

Vitamin B₁₂ and cobalt cycling among diatoms and bacteria in Antarctic sea ice microbial communities

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Abstract

Within McMurdo Sound's annual sea ice, assimilation and concentrations of vitamin B₁₂ (cobalamin), microbial community productivity, and biomass were examined among three 100-m² quadrats where light penetration was manipulated by snow cover during austral summer. From late October through December, B₁₂ concentrations (6–32 pmol L⁻¹) and assimilation rates (17–780 pmol m⁻² d⁻¹) in congelation ice covaried with primary productivity (0.0001–250 mmol C m⁻² d⁻¹) and chlorophyll *a* (0.6–36 mg m⁻²). Within ice core samples, incorporation of ⁵⁷Co-B₁₂ into the >1.0- μ m size fraction (mostly diatoms) was almost 100 times faster than into 0.2–1.0- μ m particles (mostly heterotrophic bacteria) and was dependent on light and active transport across cell membranes. Microalgal B₁₂:C cell quotas in field communities varied widely (2.6–150,000 nmol B₁₂ (mol C)⁻¹; \bar{x} = 500) and generally exceeded those of cultured temperate diatoms (0.4–55 nmol B₁₂ (mol C)⁻¹; \bar{x} = 4.1). Comparisons of dissolved B₁₂ pools in the ice and their turnover (0.02–0.6 d⁻¹) with underlying seawater suggest that this vitamin is produced in situ rather than delivered from waters below. Production and uptake of B₁₂ and uptake of cobalt, required for B₁₂ synthesis, were then examined among bacteria isolated from these communities. Only 23% of 78 bacterial isolates were incapable of B₁₂ uptake, but these clones assimilated dissolved cobalt. Intracellular B₁₂ production was evident in 9 of the 11 isolates screened and their cell quotas varied widely, 0.6–6,800 nmol B₁₂ (mol C)⁻¹. Mass balance analyses and published kinetics data independently suggest that microalgal growth in sea ice was not limited by vitamin B₁₂ in most of our field observations and that in situ bacterial B₁₂ production could potentially meet microalgal demands. Similar analyses, however, suggest that cobalt supply from underlying waters may have limited community growth and B₁₂ production.

Consistent with Liebig's law of the minimum, numerous aquatic studies have demonstrated that the nutrient in shortest supply (N, P, Si, or Fe) relative to cellular demand at any particular time or site limits system productivity (Liebig 1855). Furthermore, relative stoichiometries and speciation of these elements can influence phytoplankton community structure as a consequence of cell nutrient quotas, uptake kinetics, and specificity of individual population's uptake systems (Tilman 1982; Karl 2002). Selective growth responses within phytoplankton communities can have important implications for carbon cycling,

from influencing trophic structure to controlling carbon sequestration in the ocean's interior. For example, nutrient regimes promoting growth of large, biomineralizing (CaCO₃, SiO₂) taxa tend to select for larger herbivores and favor higher carbon export to the mesopelagic as sinking aggregates and fecal pellets (de Baar et al. 1995).

Recent research on iron limitation in high-nutrient–low-productivity regions, such as the Southern Ocean (Martin et al. 1990; de Baar et al. 1995), has spawned a growing awareness that other micronutrients may also limit phytoplankton populations, either selectively or broadly. Vitamin B₁₂ may be one such keystone micronutrient whose availability determines which major taxa dominate a microalgal community. Decades ago, investigations revealed that many eukaryotic phytoplankton species are B-vitamin auxotrophs, i.e., unable to biosynthesize one or more B vitamins (e.g., Droop 1957; Provasoli 1963; Carlucci and Silbernagel 1969). Revisiting the cultivation literature, Croft et al. (2005, 2006) reported that a high proportion of diatom (59%) and dinoflagellate (89%) species in contemporary culture collections are demonstrably B₁₂ auxotrophs and require extracellular vitamins to grow. These observations suggest that growth of many algal taxa can be limited by availability of extracellular B₁₂. The opposing view that dissolved cobalamin seldom falls to growth-limiting concentrations in the ocean has also been expressed on the basis of early laboratory studies (see

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Droop 2007 for summary), but has not been widely tested in the field. Recent field experiments in temperate coastal waters have demonstrated that growth of larger phytoplankton taxa can be preferentially stimulated by adding vitamins B₁₂ or B₁ (or both) to microcosms, even in relatively nutrient-rich natural waters (Sañudo-Wilhelmy et al. 2006; Gobler et al. 2007).

The requirement for the B₁₂ coenzyme in transmethylation reactions, methionine synthesis, and ultimately DNA replication is highly conserved among prokaryotes, protists, and animals (Warren et al. 2002; Rodionov et al. 2003). Unlike macronutrients and most micronutrients, abiotic pathways for producing cobalamin, a tetrapyrrole ring with several side chains and the rare element cobalt as central coordinating ion, are highly improbable under Earth's current biogeochemical regimes. Hence this micronutrient appears to be exclusively biogenic in origin. Consequently, many microalgal species must obtain B₁₂ directly or indirectly from other community members through a variety of processes. Alternatively, some species may circumvent extracellular B₁₂ requirements by relying on B₁₂-independent enzyme isoforms (Rodionov et al. 2003). Observations from nonaxenic cultures strongly suggest that auxotrophic microalgae acquire B₁₂ from bacterial symbionts (Provasoli 1963). However, mechanisms by which bacteria release an energetically expensive molecule, such as cobalamin, to culture media or natural waters remain subject to speculation. Viral lysis, grazing inefficiencies, programmed cell death, and active cellular export, similar to siderophore release, are some plausible mechanisms in natural systems (Provasoli 1963; Croft et al. 2006). Nonetheless, field studies directly examining B₁₂ cycling and its role in microalgal growth at the community level are few and far between (e.g., Parker 1977; Swift and Guillard 1978; Sañudo-Wilhelmy et al. 2006).

Recently, Panzeca et al. (2006) demonstrated that phytoplankton production off the Antarctic Peninsula could be stimulated by iron or B₁/B₁₂ vitamin amendments alone and stimulated even more by adding Fe, B₁, and B₁₂ together. Bertrand et al. (2007) reported a similar B₁₂-Fe colimitation and preferential stimulation of phytoplankton taxa in the open Ross Sea. Clearly, dissolved B₁₂ availability can alter production and affect planktonic community structure in the Southern Ocean. The present study examines cycling of vitamin B₁₂ and cobalt within microbial communities occupying annual sea ice that covers McMurdo Sound, Antarctica every winter (congelation ice). Sea ice microbial communities represent well-constrained systems in which "epontic" (ice-colonizing) microorganisms are more or less immobilized in brine between ice crystals in the lower 5 cm of these 2–3-m-thick ice sheets and remain between –5.0°C and –1.9°C, depending on depth, snow cover, and season (Kottmeier and Sullivan 1988). These communities lie within steeply attenuated downwelling light fields and opposing nutrient gradients established by biological drawdown and diffusive fluxes from nutrient-rich waters below (Smetacek et al. 1992; Arrigo et al. 1995). This system is ideally suited for study of B₁₂ dynamics because the dominant photoautotrophs are typically diatoms, the majority of which

are B₁₂ auxotrophs (Croft et al. 2006). The principal external nutrient source is the underlying seawater, although infiltration of meltwater from above can occur late in austral summer (Palmisano and Garrison 1993; Garrison et al. 2005). Furthermore, in situ productivity can be controlled experimentally by manipulating snow cover, thereby altering surface albedo and consequently downwelling irradiance (Sullivan et al. 1985; Grossi et al. 1987). Here we examine B₁₂ assimilation by communities in experimental plots held at three different light levels over a 6-week period. We further examine bacteria isolated from the same sea ice communities and waters below for their ability to assimilate dissolved cobalt and produce vitamin B₁₂ as well as assimilate extracellular B₁₂. Results are used to evaluate nutrient limitation and micronutrient exchange between bacteria and microalgae.

Materials and methods

Field experiments—Field studies to evaluate effects of light availability on B₁₂ assimilation and productivity in annual sea ice were conducted by manipulating snow cover at a site over a 20-m water column in McMurdo Sound, southeast of Cape Armitage, Ross Island (~77.8°S, 167°W). Three 100-m² experimental quadrats were prepared on 28 October 1981, when natural snow cover at the site was approximately 25 cm. Quadrats 1, 2, and 3 (Q-1, etc.) were maintained through December at constant snow covers of approximately 4, 80, and 25 cm of snow, respectively. Photosynthetically active radiation (between 400 and 700 nm) was measured intermittently (every 1.5 to 3 weeks) beneath the ice between 11:00 h and 13:00 h by divers using a spectroradiometer with cosine collector (MER-100, Biospherical Instruments; Palmisano et al. 1987).

For vitamin B₁₂ assimilation and primary productivity measurements, replicate cores of congelation ice were collected on 11–15 and 27 November and 3–4 December from as many as four locations within each quadrat using a SIPRE auger (Geo-Test) with 7.5-cm-diameter 316-grade stainless steel barrel. Only the ice cores' lower 5-cm segments were used for experiments because they contained 99% of total chlorophyll *a* (Chl *a*) in any given core (Palmisano and Sullivan 1983).

Vitamin B₁₂ assimilation—From each quadrat, replicate core segments were quartered longitudinally and subsamples (~40 cm³ each) were placed in individual transparent polymethylpentene jars (Nalgene), previously rinsed with filtered seawater. Jars were then filled to capacity (175 mL) with measured volumes of chilled filtered seawater (<0.22 μm). Although sampling practices and seawater diluents probably increased ambient inorganic nutrients in experimental microcosms, including iron and other metals (not measured), they diluted dissolved cobalamin inventories 4- to 20-fold (presented below). To initiate vitamin assimilation experiments, ⁵⁷Co-B₁₂ (~4.4 × 10⁻³ Bq L⁻¹ or 0.3–0.5 pmol L⁻¹ B₁₂, Amersham CT.2, ⁵⁷Co-B₁₂ 3.7–11 Bq μg⁻¹) was added to two subsamples: one live and one a formaldehyde-killed control (2% final conc.). Remaining

subsamples were used to measure ambient cobalamin concentrations. After radiotracer addition, samples were sealed and held below 0°C in darkness (<30 min) until placed by scuba divers in buoyant chambers beneath their respective quadrats. Most $^{57}\text{Co-B}_{12}$ samples were incubated in situ for 12 h, but time-course experiments were also performed on three occasions. Core segments were not melted before experiments to better mimic ice microenvironment conditions. This method may have slowed equilibration of radiotracer with unlabeled pools in seawater and sea ice phases. However, this uncertainty was preferred over deliberate alteration of the ice microenvironment and in fact, most ice melted during the -1.9°C in situ incubations.

After retrieval, any residual ice was melted in the dark at 0°C in ≤ 2 h. To determine $^{57}\text{Co-B}_{12}$ assimilation, sea ice microbes in live and killed controls from 50-mL subsamples were collected on 0.2- μm pore size Nuclepore membranes and rinsed twice with ice-cold filter-sterilized seawater. $^{57}\text{Co-B}_{12}$ activity was radioassayed on a Beckman Biomamma Counter. Assimilation rates ($\text{pmol L}^{-1} \text{d}^{-1} \text{B}_{12}$) in meltwaters were calculated by subtracting control values from live treatments, correcting for sample dilution, estimating specific activity from the sum of native unlabeled B_{12} and added radiotracer, and then dividing by total elapsed time. Calculated rates are conservative because most of the core sample volume is initially occupied by ice crystals. However, accounting for interstitial water:ice ratios in each sample was not feasible.

To determine whether microalgae or bacteria dominated B_{12} assimilation in sea ice, a detailed time-course experiment was run at Eklund Biological Laboratory on 29 December 1982 using two core segments from our field study site. Ice core samples were diluted in ice-cold 0.22- μm -filtered seawater and melted in the dark at 0°C, then dispensed into three seawater-rinsed Nalgene incubation jars and inoculated with $^{57}\text{Co-B}_{12}$ (0.24 Bq L^{-1} or $26 \text{ pmol L}^{-1} \text{B}_{12}$ final conc.). Samples were incubated in a -1.9°C water bath under simulated in situ light conditions ($0.05 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and subsampled periodically. After withdrawing the 6-h subsamples, one jar was placed in an opaque container (dark treatment) and another was inoculated with carbonyl cyanide *m*-chlorophenylhydrazine (CCCP treatment; $10^{-5} \text{ mol L}^{-1}$ final conc.), a protonophore dissipating transmembrane proton gradients, thereby disrupting photophosphorylation in chloroplasts, cell motility, and active transport of substrates. Herold (1984) demonstrated that $10^{-5} \text{ mol L}^{-1}$ CCCP completely blocked $^{57}\text{Co-B}_{12}$ assimilation in diatom cultures of *Thalassiosira pseudonana*. All jars remained in the illuminated -1.9°C water bath and were subsampled for another 42 h. Subsamples were first filtered through 1.0- μm pore size Nuclepore membranes and rinsed with equal volumes of ice-cold 0.22- μm -filtered seawater. These filtrates were then passed through 0.2- μm pore size membranes that were also rinsed with sterile seawater to capture the 0.2–1.0- μm particle fraction. Analyses and calculations were as described above and include rates for diatom-dominated ($>1.0 \mu\text{m}$) and bacteria-dominated (0.2–1.0 μm) size fractions.

Microalgal biomass and productivity measurements—Chl *a* concentrations and net primary production (NPP) were determined from dedicated ice cores collected contemporaneously with those used for $^{57}\text{Co-B}_{12}$ experiments. Methods used are similar to those described in Strickland and Parsons (1972) and are more thoroughly presented in Grossi et al. (1987). Similar to B_{12} assimilation experiments, core segments from each quadrat were placed in Nalgene jars and diluted with ice-cold 0.22- μm -filtered seawater; one to two serving as light treatment, one as killed control (2% formaldehyde final conc.), and one as dark treatment (opaque jar). Samples were inoculated with $\text{NaH}^{14}\text{CO}_3$ to a final activity of $1.7 \times 10^3 \text{ Bq L}^{-1}$ and incubated in situ for 12–24 h alongside the B_{12} assimilation experiments described above. Assimilation of $\text{NaH}^{14}\text{CO}_3$ was experimentally demonstrated to be linear for ≥ 30 h for these congelation ice communities at this time of year (Grossi et al. 1987). Incubations were terminated by addition of formaldehyde (2% final conc.). Samples were processed in the Eklund Biological Laboratory immediately upon return.

Seawater samples were collected from 1 m below the congelation ice–water interface on 17 Nov 81 using a Kemmerer polyvinyl chloride water sampler. Four light and two dark bottles were supplemented with $\text{NaH}^{14}\text{CO}_3$ ($2.5 \times 10^3 \text{ Bq L}^{-1}$ final activity) and incubated for 24 h suspended beneath Q-3. After incubation, samples were processed the same as ice core samples.

In the lab, phytopigments were extracted in 90% acetone from duplicate subsamples (2–10 mL meltwater or ≤ 1 liter for seawater) collected on glass fiber filters (Whatman GF/C) and then frozen. Chl *a* and pheopigments were determined fluorometrically as described in Strickland and Parsons (1972). Replicate subsamples for total NPP were routinely captured on 0.2- μm Nuclepore membranes and sometimes also on 1.0- μm Nuclepore membranes to assess activity of larger cells. Membranes were rinsed with chilled 0.22- μm -filtered seawater, fumed over concentrated HCl overnight to remove unincorporated radiocarbon, and radioassayed by liquid scintillation counting. Photosynthetically fixed extracellular organic ^{14}C (EO^{14}C) was also measured in subsamples of 0.2- μm filtrate by acidifying to pH 2 with 0.1 mol L^{-1} HCl and agitating uncapped on a rotary shaker for 12 h to drive off unincorporated radiocarbon. Triplicate 1-mL subsamples then were radioassayed. Fractional contribution of EO^{14}C to total organic ^{14}C may be artificially elevated because incubations were terminated by addition of formaldehyde, a treatment that may cause release of intracellular ^{14}C from microalgae.

Volumetric NPP ($\text{mmol m}^{-3} \text{d}^{-1} \text{C}$) was computed as total ^{14}C captured on 0.2- μm filters and in EO^{14}C from light bottles minus comparable pools in dark bottles, then multiplied by specific activity (dissolved inorganic carbon/ ^{14}C) and divided by incubation time. Details of these calculations and their assumptions appear in Grossi et al. (1987). Integrated daily NPP and B_{12} assimilation rates ($\text{mol m}^{-2} \text{d}^{-1}$) were calculated from volumetric rates as follows. For congelation sea ice, $\text{mmol m}^{-3} \text{h}^{-1}$ was multiplied first by a dilution correction factor ($F = \text{total sample volume divided by meltwater volume}$), by 24 h,

which approximates the observed photoperiod, and then by the average thickness of experimental ice sections (0.05 m). For underlying seawater, only volumetric rates were used because depth of photic zone was not determined and only a single depth was sampled.

Isolation and maintenance of sea ice bacteria—Although Archaea may comprise as much as 25% of the “prokaryoplankton” in the Southern Ocean (Murray et al. 1998), they have escaped detection in Antarctic sea ice (Brown and Bowman 2001). Thus, we assume that our communities and clone collection are overwhelmingly populated by the domain Bacteria and refer to them as such hereafter. Bacteria were isolated by a modified spread plate technique from sea ice samples and underlying seawater collected from our study site, details of which appear in Kobori et al. (1984a,b). Inocula were spread onto 2216E nutrient agar plates and maintained in darkness at 0°C ± 1°C. To avoid thermal shock to these psychrophilic and psychrotolerant bacteria during all manipulations, cultures and media were kept on ice and transfers were made with either chilled sterile toothpicks or pipets. Visually distinct colonies were randomly selected and restreaked to obtain clonal isolates. Clone characterization predated current ribotyping methods, so mostly phenotypic traits, including cell and colony morphology, motility, psychrophily vs. psychrotolerance, antibiotic resistance, and alkaline phosphatase activity, were catalogued. Presence and size of plasmids were also considered (Kobori et al. 1984a,b). Seventy-eight of the 155 clones were selected for study and maintained on 2216E nutrient agar slants (Difco) at 0°C ± 1°C and screened for B₁₂ and Co assimilation and B₁₂ production (described below).

Vitamin B₁₂ assimilation by bacterial isolates—To minimize contamination in all in vitro experiments and B₁₂ bioassays described below, Pyrex glassware was cleaned with Alconox detergent, rinsed thoroughly in deionized-distilled water (ddH₂O), and soaked in a Chromerge acid (conc. H₂SO₄-K₂Cr₂O₇) bath for 24 h, followed by 24 h in ddH₂O and then drying for 12 h at 260°C (Carlucci and Bowes 1972a). To examine dissolved vitamin B₁₂ assimilation among Antarctic bacterial clones, bacterial inocula were transferred to 10 mL of filter-sterilized seawater (<0.22 μm) that was freshly collected inshore from the Southern California Bight. This 18-d dark incubation at 0°C ± 1.0°C acclimated cells to environmental nutrient concentrations and exhausted carryover of trace nutrients from agar slants. After acclimation, 0.1-mL inocula were transferred to 10 mL of fresh filter-sterilized seawater amended only with ⁵⁷Co-B₁₂ (6.6 pmol L⁻¹ total B₁₂, 3.3 × 10⁻³ Bq L⁻¹ final activity) and incubated for a subsequent 10 d under the same incubation conditions. After incubation, replicate subsamples were passed through 0.2-μm Nuclepore membranes followed by two 5-mL ice-cold sterile seawater rinses. Membranes were radioassayed for ⁵⁷Co activity on a Beckman Biogamma Counter. Experimental values were corrected with 2% formaldehyde-killed controls and 0-h blank values to yield ⁵⁷Co-cobalamin assimilation rates.

Cell-specific and biomass-specific rates were determined by enumerating cells and estimating biomass produced during incubations. Subsamples were preserved with borate-buffered formaldehyde (2% final conc.) at the beginning and end of incubations. Bacterial enumeration was performed by epifluorescence microscopy on preserved material stained with acridine orange (Hobbie et al. 1977). Bacterial biomass was estimated from 200 randomly selected cells in each sample by visually sorting into eight size classes on the basis of their linear dimensions, approximated with an ocular micrometer at ×1,000 magnification. Cellular carbon biomass (C) was estimated from biovolume (V) using an allometric carbon-to-volume extrapolation function (C = 0.12V^{0.72}; Norland 1993).

Cobalt assimilation by bacterial isolates—Isolates growing in unamended seawater and displaying slow vitamin B₁₂ turnover (≥400 d) in the previous experiment clearly did not require extracellular vitamin and were considered presumptive cobalamin producers. These isolates were acclimated as described above before transferring to a seawater medium lacking B₁₂. Dissolved organics, including vitamins, were extracted from 1 liter of filtered seawater by mixing for 30 min with Norit carbon (10 g dry weight) that had been activated by three 5% NaCl rinses (Carlucci and Bowes 1972a). Norit carbon was removed by passing vitamin-free seawater through Whatman qualitative and Millipore 0.22-μm filters. Trace metals were removed from vitamin-free seawater and the NH₄Cl and NaH₂PO₄·H₂O stock solutions using an activated Chelex 100 extraction column pretreated with 2 mol L⁻¹ HNO₃, 2 mol L⁻¹ HCl, and ddH₂O rinses, according to Bruland et al. (1985). Biotin, thiamin, NH₄Cl, NaH₂PO₄·H₂O, and trace metals (including 50 nmol L⁻¹ CoCl₂ and 11.7 μm L⁻¹ ethylenediamine tetraacetic acid [EDTA]) were added to vitamin-/metal-free seawater in the same concentrations reported for f/2 medium after adjusting pH to 7.8 (Guillard and Ryther 1962). To supply organic substrates at a final concentration of ~0.8 mmol L⁻¹ C, vitamin-free casamino acids (11.0 mg L⁻¹), tryptophan (0.8 mg L⁻¹), and glucose (23.0 mg L⁻¹) were added. Subsequent experiments were run with these substrates at 10 and 100 times higher concentrations. For perspective, dissolved organic carbon concentrations in ice meltwater from our study site varied between 0.9 and 5.0 mmol L⁻¹. Complete medium was filter-sterilized (<0.22 μm) and 50-mL aliquots were transferred to sterile acid-washed flasks.

After addition of ⁵⁷CoCl₂ (7.3 × 10⁻⁵ Bq mL⁻¹ final activity), 0.1-mL bacterial inocula from preparatory incubations were transferred to the defined B₁₂-free medium. Incubations were conducted in darkness at 0°C ± 1°C on a shaker table at 100 revolutions per minute. Growth, ⁵⁷Co activity, and B₁₂ production samples were taken at 0, 4, and 10 d. Formaldehyde-killed (2% final conc.) controls were used to correct experimental values for abiotic ⁵⁷Co adsorption. Uninoculated controls were screened for possible contamination allowed by filter sterilization, but ⁵⁷Co assimilation and growth by contaminant cells were not evident under our incubation conditions. Samples for growth were fixed with formaldehyde

(2% final conc.) and counted as described above. During sampling, replicate 10-mL aliquots captured on 0.2- μm Nuclepore membranes were treated as described for B_{12} assimilation. Filtrates collected from these samples were frozen for subsequent analysis of dissolved B_{12} by means of bioassay (described below). Replicate samples for intracellular B_{12} concentrations were extracted from washed cells captured on 0.2- μm Nuclepore membranes by homogenizing with a tissue grinder, acidifying to pH 3.5 with 0.1 mol L^{-1} HCl, boiling at 100°C for 1 h, and neutralizing with 0.1 mol L^{-1} NaOH (Carlucci and Bowes 1972b). Extracted samples were immediately frozen (-20°C) and stored for bioassay.

Vitamin B_{12} concentrations—Extra- and intracellular cobalamin concentrations were measured by the bioassay technique using the diatom *T. pseudonana* clone 3H (CCMP1335), a B_{12} auxotroph (Carlucci and Bowes 1972a). Seawater for bioassays was rendered vitamin free as described above. Axenic inocula of *T. pseudonana* were incubated in Erlenmeyer flasks on an orbital shaker under a fluorescent light bank ($\sim 85 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) for 10 d at 18°C in modified *f/2* assay medium (only B_{12} omitted) that contained subsamples from field or in vitro experiments. Cell yields were routinely estimated for samples and external B_{12} standards (0.1–3.0 ng L^{-1}) by in vivo fluorescence using a Turner Designs fluorometer. In vivo fluorescence results were translated into cell and biomass yields by regressing against contemporaneous measurements of microscopic cell counts ($r^2 = 0.94$) and extracted Chl *a* ($r^2 = 0.91$) in several bioassay runs. Assay runs generating standard curves with coefficients of determination (r^2) < 0.90 were discarded and samples were rerun. Each sample was routinely run in duplicate flasks and ranges among replicates varied from 0% to 20% of the mean, but averaged $\pm 5\%$.

Statistics—All plotting and statistical analyses were performed using SigmaPlot and SigmaStat (Systat Software). For box and whisker plots, boxes enclose the 25th (lower hinge) to 75th percentiles (upper hinge) of all observations, solid lines within boxes denote medians, and whiskers represent the 5th and 95th percentiles. Central tendencies for results with wide ranges and skewed distributions are presented as geometric means, which reduce outlier biases, and as the “H-spread,” which is the data range between lower and upper hinges in box plots or the median 50% of all observations.

Results and discussion

Vitamin B_{12} assimilation in sea ice communities—Several lines of evidence demonstrate that association of $^{57}\text{Co-B}_{12}$ tracer with particles retained on Nuclepore membranes results from active biological uptake rather than passive sorption of tracer to particles. First, passively sorbed solutes tend to equilibrate almost instantaneously and can only increase through time if new sorption sites or particle-reactive solutes are introduced. In the control sample, radioactivity in particulates steadily increased throughout

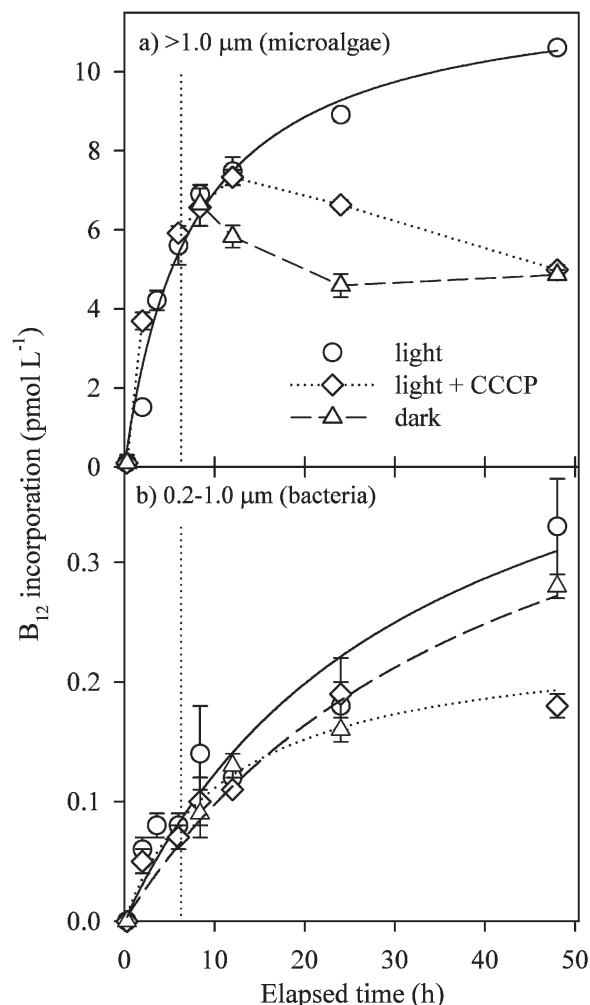


Fig. 1. In vitro time course for vitamin B_{12} incorporation determined by following $^{57}\text{Co-B}_{12}$ activity in microalgal ($>1.0 \mu\text{m}$) and bacterial-sized ($0.2\text{--}1.0 \mu\text{m}$) particles in core segments from congelation ice collected from McMurdo Sound, Antarctica on 28 December. Initially, triplicate samples were incubated under the same light conditions and after 6-h time point one sample was placed in the dark while another received a metabolic poison, the protonophore CCCP ($10^{-5} \text{ mol L}^{-1}$ final conc.; see Materials and methods).

the time course for the 0.2–1.0- and $>1.0\text{-}\mu\text{m}$ size fractions (Fig. 1). In the replicate sample placed in total darkness after 6 h, however, radioactivity in the $>1.0\text{-}\mu\text{m}$ particulate fraction actually fell by 16% over the remaining time course, while unaffected in the 0.2–1.0- μm fraction. Dosing another replicate sample with CCCP, a protonophore, after 6 h caused a 25% loss of radioactivity in the $>1.0\text{-}\mu\text{m}$ fraction and suppressed increases in the 0.2–1.0- μm fraction, although responses were somewhat delayed. We speculate that lagged responses in dark and inhibited treatments result from energy stored in excess photosynthates continuing to drive active transport in the dark and possibly slow incorporation of CCCP into membranes at low temperatures, respectively. Loss of particulate $^{57}\text{Co-B}_{12}$ tracer in dark and inhibited treatments may represent

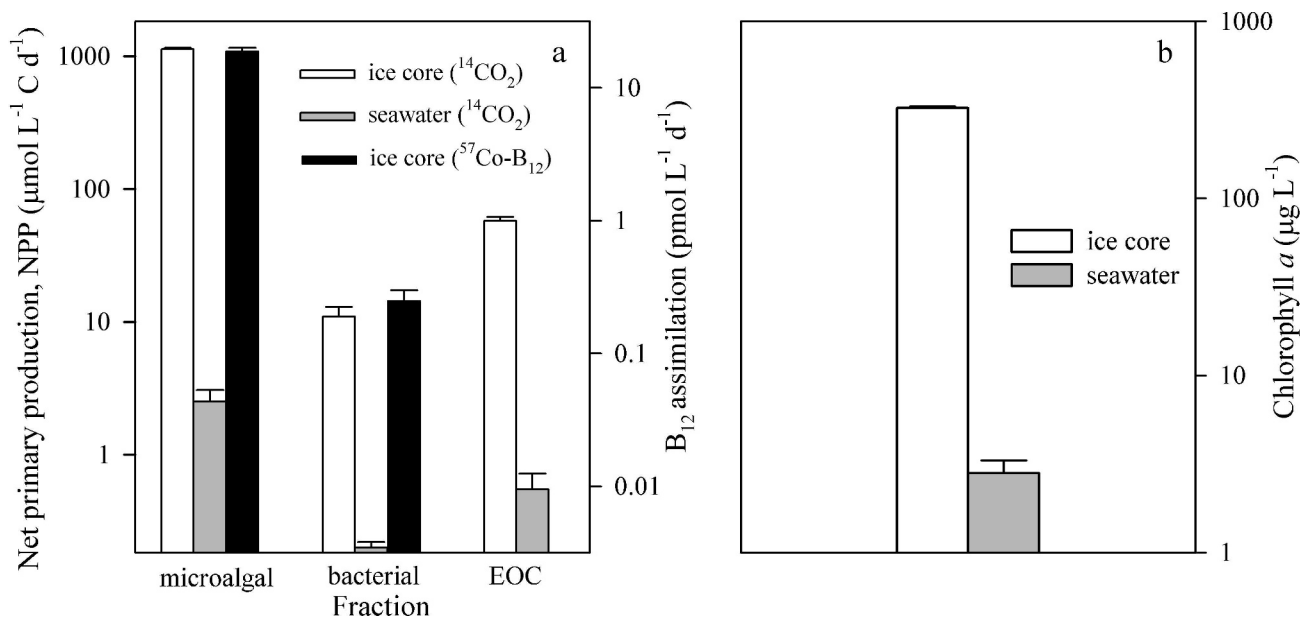


Fig. 2. (a) Contemporaneous rates of net primary production and vitamin B₁₂ assimilation in the >1.0- μm (microalgal) and 0.2–1.0- μm (bacterial) size fractions, and production of extracellular organic carbon (EOC) in sea ice cores from Fig. 1 and in seawater collected on 27 December 1 m below ice–water interface at the coring site. (b) Concentrations of Chl *a* in the ice core meltwater and underlying seawater. Error bars for ice cores represent 1 SD from mean of duplicate analyses of duplicate cores incubated in situ. Error bars for vitamin B₁₂ assimilation are 1 SE of regression slopes used for rate computation.

passive efflux from intact cells as previously observed for axenic cultures of *T. pseudonana* (Herold 1984). Alternatively, mortality-related release of intracellular pools, whether natural or CCCP-induced, may have outpaced assimilation rates in these treatments. Vitamin B₁₂ assimilated by the bacterial size fraction does not appear to be light dependent, but clearly requires energized membranes (i.e., proton gradient). We interpret these results as convincing evidence that incorporation of dissolved ⁵⁷Co-B₁₂ into particles results from energy-dependent biological assimilation, primarily fueled by photosynthesis and dependent upon energized cytoplasmic membranes and active transport across them.

Assimilation of the ⁵⁷Co-B₁₂ tracer was clearly dominated by organisms >1.0 μm (primarily diatoms) throughout the time course, consuming dissolved cobalamin at a rate of 0.8 pmol L⁻¹ h⁻¹ B₁₂ in the first 8 h (Fig. 1). Whether the apparent diminution of assimilation rates after 8 h resulted from dilution of the ⁵⁷Co-B₁₂ tracer by newly released nonradioactive B₁₂ or from limitation by another nutrient cannot be determined with available data. Assimilation by the bacterial size fraction (0.2–1.0 μm) was comparatively slow, only 0.01 pmol L⁻¹ h⁻¹ B₁₂, and accounted for less than 3% of total B₁₂ assimilation within this sea ice sample. Similarly, NPP in sea ice was dominated by organisms larger than 1.0 μm , accounting for $\geq 94\%$ of total daily CO₂ fixed (Fig. 2a). We note that this size fraction may have fixed closer to 99% of the total, because about 5% of total daily ¹⁴CO₂ fixed appeared in the EO¹⁴C pool, but its source could not be determined. The subordinate role of bacteria in these processes is consistent with a related sea ice study in which carbon biomass and

production by bacteria were only 1% and 9% those of microalgae, respectively (Kottmeier et al. 1987). For comparison, Bertrand et al. (2007) observed that their bacterial size fraction (0.2–2.0 μm) in diatom-dominated plankton communities of the open Ross Sea accounted for $\sim 30\%$ of vitamin B₁₂ assimilation. This higher proportion may simply reflect differences in relative cell sizes, biomasses, and productivity of microalgae and bacteria in planktonic vs. sea ice communities. Nonetheless, competition for dissolved vitamin B₁₂ from organisms in the 0.2–1.0- μm size fraction appeared to be minimal within congelation ice.

Microalgal biomass in sea ice overlying McMurdo Sound is typically overwhelmingly attributable to large diatom species (Palmisano and Garrison 1993). In studies from this region, pennate diatoms, such as *Amphipora kufferathii*, *Berkeleya* sp., *Fragilaria* sp., *Nitzschia stellata*, *Nitzschia closterium*, and *Pleurosigma* sp., as well as the centric diatoms, *Chaetoceros* sp. and *Thalassiosira antarctica*, account for the majority (>90%) of microalgal assemblages (Grossi 1985; Sullivan et al. 1985; Grossi et al. 1987). Our data suggest that relatively large photoautotrophs such as these not only drove primary production within congelation ice, but were also the primary consumers of dissolved B₁₂.

Through most of austral summer, sea ice near our study site (Cape Armitage) transmits <1% *I*₀ in downwelling irradiances to the water column, which supports almost no detectable primary production or phytoplankton biomass until some time in December (Sullivan et al. 1982; Grossi et al. 1987). Typical of McMurdo Sound's fast ice areas, sea ice communities dominated total system productivity

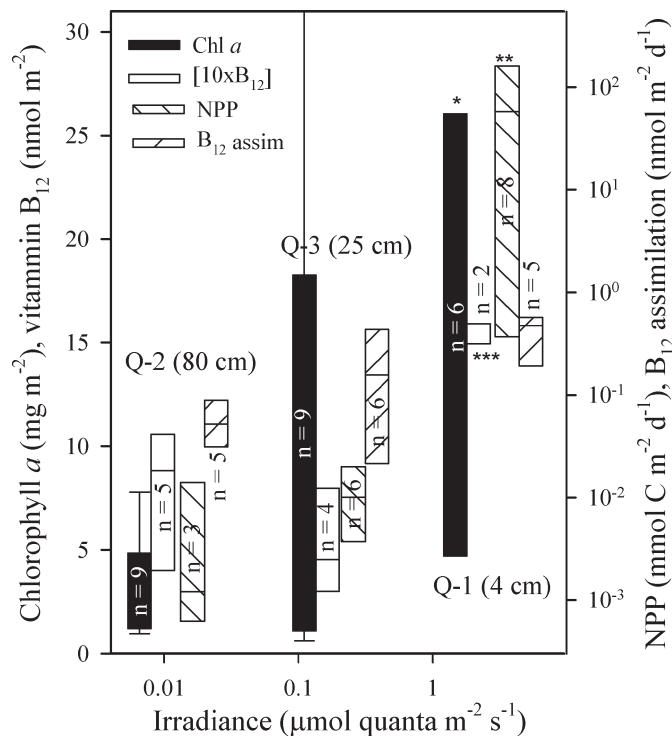


Fig. 3. Box plot of concentrations of Chl *a* and vitamin B₁₂ and rates of primary production and vitamin B₁₂ assimilation compared with average downwelling irradiance received by the bottom 5 cm of sea ice cores. Cores were obtained from three quadrats, each of which was maintained with distinct snow covers for 40 d. Analyses were made 19–22 and 38–40 d after experimental quadrats were established on 26 October. Significant differences determined by Kruskal–Wallis one-way ANOVA on ranks; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

during our 27–28 December sampling, Chl *a* biomass and NPP in the upper water column only amounting to 0.9% and 0.3%, respectively of comparable volumes of meltwater (congelation ice + interstitial water) (Fig. 2). Like sea ice, primary production in the water column was dominated (>92%) by the >1.0- μm size fraction (Fig. 2a). Interestingly, production rates of EO¹⁴C in both epontic and planktonic systems exceeded ¹⁴C assimilation in the 0.2–1.0- μm size fraction by 2.8–5.2-fold, implying slow bacterial recycling of this dissolved organic pool.

NPP in congelation ice is usually confined to the bottom 5 cm (Kottmeier and Sullivan 1988), whereas NPP in underlying plankton is likely to be broader. In this study, only a single water depth (1 m below interface) was sampled to compare with ice communities and the photic zone's extent is unknown. Therefore, we can only estimate integrated areal carbon fluxes in the underlying plankton. Through mid-December, under-ice visibilities observed by scuba were 100 to 300 m horizontally until a coastal *Phaeocystis pouchetii* bloom was advected to our site, reducing visibility to ~1 m, a typical seasonal cycle for McMurdo Sound (Arrigo et al. 1998). Thus NPP was likely confined to the upper 2–3 m of the water column at most and integrated phytoplankton production could only

amount to about 10% that of epontic microalgae. However, this imported bloom did appreciably enrich phytoplankton biomass over that observed earlier in the season (presented below), albeit unproductive. Because of the subordinate role of phytoplankton production during most of our study period, our studies of vitamin B₁₂ cycling were confined to the overlying congelation ice community.

Sea ice productivity and B₁₂ assimilation—Snow cover exerts strong control over the downwelling irradiance reaching sea ice communities and consequently on microbial biomass and productivity supported over a growing season (Grossi et al. 1987; Kottmeier et al. 1987). A mere 5 cm of snow atop annual sea ice can reduce irradiances at the ice–water interface to ~0.4% I_0 , below the widely accepted threshold (1% I_0) that permits NPP (Palmisano and Sullivan 1985). In the present study, manipulation of snow cover between 4 and 80 cm in late October induced large variations in Chl *a* biomasses (0.6–36 mg m⁻² Chl *a*) and productivities (0.0001–250 mmol m⁻² d⁻¹ C) by early December among three experimental quadrats (Fig. 3). Sea ice in these neighboring quadrats essentially started with the same communities and diverged quantitatively (and perhaps qualitatively) over the experimental period, presumably in response to varying degrees of light limitation. The darkest quadrat (Q-2 = 80 cm snow) exhibited the lowest Chl *a* biomass and productivity of the three quadrats. Variances in Chl *a* concentrations within Q-1 (4 cm snow) and Q-3 (25 cm snow) were significantly higher than in Q-2. These differences probably reflect heterogeneous community growth over the study period in response to more intense and variable light fields as well as the low sample numbers. Wide ranges in dissolved cobalamin concentrations (6–32 pmol L⁻¹ B₁₂) and assimilation rates (5.5–480 pmol m⁻² d⁻¹ B₁₂) also developed over this time period. Cobalamin concentrations were similar in the two least-illuminated quadrats and were significantly higher in the high-light quadrat (Q-1). In contrast, B₁₂ assimilation rates and microalgal biomass and growth appeared to vary proportionately with light availability (Fig. 3). We cannot exclude the possibility that measured rates of primary production and B₁₂ assimilation were artificially stimulated by unintentional contamination with iron and other micronutrients because cores were sampled with a stainless steel auger and modern trace-metal-clean techniques were not used. However, we don't expect that this potential artifact would bias the relationship between primary production and B₁₂ assimilation. Moreover, differential Chl *a* accumulations and rate measurements all clearly argue for the primacy of light as the limiting resource among these three quadrats.

Variations in dissolved vitamin B₁₂ concentrations within sea ice varied strongly ($r = 0.78$, $p < 0.001$, $n = 16$) with microalgal productivity (Fig. 4a). Vitamin B₁₂ concentrations were also positively correlated with algal biomass (Chl *a*) ($r = 0.57$, $p < 0.02$, $n = 16$) (Fig. 4a). Likewise, rates of B₁₂ assimilation appeared to be positively related to the size of dissolved cobalamin pools ($r = 0.76$, $p < 0.001$) within meltwaters (Fig. 4b). Covariations between B₁₂ assimilation rates and either Chl *a* concentrations ($r =$

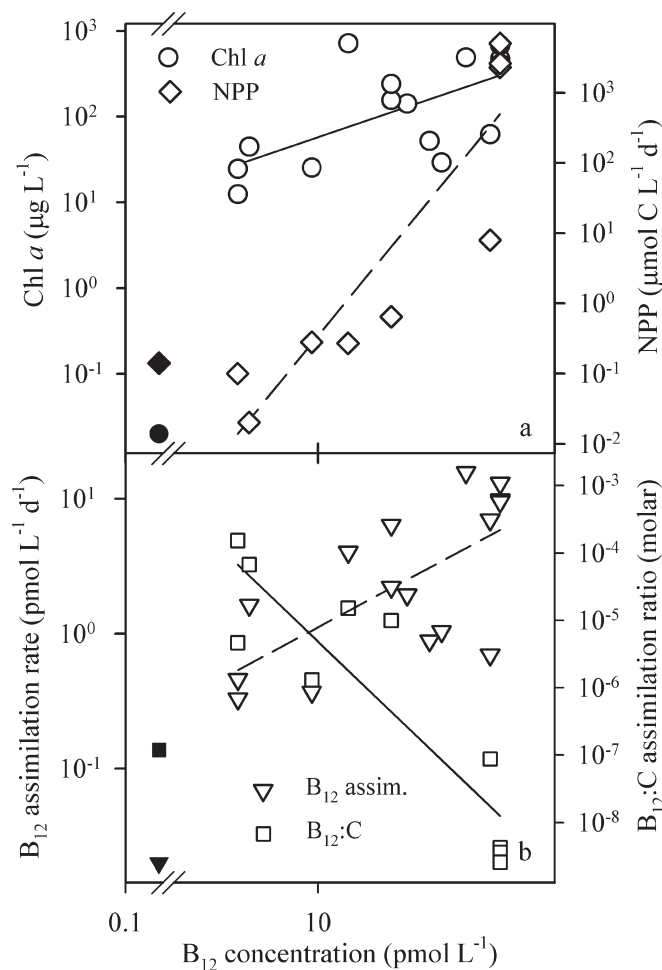


Fig. 4. Variations in (a) Chl *a* concentrations, net primary production, (b) vitamin B₁₂ assimilation, and B₁₂-to-carbon assimilation ratios (*Q*) over range of cobalamin concentrations observed within the bottom 5 cm of ice cores from quadrats 1–3 (replot of Fig. 3). Water column samples collected just below Q-3 midway through the field experiment (17 Nov) are presented for comparison (closed symbols).

0.78, $p < 0.001$) or NPP ($r = 0.77$, $p < 0.01$) were at least as strong as between the two photoautotrophic variables ($r = 0.70$, $p < 0.005$ for NPP vs. Chl *a*, $n = 24$) themselves (not presented). Turnover rates of dissolved B₁₂ pools (assimilation rate:concentration) varied from 0.02 to 0.60 d⁻¹ and were positively correlated with algal biomass ($r = 0.78$, $p < 0.001$). The B₁₂:C molar ratio (B₁₂ assimilation:carbon production), which equates to Droop's (1973) cell nutrient quota, *Q*, decreased as a function of dissolved B₁₂ concentration (Fig. 4b). These ratios varied widely from 2.6 to 150,000 nmol B₁₂ (mol C)⁻¹, yielding a mean value of 500. Meaning that on average for every mole of B₁₂ assimilated, about 2 million moles of inorganic carbon were photosynthetically transformed into biomass. Widely varying cell quotas are commonly reported for micronutrients (e.g., Carlucci and Bowes 1972b; Sunda and Huntsman 1995; Ho et al. 2003) and in the present case may reflect variations in micro- and macronutrient

availability, growth rates, and community composition over the course of this field experiment.

Interpretation of covariations among microalgal and vitamin B₁₂ parameters is complex. Clearly, assimilation of dissolved B₁₂ is responsive to rates of primary production and algal biomass. If these microalgae are, in fact, auxotrophic for cobalamin, then their growth is dependent on its availability in extracellular pools. Sources of this dissolved vitamin are, however, poorly known. Either an external reservoir exists or in situ production must be responsible for building dissolved pools and meeting algal demand through the growing season. Several studies have demonstrated that waters underlying sea ice are indeed the primary source of inorganic macronutrients to epontic communities and their transport is largely controlled by diffusion, tidal pumping, and weak advection (Smetacek et al. 1992; Thomas and Dieckman 2002). Inorganic macronutrient concentrations in this water column are typically high, e.g., ~ 10 – 35 μmol L⁻¹ NO₃⁻ and ~ 0.7 – 2.5 μmol L⁻¹ PO₄³⁻ (Arrigo et al. 1999). Although NPP in the seawater sample collected below our experimental site was not the absolute lowest measured during this 40-d field experiment, concentrations of Chl *a* and B₁₂ as well as B₁₂ assimilation were barely detectable on 17 November (Fig. 4a,b). Furthermore, the typical underlying platelet ice layer described in Arrigo et al. (1995) was absent from our site during this season. Therefore, uptake of dissolved nutrients by platelet ice and phytoplankton communities appeared to have little effect on nutrient supply to congelation ice communities from waters below.

Is it possible that the water column is a source for vitamin B₁₂? Our seawater sample yielded a dissolved B₁₂ concentration of only ~ 0.5 pmol L⁻¹. In stark contrast, dissolved pools of cobalamin in ice core meltwaters averaged ~ 20 pmol L⁻¹ B₁₂. One uncertainty in this analysis is the accuracy of dissolved cobalamin measurements by the classic bioassay method. Using the recently introduced high-performance liquid chromatography (HPLC) method of Okbamichael and Sañudo-Wilhelmy (2004), B₁₂ concentrations in the eastern North Atlantic were reported to vary between 0.1 and 2.5 pmol L⁻¹ (Panzeca et al. in press) and from 0.5 to 3.5 pmol L⁻¹ in open surface waters west of the Antarctic Peninsula (Panzeca unpubl.), very similar to our concentrations and other bioassay-based measurements in the Indian sector of the Southern Ocean, ≤ 2.4 pmol L⁻¹ B₁₂ (Carlucci and Cuhel 1977). HPLC-based B₁₂ measurements in sea ice are unavailable. However, HPLC-measured B₁₂ is reported to vary between 5 and 87 pmol L⁻¹ in productive, mid-latitude semienclosed coastal waters (Sañudo-Wilhelmy et al. 2006), not unlike our ice core estimates. We conclude that although bioassay determinations are more time-consuming and less precise, they are not necessarily less accurate than HPLC determinations. We also note that actual B₁₂ concentrations in interstitial waters may be at least an order of magnitude higher than reported, because ice crystals occupy most of the sea ice volume, accounting for 66% to 98% of congelation ice's volume (Garrison et al. 2005). Therefore, dissolved cobalamin concentrations may have actually varied from 20 to 1,600 pmol L⁻¹ B₁₂ in the

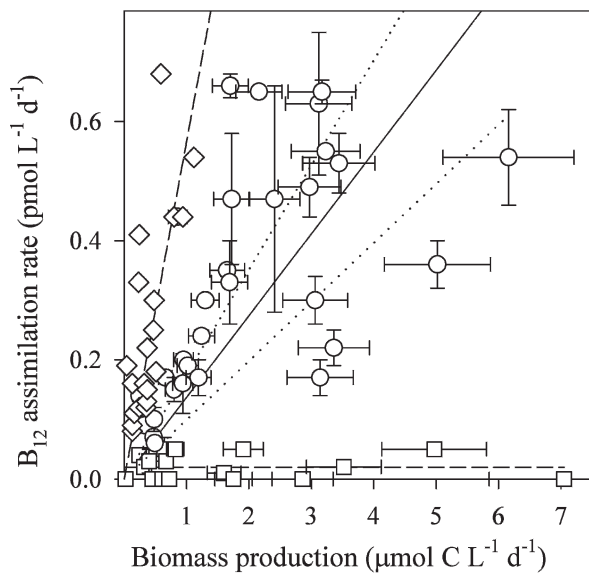


Fig. 5. Comparison of vitamin B₁₂ assimilation rates and bacterial biomass production rates for 78 clones isolated from the sea ice and water column at the study site and incubated for 10 d at 0°C ± 1.0. Means ± 1 SD presented for duplicate ⁵⁷Co-B₁₂ analyses and cell biomass estimations determined from enumerating multiple microscopic fields (10) for abundance and 200 individuals for size (see Materials and methods). Regression (solid line) and 99% confidence limits (dotted lines) include all open circles and diamonds (*n* = 60). Diamonds represent the high-B₁₂ quota subset (*n* = 21). Squares represent isolates exhibiting little or no cobalamin assimilation (*n* = 18).

interstitial waters during our study. Thus, sea ice communities are more likely to be sources of dissolved vitamin B₁₂ to underlying water rather than vice versa, especially when sea ice deteriorates in late summer. In fact, Sedwick et al. (2000) reported that melting sea ice from McMurdo Sound appears to be a rich source of accumulated Fe and Mn to surface waters of the coastal Ross Sea in summer, so B₁₂ release to planktonic systems seems equally plausible.

It appears unlikely that B₁₂ can be supplied from below at sufficient rates over the growing season to amass observed dissolved inventories and support computed turnover rates. Hence it must be synthesized and cycled internally within epontic communities. Observations suggest an interdependent relationship in which heterotrophic bacteria, either directly or indirectly, receive organic substrates from algae to support growth (Grossi et al. 1987; Kottmeier et al. 1987). In turn, members of the bacterial community synthesize cobalamin, releasing it to surrounding brine, which then supports photoautotrophic growth of auxotrophs (Parker 1977; Croft et al. 2005). Release of organic substrates and vitamin B₁₂ to the environment may result from exudation, viral lysis, autolysis, or grazing-related processes. Ultimately, productivity and B₁₂ cycling within these epontic communities must be controlled by availability of light, macronutrients, and micronutrients, such as iron and cobalt.

Vitamin B₁₂ assimilation among sea ice bacterial isolates—

Although prokaryotes are thought to be the only natural source of cobalamin, only about a third of the ~250 bacterial and archaeal taxa sequenced by the year 2000 possess the genes encoding for the >30 enzymes in the two known cobalamin synthetic pathways (Raux et al. 2000). Presumably the remaining 2/3 of prokaryotes acquire extracellular cobalamin or have evolved B₁₂-independent pathways (Rodionov et al. 2003). Experimental and genetic evidence demonstrate that some prokaryotic taxa possess salvage pathways to assimilate extracellular B₁₂ or its precursors from their environment (Burkholder and Lewis 1968; Woodson and Escalante-Semerena 2004). Our first field experiment demonstrated that organisms in the 0.2–1.0-μm size class assimilated cobalamin roughly in proportion to their biomass and productivity (Fig. 2 and Kottmeier et al. 1987). Apparently both B₁₂-producing and -consuming bacteria coexist within sea ice.

The prevalence of B₁₂ salvage capabilities among bacteria from our study site was examined among our 78 psychrophilic bacterial isolates by screening for assimilation of dissolved ⁵⁷Co-B₁₂ (6.6 pmol L⁻¹ B₁₂). Cell yields at

Table 1. Bacterial cell quotas (*Q*) for vitamin B₁₂ and cobalt on the basis of assimilation and production experiments performed on clones isolated from Antarctic sea ice and underlying seawater. Values represent rates of assimilation or production divided by rates of bacterial C biomass production for each responsive clone. Ranges, geometric means, sample size (no. of measurements), and prevalence of assimilation or production among clones presented.

	Range*	Mean*	Sample size	Clonal prevalence
				Positive : negative
Assimilation (nmol : mol)				
B ₁₂ : C	73–124,000	550	60	60 : 18
Co : C	4–70,000	880	36†	11 : 0
B ₁₂ production (nmol : mol)				
Intracellular B ₁₂ : C	0.6–6,800	34	16	9 : 2
Extracellular B ₁₂ : C	220	NA‡	11	1 : 6

* Ranges and geometric means include only responsive clones, i.e., nondetects excluded.

† Sample size does not necessarily correspond to prevalence for two reasons, either because biomass production values for some clones were unreliable or because individual clones were screened under multiple organic substrate concentrations.

‡ Not applicable, only single clone produced detectable extracellular vitamin B₁₂.

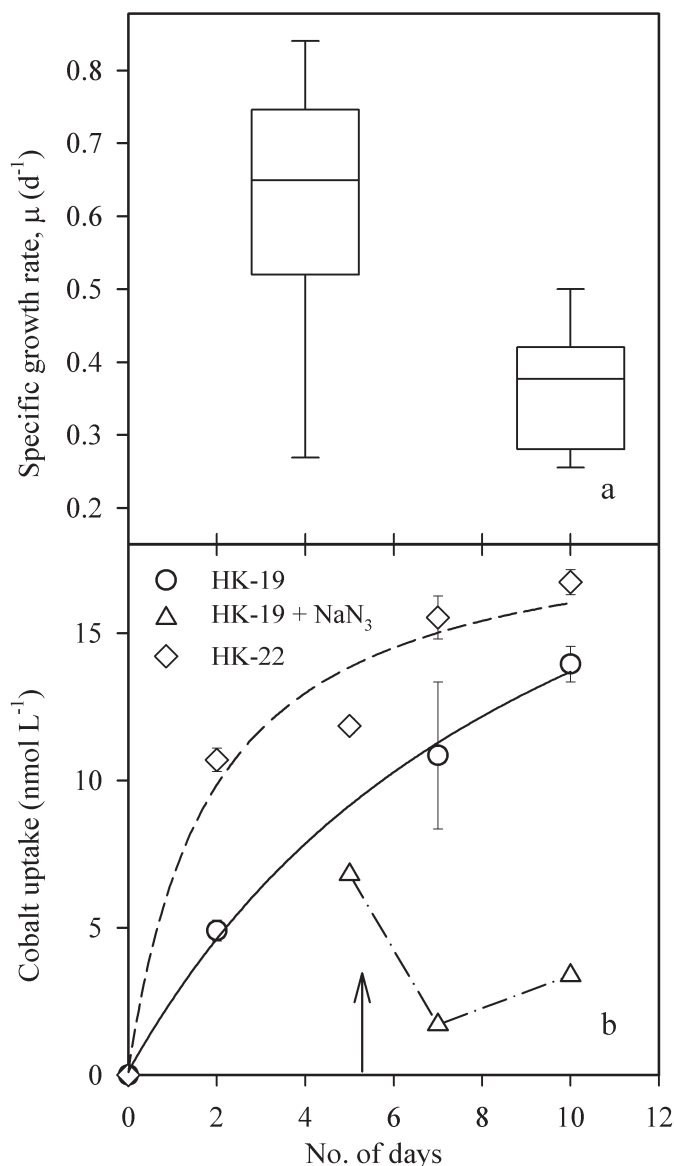


Fig. 6. Growth rates (a) of 18 Antarctic bacterial clones computed from 0–4 and 0–10 d during the vitamin B₁₂ assimilation experiment presented in Fig. 5 and (b) cobalt assimilation (⁵⁷Co uptake) time courses by two of these Antarctic bacterial clones. Seawater medium and incubation conditions in both experiments were similar. Sodium azide (NaN₃) (40 mmol L⁻¹ final conc.) was added to a replicate culture of clone HK-19 on day 5 (indicated by arrow). Means \pm 1 SD presented for duplicate Co uptake measurements.

the end of these 10-d, low-nutrient, low-temperature incubations varied from 0.02×10^6 to 3.9×10^6 cells mL⁻¹ and growth rates calculated from them varied between 0.01 and 0.84 d⁻¹ (mean = 0.39 d⁻¹). Among most isolates, rates of B₁₂ assimilation covaried with cell biomass production (Fig. 5). For 18 randomly selected isolates, cell production was also measured at 4 d. In all cases, specific growth rates calculated at 4 d were significantly higher ($p < 0.001$) than those at 10 d,

indicating that growth was slowing during the 4–10-d period, possibly from substrate exhaustion (Fig. 6a). Therefore, rates of biomass production and cobalamin assimilation reported here are conservative estimates.

Among responsive members of our bacterial culture collection, B₁₂:C ratios (Q) computed from B₁₂ assimilation and carbon biomass production rates varied widely; 73–124,000 nmol B₁₂ (mol C)⁻¹ (Table 1). Comparing B₁₂ assimilation and carbon biomass production in Fig. 5 suggests three levels of response to dissolved B₁₂ within our culture collection: (1) a no-uptake group (squares; $n = 18$), (2) a median group (circles; $n = 39$), and (3) a high-B₁₂ assimilation group (diamonds; $n = 21$). Even though grown simultaneously in the same medium, a common B₁₂:C ratio is not apparent among these clones. However, the H-spread (median 50% of all observations) was relatively narrow, ~ 200 –700 nmol B₁₂ (mol C)⁻¹ and the mean was 550. Whether these isolates actually had distinct cobalamin cell quotas or observed variance is a product of varying uptake efficiencies or experimental imprecision cannot be determined from existing data. It is plausible that the 18 isolates showing little or no detectable incorporation of ⁵⁷Co-B₁₂ (Fig. 5), some of which exhibited relatively high growth yields ($>1.0 \times 10^6$ mL⁻¹), lack cobalamin transporters and were cobalamin producers (B₁₂ prototrophs).

At the very least, these experiments inform about prevalence of salvage pathways for B₁₂ among members of this culture collection. More than 75% of our clones exhibited the ability to assimilate extracellular B₁₂. However, these 60 B₁₂-assimilating clones are just as likely to be B₁₂ prototrophs as auxotrophs. From an energetics perspective, cells capable of utilizing extracellular pools of this costly coenzyme or its precursor and downregulating de novo synthetic pathways are at a competitive advantage over those that do not (Woodson and Escalante-Semerena 2004). Within a Rhône estuary culture collection ($n = 232$), for example, only 3.5%, 10%, and 14% of clones were truly auxotrophic for cobalamin, biotin, and thiamin, respectively, i.e., no growth without extracellular B vitamin (Berland et al. 1976). In Burkholder and Lewis' (1968) collection ($n = 665$) of B-vitamin auxotrophs isolated from temperate coastal waters and sediments, $>20\%$ of clones required extracellular cobalamin, either alone or combined with other B vitamins. We conclude that salvage pathways for B₁₂ assimilation were commonly expressed among bacteria at our site and that B₁₂ auxotrophy among marine prokaryotes is not rare.

Constraints on analyses of clone collections such as ours and those cited above are that cultures are selective by nature, they grossly undersample natural diversity, and a single ecotype may be represented by multiple clones within a collection. Beyond recognition, little can be done about the first two concerns with existing data. Characterization of our clones predated modern ribotyping methods, so mostly phenotypic traits, including B₁₂ assimilation, were catalogued. Comparisons of the eight observed attributes among the 78 clones revealed four doublets, suggesting that at least four ecotypes are represented twice in our collection. So clearly, our representation of polar bacteria

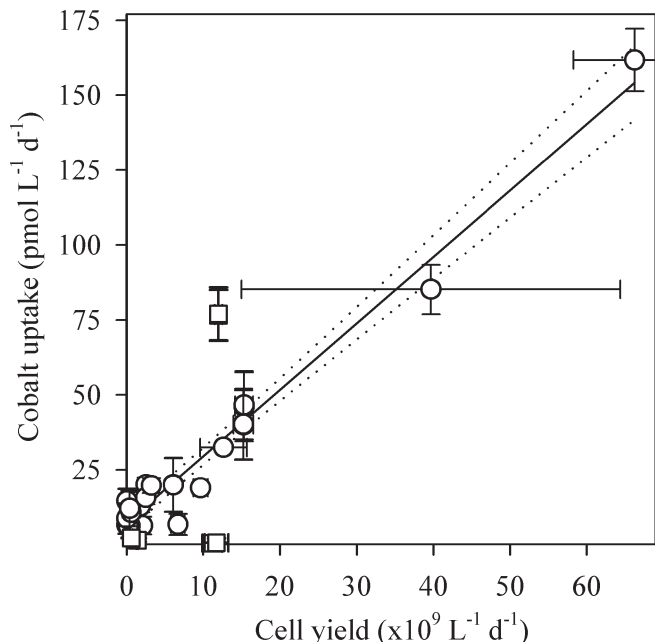


Fig. 7. Variations in cobalt uptake and cell yields for 11 Antarctic bacterial isolates cultivated for 10 d at $0^{\circ}\text{C} \pm 1.0$ with ^{57}Co (50 nmol L^{-1} total Co concentration) and several organic substrate concentrations. Means ± 1 SD presented for duplicate ^{57}Co uptake analyses and multiple microscopic fields (10) enumerated per filter for cell yields. Regression (solid line) and 99% confidence limits (dotted lines) include all open circles; $\text{pmol Co} = 7.4 + 2.2 \times 10^9 \text{ cells}$, $r^2 = 0.97$, $n = 30$.

is confined to cultivable taxa and our assessment of prevalence is biased by clonal redundancy.

Cobalt uptake and vitamin B_{12} production among bacterial isolates—The cobalamin molecule contains an atom of cobalt and thus its synthesis depends upon acquisition of extracellular Co (Warren et al. 2002). Therefore, 11 randomly selected clones exhibiting little or no B_{12} assimilation (Fig. 5) as well as one B_{12} -assimilating clone were screened for cobalt uptake in seawater medium devoid of cobalamin. To determine whether these clones actually used assimilated Co to produce B_{12} , cobalamin production was assessed in filtrates (extracellular pool) and in cell lysates (intracellular pools). As seen from the two examples in Fig. 6b, incorporation of ^{57}Co into particles continued throughout these 10-d, low-temperature growth experiments. Addition of the respiratory poison NaN_3 to a replicate sample on day 5 demonstrated that accumulation of ^{57}Co activity in particles is dependent on actively metabolizing and growing cells (Fig. 6b). Similar to growth in the previous experiment (Fig. 6a), uptake rates slowed in the latter half of the incubation. Although rates calculated from 10-d end points are conservative estimates of true rates, estimates of cell quotas ($\text{Co} : \text{C}$ or $B_{12} : \text{C}$) should be reasonably accurate because first-order uptake and growth rate constants (d^{-1}) both declined by 40–47% in the latter half of the incubations.

In general, cobalt assimilation was proportional to cell production in experiments run under varying organic substrate conditions and saturating metals concentrations,

e.g., Co and Zn were 50 and 80 nmol L^{-1} , respectively (Fig. 7). Although Co assimilation was observed for all 11 clones, rates observed for three trials were off the trend line (Fig. 7). From remaining data, an average cellular Co quota of $2.2 \times 10^{-21} \pm 0.2 \text{ mol cell}^{-1}$ or $\sim 1,300 \pm 44$ atoms of Co cell^{-1} is derived from the regression slope. Carbon-specific quotas, however, were more variable, with the H-spread lying between 250 and 2,500 $\text{nmol Co (mol C)}^{-1}$ and a mean of 880 (Table 1). Cobalt speciation potentially affects its bioavailability to planktonic organisms and may influence accuracy of calculated rates, but is not explicitly considered in this study. According to Saito et al. (2002), free Co^{2+} should be about 0.1% of total Co and the remainder complexed with organic chelators at the concentrations of EDTA used in our seawater medium. Their study also demonstrated that the cultured cyanobacterium, *Prochlorococcus marinus*, easily accessed both ligand-bound Co and free Co^{2+} ions, which may also be the case for other prokaryotes. Thus, calculating specific activity on the basis of total cobalt and tracer added, irrespective of speciation, is the most conservative assumption.

Berland et al. (1976) reported that 26% of bacterial clones in their collection released vitamin B_{12} into their media, the cause of which was unexplored. In our first Co assimilation experiment, seven clones were screened for production of extracellular cobalamin, which was only detected in the medium of a single clone after 10 d and was produced at a biomass-specific rate of $220 \text{ nmol } B_{12} (\text{mol C})^{-1}$. Given the sparse occurrence of extracellular B_{12} release among our clones, filtrates from additional clones were not screened for extracellular B_{12} . In contrast, production of intracellular cobalamin was detectable in 9 of 11 clones and varied widely from 0.6 to 6,800 $\text{nmol } B_{12} (\text{mol C})^{-1}$ of biomass produced and averaged 34. Interestingly, two clones exhibiting no detectable intracellular B_{12} production grew well under experimental conditions, ranking first and seventh in cell biomass production. We are unable to resolve whether these clones actually lacked cobalamin or it escaped detection by our methods. However, the fact that all these heterotrophic clones assimilated ^{57}Co and that a wide range in $B_{12} : \text{C}$ ratios was apparent suggest that extraction, preservation, and detection efficiencies of intracellular B_{12} varied.

Total cobalamin production ($\text{pmol L}^{-1} \text{ d}^{-1} B_{12}$) was not significantly correlated with bacterial number or biomass produced ($p > 0.25$) by the nine B_{12} -producing clones (not presented). However, Co assimilation and intracellular B_{12} production clearly did covary with one another ($r = 0.88$, $p < 0.001$), albeit nonlinearly (Fig. 8). Interestingly, clones with the lowest $B_{12} : \text{C}$ ratios exhibited higher relative cobalt quotas, i.e., Co excesses. Given that all clones were grown under identical conditions presumably in a medium that was trace-metal-replete ($f/2$), these diverging cell quotas suggest interspecific variations in assimilation and biosynthetic efficiencies and possibly differences in cobalt and cobalamin requirements.

Potential nutrient limitation in epontic communities—Previous field measurements suggest that growth of phytoplankton can be B-vitamin limited at times (Carlucci

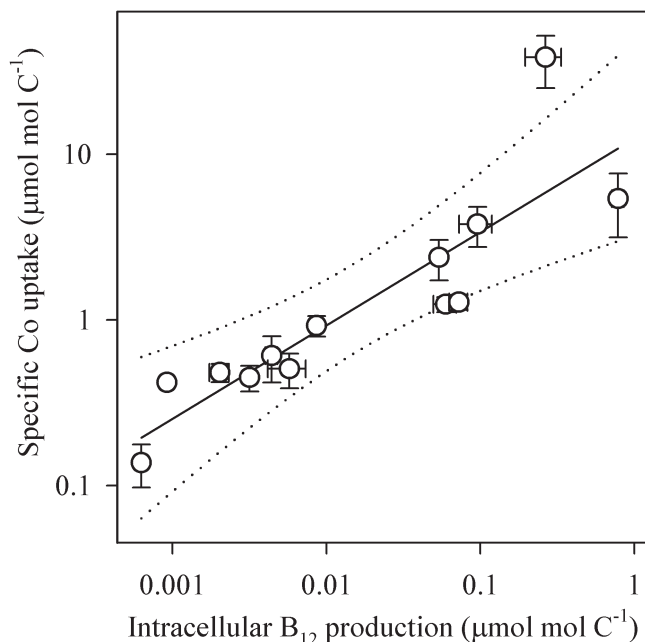


Fig. 8. Relationship between biomass-specific Co uptake and intracellular vitamin B₁₂ production among nine Antarctic bacterial isolates. Means \pm 1 SD presented for duplicate analyses. Regression (solid line) and 99% confidence limits (dotted lines) presented for results from two experiments that included some of the same clones; $\text{Co} = 12.4\text{B}_{12}^{0.56}$, $r^2 = 0.77$, $n = 16$.

and Cuhel 1977; Panzeca et al. 2006; Bertrand et al. 2007). Is the same true for sea ice communities? Maestrini et al. (1986) demonstrated that microalgal growth in Canadian Arctic fast ice was nutrient-limited for part of the season and was far more responsive to trace metal enrichments than addition of any B vitamins. Perhaps metal availability exerts more control than B vitamins in sea ice communities as well. To assess potentials for vitamin B₁₂ and cobalt limitation among epontic microalgae in McMurdo Sound, we first compared estimates of B₁₂ cell quotas from our field communities with those derived from diatom cultures. The H-spread for ratios in field samples fell between 4 and 15,000 nmol B₁₂ (mol C)⁻¹. These ratios significantly exceed those derived from studies of diatom cultures ($p < 0.005$; Mann-Whitney rank sum), which varied from 0.4 to 55 nmol B₁₂ (mol C)⁻¹ and averaged 4.1 (Table 2) for cells grown under a range of vitamin availabilities. However, the H-spread for diatom cultures actually fell within a very narrow range; 2–9 nmol B₁₂ (mol C)⁻¹. Given how widely C:N:P:metals ratios vary in the phytoplankton literature (e.g., Sunda and Huntsman 1995; Ho et al. 2003; Quigg et al. 2003), it is remarkable that most pure culture estimates of diatom B₁₂ quotas only varied by a factor of \sim 5, because methods to derive cell quotas and nutrient status of cultures varied among studies. In our field communities, only ratios from the most productive quadrat, Q-1 (2.6–88 nmol B₁₂ [mol C]⁻¹), were within the same order of magnitude as the range for pure cultures.

Our direct measurements of B₁₂ assimilation rates and dissolved inventories during field experiments yield cobalamin turnover rates lying between 0.02 and 0.60 d⁻¹ with a

mean of 0.20 d⁻¹, suggesting that surplus B₁₂ was available on most dates. If we assume that all measured B₁₂ is assimilated, apply the mean cell quota of 4.1 nmol B₁₂ (mol C)⁻¹ for cultured diatoms to epontic populations, and assume a 50 g C (g Chl *a*)⁻¹ conversion (Grossi et al. 1987), dissolved B₁₂ pools could potentially support as much as 40-fold higher diatom biomass ($\bar{x} = 8.2\text{-fold} \pm 3.3$) in sea ice. The relatively high B₁₂:C ratios observed in Q-2 and Q-3 is probably not indicative of higher vitamin requirements of these communities, but rather is evidence of luxury consumption of B₁₂ as so elegantly demonstrated for *Monochrysis lutheri* by Droop (1973). Although data are unavailable for isolated polar diatom species, published half-saturation growth constants (K_s) for temperate diatoms are 3–200 times lower than dissolved B₁₂ concentrations in sea ice (6–32 pmol L⁻¹) (Table 2). If B₁₂-dependent kinetics of epontic species are similar, then Monod kinetics predict that growth rates should be limited by resources other than B₁₂ availability, because B₁₂ concentrations tended to be in the saturating portion of published growth curves. The relatively low B₁₂:C ratios observed in Q-1 (least light-limited) suggest that sea ice communities may have approached B₁₂ limitation only during the height of production. However, all available results are consistent with the hypothesis that dissolved cobalamin was not growth-limiting to epontic microalgae during most of our observation period.

Maintenance of observed dissolved vitamin inventories and microalgal production requires comparable regeneration rates by microbial loop processes, which equates to B₁₂ releases varying between 0.3 and 16 pmol L⁻¹ d⁻¹ B₁₂ and averaging 2.4 pmol L⁻¹ d⁻¹ B₁₂. Pathways through which cobalamin enter dissolved pools remains the subject of much speculation (Karl 2002; Croft et al. 2005, 2006). Results presented above and by Berland et al. (1976) support the hypothesis that the minority of healthy bacteria actually exude or leak cobalamin to their environment as first proposed by Provasoli (1963). Therefore, mortality and senescence processes (viral lysis, predation, and programmed cell death) must be invoked to explain the majority of extracellular B₁₂ inventories. Unfortunately, in situ rates of cobalamin production and release are not yet known for any aquatic system. Consequently, the only alternative is to extrapolate potential in situ rates from our laboratory studies of B₁₂ production by bacterial isolates and published estimates of bacterial production within sea ice.

To estimate rates of B₁₂ production by bacteria in epontic communities we used intracellular B₁₂:C ratios from our culture collection (Table 1) and maximal bacterial biomass and specific growth rates of 3.7 $\mu\text{mol L}^{-1} \text{C}$ and 0.8 d⁻¹ observed within McMurdo congelation ice during a related study (Kottmeier et al. 1987). Potential rates of community B₁₂ production in sea ice meltwater varied from 0.005 to 50 pmol L⁻¹ d⁻¹ B₁₂ with a mean of 0.3. Whereas mean production was only about 12% of mean demand (2.4 pmol L⁻¹ d⁻¹ B₁₂) observed in our field study, B₁₂:C ratios in 5 of the 11 clones were sufficient to support production rates matching or exceeding the mean microalgal consumption. Considering observed variability in cell

Table 2. Ranges in cell quotas (Q) and Monod growth kinetic parameters for McMurdo sea ice microalgae (>90% diatoms by biomass) and cultured diatoms. Geometric means presented in parentheses.

Organism	$B_{12}:C^*$ (nmol:mol)	$Co:C^*$ (nmol:mol)	K_s for B_{12}^\dagger (pmol L ⁻¹)	Source
Sea ice microalgae	2.6–150,000 (500)‡	ND§	ND	This study
Isolated diatoms				
<i>T. pseudonana</i> 3H	9.2–25 (14)	ND	0.34	This study; Guillard and Ryther (1962)
<i>T. pseudonana</i> (<i>C. nana</i>)	2.4–3.5 (2.9)	ND	0.15–2.1	Carlucci and Silbernagel (1969)
<i>T. pseudonana</i> 66a	5.1–5.3 (5.2)	ND	0.73	Carlucci (1973); Herold (1984)
<i>T. pseudonana</i> 3H (<i>C. nana</i>)	0.6–0.7 (0.65)	ND	0.17–0.26	Swift and Taylor (1974)
<i>S. costatum</i>	0.4–55 (4.1)	ND	ND	Carlucci and Bowes (1972b); Messina and Baker (1982)
<i>S. costatum</i>	4		0.19	Droop (2007)
<i>P. tricornutum</i>	2.6–8.4 (4.4)	ND	ND	Carlucci and Bowes (1972b)
Seven species	4–13 (7.2)	ND	ND	Guillard and Cassie (1963)
Six species¶	ND	2.6–3,700 (100)	ND	Sunda and Huntsman (1995); Ho et al. (2003)

* Where necessary, data were normalized to carbon by using N cell⁻¹ presented in Guillard and Cassie (1963) for particular species and assuming a C:N:P ratio of 65:9:1 for diatoms (Quigg et al. 2003). In cases where cell volume yields (V , μm^3) were reported, carbon content was computed using $\text{pg C} = 0.109V^{0.991}$ (Montagnes et al. 1994).

† Monod half-saturation constant for growth.

‡ Median computed from relative assimilation rates of B_{12} and CO_2 in all field experiments as determined from ^{57}Co - B_{12} and ^{14}C -bicarbonate tracers.

§ Not determined.

|| On the basis of studies with *Chaetoceros* sp., *T. pseudonana* 3H, *Cyclotella nana* (*T. pseudonana*), *T. fluviatilis*, *Skeletonema* sp., *S. costatum*, *Fragilaria* sp.

¶ On the basis of studies with *Thalassiosira oceanica*, *T. pseudonana*, *T. eccentrica*, *T. weissflogii*, *Ditylum brightwelli* and *Nitzschia brevisrostris*. Values from Ho et al. (2003) were derived from cells grown at a single concentration of Co^{2+} and Zn^{2+} (20 pmol L⁻¹ each). Values from Sunda and Huntsman (1995) were selected from incubations conducted at Co^{2+} and Zn^{2+} concentrations mostly closely resembling average southern Ross Sea values, where total Co \cong 25 pmol L⁻¹ and Zn \cong 3.3 nmol L⁻¹ (Fitzwater et al. 2000) and $\text{Co}^{2+} \cong$ total Co $\times 10^{-2.94}$ and $\text{Zn}^{2+} \cong$ total Zn $\times 10^{-2.88}$ (Saito et al. 2002).

quotas for both diatoms and bacteria, and uncertainties associated with actual bacterial community composition, the near agreement between estimates of cobalamin demand and production is surprising. Whether B_{12} production and release by far more diverse bacterial communities within sea ice actually keeps pace with microalgal demands remains to be demonstrated empirically.

The disparity between dissolved B_{12} concentrations in underlying water and congelation ice (0.5 vs. 6–32 pmol L⁻¹ B_{12}) suggests that epontic communities build and conserve cobalamin inventories through the growing season. Ultimately, production of B_{12} within sea ice may be limited by cobalt availability, which is primarily delivered from underlying seawater. Although cobalt limitation was not evident in the open Ross Sea (Bertrand et al. 2007), cobalt can be growth-limiting in other marine settings, e.g., in the Kattegat and Skagerrak, in the tropical North Pacific, and eastern North Atlantic (Granéli and Risinger 1994; Saito et al. 2005; Panzeca et al. 2008). In the southern Ross Sea, measured cobalt concentrations in the upper 100 m are quite low, varying from <5 to 38 pmol L⁻¹ and often exhibit nutrient-like profiles with depletions in the photic zone (Fitzwater et al. 2000). Dissolved cobalt concentrations in sea ice pore waters are unknown. However, upper limits for the beginning of the growing season can be estimated from seawater concentrations and brine concentrating factors, and by assuming that Co behaves conservatively with salinity (Sañudo-Wilhelmy et al. 2002). In the bottom 5 cm of McMurdo sea ice, the

minimum temperature and maximum salinity of brine tend to be about -5°C and 150, respectively (Kottmeier and Sullivan 1988; Thomas and Dieckman 2002). Hence, conservative solutes would be concentrated as much as fivefold and dissolved Co concentrations could be as high as ~ 190 pmol L⁻¹.

Among six diatom species surveyed in the literature, Co cell quotas appear to span a wide range, from 0.5 to 20,000 nmol Co (mol C)⁻¹ depending upon dissolved Co and Zn concentrations (Sunda and Huntsman 1995; Ho et al. 2003). Because Co and Zn can be used interchangeably by some enzymes and may be colimiting under some conditions (Saito et al. 2008), we only used experimental values for cultures exposed to total Co and Zn concentrations most closely resembling southern Ross Sea (12–38 pmol L⁻¹ Co and 0.24–5.7 nmol L⁻¹ Zn; Fitzwater et al. 2000) to estimate cell quotas for Antarctic diatoms. Nonetheless, cell ratios still varied widely, 2.6 to 3,700 nmol Co (mol C)⁻¹ and averaged 100 (Table 2). Using mean to maximum primary production rates (0.8–5.1 mmol L⁻¹ d⁻¹ C) from our field experiments and the mean diatom ratio from Table 2 yields estimates of microalgal cobalt demand in our sea ice samples as high as ~ 500 pmol L⁻¹ d⁻¹ and averages 80 pmol L⁻¹ d⁻¹. At these levels, diatom demand could potentially draw down dissolved cobalt pools initially trapped in brine in 0.4 to 2.4 d and exceed turnover of cobalamin by 4- to 20-fold. Intracellular Co within diatoms alone represents a pool of between 5 and 300 pmol L⁻¹ and averages 35 pmol L⁻¹. A similar exercise with bacterial data is not justified because

Co:C ratios from our culture collection (Table 1) were derived from Co assimilation in Co-replete media, containing at least 250-fold higher Co concentrations than expected in sea ice meltwaters.

In summary, our field studies have demonstrated that inventories and assimilation of vitamin B₁₂ are coupled to microalgal biomass and productivity, all of which appear to be strongly controlled by light availability in sea ice communities. Independent analyses of cobalamin turnover in field samples, comparisons of B₁₂:C cell quotas in field communities and cultured diatoms, and published growth kinetics data all strongly suggest that surplus vitamin B₁₂ was available to microalgae during most of our sampling campaign. Relative concentrations of B₁₂ in ice meltwater and underlying seawater illustrate that inventories of extra- and intracellular B₁₂ must build up through the growing season and recycle internally. Our study of cell-specific cobalamin production in a limited number of bacterial isolates and published *in situ* biomass production rates yielded estimates of B₁₂ production that were not vastly different from observed microalgal assimilation within sea ice. We cannot exclude the possibility that epontic communities were vitamin B₁₂-limited earlier in the growing season or under the highest light conditions. Nonetheless, our computations suggest that B₁₂ supply balanced or exceeded demand during the height of the growing season.

In the sea ice ecosystem, diffusive influx from underlying water appears to be the most plausible external source of cobalt. Considering that reported Co concentrations are usually <50 pmol L⁻¹ for the open Ross Sea (Fitzwater et al. 2000; Bertrand et al. 2007), passive fluxes into interstitial waters of sea ice must be very slow. Our estimates of cellular demands for cobalt, primarily by diatoms, suggest that dissolved cobalt trapped in brine channels during ice formation would be rapidly depleted as the growing season commenced. Concentrations in interstitial waters may increase through time as a consequence of continuous influx driven by biological drawdown of dissolved pools, and effective retention and recycling by active microbial communities through the growing season. Our results demonstrate that both vitamin B₁₂ and Co are actively cycled within this ecosystem, even at -1.9–0°C. Clearly, our reductionist approach grossly oversimplifies the physical and ecological complexity of natural sea ice communities by not accounting for ice structure, brine concentrations, other potential limiting nutrients (N, P, Si, Fe), nondiffusive transport mechanisms, and the potential diversity of responses within natural microbial communities. This and previous field studies have demonstrated the primacy of light limitation on epontic community production. However, considering the rarity of both micronutrients and chemical instability of free vitamin B₁₂ (Vandamme 1989), the potential for colimitation by either B₁₂ or Co can not be dismissed. Growth limitation by these micronutrients, *in situ* cobalamin and Co cycling, prevalence of B₁₂ auxotrophy, and absolute B₁₂ requirements among marine microalgae and prokaryotes are all fertile areas for future research.

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