive diatom blooms (Martin et al., 1990; Sedwick et al., 2000; Rose et al., 2009; Feng et al., 2010). However, other experiments have shown that field populations of *P. antarctica* may also benefit from iron addition (Olson et al., 2000; Coale et al., 2003; Bertrand et al., 2007). It has previously been suggested that elevated dissolved iron concentrations in the springtime, supplied by winter mixing and sea



ice melt, are progressively depleted via phytoplankton uptake, thus resulting in low dissolved iron concentrations and iron limitation of primary productivity during the summer (Sedwick et al., 2000; Coale et al., 2005). However, more recent work revealed relatively low dissolved iron concentrations in the Ross Sea polynya during the spring, thus challenging the conceptual model of a gradual progression toward iron limitation of phytoplankton growth (Sedwick et al., in review).

There is increasing evidence that the availability of vitamin B₁₂ can also play a role in shaping phytoplankton dynamics in Antarctic coastal ecosystems (Panzeca et al., 2006; Bertrand et al., 2007) as well as other environments including the Gulf of Alaska (Koch et al., 2011), Long Island embayments (Sañudo-Wilhelmy et al., 2006; Gobler et al., 2007), the Sargasso Sea (Menzel and Spaeth, 1962), and the Gulf of Maine (Swift, 1981). Shipboard bottle incubation experiments performed in the Ross Sea during austral summer 2005 demonstrated that vitamin B₁₂ became the limiting nutrient once iron was replete, but only in areas with low bacterial abundance (Bertrand et al., 2007). This scenario can be referred to as periodic secondary B₁₂ limitation, or vitamin and iron colimitation (Saito et al., 2008). The addition of B₁₂ and iron also increased the percentage of diatoms in the bottle incubations relative to iron addition alone, suggesting that vitamin B₁₂ may help govern rates of primary production as well as phytoplankton species composition.

Vitamin B₁₂ is produced by some bacteria and archaea, while other prokaryotes as well as most eukaryotic phytoplankton require an exogenous supply of B₁₂ to sustain growth (Roth et al., 1996; Rodionov et al., 2003; Croft et al., 2005; Tang et al., 2010). Those phytoplankton and bacteria that do not require vitamin B₁₂ possess alternative biochemical pathways for accomplishing essential reactions, including use of a di-ferric form of ribonucleotide reductase (type I) rather than the B₁₂-requiring version (type II),

and the use of zinc-requiring methionine synthase (MetE) rather than the version that requires vitamin B₁₂ and zinc (MetH) (Zhang et al., 2009; Helliwell et al., 2011).

Biosynthesis of vitamin B₁₂ requires over 30 enzymatic steps and appreciable amounts of energy, carbon, nitrogen, and transition metals including cobalt, zinc, and in some cases iron (Roth et al., 1996; Raux et al., 2000). Though some B₁₂ biosynthesis genes, potentially from an undescribed clade of gamma proteobacteria, have been detected in microbial communities from the Ross Sea (Bertrand et al., 2011), the genetic potential for vitamin B₁₂ production remains largely uncharacterized in the Ross Sea or in any other marine environment. This is in part because 16S rRNA profiling, which is commonly used for bacterial community structure analysis, does not provide information about vitamin B₁₂ production potential, since the presence of the biosynthesis pathway among bacterial and archaeal lineages is widespread and extremely variable. An exception to this is the marine cyanobacteria, where all available sequenced genomes appear to contain the B₁₂ biosynthetic pathway (Rodionov et al., 2003), and a range of strains have been shown to produce significant amounts of B₁₂ (Bonnet et al., 2010). The Ross Sea and other Antarctic marine systems lack appreciable cyanobacterial populations (Caron et al., 2000; Marchant, 2005; Bertrand et al., 2011), leaving Antarctic marine systems potentially deficient in a source of this vitamin, making these regions particularly vulnerable to B₁₂ limitation. In addition, genome sequences from representatives of the abundant heterotrophic bacterial clade SAR11 suggest that in addition to not producing vitamin B₁₂ (Giovannoni et al., 2005), these microbes do not require the vitamin and do not possess known B₁₂ uptake pathways (Zhang et al., 2009), implying that B₁₂ independence may be part of the ecological niche of these abundant heterotrophic bacteria.

Either through cycling of the microbial loop (Karl, 2002; Droop, 2007) or through direct symbiotic interaction (Croft et al., 2005), bacteria and archaea must be the ultimate source of vitamin B₁₂ to auxotrophic (vitamin-requiring) phytoplankton. Therefore, in areas of the ocean where vitamin B₁₂ has been shown to be an important factor in determining phytoplankton growth or community composition, understanding the controls on primary productivity demands consideration of bacterial growth and dynamics. Although bacterial abundance and biomass can reach bloom proportions in the Ross Sea, bacterial production there is relatively low, especially during the austral spring (Ducklow et al., 2000, 2001). Ross Sea bacterial production is thought to be controlled by the availability of dissolved organic matter (DOM), with little direct impact of temperature (Ducklow et al., 2001). However, the role of iron in controlling bacterial production has yet to be fully investigated. Although it is possible for marine bacterial growth rates and efficiency to be limited by iron availability in cultures (Tortell et al., 1996), there has been little study of bacterial iron limitation in the field. Bacterial growth in the Southern Ocean has been observed to be DOM limited (Hall and Safi, 2001; Oliver et al., 2004), iron limited (Pakulski et al., 1996), and iron and DOM colimited (Church et al., 2000). Availability of DOM has been found to be the primary limiting factor for bacterial production in other iron limited HNLC (high nutrient low chlorophyll) areas (Hale et al., 2006), although in some regions

iron quickly becomes limiting when organic carbon limitation is alleviated (Kirchman et al., 2000). In addition, culture-based evidence suggests that low iron availability can induce the production of highly recalcitrant DOM by Antarctic phytoplankton and thus that iron and DOM limitation could be synergistically limiting bacterial growth (Becquevort et al., 2007).

In this study, we sought to investigate several parameters relevant to the cycling of iron and vitamin B₁₂ in the southern Ross Sea. The results presented here indicate that availability of iron can regulate phytoplankton growth rates, impact bacterial growth, and influence B₁₂ uptake rates during the austral spring.

MATERIALS AND METHODS

STUDY AREA AND WATER COLLECTION

These experiments were conducted in the Ross Sea during the CORSACS 2 cruise in austral spring 2006 (NBP0608). Additional samples were collected during the CORSACS 1 cruise in the austral summer of 2005–2006 (NBP06081). For the bottle incubation experiments, water was collected from ~3 m depth using a trace metal clean (TMC) Teflon diaphragm pumping system (Bruland et al., 2005; Sedwick et al., in review). All sample and incubation bottles used were detergent and acid-cleaned [0.1% citranox for 48 h, 10% HCl (Baker, Instra-analyzed) for 7 days, clean pH 2 water (dilute HCl) rinsed]. Sample bottles were filled and manipulated in a positive-pressure TMC environment constructed with laminar flow hoods and plastic sheeting to avoid trace metal contamination.

SHIPBOARD BOTTLE INCUBATION STUDY

A bottle incubation experiment using whole seawater with additions of 1 nM added iron, 500 pM added cobalt, 1 nM added zinc, and 100 pM added vitamin B₁₂ was started in the Ross Sea on December 3, 2006 at 76.50°S 179.99°W, hereafter referred to as Station SP3 (Figure 1), and carried out for 6 days. Whole seawater was dispensed into a 50 L TMC mixing carboy and from there into 2.2 L polycarbonate (PC) bottles in triplicate for each treatment (+Co, +Fe, +B₁₂, +Zn, +B₁₂Fe, +CoFe, +ZnFe, and unamended control). The bottles were tightly capped and placed outdoors in deckboard flow-through incubators at ~20% ambient light, shielded with neutral density screening. Ambient temperature (−2 to 1°C) was maintained by a constant flow of surface seawater through the incubators. Approximately every 60 h, nutrients and chlorophyll *a* (Chl *a*) were measured. At the beginning and the end of the experiment, samples were taken for phytoplankton microscopy, pigment analysis, particulate organic carbon (POC) analysis, and bacterial cell counts.

TRACE NUTRIENT SUPPLEMENTATION

In all cases, iron was added as FeCl₃ (Fluka), cobalt was added as CoCl₂ (Fluka), and zinc was added as ZnSO₄ (Fisher), all in weakly acidified (pH 3, SeaStar HCl) Milli-Q water. Vitamin B₁₂ (Sigma, Plant-cell culture tested cyanocobalamin, 99%) was added as a solution in Milli-Q water, purified for trace metals by passing through a column of 2–3 mL of prepared Chelex-100 beads (BioRad; Price et al., 1988/1989).

BACTERIAL REGROWTH EXPERIMENT

A TMC bacterial regrowth experiment was conducted at station SP3 (Figure 1). Two liters of water, collected in a 2.5 L PC bottle as described above, was filtered using an acid-cleaned 0.2 μm cartridge filter and supplemented with 10% 0.65 μm filtered water, containing a portion of the free-living bacterial community from the same location (syringe filtered with an acid-cleaned PC filter membrane in an acid-cleaned 25 mm Swinnex filter holder). Triplicate aliquots of 250 mL each were removed into acid-cleaned polyethylene bottles as unamended controls. 1 nM Fe (as above) was added to the remaining mixture, and three aliquots of 250 mL each were removed into acid-cleaned polyethylene bottles for iron addition treatments. Sample aliquots (27 mL) were preserved with 10% filtered formalin and retained for bacterial cell enumeration. The experimental treatments were all incubated in the dark at 0°C. After 3 days, 10 mL was removed from each treatment, preserved with 10% filtered formalin, and reserved for bacterial cell enumeration.

NUTRIENT ANALYSIS

Nutrients, including nitrate plus nitrite, nitrite, phosphate, and silicic acid were measured in the incubation experiments approximately every 60 h, in 0.2 μm filtered subsamples from each bottle in the bottle incubation study. Analysis was performed at sea using a Lachat QuickChem Autoanalyzer. Minimum detectable levels were 0.04 μM phosphate, 0.09 μM nitrate plus nitrite, 0.01 μM nitrite, and 0.38 μM silicic acid.

BIOMASS ANALYSIS

Over the course of the bottle incubation experiment, total Chl *a* was measured approximately every 60 h using 90% acetone extracts and the non-acidified fluorometric method (Welschmeyer, 1994), employing a Turner Designs TD700 fluorometer and Whatman GF/F filters. Bacteria were enumerated using a previously published method involving DAPI (4′-6-Diamidino-2-phenylindole) staining (Porter and Feig, 1980). Samples for phytoplankton community composition analysis were preserved with 1% glutaraldehyde, filtered on to 2 μm pore size PC filters, and analyzed by using ~2000 individual counts at 100× magnification via standard epifluorescence microscopy techniques coupled with DAPI staining. Obtaining accurate cell concentration estimates was not possible by our method due to the high abundance and extensive clumping of *Phaeocystis* cells, so these counts can only be used to assess community composition, not abundance. Phytoplankton pigment samples (0.25–1 L) were filtered on to Whatman GF/F filters, flash frozen in liquid nitrogen, and stored at −80°C prior to analysis by HPLC (Zapata et al., 2000; DiTullio and Geesey, 2002). Samples for POC analysis (100–200 mL) were filtered onto pre-combusted (450°C, 2 h) Whatman GF/F glass fiber filters, which were then analyzed using a Carlo Erba NA1500 elemental analyzer/Conflo II device and a Finnigan Delta Plus mass spectrometer at the Stanford University Stable Isotope Biogeochemistry Laboratory. Elemental compositions were measured using the mass 44 beam intensity (V) on the Delta Plus, calibrated against the mass 44 beam intensity of at least five certified reference standards that were analyzed throughout the course of each run

of 40 samples. Relative reproducibility of the acetanilide standard averaged 0.65% for carbon.

VITAMIN B₁₂ UPTAKE

Measurement of short-term B₁₂ uptake using tracer level additions of the vitamin is difficult given the low picomolar concentrations of B₁₂ in natural waters (Menzel and Spaeth, 1962; Panzeca et al., 2009). For this reason, we chose to measure “potential uptake” rates with the addition of ⁵⁷Co-labeled B₁₂ (0.09 pM) and 40 pM unlabeled B₁₂. This measurement is practical in that it can allow for short-term rate assessments and does not require the measurement of water column vitamin concentrations. The measurements reflect rates of uptake at the specific concentration of the vitamin added and not *in situ* rates. As such, they are useful for comparing uptake rates between experimental treatments, but cannot be used to estimate the true rate of removal of B₁₂ from the water column by phytoplankton and bacteria.

⁵⁷Co-labeled cyanocobalamin was used to measure the rate of vitamin B₁₂ uptake at the site of the bottle incubation study and in the community present at the end of the bottle incubation experiments, as well as at other sites within the Ross Sea. Uptake by the >2 and >0.2-μm size fractions was measured according to previously published protocols (Bertrand et al., 2007). Briefly, approximately 0.09 pM ⁵⁷Co B₁₂ (360 μCi/g ⁵⁷Co B₁₂) and 40 pM unlabeled vitamin B₁₂ were added to duplicate or triplicate 160 mL PC bottles filled with whole sea water from specific Ross Sea locations or with aliquots of whole incubation experiment water from the different incubation treatments described above. After 24 h, the samples were filtered through 0.2 or 2 μm PC filter membranes and rinsed with 1–2 mL of 0.4 μm- filtered seawater each. The ⁵⁷Co radioactivity on each filter was determined using a Canberra Germanium Gamma detector. Since natural concentrations of vitamin B₁₂ in pristine environments are believed to lie in the low picomolar range (Menzel and Spaeth, 1962; Swift, 1981; Panzeca et al., 2009) and the ⁵⁷Co-labeled B₁₂ was added in sub-picomolar concentrations, the total concentration of B₁₂ can be approximated as the amount of unlabeled B₁₂ added in these experiments (~40 pM). Using the percent uptake of radiolabeled B₁₂ and this total concentration, total vitamin B₁₂ uptake per day was calculated for each filter size, as described by the equation B₁₂ uptake/L/day = counts of ⁵⁷Co B₁₂ on filter/L/day × 1/counts of ⁵⁷Co B₁₂ added × total B₁₂ concentration. The 0.2-μm filter activity was considered indicative of total community uptake. The 2-μm filter represented eukaryotic uptake from the >2-μm size fraction while the 0.2-μm filter minus the 2-μm filter was considered a proxy for the bacterial community uptake rate. Control studies were conducted at several locations during the CORSACS 1 cruise by killing the community with glutaraldehyde (1% final concentration, B₁₂ added 3 h after glutaraldehyde added) or with heat (microwaving a 180-mL sample for 5 min, shake, repeat three times; B₁₂ added 3 h after heat-killed). Killed controls were incubated at 0°C in the dark. A titration experiment was conducted at station NX 14 (37 m depth, November 18, 2006, 75.93° S, 178.36° E) where varying amounts of non-radiolabeled B₁₂ was added to whole seawater and 24 h uptake rate measurements were taken to determine how these added concentrations affected uptake rate by the >2-μm size fraction. In addition, a time course experiment with 40 pM added B₁₂ was

conducted at station NX 17 (17 m depth, November 26, 2006; 76.50° S, 178.55° E) to examine changes in uptake rate in the >2-μm size fraction between 10 min and 50 h.

STATISTICS

Student's unpaired *t*-test was used to determine the statistical significance of differences between treatments. *T* and *p* values are reported and differences are discussed at the 95% confidence level.

RESULTS AND DISCUSSION

PHYTOPLANKTON AND BACTERIAL GROWTH LIMITATION BY IRON

In the incubation experiment conducted at Station SP3, bottles of unfiltered surface seawater were supplemented in triplicate with iron, cobalt, zinc, and vitamin B₁₂ (Figure 2). Iron addition increased Chl *a* production as well as nitrate and phosphate drawdown relative to the unamended control treatments, suggesting that primary production in this area was limited by iron availability. Iron limitation of phytoplankton growth in the Ross Sea has previously been observed during summer, but not during spring (Martin et al., 1990; Sedwick et al., 2000; Coale et al., 2003). This finding represents the first evidence of iron limitation in the Ross Sea during the spring, and reflects the low surface dissolved iron concentration measured at station SP3 (0.035 nM), which is considerably lower than previously reported springtime values for this region (Sedwick et al., 2000; Coale et al., 2005; Sedwick et al., in review). These observations require that the conceptual model of seasonal iron limitation in the Ross Sea, where iron becomes gradually depleted from wintertime maxima and phytoplankton populations become iron limited only in summer (e.g., Sedwick et al., 2000), be revised to allow that these waters may become iron limited relatively early in the growing season (Sedwick et al., in review). The observation of springtime iron limitation in this *P. antarctica*-dominated community also demonstrates that *P. antarctica* can be prone to iron limitation in Ross Sea natural communities despite low cellular iron requirements (Garcia et al., 2009; Sedwick et al., in review).

Silicic acid drawdown was negligible in all experimental treatments, suggesting that diatoms comprised only a minor portion of the community. This finding is supported by microscopy-determined phytoplankton community analyses (Table 1) and by pigment analyses (Figure 3). For all treatments, *P. antarctica*-dominated the community at the beginning and end of the incubation experiment. However, based on the microscopy-determined community composition, both diatom and *Phaeocystis* abundance must have increased their growth rates comparably in response to iron addition since the diatoms remained an approximately constant proportion of the community throughout the experiment (Table 1). Likely because of their small initial abundance and thinly silicified nature (e.g., *Pseudonitzschia* spp.), diatom biomass remained relatively low and did not decrease in silicic acid concentrations despite an iron-induced increase in growth.

Figure 3 shows ratios of particulate 19-hexanolyoxyfucoxanthin (Hex), a haptophyte indicator pigment indicative of *P. antarctica* in the Ross Sea, to the pigment fucoxanthin (Fuco), which is primarily indicative of diatoms (DiTullio and Smith, 1996). Hex:Fuco ratios >2 indicate relative dominance of *Phaeocystis*; all samples

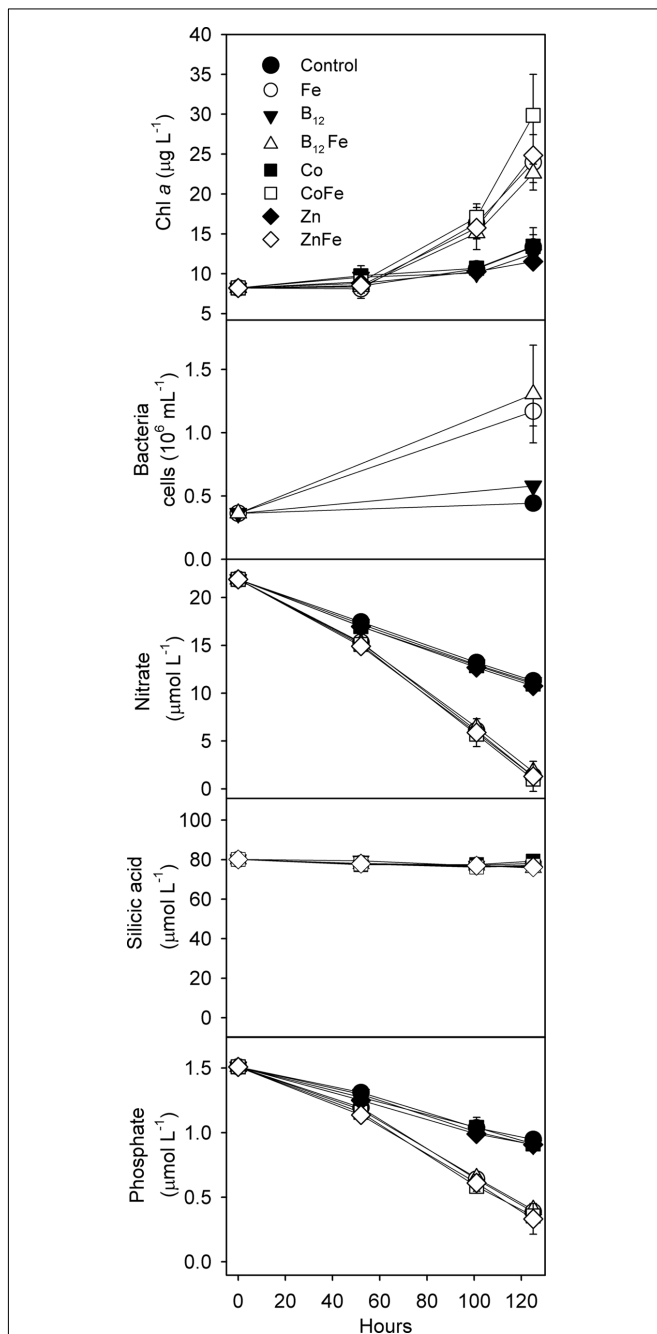


FIGURE 2 | Results of a trace metal clean whole-water bottle incubation experiment demonstrating that both phytoplankton and bacteria were iron limited. This experiment was conducted during the austral spring of 2006 at station SP3 in the Ross Sea; Chl *a*, bacterial cells, and major nutrient concentrations over time are shown. Values shown are means of triplicate treatments with error bars representing one SD. Where error bars are not visible, they fall within the bounds of the symbol. Iron stimulated enhanced Chl *a* production as well as major nutrient drawdown. No significant additional stimulation of Chl *a* production was observed upon the addition vitamin B₁₂ and iron, zinc and iron, or cobalt and iron, relative to iron alone, suggesting that neither Co, Zn nor B₁₂ was secondarily limiting phytoplankton growth. The addition of iron also, either directly or indirectly through increased availability of fixed organic nutrients, caused increased bacterial growth.

from the incubation experiment had Hex:Fuco ratios >4 , indicating strong haptophyte dominance. However, the iron-amended incubation samples had a $35 \pm 7\%$ lower Hex:Fuco ratio than the unamended incubation treatments at the end of the experiment (Figure 3). The most likely explanation for this, given the absence of an increase in silicic acid drawdown that should accompany a significant increase in diatom growth, is that *Phaeocystis* increased its fucoxanthin production upon alleviation of iron limitation, as previously observed (Van Leeuwe and Stefels, 1998). The 1-nM iron additions used here have been shown to decrease Hex:Fuco production in *Phaeocystis* approximately 10-fold (DiTullio et al., 2007). It is also possible that the growth of very thinly silicified diatoms were fueled by iron addition, which would not result in a significant silicic acid drawdown (Hutchins et al., 2001). However, the N/P drawdown ratios in both the iron-amended ($N/P = 18.0 \pm 0.4$) and unamended ($N/P = 18.4 \pm 0.4$) treatments were similar (Figure 2). Since diatoms display a much lower N:P drawdown ratio than *P. antarctica* populations in the Ross Sea (diatoms: 9.7 ± 0.3 ; *P. antarctica* 19.2 ± 0.6 ; Arrigo et al., 1999, 2000), these measured ratios suggests that *P. antarctica*, rather than diatoms, were primarily responsible for nutrient drawdown regardless of iron nutritional status. Taken together, these community analyses suggest that dissolved iron availability was not an important factor in driving community structure in this experiment since *P. antarctica* and diatoms remained in approximately equal proportions throughout. The increase in *Phaeocystis* growth upon iron addition in this experiment is consistent with culture experiments that suggest iron half saturation constants for *P. antarctica* growth are well above the very low concentration of ambient dissolved iron (0.035 nM) that was measured in seawater at station SP3 (Garcia et al., 2009; Saito et al., in preparation).

The paired additions of vitamin B₁₂ with iron, zinc with iron, or cobalt with iron did not increase Chl *a* production or nutrient drawdown relative to the addition of iron alone or appreciably change Hex:Fuco ratios beyond those changes induced by iron. While these results initially appear to be in contrast to our previous observations of B₁₂ and Fe colimitation of phytoplankton growth in the Ross Sea during summer (Bertrand et al., 2007), we believe that the results of these spring and summer experiments are consistent based on two observations. First, the initial bacterial abundance in this bottle incubation experiment was high ($3.6 \pm 0.05 \times 10^5 \text{ cells mL}^{-1}$) relative to areas in the Ross Sea where strong vitamin B₁₂ and iron colimitation was observed in the summer ($\sim 6 \times 10^4 \text{ cells mL}^{-1}$), as illustrated in Figure 4. If bacteria are indeed the major source of B₁₂ in this system, then greater bacterial abundance during spring could result in greater availability of vitamin B₁₂. Second, our previous summer experiments showed that B₁₂ addition did not stimulate the growth of *P. antarctica*, the dominant phytoplankton in this experiment. Although it is possible that *P. antarctica* does not require vitamin B₁₂ for growth, the lack of a *P. antarctica* response might also reflect the high bacterial abundances associated with *Phaeocystis* colony mucilage (Putt et al., 1994), which may provide a localized source of B₁₂. The lack of evidence for zinc or cobalt limitation or colimitation with iron in this experiment is also comparable with other Ross Sea studies where zinc additions increase Chl *a* production in some but not all locations (Cochlan et al., 2002; Coale et al., 2003)

Table 1 | Phytoplankton community composition during the incubation experiment at station SP3, given as percentage of total individual cells in the community, in the initial community and at the end of the incubation in the control, and iron added treatments. For the initial community and the control treatment, only single counts were conducted. For the final timepoint in the +Fe treatment, single counts on triplicate bottles were performed; ± 1 SD is shown.

	<i>Phaeocystis</i>	Chain forming diatoms	Single cell diatoms	Cryptophytes	Dinoflagellates
Initial	80.9	3.2	12.6	2.4	0.9
Final, Cont	86.1	1.4	11.8	0.5	0.3
Final, +Fe	84.0 \pm 1.5	1.6 \pm 1.2	13.4 \pm 0.6	0.7 \pm 0.1	0.2 \pm 0.1

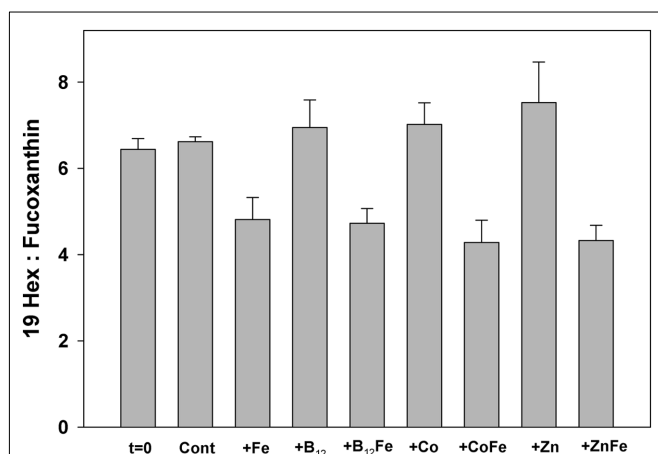


FIGURE 3 | Ratios of particulate 19-hexanoxyfucoxanthin (19-Hex) to fucoxanthin (Fuco), measured by HPLC analysis, at the beginning of the incubation experiment and at the end of each treatment. Bars are means of three biological replicates and error bars are one SD. In the Ross Sea 19-Hex predominantly represents *Phaeocystis* and Fuco predominantly represents diatoms. All samples throughout the experiment had ratios in excess of four, illustrating strong *Phaeocystis* dominance.

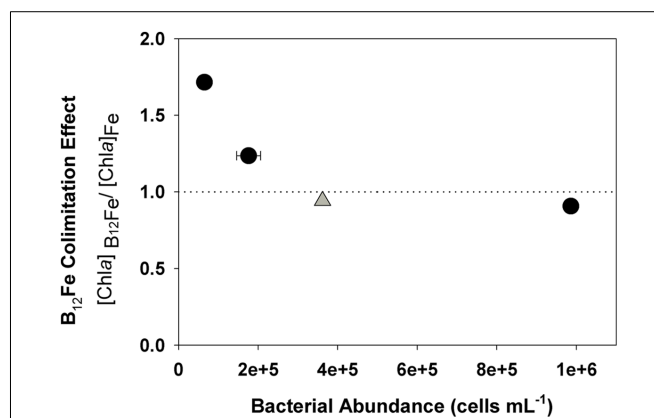


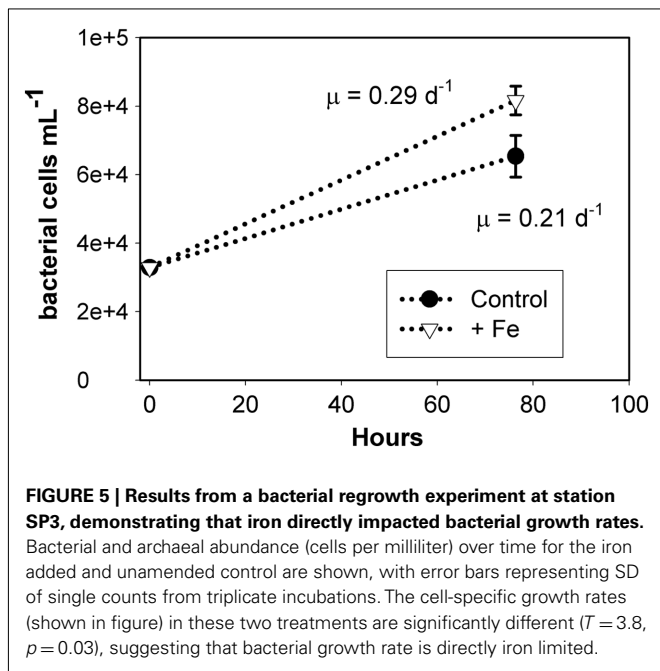
FIGURE 4 | Bacterial abundance at the beginning of an incubation experiment during the austral spring in the Ross Sea (gray diamond, SP3, this study) and three bottle incubations from the austral summer of 2005 in the Ross Sea (Bertrand et al., 2007, black circles) relative to an index of vitamin B₁₂ colimitation (ratio of final Chl a concentrations in +B₁₂Fe treatments to final Chl a concentrations in the +Fe treatments), where a ratio of one indicates no stimulation of Chl production by B₁₂ and a ratio of two means a doubling of Chl production upon the addition of B₁₂. Stimulation of phytoplankton growth by vitamin addition appears to have an inverse relationship with initial bacterial abundance. This data shows that the lack of vitamin limitation observed at station SP3 is consistent with previous results in that the initial bacterial community was larger than those present in experiments when the vitamin was limiting. Where error bars are not visible, they were smaller than the symbols.

and cobalt additions did not increase Chl *a* production (Bertrand et al., 2007), all consistent with the high concentrations of labile Co in this region (Saito et al., 2010) and the interreplacement of cobalt and zinc in *P. antarctica* (Saito and Goepfert, 2008).

Iron additions also substantially increased bacterial abundance in our bottle incubation experiment (~ 3 -fold greater increase in bacterial abundance over 5 days in the +Fe treatment versus the control; Figure 2) indicating that bacterial growth was limited by iron, either directly, or through iron-induced changes in organic matter production by phytoplankton. While iron addition increased phytoplankton abundance and potentially organic matter production, iron additions might also have altered the composition of the organic matter since culture experiments have shown that high iron concentrations (2 nM) substantially increase the lability of *P. antarctica*-derived organic matter (Becquevort et al., 2007).

In order to investigate whether bacterial communities were limited by iron availability directly or by the supply of organic matter from phytoplankton, an additional bacterial regrowth experiment was conducted in which grazers and phytoplankton were excluded by filtration (0.65 μ m pore size), and the remaining bacterial community was diluted and allowed to grow with and without 1 nM

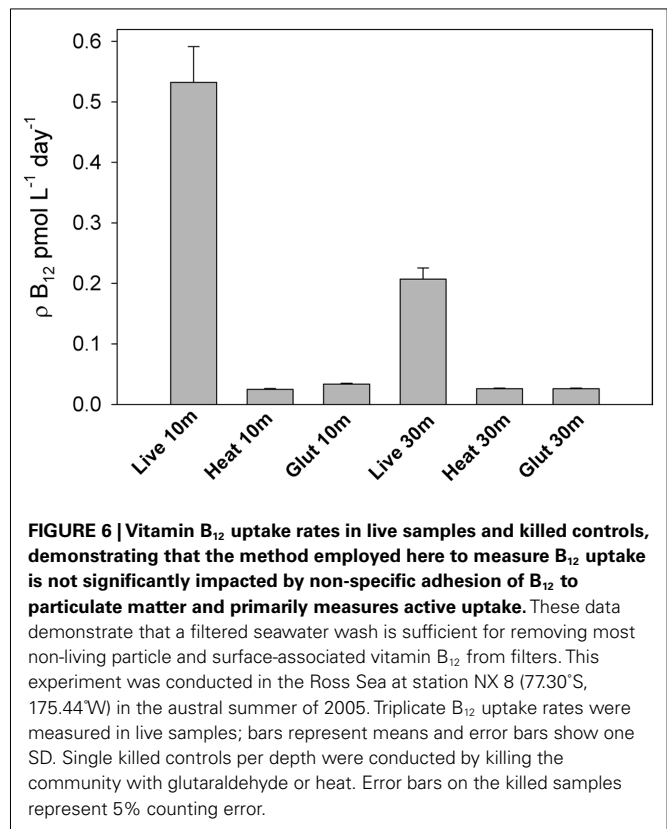
iron amendments. As shown in Figure 5, iron addition increased the cell-specific growth rate to 0.29 ± 0.01 day⁻¹ over a rate of 0.21 ± 0.03 day⁻¹ in the unamended control treatment (error is one SD about the mean of biological triplicate measurements). The filtration employed to remove the grazing and phytoplankton community may have increased DOM concentration and changed DOM composition, but any such changes would have been uniform across the treatments. We thus conclude that dissolved iron addition directly enhanced bacterial growth rates in this assemblage and that bacterial growth rates in these waters may be subject to colimitation by the availability of DOM and iron. While these growth rates are within the range of previously reported values for the Ross Sea (0.25 day⁻¹; Ducklow, 2000), they may deviate from *in situ* rates due to sample manipulation involved in the experiment. These results suggest that iron availability directly impacts bacterial growth rates in the Ross Sea and highlight that bacteria compete with phytoplankton for dissolved iron.



VITAMIN B₁₂ UPTAKE: IRON INFLUENCES AND BACTERIAL CONTRIBUTION

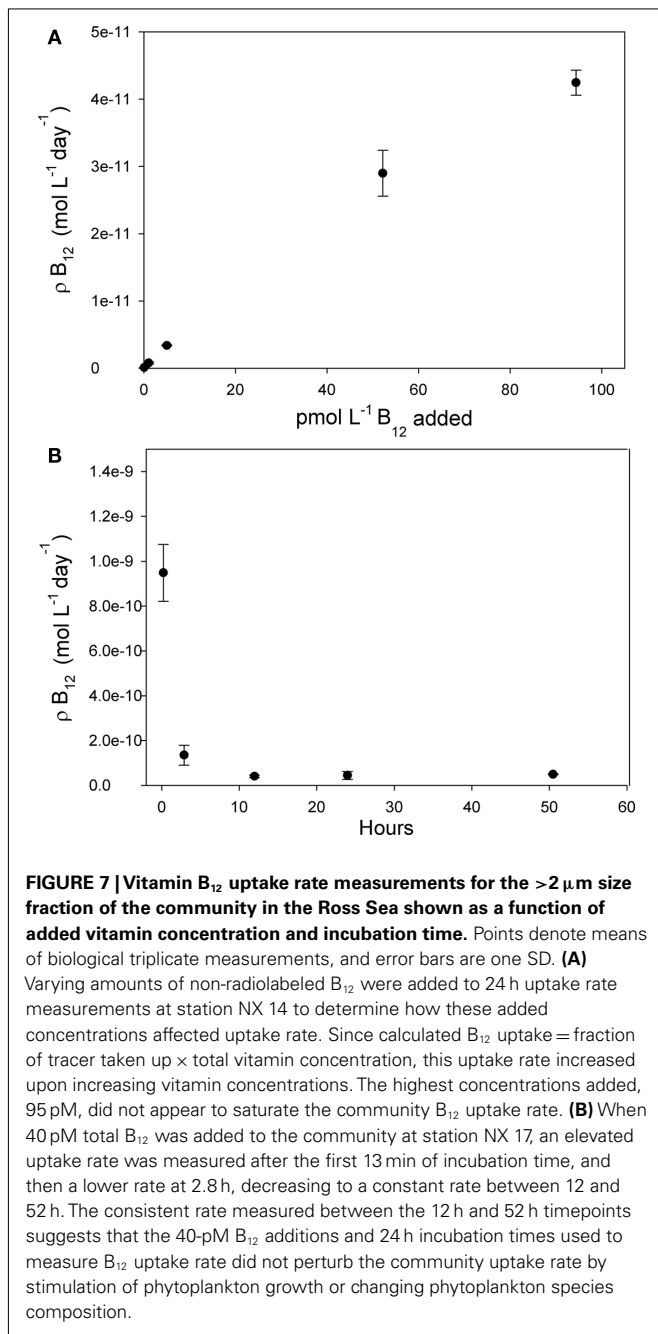
Uptake of B₁₂ by biota is considered a major sink for the vitamin in natural waters (Karl, 2002). In this study, we used radiotracer additions to examine the effect of iron amendments on vitamin B₁₂ uptake rates by microbial communities in the incubation experiment described above and in several proximal locations. Heat and glutaraldehyde-killed treatments verified that this measured B₁₂ uptake occurred through active rather than passive mechanisms (Figure 6), thus reflecting biological processes rather than adhesion to particulate matter. The technique used to measure these rates involved the addition of large amounts of vitamin B₁₂ (~40 pM). This non-tracer level addition was chosen to facilitate a comparison of uptake rates between experimental treatments without the need for measuring ambient vitamin B₁₂ concentrations in this small-volume incubation experiment. Since calculated B₁₂ uptake = fraction of tracer taken up × total vitamin concentration, these ~40 pM additions significantly increased the calculated uptake rates compared ambient B₁₂ concentrations (Figure 7A). These calculated rates thus do not represent *in situ* uptake, but rather rates at this relatively high B₁₂ concentration.

Typical models for nutrient uptake predict that progressive additions of higher nutrient concentrations increase nutrient uptake rates until the community's uptake machinery is saturated. Interestingly, the ~40 pM B₁₂ additions used here did not saturate phytoplankton community uptake rates (Figure 7A), and even up to 95 pM of added B₁₂ did not appear to saturate these rates. If we assume a Michaelis–Menten type kinetic relationship between uptake rate and added vitamin concentrations, plotting the data in Figure 7A in double reciprocal form (data not shown) yields a half saturation constant of 83 pM for B₁₂ uptake, which is much higher than typical ambient B₁₂ concentrations.



The technique we have applied can be used to compare calculated uptake rates in different experimental treatments assuming that the composition of the community in each experimental treatment does not appreciably change as a result of adding high B₁₂ concentrations over the course of the 24-h measurement. This assumption is supported by the observation that the uptake rates measured between 12 and 52 h were the same (Figure 7B) since 12 h is not enough time for a community composition change to occur given the typical generation times in polar waters. Interestingly, the uptake rate measured after 13 min was much faster than at the later timepoints, which is consistent with an uptake model whereby cell surface B₁₂-binding proteins are quickly saturated by the high concentrations of added B₁₂, then, in a second slower step, the vitamin is transferred into the cell. This is analogous to experimental results showing that the rate of zinc uptake by diatoms was elevated in the first few minutes after the addition of high Zn concentrations, which was attributed to Zn binding to cell surface sites (Sunda and Huntsman, 1992) and is also consistent with results from B₁₂ uptake studies in *Ochromonas* cultures showing that vitamin uptake also occurs sequentially, first rapidly and then a second slower phase (Bradbeer, 1971). The slight depression in B₁₂ uptake rate between the 3 and 12-h timepoints (Figure 7B) is consistent with cells experiencing minor negative feedback in uptake rates after >3 h of exposure to high B₁₂ concentrations, as previously observed for other micro and macronutrients (Sunda and Huntsman, 1992).

There are few studies of B₁₂ uptake rates in natural phytoplankton communities to date (Bertrand et al., 2007; Taylor and



Sullivan, 2008; Koch et al., 2011). Such measurements are in early stages of development. Even in laboratory-based culture studies, measurements of B₁₂ uptake are complex and have yielded results that are not easy to interpret (Droop, 1968). Measurements presented here offer an initial look at controls on B₁₂ uptake in field populations, but further study is required to develop methodology for measuring *in situ* rates and to better constrain factors affecting cellular B₁₂ uptake machinery.

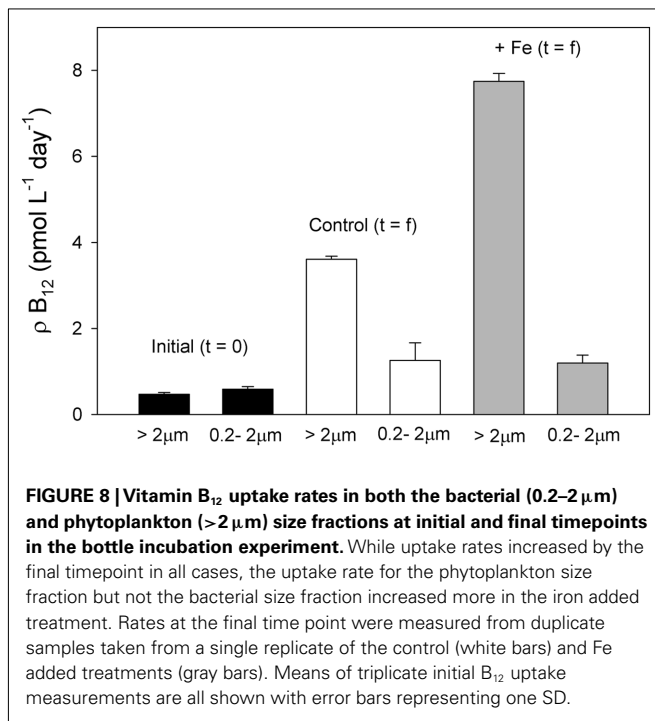
Phytoplankton size fraction B₁₂ uptake

B₁₂ uptake was measured for two separate size fractions of the community, 0.2–2 and >2 μm. The 0.2–2-μm size fraction

includes largely non-photosynthetic bacteria and archaea since there are no appreciable cyanobacterial populations in the Ross Sea (Caron et al., 2000; Marchant, 2005) and picoplanktonic phytoplankton were not observed by our microscopy methods. The >2-μm size fraction includes eukaryotic phytoplankton, which comprise the vast majority of photosynthetic biomass in this region (Scott and Marchant, 2005).

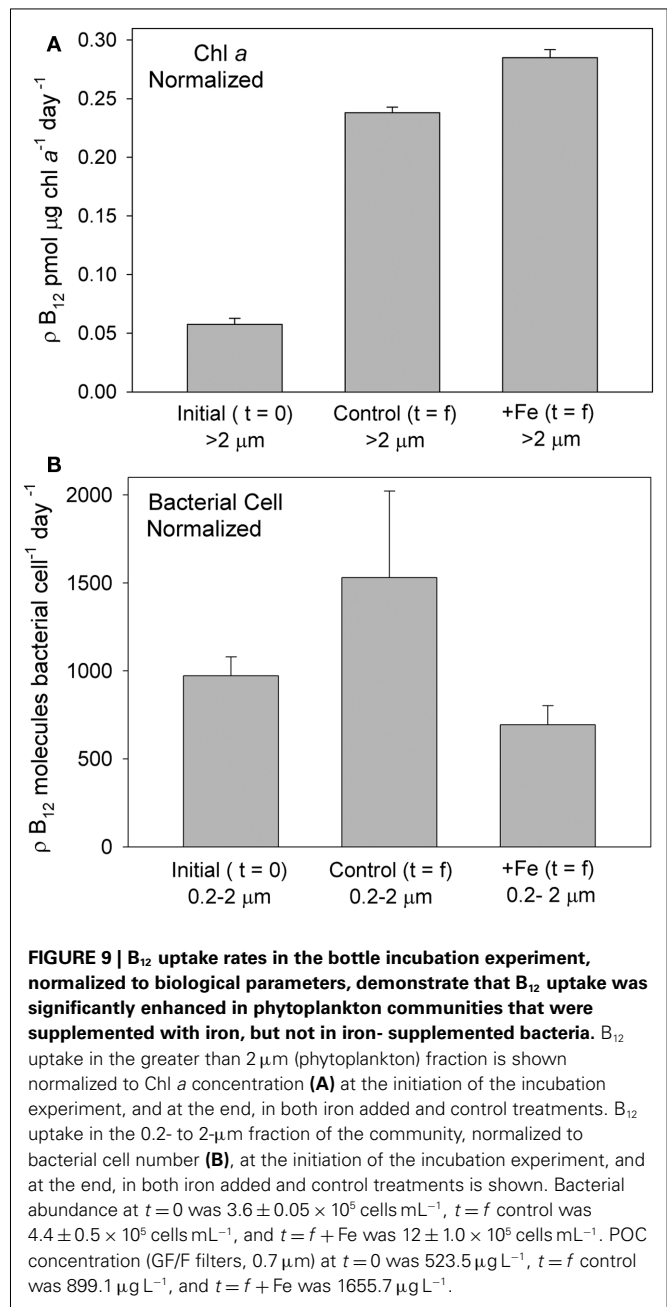
At the end of the incubation experiment, the iron-amended treatment exhibited a substantially higher B₁₂ uptake rate in the phytoplankton size fraction relative to the control (Figure 8). When normalized to Chl *a*, this uptake rate was still higher in the iron-amended treatment than in the control at the final timepoint (significantly different; $T = 7.99$, $p = 0.015$, Figure 9A). This normalized value is a measure of how community vitamin uptake rates change relative to Chl *a* changes. It is unlikely that this increase in chlorophyll-normalized B₁₂ uptake rate is solely a function of differences in cellular pigment concentration between treatments. This is because cells in the iron-amended treatment might be expected to contain more Chl *a* per cell, since Chl deficiency (chlorosis) is often associated with iron limitation (Zettler et al., 1996). If a Chl-deficient cell (i.e., in the control treatment) had the same cell-specific B₁₂ uptake rate as a Chl-replete cell (i.e., in the +Fe treatment), then the Chl *a*-normalized B₁₂ uptake rate should be *higher* in the control treatment. In fact, we observed the opposite trend: Chl-normalized B₁₂ uptake rate was greater for the Fe-amended treatments, suggesting that this trend reflects an increase in community B₁₂ uptake rate rather than changes in pigment composition. This result suggests that vitamin B₁₂ uptake by the phytoplankton community increased after iron addition due to (1) a change in species composition, (2) an increase in cellular vitamin transporters, or (3) an increased use of energy allocated to active vitamin transport systems in order to increase vitamin uptake rates in step with growth rates. The first scenario is unlikely, given that the community remained heavily dominated by *P. antarctica* in all treatments (Table 1, Figure 3), suggesting that the latter two mechanisms may be important.

In addition to revealing an effect of iron on B₁₂ uptake, our experiment showed a 4–6 × lower Chl *a*-normalized B₁₂ uptake rate at the beginning of the incubation experiment relative to the final timepoint, in both the control and iron-amended treatments (Figure 9A). Although both the initial and final uptake measurements were conducted under identical conditions in the deckboard incubator, the initial timepoint measurement was taken immediately upon transfer from *in situ* conditions to the shaded incubator while the final timepoint measurements were taken after a 5-day acclimation inside the bottle incubation experiment. Thus, the large change in uptake rate might partly reflect “bottle effects,” or the impact of altered irradiance. If the conditions favored faster growth in the final stages of the experiment, then these enhanced uptake rates could reflect increased growth rate and overall energy available to cells, resulting in an increase in energy allocated to active vitamin transport systems that is unconnected to vitamin nutritional status. However, an increase in available energy at the end of the incubation experiment is unlikely, particularly in the case of the control treatment, since that community was becoming increasingly iron limited. These higher uptake rates may thus in



part reflect stress experienced by the phytoplankton due to depletion of available vitamin supplies. Although there is an internal source of vitamin B₁₂ in these incubations (bacterial growth), the vitamin sinks due to bacterial and phytoplankton uptake and photochemical degradation (Carlucci et al., 1969) are likely to be considerable. On balance, this may result in progressive depletion of B₁₂, mirroring the nitrate and phosphate drawdown observed in this experiment (Figure 2). Such depletion could induce an upregulation of vitamin transporters, which would be reflected in elevated B₁₂ uptake rates.

The observed changes in B₁₂ uptake rate are consistent with the phytoplankton communities becoming stressed for lack of this vitamin by the end of the incubation experiment, which would result in increased calculated uptake rates. However, the bottle incubation study did not show an increase in phytoplankton biomass upon vitamin B₁₂ addition, leading to the conclusion that there was no colimitation by vitamin B₁₂ and iron at this time and location. This observation suggests that bottle incubations may not be sensitive to some subtle physiological states of nutrient stress that are nonetheless relevant for biogeochemical cycling and that might indicate susceptibility to nutrient limitation. Nutrient uptake studies like these, as well as the application of protein or transcript-based biomarker assays for gene products that are abundant under defined states of nutrient stress (LaRoche et al., 1996; Webb et al., 2001; Dyhrman et al., 2002), can complement bottle incubation studies and provide more subtle information about the limitation and colimitation experienced by microbial communities. The dramatic increase in vitamin uptake rates from the beginning to end of this incubation experiment and upon increasing B₁₂ concentrations (Figure 7) suggest that phytoplankton can exert considerable control over the cellular machinery that is used to take up vitamin B₁₂. This implies that future studies to



document the cellular response of phytoplankton to vitamin stress and to develop protein or transcript-based biomarkers for vitamin limitation may be feasible.

Bacterial size fraction uptake

The initial B₁₂ uptake rates at the beginning of the incubation experiment were slightly greater for the 0.2–2-μm size fraction than for the greater than 2 μm fraction (Figure 8, not significantly different; $T = 2.34$, $p = 0.078$). This observation suggests that in addition to being the source of vitamin B₁₂, the bacterial and archaeal community size fraction is also an important sink. This evidence, along with that offered in another study (Koch et al., 2011) favors a departure from the Karl (2002) model of vitamin

B₁₂ cycling, which suggests that eukaryotes dominate B₁₂ uptake. Our results are also consistent with the fact that, according to currently available genomic sequencing information, many Antarctic marine bacterial strains appear to require an exogenous source of B₁₂ (Bertrand et al., 2011).

The rate of B₁₂ uptake by the 0.2–2- μ m size fraction normalized to the size of the bacterial community at the initial and final timepoints for the iron-amended treatment were not significantly different ($T = 1.19$, $p = 0.41$), while uptake rates in the initial versus final community in the control treatment were significantly different ($T = 3.44$, $p = 0.04$), with a higher B₁₂ uptake rate in the final community (Figure 9B). Since many bacterial cells, including the abundant SAR11 clade, do not take up vitamin B₁₂ (Zhang et al., 2009), these rate measurements that are normalized to total cell abundance should not be taken as reflections of individual cell uptake rates but rather as a measure of the change in vitamin uptake rate relative to the change in bacterial community size. Although our experimental uncertainties are substantial, this trend may suggest a shift in community composition in the control treatment (under iron limitation) toward bacteria that take up exogenous B₁₂, or enhanced cellular B₁₂ uptake rate within that community. A similar shift or increase in uptake rate does not seem to have occurred in the iron replete treatments. This suggests that when iron availability is low, vitamin B₁₂ uptake by bacteria may increase. This hypothesis is consistent with reports that part of bacterial B₁₂ demand arises from use in ribonucleotide reductase (RNR) enzymes, which use either iron or vitamin B₁₂ and that, in some bacteria, the transcription of the gene encoding the iron-requiring RNR is only expressed when the B₁₂ cell quotas are low (Borovok et al., 2006).

IMPLICATIONS

Iron influences on B₁₂ production

The results of our incubation and regrowth experiments suggest that dissolved iron availability can help regulate net bacterial productivity in the Ross Sea. Since vitamin B₁₂ production rates are tied to bacterial productivity, our results further suggest that vitamin B₁₂ production could be influenced by the availability of dissolved iron. Although there are likely many factors contributing to B₁₂ production rates, vitamin availability to phytoplankton appears to scale with bacterial abundance since small bacterial communities were observed where B₁₂ limited primary production (Figure 4). Constraining the rate of B₁₂ production and the factors that regulate it presents challenges similar to those involved constraining the cycling of other trace level nutrients, such as ammonia. Knowing the concentration of vitamin B₁₂ may not tell us much about its production rate, given its rapid uptake by the biota and its short photochemical half life in the surface ocean (Carlucci et al., 1969). Since the rate at which individual organisms conduct vitamin biosynthesis may be variable, knowing the number of community members that can produce the vitamin at any one time may also tell us little about the gross B₁₂ production rate (Röling, 2007). The rate of vitamin transfer from the particulate to the dissolved pool, and not simply the rate of biosynthesis, must also be considered. This flux will be a function of the production rate within the local microbial communities; the rate of population turnover by grazing, viral lysis, and other factors, as well as the efficiency of the microbial loop. The addition of the vitamin

due to mixing with other water masses is likely small (Panzeca et al., 2009), but must also be considered. These issues are beyond the scope of this paper, but are important for understanding the biogeochemistry of vitamin B₁₂.

Persistence and timing of phytoplankton and bacterial blooms in iron fertilization events

The results of this study suggest that DOM consumption rates, insofar as they are related to bacterial growth, may be directly influenced by dissolved iron availability, and that B₁₂ production and consumption in the Ross Sea are also likely impacted by iron. When taken together, these results present the possibility that during a natural or engineered iron fertilization event, B₁₂ production may increase and bacterial B₁₂ demand may decrease, thereby resulting in enhanced availability of B₁₂ to phytoplankton. It is possible that this enhanced availability could alleviate B₁₂ colimitation of phytoplankton communities, resulting in increased primary production. However, the relative timing of these responses will determine the impact of synergistic vitamin and iron dynamics on iron-induced blooms. If the bacterial response to iron addition lags the phytoplankton response (more likely if the bacterial community is DOM limited), then the phytoplankton community is less likely to receive increased B₁₂ supply to meet its enhanced B₁₂ demand (Figure 7). This is likely to have occurred in the summertime Ross Sea experiments that observed B₁₂ stimulation (Bertrand et al., 2007). However, if the bacterial response to dissolved iron addition is concurrent with the phytoplankton response (more likely if bacteria are primarily iron limited), then bacterial vitamin B₁₂ production may keep pace with the increasing phytoplankton demand, preventing secondary vitamin B₁₂ limitation. The balance between enhanced DOM consumption and altered vitamin B₁₂ dynamics should be considered when evaluating the impact of bacterial communities during iron fertilization.

CONCLUSION

This study provides direct evidence of iron limitation of phytoplankton growth in the Ross Sea during spring, thus challenging the idea that this Antarctic shelf ecosystem is iron replete in the early growing season. In addition, bacterial growth in this location was shown to be directly iron limited or colimited by iron and DOM. These results imply that iron availability may limit vitamin B₁₂ production, since bacteria and archaea are the only known source of B₁₂ production in the water column. The bacterial size fraction of the microbial community also comprised a significant sink of vitamin B₁₂ in the water column, with uptake measurements suggesting that this sink may be reduced under iron replete conditions. Despite a lack of enhanced Chl production upon vitamin B₁₂ addition to the bottle incubation study here, vitamin B₁₂ uptake rate measurements suggest that phytoplankton may have experienced stress due to B₁₂ deficiency, particularly when iron limitation was alleviated.

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