Limnol. Oceanogr., 58(4), 2013, 1431–1450

doi:10.4319/lo.2013.58.4.1431

Methionine synthase interreplacement in diatom cultures and communities: Implications for the persistence of B_{12} use by eukaryotic phytoplankton

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Abstract

Three proteins related to vitamin B_{12} metabolism in diatoms were quantified via selected reaction monitoring mass spectrometry: B_{12}-dependent and B_{12}-independent methionine synthase (MetH, MetE) and a B_{12} acquisition protein (CBA1). B_{12}-mediated interreplacement of MetE and MetH metalloenzymes was observed in *Phaeodactylum tricornutum* where MetH abundance was highest (0.06 fmol μg^{-1} protein) under high B_{12} and MetE abundance increased to 3.25 fmol μg^{-1} protein under low B_{12} availability. Maximal MetE abundance was 60-fold greater than MetH, consistent with the expected ~ 50–100-fold larger turnover number for MetH. MetE expression resulted in 30-fold increase in nitrogen and 40-fold increase in zinc allocated to methionine synthase activity under low B_{12}. CBA1 abundance was 6-fold higher under low-B_{12} conditions and increased upon B_{12} resupply to starved cultures. While biochemical pathways that supplant B_{12} requirements exist and are utilized by organisms such as land plants, B_{12} use persists in eukaryotic phytoplankton. This study suggests that retention of B_{12} utilization by phytoplankton results in resource conservation under conditions of high B_{12} availability. MetE and MetH abundances were also measured in diatom communities from McMurdo Sound, verifying that both these proteins are expressed in natural communities. These protein measurements are consistent with previous studies suggesting that B_{12} availability influences Antarctic primary productivity. This study illuminates controls on expression of B_{12}-related proteins, quantitatively assesses the metabolic consequences of B_{12} deprivation, and demonstrates that mass spectrometry–based protein measurements yield insight into the functioning of marine microbial communities.

Vitamin B_{12}, also known as cobalamin, is a cobalt-containing organometallic complex that is required by an estimated half of all eukaryotic phytoplankton (Croft et al. 2005; Tang et al. 2010). Since no eukaryotic organism is known to produce B_{12} (Warren et al. 2002), marine bacteria and archaea must therefore supply auxotrophic (vitamin-requiring) phytoplankton with B_{12} through production and release into the water column upon death and cell lysis (Karl 2002; Droop 2007; Bonnet et al. 2010) or through direct interactions (Croft et al. 2005). In the open ocean, B_{12} is present in femtomolar to low picomolar concentrations (Sañudo-Wilhelmy et al. 2012) and is depleted in irradiated surface waters, largely due to biological utilization (Menzel and Spaeth 1962). It has long been hypothesized that B_{12} availability could influence marine primary production (Cowey 1956) and phytoplankton species composition (Droop 1957; Hutchinson 1961; Swift and Taylor 1972). More recent field work suggests that the vitamin does in fact affect phytoplankton growth in many areas of the ocean, including the Ross Sea (Bertrand et al. 2007), the Antarctic Peninsula sector of the Southern Ocean (Panzeca et al. 2006), the North Pacific (Koch et al. 2011), and Long Island embayments (Sañudo-Wilhelmy et al. 2006; Gobler et al. 2007).

B_{12} demand in eukaryotic algae like diatoms is currently attributed to its use in the enzyme methionine synthase (Croft et al. 2005; Helliwell et al. 2011), which is responsible for generating methionine from homocysteine and 5-methyltetrahydrofolate (Banerjee and Matthews 1990). This serves to produce both methionine and tetrahydrofolate and is thus an essential component of cellular one-carbon metabolism. Some eukaryotic algae only have the B_{12}- and zinc-requiring version of this enzyme (MetH) encoded in their genomes (e.g., the diatom *Thalassiosira pseudonana*). Such phytoplankton strains have been reproducibly demonstrated to have an absolute B_{12} requirement for growth (Guillard and Ryther 1962; Croft et al. 2005; Bertrand et al. 2012). Other algal genomes encode both MetH and an additional zinc-requiring enzyme that accomplishes the same reaction, but without B_{12} (MetE; e.g., the diatom *Phaeodactylum tricornutum*). In *Escherichia coli*, the turnover number for MetE (kcat) = 0.12 ± 0.03 s^{-1} (Taurog et al. 2006) whereas MetH k_{cat} = 8–13 s^{-1} (Taylor and Weissbach 1973), suggesting that MetH is a much more efficient enzyme than MetE. Interestingly, no eukaryotic marine microbes have been identified to date that have only the B_{12}-independent *metE* methionine synthase gene, suggesting that, despite low and potentially highly variable marine B_{12} concentrations (Sañudo-Wilhelmy et al. 2012), it is unfavorable for marine microbial eukaryotes to lose the ability to use B_{12} for methionine synthase activity. The presence of *metE* in addition to *metH* appears to result in the lack of an absolute B_{12} requirement in the diatom *P. tricornutum*, where *metE* is only transcribed

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and translated into protein when B_{12} is unavailable (Helliwell et al. 2011; Bertrand et al. 2012). However, some marine microbial eukaryote genomes encode another enzyme, methylmalonyl coA mutase, which is thought to require B_{12} for activity. The presence of this gene in genomes does not appear, however, to result in a measurable physiological B_{12} requirement under culture conditions examined thus far (Croft et al. 2006; Helliwell et al. 2011). Despite B_{12}'s important role in phytoplankton metabolism, mechanisms for its acquisition are not completely elucidated. One protein, cobalamin acquisition protein 1 (CBA1), was recently identified and shown to be involved in B_{12} uptake in diatoms (Bertrand et al. 2012). This protein was found to be more abundant under B_{12} stress and to facilitate B_{12} transport.

It is notable that B_{12} requirements do not follow phylogenetic lines (Croft et al. 2006; Helliwell et al. 2011) and that there are even strain-level differences in B_{12} requirements among eukaryotic phytoplankton (Tang et al. 2010). This suggests that taxonomically based analyses of marine microbial community composition will be unable to determine the proportion of the community that has an absolute B_{12} requirement. This observation also supports the notion that B_{12} auxotrophy has arisen multiple times throughout algal evolutionary history, likely driven by variations in environmental B_{12} availability (Helliwell et al. 2011).

Bioassay bottle incubation experiments have refocused attention on the potentially important role of B_{12} in influencing primary productivity (Panzeca et al. 2006; Bertrand et al. 2007). However, these experiments may be prone to artifacts in part because they necessarily separate microbial communities from relevant physical processes including mixing and variable light conditions. The application of protein or transcript-based biomarker assays for gene products with abundance patterns that are driven by B_{12} nutritional status would complement bottle incubation bioassays and may provide more subtle information about the potential for vitamin starvation in microbial communities. Such biomarkers provide the added benefit of being in-situ measurements, requiring only sample collection rather than significant manipulation of communities for bioassays. These biomarker measurements also have the potential to be high throughput and offer enhanced spatial and temporal resolution at sampling rates that are not feasible using traditional bioassays. While such in-situ biomarkers have been developed and used for iron (LaRoche et al. 1996; Webb et al. 2001; Chappell et al. 2012) and phosphorus (Dyhrman et al. 2002), there has been no previous inquiry into these kinds of developments for vitamin B_{12}.

In this study, quantitative selected reaction monitoring (SRM) mass spectrometry–based methods were developed and utilized to measure the abundance of MetE, MetH, and CBA1 proteins in cultured and natural diatom communities. SRM mass spectrometry can be highly sensitive and quantitative, offering potential for measuring low-abundance targets, such as specific peptides, in complex samples (Stemmann et al. 2001; Lange et al. 2008). In contrast to global proteomic techniques, such as those employed to identify and detect changes in relative abundance of the B_{12} deprivation–induced proteins measured here (Bertrand et al. 2012), SRM targets only a subset of proteins for analysis and detects them in a quantitative mode. In this application of SRM, peptides are first separated by reverse-phase liquid chromatography then introduced to a triple quadrupole mass spectrometer via electrospray ionization. These peptides, in a predetermined mass window, are collected in the mass spectrometer's first quadrupole. Those ions are then fragmented and the generation of specific, predetermined product ions is monitored. The amount of the product ions detected is proportional to the abundance of the peptide of interest in a sample. This abundance is calibrated using stable isotope–labeled versions of each peptide, added to samples in known quantities as internal standards (Stemmann et al. 2001). These methods confidently measure the concentration of peptides in complex mixtures of extracted and digested peptides. In order to prepare samples for mass spectrometry, sample processing steps are required that have the potential to lead to some peptide loss. Previous studies and method development performed for this study have shown, however, that there is typically minimal loss and differential protein digestion during sample preparation, suggesting that these quantitative measurements can be used to determine the concentration of specific peptides produced per unit biomass (Keshishian et al. 2007).

SRM mass spectrometry was applied here in order to measure B_{12}-related proteins in diatom cultures over time, under B_{12}-limited conditions and upon pulsed resupply of the vitamin, revealing clear interreplacement (Sunda and Huntsman 1995) of MetE and MetH proteins as a function of B_{12} availability. These measurements enabled a quantitative estimation of the metabolic cost of B_{12} deprivation to diatoms, similar to a recent application of SRM in determining the iron metabolic use in a marine nitrogen-fixing cyanobacterium (Saito et al. 2011*b*). This technique was also used to measure MetE and MetH abundance in diatom communities from surface seawater in McMurdo Sound of the Ross Sea in the Southern Ocean, verifying that both of these proteins are utilized by natural populations of diatoms and that they are of utility as biomarkers for evaluating the metabolic status of microbial communities with respect to vitamin B_{12}.

Methods

Laboratory culturing and sampling—Axenic cultures of *P. tricornutum* CCMP 632 were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton and maintained using sterile and trace metal clean technique. Axenicity was monitored throughout using marine purity broth tests (Saito et al. 2002). All cultures were grown at 16°C under a constant light level of 150 μmol quanta m^{-2} s^{-1} in polycarbonate bottles; these experiments were distinct from those used in a previous study that identified B_{12}-responsive proteins (Bertrand et al. 2012). All media preparation and culture manipulation was conducted in a class 100 clean room facility. Media was prepared in a 0.2 μm filtered oligotrophic seawater base collected in a trace metal clean manner,

microwave sterilized, and supplemented with macronutrients at f/2 concentrations and with vitamins and ethylenediaminetetraacetic acid (EDTA)–buffered trace metals at concentrations as previously described by Sunda and Huntsman (1995), except for modulations of vitamin B_{12} concentrations (Sigma, plant cell culture tested, $> 98\%$). Triplicate bottles with 100 pmol L^{-1} added B_{12} or no added B_{12} (1.4 liters in 2 liter bottles and 2.4 liters in 3 liter bottles) were inoculated (1.3%) with *P. tricornutum* cells that had been acclimated through three transfers in B_{12}-deplete media with at least two doublings per transfer. After 4.5 d of growth, the B_{12}-deplete cultures were split into two and placed in sterile 2 liter polycarbonate bottles. One of each of the bottles was then supplemented with 100 pmol L^{-1} B_{12} and all cultures were returned to the incubator for growth. Culture growth was monitored daily via cell counts, using a Palmer Maloney nanoplankton counting chamber, counting at least 10 fields of view or 200 individuals at 400× magnification with light microscopy (Carl Zeiss). Proteins were sampled via gentle filtering (< 15 kPa vacuum) of 135–250 mL of culture onto 47 mm Supor 0.45 μm filters (Pall), flash freezing in liquid nitrogen, and storing at -80°C. Proteins were sampled after 3 d of growth, and again 24 h after refeeding the cultures with B_{12}.

Protein extraction and digestion from cultures—Cells were rinsed from the filters and resuspended in 600 μL B-PER reagent (Thermo Scientific) supplemented with 5 mmol L^{-1} EDTA and 1 mmol L^{-1} phenylmethanesulfonylfluoride (a serine protease inhibitor). Samples were incubated at room temperature for 20 min with occasional gentle vortexing. The cells were then sonicated with a microtip (Branson digital sonifier) on ice, twice for 1 min at constant duty cycle with a 5 min pause on ice between sonication steps. Samples were centrifuged for 30 min at 14,100 relative centrifugal force (RCF) and 4°C, and supernatants were precipitated overnight in 50% acetone 50% methanol 0.5 mmol L^{-1} HCl at -20°C. Precipitated protein was collected by centrifugation at 14,100 RCF for 30 min at 4°C and dried by speed vacuum at room temperature. Protein was resuspended in 100 μL of the extraction buffer for 30 min at room temperature. Aliquots were taken for protein concentration determination by detergent-compatible (DC) protein assay using bovine serum albumin as a protein standard (BioRad). Proteins were stored at -80°C until digestion.

Field sampling—Surface ($\sim$ 3 m) seawater in a volume of 250–500 liters was pumped into a 250 liter carboy and then onto 293 mm diameter 3 μm pore size polyethersulfone filters (Versapore, Pall) through a hole drilled in sea ice (Sta. 399, 400, 401) or just over the ice edge (Sta. 402) from 18 Nov 2009 to 23 Nov 2009. Station locations were: 402: 77.6442°S, 165.7178°E; 401: 77.6501°S, 165.7497°E; 400: 77.6587°S, 166.1680°E; 399: 77.6730°S, 166.4238°E. These samples were included in the J. Craig Venter Institute's Global Ocean Sampling project (GOS numbers 399–402); established sampling and filtering protocols were used. Filters were frozen in liquid nitrogen, kept on dry ice for shipping, and stored in the laboratory at -80°C. Two chlorophyll samples per station were taken from the 250 liter carboy and measured according to Joint Global Ocean Flux Study protocols. A 45 min net tow sample was taken through an ice hole at Sta. 399. Live light micrographs of the net tow sample were taken at 200× magnification on a Zeiss microscope using a Nikon CoolPix 995 and ambient lighting in the field.

Field sample protein extraction—Filters were cut in half, with half retained for future analysis and half used for protein extraction. For protein extraction, the filter half was cut into strips and incubated on ice for 15 min in 24 mL of extraction buffer (0.1 mol L^{-1} Tris HCl, pH 7.5; 5% glycerol, 1% sodium dodecyl sulfate, 10 mmol L^{-1} EDTA) was periodically gently vortexed, then heated to 92–98°C in an Eppendorf Thermomixer for 15 min, gently vortexed for 1 min, then mixed at 20°C for 2 h in a Bambino hybridization oven (Boekel Scientific), with gentle vortexing for 30 s every 30 min (Saito et al. 2011*a*). The extraction buffer was then collected and centrifuged at 7000 RCF at 10°C for 20 min. The supernatant was collected, filtered with a 5 μm polycarbonate filter, and concentrated via ultrafiltration in Vivaspin6 5000 Da molecular weight cut-off polyethersulfone membrane in centrifugal concentrators (Sartorius Stedum) spinning at 2100 RCF at 12°C until the volume was reduced to $\sim$ 1200 μL per sample ($\sim$ 3 h). Protein was precipitated overnight at -20°C in five volumes of ice-cold 50% acetone, 50% methanol with 0.5 mmol L^{-1} HCl. Samples were spun at 13,000 RCF at 4°C for 20 min; supernatants were aspirated and remaining pellets were dried at room temperature in a speed vacuum and resuspended in 100 μL of extraction buffer. Aliquots were taken for protein concentration determination by DC assay as described for culture samples above. Approximately 1000 μg of protein was extracted from each of these filters, which was in 500-fold excess of that required for mass spectrometry analyses conducted as described below.

Protein digestion—Both culture and field protein samples were digested into peptides following the tube gel procedure (Lu and Zhu 2005) with minor modifications. Briefly, samples (in known amounts of 25–100 μg total protein) were immobilized in 15% acrylamide in pH 7.5 Tris buffer, fixed with 10% acetic acid and 50% ethanol, washed successively with 10% acetic acid and 50% methanol, then acetonitrile and 25 mM ammonium bicarbonate (pH 8), and then cut into 1 mm^3 pieces. Samples were reduced with 10 mmol L^{-1} dithiothreitol at 56°C for 1 h, alkyated with 30 mmol L^{-1} iodoacetamide for 1 h, and then washed in 25 mmol L^{-1} ammonium bicarbonate (pH 8) and digested with trypsin (Promega Gold Mass Spectrometry Grade, Promega Inc.) in 25 mmol L^{-1} ammonium bicarbonate at pH 8 for 16 h at 37°C (1 : 20 ratio trypsin to total protein). The peptides were extracted from the gel cubes by successive additions of 50% acetonitrile and 5% formic acid in water. The extracted peptides were combined and concentrated by speed vacuum for about 3 h to < 20 μL, diluted with 2% acetonitrile and 0.1% formic acid, and stored at -80°C.

Total bulk protein concentrations in the mass spectrometry samples were determined post–tryptic digestion via DC assay using a tryptic digest of bovine serum albumin as a protein standard. These compared well to peptide concentrations expected assuming full protein recovery from the tube gel digestion procedure (data not shown).

Labeled peptide handling and mass spectrometry—The protein digestions were analyzed by high-pressure liquid chromatography (HPLC) coupled to mass spectrometry as follows: a Paradigm MS4 system (Michrom Bioresources) was employed for HPLC separation. Samples were injected onto a peptide Cap Trap in-line with a reversed-phase Magic C18 AQ column (0.2 × 50 mm, 3 μm particle size, 200 Å pore size, Michrom Bioresources). The chromatography consisted of a hyperbolic gradient from 5% buffer A to 95% buffer B for 40 min, where A was 0.1% formic acid (Michrom Ultra Pure) in water (Fisher Optima) and B was 0.1% formic acid in acetonitrile (Fisher Optima) at a flow rate of 4 μL min^{-1}. An Advance nanocapillary electrospray source (Michrom Bioresources) was used to introduce the sample into the Thermo Vantage TSQ Triple Quadrapole Mass Spectrometer (TSQ). The TSQ was programmed to perform SRM as previously described for cyanobacterial peptides (Saito et al. 2011*b*), this time using peptides relevant to B_{12} metabolism in diatoms (Table 1). Isotopically labeled versions of each peptide (Sigma-Aldrich) were employed as internal standards (Stemmann et al. 2001). These peptides were handled according to the manufacturer's instructions where 20 μL of one of several solvent mixtures, depending on peptide hydrophobicity and charge, were added to 1 nmol lyophilized vials. The solvent mixtures employed were: 5% acetonitrile 95% water, 90% acetonitrile 1% formic acid 9% water, 95% acetonitrile 0.5% formic acid 5% water, water alone, or 10% formic acid.

The SRM reaction conditions for monitoring each peptide were optimized during direct infusion of each heavy isotope–labeled peptide (48 fmol μL^{-1} in 20% acetonitrile, 80% water, 0.1% formic acid) at 4 μL min^{-1} into the TSQ, optimizing the S-lens value for each parent ion (2+ and 3+) and the collision energy for generating each possible product ion. The most abundant three to five product ions generated from peptide parent ions were chosen for inclusion in the SRM used for quantitative analysis. Q1 operated in 0.2 full width at half maximum (FWHM) resolution and Q3 operated in 0.7 FWHM resolution, with a cycle time of 1.6 s. SRM was scheduled based on peptide pair chromatographic retention times, as shown in Table 1.

Linear response of each peptide monitored by these SRMs was validated by creating a standard curve from 0.2 to 240 fmol of each heavy isotope–labeled reference peptide in 2 μg of *P. tricornutum* tryptic peptide sample matrix. For quantitative analyses, 20 fmol of heavy isotope–labeled versions of each peptide were added to diatom peptide extracts (1 or 2 μg bulk protein) and the amount of the peptide of interest produced by the diatom (called the native peptide version herein) was calculated as a ratio to the size of the peak produced by 20 fmol of the heavy-labeled version of that peptide. The peptides measured and the reactions monitored for each are given in Table 1. Limits of detection (LOD) and limits of quantitation (LOQ), as defined by the American Chemical Society (MacDougall et al. 1980) are also given in Table 1 and were calculated for each peptide as follows: the intensity of the background peak for each reaction monitoring a native peptide was calculated in three blank mass spectrometry runs (no sample injection) and the femtomole of native peptide represented by this background intensity was calculated as a ratio to the intensity of the peak generated by the addition of 20 fmol of heavy peptide in 2 μg total protein sample. LOD = Sb + 3σ, where Sb is the mean signal blank, in fmol per μg protein added, and σ is the standard deviation about the mean of Sb; LOQ = Sb + 10σ. Any values below these LOQ concentrations are reported as zero or nondetectable in this study.

Statistical tests of difference—All statements of comparison were supported with Student's unpaired *t*-tests (α = 0.05); *p*-values are reported in the text of the Results section for each comparison.

Results

Peptide abundance through B_{12} starvation and recovery in diatom cultures—Cultures of *P. tricornutum*, grown through several transfers in B_{12}-deplete media, grew to 15 ± 7% lower cell density (densities significantly different; $p = 0.03$) and at a 7 ± 3% slower rate compared to control treatments supplemented with 100 pmol L^{-1} added B_{12} (Fig. 1; rates significantly different; $p = 0.002$). In addition, B_{12} resupply to vitamin-starved cultures resulted in an 11 ± 4% increase in cell number over 24 h (final cell numbers significantly different; $p = 0.00002$). These data suggest that although this diatom possesses genes encoding both MetE and MetH and therefore does not require B_{12} absolutely, it receives a growth benefit in the presence of the vitamin. The same enhancement of growth under high B_{12} has also been documented in centric diatoms that do not have absolute B_{12} requirements (Swift and Guillard 1978).

Peptides selected for quantifying MetE, MetH, and CBA1 protein abundance are given in Table 1. Standard curves for heavy-labeled versions of these peptides are shown in Fig. 2, demonstrating that these peptides respond linearly over four orders of magnitude. The intensity of the mass spectrometry response varied between peptides (Fig. 2, y-axis). This supports the use of heavy isotope–labeled versions of each peptide of interest as internal standards for proper quantitation.

Concentrations of peptides diagnostic of MetE, MetH, and CBA1 were measured in *P. tricornutum* samples 3 d after inoculation and again 24 h after B_{12} resupply (Fig. 3). The abundance of all three proteins measured was greater at the later time point (significantly different abundances; MetH $p = 0.001$; MetE $p = 0.0005$; CBA1 $p = 0.0001$), suggesting that these proteins comprise a larger fraction of total protein at the onset of stationary phase compared to during exponential growth. MetE, B_{12}-independent methionine synthase, was more abundant under low B_{12}

Table 1. Peptide sequences, names, selected reaction monitoring parameters, limits of detection, and limits of quantification.

Protein	Peptide	Peptide name	Parent ion (+z)	Parent (*m/z*)	Product (*m/z*)	Collision energy	Start time (min)	Stop time (min)	S-lens value	LOD (fmol)	LOQ (fmol)
Pt48322 CBA1	FFS[V_C13N15]FFNK	Pt48322_1heavy	2	521.2686	747.3930	15	16.80	18.80	110		
			2	521.2686	660.3610	15	16.80	18.80	110		
			2	521.2686	555.2900	16	16.80	18.80	110		
	FFSVFFNK	Pt48322_1native	2	518.2682	741.3930	15	16.80	18.80	110	0.0006	0.0013
			2	518.2682	654.3610	15	16.80	18.80	110		
			2	518.2682	555.2900	16	16.80	18.80	110		
Pt48322 CBA1	EHTANQ[V_C13N15]VEAAESR	Pt48322_2heavy	2	773.8712	1280.6335	21	10.10	12.10	136		
			2	773.8712	1179.5858	24	10.10	12.10	136		
			2	773.8712	866.4472	25	10.10	12.10	136		
			2	773.8712	761.3788	25	10.10	12.10	136		
	EHTANQVVEAAESR	Pt48322_2native	2	770.8712	1274.6335	21	10.10	12.10	136	0.0120	0.0231
			2	770.8712	1173.5858	24	10.10	12.10	136		
			2	770.8712	860.3788	25	10.10	12.10	136		
			2	770.8712	761.3788	25	10.10	12.10	136		
MetE, Pt	GS[L_C13N15]SEVDLIK	pMetE_1heavy	2	534.3065	803.4509	14	13.2	15.2	124		
			2	534.3065	716.4189	13	13.2	15.2	124		
			2	534.3065	587.3763	21	13.2	15.2	124		
	GSLSEVDLIK	pMetE_1native	2	530.7979	803.4509	14	13.2	15.2	124	0.0004	0.0008
			2	530.7979	716.4189	13	13.2	15.2	124		
			2	530.7979	587.3763	21	13.2	15.2	124		
MetE, broad	VIQVDEPA[L_C13N15]R	bMetE_1heavy	2	573.8332	934.5066	16	11.9	13.9	135		
			2	573.8332	806.448	21	11.9	13.9	135		
			2	573.8332	707.3796	20	11.9	13.9	135		
	VIQVDEPALR	bMetE_1native	2	570.3246	927.4894	16	11.9	13.9	135	0.0004	0.0008
			2	570.3246	799.4308	21	11.9	13.9	135		
			2	570.3246	700.3624	20	11.9	13.9	135		
MetH, Pt	IGIT[V_C13N15]IDTVK	pMetH_1heavy	2	532.8264	951.5615	18	13.6	15.6	116		
			2	532.8264	781.456	18	13.6	15.6	116		
			2	532.8264	575.3399	16	13.6	15.6	116		
			2	532.8264	894.5401	17	13.6	15.6	116		
			2	532.8264	680.4083	18	13.6	15.6	116		
	IGITVIDTVK	pMetH_1native	2	529.8264	945.5615	18	13.6	15.6	116	0.0005	0.0010
			2	529.8264	775.456	18	13.6	15.6	116		
			2	529.8264	575.3399	16	13.6	15.6	116		
			2	529.8264	888.5401	17	13.6	15.6	116		
			2	529.8264	674.4083	18	13.6	15.6	116		
MetH, diatoms	ISGGISN[L_C13N15]SFGFR	dMetH_1heavy	2	681.3679	1161.6124	21	16.15	18.15	149		
			2	681.3679	934.4855	20	16.15	18.15	149		
			2	681.3679	613.3093	24	16.15	18.15	149		
	ISGGISNLSFGFR	dMetH_1native	2	677.8593	1154.5953	21	16.15	18.15	149	0.0003	0.007
			2	677.8593	927.4683	20	16.15	18.15	149		
			2	677.8593	613.3093	24	16.15	18.15	149		

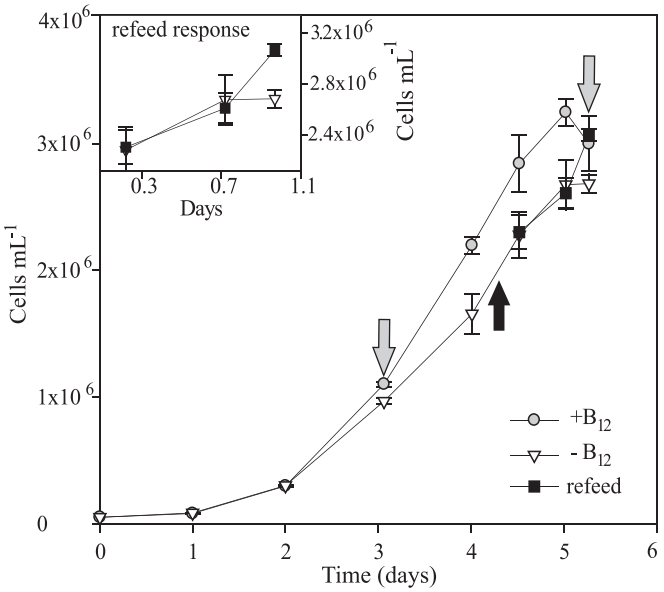

Fig. 1. Growth of *Phaeodactylum tricornutum*, with and without added B_{12} and upon resupply of B_{12} to starved cultures, shown as cells per milliliter over time. Symbols are means of single counts from triplicate cultures, with error bars representing 1 standard deviation. The results of the resupply are repeated as an inset, with the x-axis as time in days since B_{12} was resupplied. Gray arrows indicate locations where protein samples were taken, and the black arrow indicates where the low-B_{12} culture was split and half was resupplied with the vitamin. The growth rate of cultures during exponential growth (days 1–3) was 1.26 ± 0.01 d^{-1} for the $+B_{12}$ culture and 1.17 ± 0.02 d^{-1} for the $-B_{12}$ culture, showing that despite the fact that *P. tricornutum* grows well in the absence of B_{12}, it receives a growth rate enhancement when it utilizes the vitamin. This is supported by results from the resupply of 100 pmol L^{-1} B_{12} to $-B_{12}$ cultures which showed an increase in cell number as a result of the vitamin addition.

at both time points (significantly different abundances; early $p = 2 \times 10^{-9}$; late $p = 1 \times 10^{-10}$). Peptides from MetH, the B_{12}-dependent methionine synthase isoform, were present in low quantities, relatively close to our limits of quantitation (Table 1; Fig. 3). Despite these low concentrations, differences in MetH expression between high- and low-B_{12} treatments were resolved (Fig. 3). MetH was more abundant in the $+B_{12}$ treatments than $-B_{12}$ (significantly different; early $p = 0.002$; late $p = 0.0002$) and increased upon B_{12} resupply to levels measured in the $+B_{12}$ treatment (significantly different; $p = 0.0001$). Mass spectral data supporting this conclusion are shown in Fig. 4.

CBA1, a protein involved in B_{12} acquisition (Bertrand et al. 2012), was more abundant under low-B_{12} conditions compared to replete conditions at each time point (statistically different; early $p = 0.0002$; late $p = 0.0005$). However, concentrations of CBA1 in the low-B_{12} condition during log-phase growth are similar to those in replete cells during the onset of stationary phase (not different $p = 0.5$), suggesting that abundance of this protein is driven by growth phase and B_{12} availability. These two factors may be correlated, as described below. In addition, the abundance of CBA1 increased by an average of 2.5-fold post–B_{12} resupply, suggesting that CBA1 production responds not only to sustained low levels of B_{12} availability but also to pulses of high B_{12} availability (concentrations different after refeed; $p = 0.005$).

MetE and MetH peptide abundance measurements in McMurdo Sound—In order for peptides developed as biomarkers in cultured diatoms to be of use as biomarkers in the field, the degree of phylogenetic conservation for each peptide among strains and species must be considered. This is because our mass spectrometry approach is capable of measuring exact peptide masses such that only precisely matching sequences can be detected in an individual assay; it also cannot distinguish between exact sequences that are commonly expressed by different species or strains. Of the peptides measured in this culture study, two peptides, bMetE1 and dMetH1, are conserved across their respective proteins in diatoms (Table 2) and so were hypothesized to be of utility in this study for measuring MetE and MetH presence in mixed, natural diatom communities.

Peptides bMetE1 and dMetH1 were detected and quantified in samples from McMurdo Sound of the Ross Sea, Antarctica (Fig. 5). dMetH1, ISGGISNLSFGFR, preceded by an N-terminal lysine or arginine residue, which is required as part of the peptide cleavage site, is present only in available MetH sequences from diatoms (Table 2). This peptide contains one of the conserved residues for homocysteine binding, but this location is otherwise quite variable among other MetH sequences. Based on this information, we can deduce that dMetH1 abundance may be a proxy for diatom MetH protein expression. Peptide bMetE1 (VIQVDEPALR) is part of the conserved tetrahydrofolate (THF) binding region in MetE and is present in available diatom MetE sequences as well as a range of bacteria and fungi (Table 2). This suggests that measurement of bMetE1 abundance can be considered a proxy for some subset of community MetE production, perhaps inclusive of all diatoms, though this should be validated through comparison to additional diatom MetE sequences as they become available.

For these field measurements, protein was extracted from 3 μm filters containing particulate matter from surface water ($\sim$ 3 m depth) from Sta. 399–402. Samples 399–401 were collected from underneath first-year sea ice and sample 402 was collected at the sea ice edge. Phytoplankton communities in this region were strongly diatom dominated (Fig. 5F). Given these large filter sizes, the community composition, and the phylogenetic specificity of these biomarker peptides as described above, bMetE1 and dMetH1 detected in this study likely originated predominantly from diatoms with some fraction of the MetE peptides being from bacteria or archaea associated with larger particles.

The identification of these MetE and MetH peptides in field samples is supported by the chromatograms and product ion distributions for these two peptides in samples from Sta. 399, shown in Fig. 5C,D. Since the product ion distributions and retention times are similar for the heavy and native versions of both peptides, bMetE1 and pMetH1 were positively identified. This is in contrast to the measurement of bMetE1 at Sta. 402, which was problematic and hence marked with an X in Fig. 5B. Despite

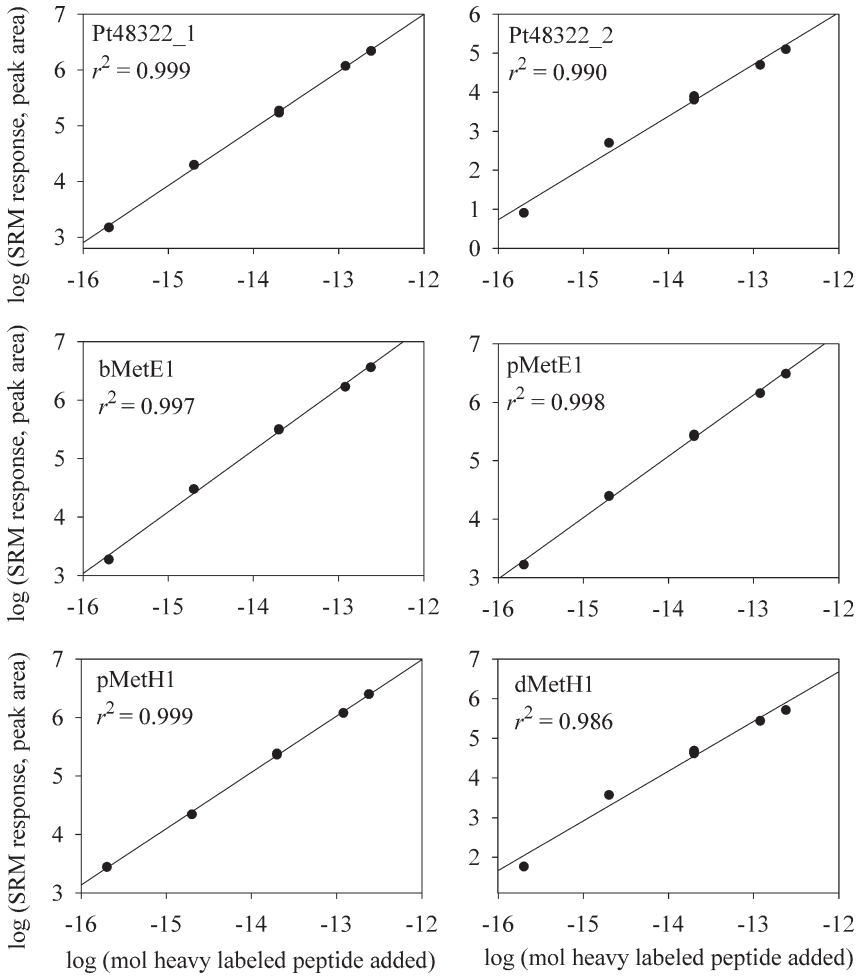

Fig. 2. Standard curves for selected reaction monitoring (SRM) detection of diatom peptides. The log of the intensity of the SRM response (peak area, sum of product ion intensities) is plotted against the log of moles of stable isotope–labeled (heavy) version of each peptide added (Table 1). Linear regressions are shown in the solid line and the coefficients of variance for each are given. For each peptide, the response is linear over four orders of magnitude, and the lowest amount of labeled peptide added was 0.2 fmol. These data verify the linear behavior of each peptide employed here for measurement of diatom proteins.

having a peak at the same retention time as the heavy-labeled version of bMetE1, the product ion distribution generated for the SRM designed to monitor the native peptide is quite different from the heavy-labeled version at that retention time (Fig. 6). This suggests that another parent ion, not generated by peptide VIQVDEPALR but falling into its Q1 mass window, generates a product ion of *m*/*z* 927.489 and thus contaminated the SRM measurement of peptide VIQVDEPALR in this sample. As a result, the size of this peak cannot be used to quantify the amount of bMetE1 in this sample. There was little evidence for such contamination in the bMetE1 measurements at other locations since the product ion distributions in the native peak and heavy peak are similar (Fig. 5).

Discussion

The quantitative proteomic assessments conducted in this study reveal three major findings: (1) the B_{12}-independent MetE methionine synthase enzyme directly replaced the B_{12}-requiring methionine synthase enzyme MetH as a function of B_{12} availability; (2) changes in protein abundances of MetE, MetH, and the B_{12} acquisition protein CBA1 induced by B_{12} deprivation suggest that there is a metabolic cost to growth without B_{12}, which may potentially contribute to the persistence of B_{12} requirements in marine microbial eukaryotes; and (3) that peptide abundance patterns can be used to gain insight into vitamin B_{12} nutritional status of natural diatom communities. Beyond these major findings, we also use these quantitative peptide abundance measurements to make inferences about the molecular biology of diatoms, including regulation of protein expression patterns and the possibility for allelic differential expression, as discussed below.

Peptide abundance patterns in cultured diatoms—MetE and MetH: The quantitative peptide abundance measurements described in this study demonstrate that there is a

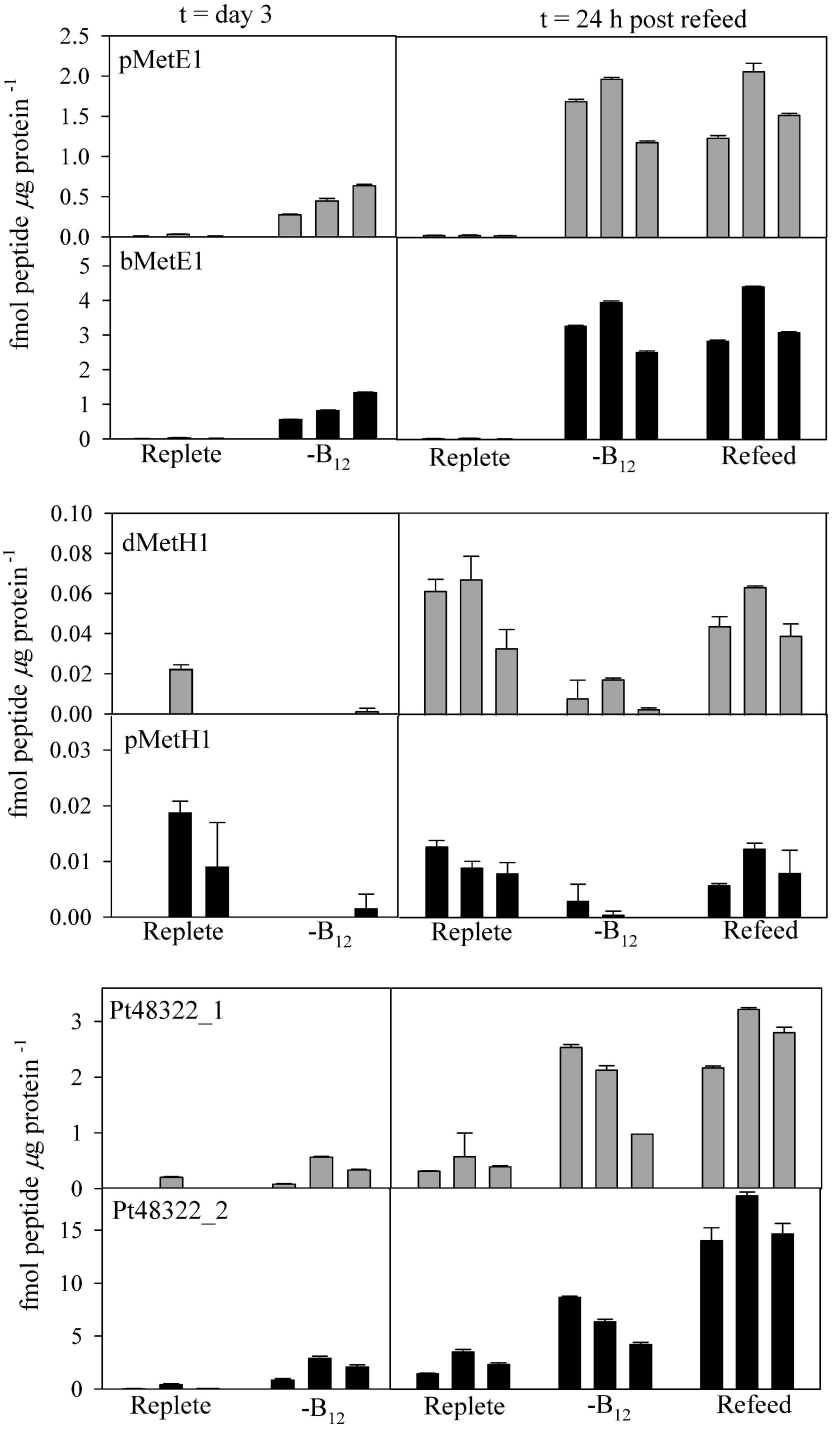

Fig. 3. Abundance of B_{12}-related peptides in cultures of *P. tricornutum*. Concentrations of two peptides diagnostic of each protein of interest, CBA1, MetH, and MetE, are given normalized to total protein for triplicate cultures of $+B_{12}$ and $-B_{12}$ treatments after 3 d, and $+B_{12}$, $-B_{12}$, and refeed treatments 24 h after the refeed. Bars are means of technical triplicate measurements with error bars as 1 standard deviation. MetE, B_{12}-independent methionine synthase, was much more abundant under low B_{12} at both time points and did not decrease in abundance 24 h after B_{12} was resupplied. MetH, B_{12}-dependent methionine synthase, was present in low quantities that were close to our detection limit, was more abundant in the $+B_{12}$ treatments, and increased upon B_{12} resupply. Pt48322 (CBA1), a B_{12} acquisition protein (Bertrand et al. 2012), was more abundant under $-B_{12}$ conditions and increased further upon B_{12} resupply.

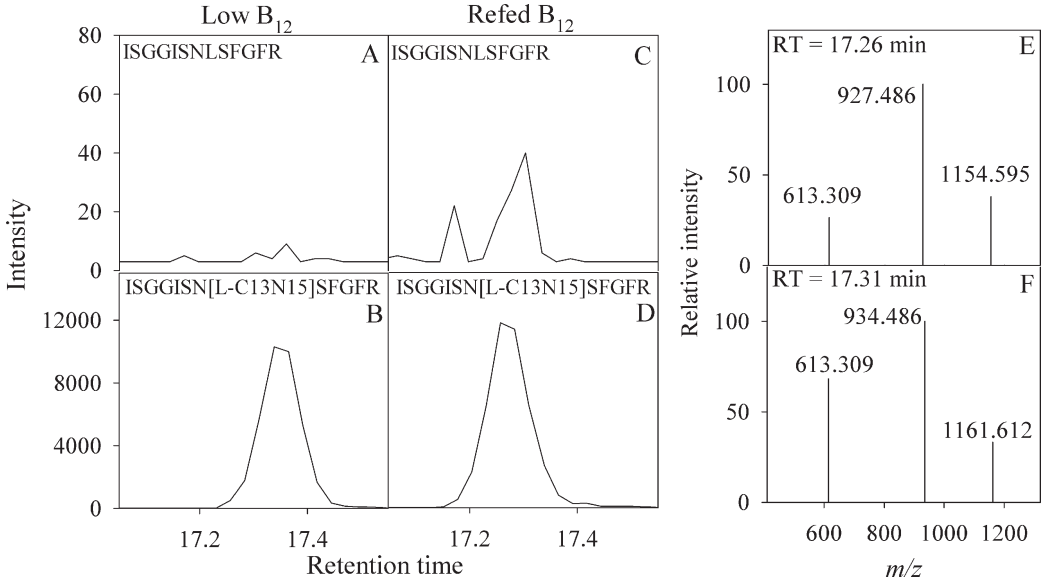

Fig. 4. Chromatograms showing the intensity of the SRM response for the native and heavy-labeled version of peptide dMetH1 (ISGGISNLSFGFR), diagnostic of MetH, from *P. tricornutum* cultures. (A–D) Chromatograms from a low-B_{12} culture and a culture resupplied with B_{12}, both 24 h after the resupply, are shown. Intensity is the sum of the abundance of specific product ion peaks generated from the parent ion for each peptide. For ISGGISNLSFGFR (top), the SRM monitored is parent 677.859 to products 613.308–613.310, 927.467–927.469, 1154.594–1154.596, and for ISGGISN[L_C13N15]SFGFR (bottom) the SRM monitored is parent 681.368 to products 613.308–613.310, 934.485–934.487, 1161.611–1161.613. (E, F) are the product ion distributions for native dMetH1 (top) and heavy-labeled dMetH1 (bottom) peaks in the resupplied culture (chromatograms shown in C, D). The product ion distributions and retention times are similar between the heavy and native versions of this peptide in this sample, which support the positive identification and quantification of dMetH1 in the resupplied culture.

clear switch in metalloenzyme utilization from conditions of high B_{12} (replete growth) to low B_{12}, where MetE is abundant under low B_{12} and MetH is abundant under high B_{12} in the non–B_{12}-requiring diatom *P. tricornutum* (Fig. 3). In this diatom, methionine synthase isoform interreplacement appears to be governed by B_{12} availability.

Comparing protein abundance trends observed in this study with available data on transcript abundance yields insight into possible regulation mechanisms and relative gene product lifetimes. The abundance patterns observed here for MetE protein (Fig. 3) are consistent with *metE* gene expression patterns in *P. tricornutum* cultures; *metE* expression was repressed under high B_{12} (Helliwell et al. 2011; Bertrand et al. 2012), which may be in part controlled by a two-component sensor that appears to be co-regulated with *metE* (Bertrand et al. 2012). These data suggest that the pattern we observe here in MetE protein abundance is transcriptionally regulated. However, whereas Helliwell

Table 2. Known origins of peptides pMetH and bMetE from sequence databases including National Center for Biotechnology Information and Joint Genome Institute obtained by blastP searching the peptide sequences against these databases with an Expect (E-value) threshold of 10. Single amino acid changes are underlined and in bold text. (K/R) indicates that the amino acid in this position could be either a lysine or an arginine.

	dMetH1, (K/R)ISGGISNLSFGFR		bMetE1, (K/R)VIQVDEPALR	
	Species and protein	Sequence	Species and protein	Sequence
Genomes, proteins with exact match	*Phaeodactylum tricornutum*, MetH 23399	(K)ISGGISNLSFGFR	*Phaeodactylum tricornutum*, MetE 28056	(K)VIQVDEPALR
	Thalassiosira pseudonana, MetH 693	(K)ISGGISNLSFGFR	*Fragilariospis cylindrus*, MetE 228154	(R)VIQVDEPALR
	Fragilariopsis cylindrus, MetH 207237	(K)ISGGISNLSFGFR	>125 genomes including fungi and bacteria, with many from *Clostridium* and *Bacillus* groups	
Genomes, proteins with one mutation	*Ectocarpus siliculosus*, MetH 0279_0006	(K)ISGG**V**SNLSFGFR		
	Aureococcus anophagefferens, MetH 15101	(K)ISGG**V**SNLSFGFR		
	Plesiocystis pacifica, SIR-1 MetH	(R)ISGGISNLSF**S**FR		
	Frankia alni, ACN14a MetH	(R)ISGGISNLSF**S**FR		
	Frankia sp., *EUN1f* MetH	(R)ISGGISNLSF**S**FR		

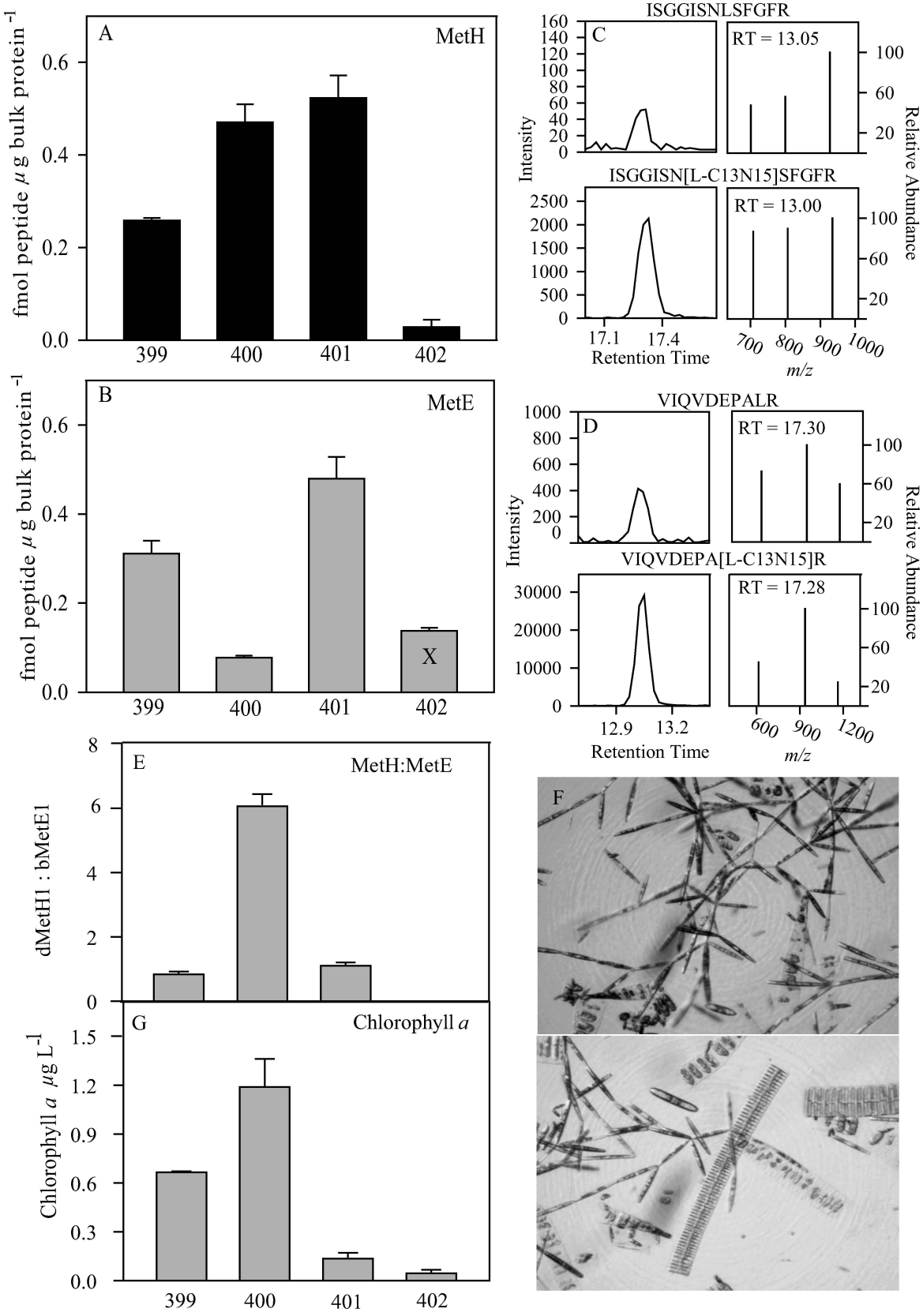

Fig. 5. Peptides diagnostic of MetE and MetH were detected and quantified in phytoplankton samples from McMurdo Sound of the Ross Sea, Antarctica. Protein extracted from 3 μm filters containing particulate matter from surface water from Sta. 399–402 had detectable (A) MetH and (B) MetE peptide present. Bars are means of technical triplicate measurements, with error bars representing 1

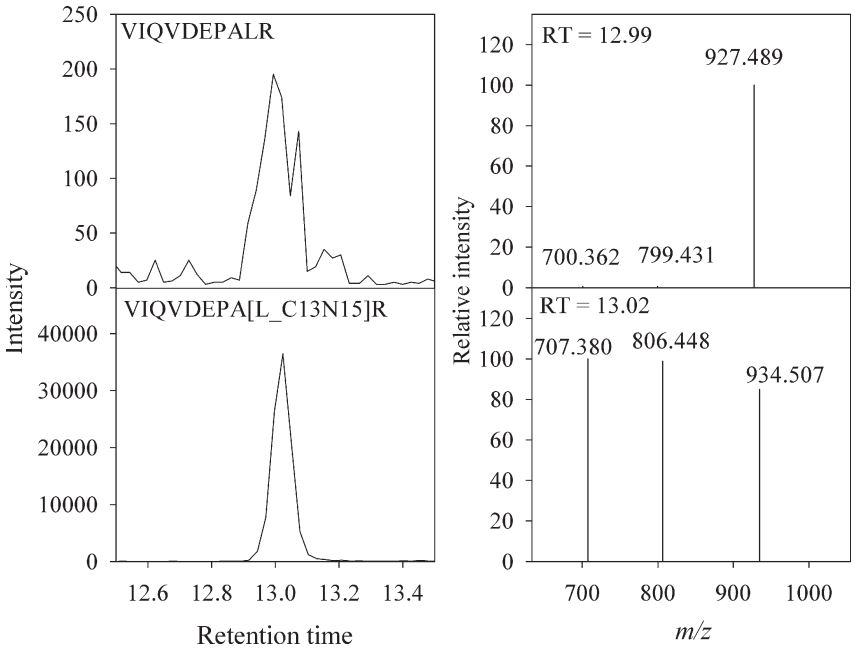

Fig. 6. The bMetE1 measurement at Sta. 402 was contaminated. Chromatograms on the left show the intensity of the SRM response measured as the native (top) and heavy-labeled version (bottom) of peptide bMetE1 (VIQVDEPALR) at Sta. 402. The product ion distributions for the native and heavy-labeled peaks are shown to the right of their corresponding chromatograms. Note that the product ion distribution for the native peptide was vastly different than the heavy-labeled version at the same retention time, suggesting that another parent ion, not generated by peptide VIQVDEPALR but falling into its Q1 mass window, generates a product ion of mass 927.489, thus contaminating the SRM measurement of peptide VIQVDEPALR in this sample. There was little evidence for such contamination in the bMetE1 measurements at other locations.

et al. (2011) showed that *metE* transcript abundance decreased within 2 h of B_{12} resupply, here we show that MetE protein abundance did not decrease within 24 h of B_{12} resupply (Fig. 3). Thus, despite the cessation of active *metE* transcript production, protein levels did not appear to concurrently decrease. It is possible that continued presence of MetE protein may be of some benefit to cells as they are accumulating B_{12} from this pulsed addition, or it may be that there is no cellular benefit to or mechanism for specifically degrading the protein.

Peptide data presented here indicate that MetH protein abundance decreased in response to B_{12} scarcity while a high abundance of MetH was induced by increased B_{12} availability to *P. tricornutum*. However, RNA-seq results suggested that *metH* transcript abundance was not dramatically affected by B_{12} availability in *P. tricornutum* (Bertrand et al. 2012). Together these results point to posttranscriptional regulation of MetH expression that depends, in some fashion, on B_{12} availability. The full recovery of MetH protein concentrations to replete levels 24 h post-resupply implies that the entirety of methionine production and recycling demand could be supplied by the more efficient MetH after this time and that MetH expression is more tightly coupled than MetE expression to short-term B_{12} availability in diatoms.

CBA1: Low B_{12} availability also resulted in higher absolute CBA1 concentrations (Fig. 3), as observed previously using relative quantitation methods (Bertrand et al. 2012). Interestingly, concentrations of CBA1 in the low-B_{12} condition during log-phase growth are similar to those in replete cells during the onset of stationary phase, suggesting that CBA1 abundance may be driven by both growth phase

←

standard deviation. Chromatograms showing the intensity of the SRM response for the native and heavy-labeled versions of peptide dMetH1 (C) ISGGISNLSFGFR and peptide bMetE1 (D) VIQVDEPALR in the sample from Sta. 399 are given, along with product ion distributions generated by the peptides. The product ion distributions and retention times were similar for the heavy and native versions of both peptides, leading to the positive identification and quantification of dMetH1 and bMetE1 in these field samples. (B) The measurement for bMetE1 at Sta. 402 appears contaminated (Fig. 6), so it is marked with an X to denote analytical difficulties. The ratio of dMetH1 to bMetE1 abundance at the three stations without bMetE1 contamination are given in panel E. (E) Means of duplicate chlorophyll *a* measurements at each location are given, with error bars as 1 standard deviation. (F) All samples were dominated by diatoms, as shown in the light micrograph from a net tow sample taken at Sta. 399, and supported by the fact that all filters were brown in color. Diatoms observed in this net tow include *Nitzschia stellata*, *Fragilariopsis cylindrus*, and *Amphiprora kufferathii*. The dimensions of the micrographs are roughly 800 μm $\times$ 500 μm (upper) and 900 μm $\times$ 600 μm (lower).

and B_{12} availability (Fig. 3). We can, through estimates of B_{12} acquisition over this period of culture growth, hypothesize as to whether B_{12} availability and growth phase are correlated in this experiment. Using a conservatively high cellular B_{12} quota of 2.5×10^{-8} pmol B_{12} per cell (which is among the higher quotas measured for other phytoplankton groups by Tang et al. [2010] and comparable to cellular quotas measured by Droop [1974] under conditions of excess B_{12}), we estimate that B_{12} drawdown by onset of stationary phase would approach 70% of the initial vitamin added. If these numbers are realistic for *P. tricornutum* in batch culture, it suggests that B_{12} availability may be sufficiently depleted at the onset of stationary phase to result in increased CBA abundance relative to earlier time points. Alternatively, the observed CBA1 abundance increase with time could suggest that B_{12} demand changes as a function of growth stage in diatoms. This may in part be explained by the higher concentrations of the B_{12}-requiring MetH protein detected at the onset of stationary phase (Fig. 3) since higher MetH concentrations would result in more enzyme active sites that require population by B_{12}. However, it is unclear why diatoms would need enhanced methionine synthase capacity when cells are entering stationary phase. Methionine sinks include protein synthesis, which would presumably be decreasing towards stationary phase, as well as S-adenosyl methionine (SAM) and other metabolite production. Variability in phytoplankton cellular demand for SAM under different growth phases is not known, and similarly, the percentage of methionine production shunted to SAM synthesis vs. protein has not yet been measured in algae. Interestingly, human cancer cells, which are defined by their unchecked growth stage, have been found, in some cases, to be unable to convert homocysteine into methionine (the human genome contains only the B_{12}-requiring MetH methionine synthase isoform; Breillout et al. 1990). Additionally, B_{12} transport is elevated in tumor cells (Gupta and Jain 2008; Waibel et al. 2008); together these observations suggest that there may be relevant biological phenomena yet to be uncovered regarding eukaryotic cells and the interactions between their cellular growth phases and methionine and vitamin B_{12} nutrition.

The abundance of CBA1 also increases by an average of 2.5-fold post–B_{12} resupply, suggesting that CBA1 production responds not only to low B_{12} availability but also to pulses of high B_{12} availability. This is consistent with CBA1 having a functional role in B_{12} acquisition. Since B_{12} degrades relatively quickly in seawater due to photodegradation (Carlucci et al. 1969) and is also rapidly consumed by biota (Bertrand et al. 2011), phytoplankton cells likely experience selection pressure to acquire the vitamin quickly and respond efficiently upon sensing B_{12} availability. In addition, there is likely a considerable period of time required for cells to effectively up-regulate B_{12} acquisition machinery and then take up enough of the vitamin to satiate B_{12} quotas for growth using MetH. The increase in CBA1 24 h post-resupply of B_{12} may reflect this process. Increased CBA1 production under pulsed B_{12} addition may also be analogous to the production of iron ligands in surface seawater upon iron addition, which is thought to be a biological response to increase microbial access to the added iron (Rue and Bruland 1997).

Implications for cellular resource allocation and persistence of B_{12} use—Marine microbial eukaryotes have three theoretical scenario "choices" regarding B_{12} metabolism and methionine synthesis: (1) maintain B_{12}-requiring *metH* and B_{12} acquisition machinery in their genomes, making them B_{12} auxotrophs, as in *T. pseudonana*; (2) maintaining *metH*, *metE*, and B_{12} acquisition machinery in their genomes, allowing them the flexibility of growth without B_{12} while maintaining the capability for more efficient growth in the presence of the vitamin, as in *P. tricornutum*; or (3) removing *metH* and perhaps B_{12} use altogether by using only *metE* for methionine synthase activity, as is characteristic of terrestrial plants. In this last scenario, if MetH is the major use for B_{12} in these organisms, deletion of the *metH* gene from the genome could presumably allow for loss of the B_{12} acquisition system without negative consequence for the cell. Interestingly, this scenario No. 3 has yet to be observed in the > 10 available marine eukaryotic phytoplankton genomes sequenced thus far—they all possess the *metH* gene (Helliwell et al. 2011; Bertrand and Allen 2012), suggesting that there has not been sufficient selection pressure on these organisms to result in the discontinuation of B_{12} use altogether. This implies that there is a benefit, perhaps in terms of cellular resource or energy allocation, which may explain the continued use of vitamin B_{12} despite its low and variable concentrations in marine waters (Sañudo-Wilhelmy et al. 2012).

We can use the quantitative protein abundance measurements in this study to examine the resource conservation benefit of B_{12} utilization through estimating changes in cellular resource allocation under scarce and abundant B_{12} availability (Table 3). We begin by examining the resources devoted to the two methionine synthase isoforms, and later we will add the cost of B_{12} acquisition to the discussion. Maximal MetE enzyme concentrations in *P. tricornutum* were 60-fold higher than maximal MetH enzyme concentrations (Table 3). This is consistent with reports that the catalytic activity (k_{cat}) for MetE is $\sim$ 50–100-fold less than for MetH in *E. coli* (Taylor and Weissbach 1973; Taurog et al. 2006), and implies that much more MetE enzyme than MetH enzyme is required to produce comparable methionine synthase activity. Moreover, use of the MetE-isoform for methionine synthesis activity in *P. tricornutum* required 30 ± 9 times more nitrogen and 42 ± 5 times more zinc compared to the predominantly MetH-based methionine synthase activity found under high-B_{12} conditions (Table 3; Fig. 7). This implies that cellular metabolism is more efficient and elemental resources are conserved under conditions of ample B_{12} availability (based on the assumption that MetE and MetH each utilize 1 mol Zn mol^{-1} protein [Pejchal and Ludwig 2005]).

However, the significant efficiency advantages of using B_{12}-requiring MetH must be considered together with the added costs of maintaining B_{12} acquisition machinery. Before discussing this, we should point out that for our purposes here we assume that MetH comprises the entirety

Table 3. Estimation of the metabolic cost of low B_{12} availability compared to a hypothetical condition in *P. tricornutum* where the diatom does not use or acquire B_{12} (no MetH or CBA1 expression). Direct measurements of B_{12}-related protein concentration (via peptides bMetE1, dMetH1, and p48322_2) were used to calculate the percentage of protein nitrogen devoted to the production of these proteins. The metabolic cost of low B_{12} availability can be estimated by the increase in nitrogen expenditure required to substitute MetE for MetH and enhance CBA1 production. Since CBA1 is a secreted protein, the measured increase in N devotion to producing this protein is likely a lower bound. Percent protein nitrogen accounted for by MetE, MetH, and CBA1 production was estimated assuming that cellular protein is 17 ± 1 weight percent nitrogen (Sterner and Elser 2002). The fold increase in N allocation to B_{12} metabolisms is estimated by dividing the sum of N used for CBA1, MetH, and MetE in low-B_{12} conditions by their sum under replete conditions. The fold increase in N allocation to methionine synthase is estimated by dividing the sum of N used for MetE and MetE in low-B_{12} conditions by their sum under replete conditions. The estimate of fold-change in N allocation under the hypothetical no-B_{12} condition vs. B_{12}-replete condition and vs. the B_{12}-starved condition was estimated by dividing the N allocation to MetE under low B_{12} by the allocation of N to CBA1, MetH, and MetE under B_{12}-replete conditions and B_{12}-starved conditions, respectively.

	Replete fmol specific protein, μg^{-1} total protein	Low B_{12} fmol specific protein, μg^{-1} total protein	mol N mol^{-1} protein	Replete fmol N in specific protein, μg^{-1} total protein	Low B_{12} fmol N in specific protein, μg^{-1} total protein	Replete % protein N in specific protein	Low B_{12} % protein N in specific protein
MetE (28056)	0.025±0.009	3.25±0.723	1018	25.3±8.68	3310±735	0.00021±0.00007	0.0273±0.00628
MetH (23399)	0.054±0.018	0.010±0.007	1613	86.7±29.43	15.4±12.1	0.00071±0.00025	0.00013±0.00010
CBA1 (48322)	2.43±1.05	6.43±2.25	544	1330±569	3500±1220	0.0109±0.00474	0.0289±0.0102
					Sum CBA1, MetE, MetH	0.0119±0.00475	0.0563±0.0120
					Sum MetE, MetH	0.00092±0.00026	0.0275±0.0063
						Fold increase in CBA1 and MetSyn N use (Low B_{12} vs. Replete)	4.75±2.13
						Fold increase in MetSyn N use (Low B_{12} vs. Replete)	29.7±8.81
					No CBA1, MetH hypothetical diatom:	Fold increase in N use for MetE (Low B_{12}) vs. CBA1+MetH (Replete)	2.33±1.05
						Fold *decrease* in N use for MetE (Low B_{12}) vs. CBA1+MetE (Low B_{12})	2.06±0.63

of diatom B_{12} demand and that without it, the CBA1 B_{12} acquisition protein is no longer of utility. Our physiological results here suggest that, given the absence of a B_{12} requirement in *P. tricornutum*, this is a reasonable assumption (Fig. 1). However, another B_{12}-requiring enzyme is present in the *P. tricornutum* genome, methylmalonyl coA mutase, and hence it is conceivable that there may be growth conditions under which this enzyme imposes a B_{12} requirement; these are not considered here. Using this assumption, along with our quantitative protein measurements, we can examine the added costs of B_{12} acquisition. In Fig. 7 we show the sum of the components for nitrogen costs associated with the B_{12} acquisition protein CBA1 and both methionine synthase isoforms, under low and high B_{12} abundance. While this figure demonstrates that the large nitrogen savings in substituting the more efficient B_{12}-requiring methionine synthase described above, it also shows that this savings is offset, in part, by a large nitrogen cost associated with the CBA1 acquisition system, particularly when B_{12} becomes scarce. Given this, we can now consider the hypothetical scenario in which the diatom evolves to express only MetE and therefore does not express MetH or CBA1, regardless of vitamin availability. Using our protein measurements, we estimate that 2-fold more cellular nitrogen resources are needed for a hypothetical MetE-only diatom vs. the wild-type diatom under high-B_{12} conditions (scenario No. 3 above, Table 3; significantly different; $p = 0.03$). Interestingly, however, if we examine nitrogen use under conditions of B_{12} scarcity where in the wild-type diatom CBA1 increases 2.6-fold, the hypothetical diatom that only uses MetE (scenario No. 3) uses 2-fold less nitrogen relative to the wild-type B_{12}-utilizing diatom (significantly different; $p = 0.03$; Table 3). This implies that there is a gradient where, when the vitamin is sufficiently abundant, it is advantageous to retain use of MetH and CBA1, but as the vitamin becomes scarce, nitrogen resources required for a B_{12}-independent lifestyle (MetE only) eventually become less than those required for B_{12} acquisition.

However, when these costs are considered on a whole cell basis, the overall cellular protein nitrogen content savings under ample B_{12} availability is estimated to be quite low, 0.044% (Table 3: difference in sum of MetE, MetH, and CBA1 between low and high B_{12}). It is important to recognize, though, that nitrogen is not the only potentially limiting resource associated with this switch in metabolism. Zinc and carbon use will place additional costs on the scenario No. 3 B_{12}-independent lifestyle, the former of which can be particularly scarce in the upper water column of many marine environments (Bruland 1989; Lohan et al. 2002). Additionally, the higher growth rate observed in the B_{12}-utilizing relative to B_{12}-starved *P. tricornutum* (Fig. 1; Discussion above) implies that growth rates are higher due to the enhanced efficiency of the B_{12}-requiring MetH enzyme, which could be particularly important under the bloom conditions favored by most diatoms. We hypothesize that the combined resource allocation and enzyme efficiency benefits of using B_{12} may help explain the enhanced growth rate observed in non-auxotrophic diatoms under high B_{12} in this study (Fig. 1) and observed in previous studies (Swift and Guillard 1978). Moreover, given that growth rates are important drivers of ecological success, it seems plausible that the persistence of B_{12} utilization and MetH expression in marine eukaryotic microbes may be in part explained by the resource conservation benefits documented here, particularly under conditions of multiple resource scarcity (colimitation),

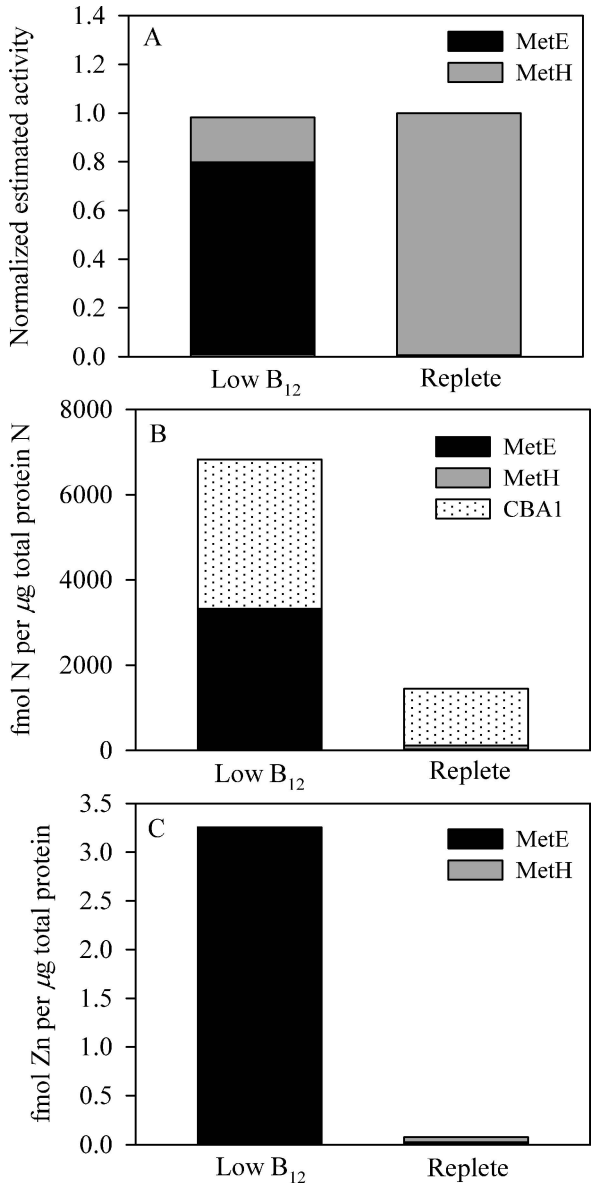

Fig. 7. (A) In order to achieve the same methionine synthase activity, diatoms require (B) more nitrogen and (C) zinc when B_{12} is scarce. Enzyme activity attributable to each MetE and MetH was estimated for each condition (replete vs. low B_{12}) using a ratio of MetH to MetE efficiency of 75 : 1 and then setting the total methionine synthase activity in the replete treatment equal to 1 (A). When comparing these efficiencies to total protein nitrogen and zinc allocated to each metabolism (B, C), it appears that methionine synthase activity is accomplished with a far smaller amount of nitrogen and zinc under B_{12}-replete conditions, suggesting that considerable resource use efficiency results from utilizing B_{12}.

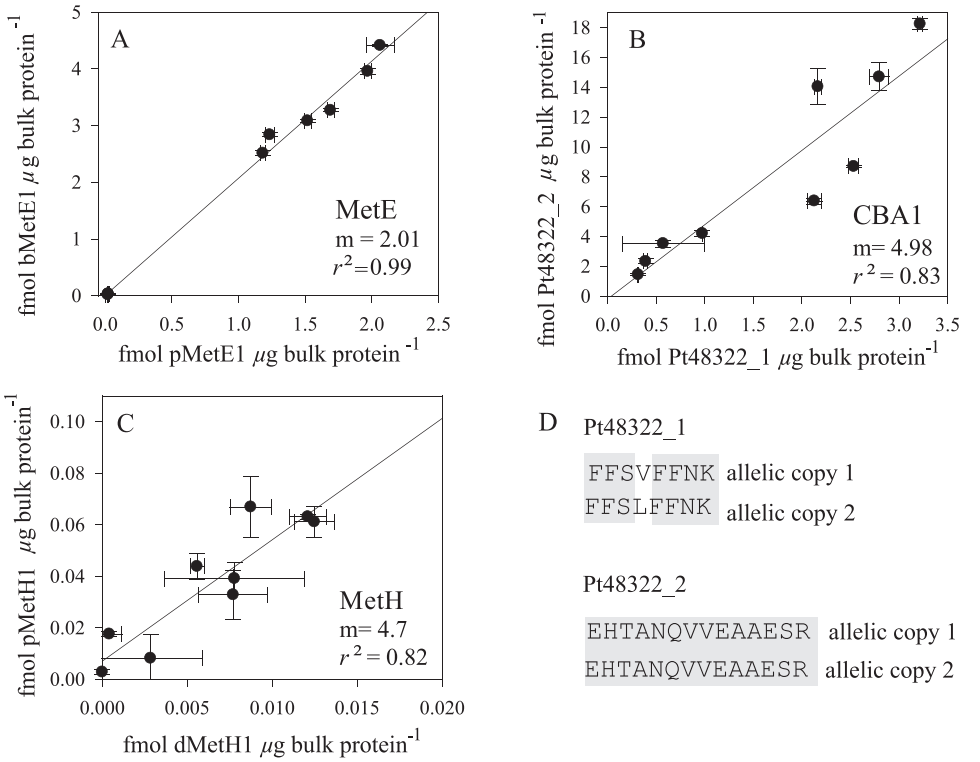

Fig. 8. Comparison of the abundance of peptides diagnostic of the same proteins. (A–C) The abundance of each peptide (means of technical triplicate measurements with error bars representing 1 standard deviation) is given vs. the abundance of a different peptide from the same protein in each *P. tricornutum* culture sample. Linear regressions are shown in the solid line and the coefficients of variance (r^2) and the slope (m) for each are given. The abundance of peptides measuring the same protein were linearly correlated, as expected. There are several possible explanations for the deviation of the slope from unity, some analytical and some biological. (D) One biological explanation for part of this slope variability is described, where one peptide used for these measurements is encoded by the CBA1 gene on only one of the diatom's chromosome copies, whereas the gene copy on the second copy of the chromosome encodes a peptide that differs by one neutral amino acid change (Bertrand et al. 2012). The second peptide is encoded by the genes on both chromosomes (allelic copies). Conserved amino acids are shaded in gray, and the differing amino acid is highlighted in white.

notably with nitrogen and zinc. While nitrogen and possibly zinc starvation are relevant ecological phenomena (Saito et al. 2008), future studies of fitness and resource allocation done under such starvation conditions are required to fully evaluate the import of B_{12} starvation under these scenarios. These results complement a recent study that suggests B_{12} utilization for MetH activity makes the green alga *Chlamydomonas reinhardtii* more resistant to heat stress than when the alga uses MetE for methionine synthase activity under B_{12} starvation (Xie et al. 2013). Together these results suggest that high B_{12} availability may, through multiple mechanisms, improve the ability of phytoplankton to respond to stressful conditions.

Our resource use calculations above suggest that conditions of *prolonged* B_{12} scarcity have the potential to create selection pressure to delete B_{12} utilization from genomes. However, as observed in marine eukaryotic phytoplankton genomes to date, the resource savings and kinetic advantages of B_{12}-dependent growth combined with current levels of marine B_{12} availability appears to exert pressure to retain B_{12} metabolism. Additional benefits of using B_{12}, including thermal stress tolerance (Xie et al. 2013), may also play a role in the maintenance of B_{12} use in phytoplankton. This is in apparent contrast to terrestrial plants, which are not known to utilize B_{12}-dependent enzymes. This difference between aquatic and terrestrial environments could be related to differences in availability of B_{12} in heterogeneous soils vs. advectively and diffusively mixed marine waters, though this requires further study.

Possible evidence for diatom allelic differential expression—The peptides measured as biomarkers for these proteins (MetE, MetH, and CBA1) all displayed exactly the same trends across treatments (Fig. 3), but had somewhat different peptide abundances. This is illustrated in Fig. 8 where the abundance of the two peptides diagnostic of each protein are plotted against each other. These measurements are strongly linearly correlated, as expected, but the slopes of the lines were not unity, which would be expected if these two peptides were measured in equal concentrations. Since these measurements were so tightly correlated, it is not likely that the deviation in slope

arose from variability in peptide extraction or digestion efficiency, as we expect variability induced by these processes to be more random. There are three possible explanations for the deviation of these slopes from unity; the first is analytical and the second two are biological.

One source of analytical error that could explain these trends comes from the use of isotopically labeled internal standard peptides for absolute quantitation. These peptides arrived from the manufacturer lyophilized and in discrete vials containing 1 nmol of the peptide. In order to use them for these measurements, they were resuspended and solubilized. If a peptide was not completely solubilized upon resuspension, this would result in overestimation of the abundance of the corresponding native peptide, but would still allow for linear standard curves, as shown in Fig. 2. Despite careful efforts in this study to completely dissolve each peptide, this is a possible partial explanation for some of the variability observed between the two peptides measured for each protein.

The second, biological, explanation for differences in peptide intensity is related to allelic variation. These diatom genomes are diploid, meaning there are two copies of each chromosome and thus two copies of each gene per cell. It is therefore possible that there is some sequence variability (typically single nucleotide polymorphisms) between two gene copies that can result in amino acid differences in peptides used for these measurements. This has been shown to be the case for protein CBA1 (Bertrand et al. 2012). One peptide is encoded by the CBA1 gene on only one of the diatom's chromosomes (Pt48322_1), whereas the second peptide (Pt48322_2) is encoded by both copies of the gene, as shown in Fig. 8D.

This scenario has not been tested for either the MetE or the MetH peptides, but it is notable that the more abundant, bMetE1, peptide is conserved among many organisms and comprises a portion of the THF binding region of the protein, suggesting that there is strong purifying selection pressure to maintain peptide conservation. In contrast, the less abundant peptide, pMetE1, is not conserved across organisms, suggesting that there is reduced selection pressure for peptide conservation between alleles. It is therefore possible that, as seen in CBA1, pMetE1 is measuring one variant of the protein and bMetE1 is measuring both.

The slope of the regression of CBA1 peptides is nearly 5 (Fig. 8), suggesting that one allelic copy is more highly expressed than the other. This is a phenomenon called allelic differential expression (ADE). ADE, allele dosage, and regulation are not well characterized in algae, but recent studies in humans and yeast estimate that 10–50% of genes show ADE (Gagneur et al. 2009). These peptide abundance measurements suggest that ADE may be an important phenomenon in algae and that SRM analysis offers a targeted analytical avenue through which ADE of specific proteins can be assessed. Additionally, it is possible that differentially detected peptides could be posttranslational modified with different functional groups, such as phosphate. This type of modification would prevent the peptide from being detected in our current analysis.

Making and interpreting peptide abundance measurements in natural diatom communities—Using culture-based protein abundance patterns to interpret field measurements: One of the major goals of this study was to develop a model of expected expression patterns of these three B_{12}-related proteins in environmental samples as a function of diatom vitamin nutritional status. To a first approximation, MetH presence indicates that B_{12} is likely being utilized for methionine regeneration since MetH expression occurs when absolutely B_{12}-requiring strains are active or when non-requiring strains are expressing MetH rather than MetE and are thus not B_{12} starved. High MetE and CBA1 abundance may be interpreted as indicating some level of starvation for B_{12} within diatom communities. However, the lack of MetE degradation upon B_{12} resupply to *P. tricornutum* is notable (Fig. 3) and suggests that the detection of MetE protein reflects immediate or recent B_{12} starvation. Given that the resupplied treatment was growing at a rate of $0.9 \pm 0.4\ d^{-1}$, measurements of MetE reflect the cell's status with respect to B_{12} nutrition over at least one doubling. The cultures went through approximately three doublings from the B_{12}-starved inoculum to the first time point where MetE concentrations were depleted in the B_{12}-replete cultures (Fig. 3). These data together suggest that high MetE expression reflects B_{12} starvation within 1–3 doublings in *P. tricornutum*. To better constrain what MetE measurements in the environment reflect about phytoplankton nutritional status, further inquiry is needed to determine the range in optimal B_{12} quotas for MetH use as well as the time span, relative to growth rate, in which different species can satiate B_{12} quotas and reflect that satiation in depression of MetE abundance.

It is likely that differences in diatom community structure will drive changes in CBA1 abundance since different diatom strains may produce different amounts of CBA1 in response to the same B_{12} levels. The variability in CBA1 abundance as a function of growth stage, B_{12} availability, and response to resupply in these cultures must also be carefully considered when interpreting protein expression patterns in field communities. Culture results suggest that CBA1 abundance is likely to remain high or even become increasingly elevated in a community for some period of time even after the B_{12} quota is satisfied. Calibrating this response for different diatom strains may aid in interpretation of field abundance patterns.

MetE and MetH peptide abundance patterns in McMurdo Sound—Peptides used for measuring these protein abundance levels in diatom cultures (dMetH1, bMetE1; Table 2) were applied to the study of B_{12} metabolism in field communities. These field measurements are interpreted here, based on currently available culture data, to assess the status of marine microbial communities with respect to vitamin B_{12} nutrition. The location chosen for initial analyses of the abundance of these peptides in the ocean was McMurdo Sound of the Ross Sea in the Southern Ocean, where B_{12} has previously been shown to influence phytoplankton growth (Bertrand et al. 2007). These data

can be reassessed in the future, if necessary, as additional culture data from polar isolates become available.

These peptides were detected and quantified in McMurdo Sound samples (Fig. 5). However, as shown in Fig. 6, measurements of bMetE peptide were problematic at one location. In order to accurately quantify bMetE in sample 402, the SRM reaction used to detect this peptide could be altered to exclude the contaminating peak *m/z* 927.489 from the measurement or the chromatography could be modified in order to separate the analyte and contaminant peaks. Alternately, a different peptide that is also diagnostic of MetE in diatoms could be measured. This highlights the importance of carefully examining the product ion distributions generated in SRM measurements, particularly in complex field samples, and also emphasizes the value of using stable isotope–labeled versions of these peptides as internal standards to verify both the retention times of analyte peptides and their product ion distribution.

The detection of MetH protein, most likely from diatoms, in McMurdo Sound biomass represents molecular-level confirmation of the use of vitamin B_{12} for methionine generation by these natural communities (Fig. 5A). It is striking that the concentration of this peptide in bulk protein from this region is severalfold greater than the concentrations measured in this culture study (Fig. 3), suggesting that MetH expression in Antarctic diatoms may be higher relative to these cultured, temperate diatoms. Since Antarctic diatoms are known to produce relatively large amounts of the osmolyte dimethylsulfoniopropionate (Krell et al. 2007), which is synthesized from methionine, potentially using SAM in subsequent steps (Gage et al. 1997), their metabolism may demand more methionine synthase activity.

The abundance of the bMetE1 peptide in McMurdo Sound samples (Fig. 6B) likely reflects the concentration of MetE in a subset of organisms, including diatoms (Table 3), that dominate this community (Fig. 5). It is unclear, however, whether measurements of this peptide as a proxy for MetE are inclusive of all diatom MetE functional diversity. bMetE1 abundance normalized to bulk protein is within the lower range of abundances measured in B_{12}-starved cultures and well above those measured in B_{12}-replete cultures. Presuming that members of the natural community in McMurdo Sound regulate MetH and MetE abundance similarly to *P. tricornutum*, these data are consistent with a portion of the > 3 μm community being starved for B_{12} within roughly three doubling times of the filtering event. Though this data set is small, it is intriguing that the abundance of bMetE1 is strongly negatively correlated with chlorophyll *a* (Chl *a*) concentration ($r^2 = 0.99$). This suggests that high MetE abundance, which appears to indicate B_{12} starvation, is found where biomass is low (Fig. 9). Though there are many other factors that influence photosynthetic biomass in this region, including iron supply, irradiance, and seasonal mixing (Martin et al. 1990; DiTullio and Smith 1996; Sedwick and DiTullio 1997), such a correlation is consistent with the idea that B_{12} availability may exert an influence on phytoplankton biomass production. Since these samples were taken from the water column

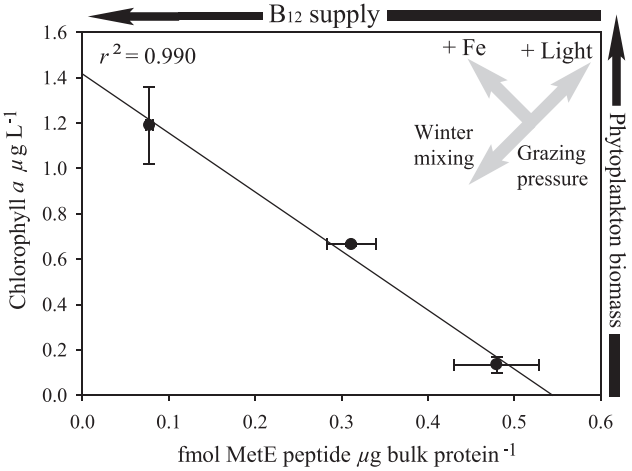

Fig. 9. There is a significant negative relationship between bMetE1 peptide abundance and chlorophyll concentrations. This trend is consistent with the suggestion that B_{12} starvation may influence the extent of photosynthetic biomass in this region since, in cultures, MetE appears to be abundant only when diatoms are starved for B_{12}. Other factors controlling phytoplankton growth in this region are known to include Fe supply, light and mixed-layer depth (winter mixing), and grazing pressure. These processes may also influence B_{12} supply (not measured here) since enhanced iron availability to bacteria may increase B_{12} availability (Bertrand et al. 2011), increased light may enhance B_{12} photodegradation (Carlucci et al. 1969), winter mixing could bring deliver B_{12} (Menzel and Spaeth 1962), and increased grazing pressure may release more B_{12} to the water column (*see* inset schematic; gray vectors depict how factor may influence phytoplankton biomass [schematic y-axis] and B_{12} availability [schematic x-axis]).

underneath sea ice, it is likely that light is also limiting phytoplankton growth, perhaps uniformly across these three samples since the trend between our indicator of B_{12} starvation and phytoplankton biomass is well correlated. Additional measurements of these proteins, particularly across the large dynamic range of chlorophyll concentrations in the Ross Sea, would be useful for more fully evaluating this trend and its relationship to other factors that affect photosynthetic biomass and vitamin B_{12} supply. Determining constraints on B_{12} production and persistence in the water column are of great import for future studies evaluating the role of phytoplankton B_{12} starvation. For instance, in this region, increased iron availability not only stimulates primary production but may also boost B_{12} supply by enhancing bacterial and archaeal growth (Bertrand et al. 2011).

Despite the apparent use of MetE by some diatoms in McMurdo Sound, the abundance of MetH suggests that some members of this community must have access to vitamin B_{12} in sufficient quantity to use the vitamin for methionine generation. This may be a reflection of the range of B_{12} quotas at which different diatoms become starved for the vitamin or may also suggest that there is a subset of diatoms with localized B_{12} sources, such as associated bacterial communities, which could promote MetH utilization. This further emphasizes the potential role of B_{12} in defining niche dimensions in marine microbial

communities. Notably, the field location where dMetH1 : bMetE1 ratios are highest (Sta. 400) has the greatest Chl *a* concentration (Fig. 5E,G). This is consistent with the community growing to higher densities when B_{12}-dependent MetH is used as the dominant mode of methionine generation, as seen here with *P. tricornutum*. This trend also supports the notion that growth under high B_{12} allows diatoms to function more efficiently, with reductions in resource devotion to methionine synthesis and regeneration under high B_{12} (Table 3). Future studies are required to further investigate the relative roles of MetE and MetH utilization in polar communities and their effect on primary productivity.

Based on the abundance profiles for protein CBA1 in cultured diatoms, this protein represents a promising target for quantitation, along with MetE and MetH, for assessing community metabolic status with respect to vitamin B_{12}. However, comparative analysis of CBA1 sequences from available diatom genomes reveals that the peptides used to measure CBA1 in diatom cultures in this study are not conserved across species, suggesting that it would be useful to measure these peptides only in regions where this cultured diatom species comprises a substantial portion of the community. Since *P. tricornutum* is a temperate species found in coastal systems (De Martino et al. 2007), peptide standards based on these genomes are not likely to be detectable in McMurdo Sound and were therefore not used for field sample analyses. Future work examining the diversity of CBA1 coding sequences from this region, via metagenomic and metatranscriptomic sequencing efforts, could enable identification of CBA1 peptide biomarkers that may be detectable and could yield insight into vitamin nutritional status of these diatom communities. Since CBA1 amino acid sequences are much more variable than those encoding MetE and MetH, multiple peptides would need to be measured via SRM mass spectrometry to assess total community CBA1 abundance. Alternatively, immunochemical approaches may be successful if a single antibody that reacts to a broad range of CBA1 peptides can be developed. However, making multiple mass spectrometry–based measurements of CBA1 peptides may illuminate controls on taxon-specific CBA1 expression in microbial communities, which could offer additional insight into the importance of B_{12} in controlling marine microbial community composition.

The data presented here suggest that the development of SRM-based peptide measurements for a range of proteins in environmental samples may prove useful for asking questions of biogeochemical relevance in a manner that is independent of the "bottle effects" that likely alter rate measurements and bioassays. One of the major benefits of SRM-based peptide analyses such as those described here is their ability to directly measure the abundance of multiple proteins simultaneously in field populations. Since proteins conduct reactions at theoretically predictable rates, these types of measurements may eventually allow for estimation of rates of biochemical reactions in the environment and could aid in the evaluation of roles of specific functional groups in key biogeochemical processes.

These lines of inquiry revealed distinct metalloenzyme interreplacement in diatom cultures that resulted in an approximately 30-fold increase in nitrogen and 40-fold increase in zinc allocated to methionine synthesis and regeneration under low B_{12} availability (Fig. 7). This metabolic cost of B_{12} starvation may help to explain the depression in *P. tricornutum* growth under low B_{12} (Fig. 1). Additional culturing studies under low-N and low-Zn conditions should be conducted to further assess the effect of these metabolic savings during resource scarcity and to improve our understanding of the factors responsible for maintaining the presence of the B_{12}-dependent methionine synthase isoform in eukaryotic phytoplankton. Increased efficiency of growth under B_{12}-replete conditions may also be reflected in enhanced Chl *a* concentrations in field locations with low MetE expression (Fig. 9). This correlation warrants further exploration as a potential indicator of the importance of B_{12} deprivation in Antarctic phytoplankton communities.

Acknowledgments

We thank Jeff McQuaid and Abigail Noble for field sampling efforts, and we acknowledge Chris Dupont, Cathy Drennan, Sonya Dyhrman, Dianne Newman, and Ben Van Mooy for helpful discussions. We thank two anonymous reviewers for their helpful insights. This work was supported by National Science Foundation (NSF) Antarctic Sciences awards 0732665, 1103503, and 0732822; NSF Division of Ocean Science awards 0752291, 0928414, and 1031271; The Gordon and Betty Moore Foundation; Center for Microbial Oceanography Research and Education; an NSF Graduate Research Fellowship (2007037200); and an Environmental Protection Agency Science To Achieve Results (EPA-STAR) Fellowship to E.M.B. (F6E720324).

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Associate editor: John Albert Raven

Received: 27 September 2012
Accepted: 25 March 2013
Amended: 07 April 2013