



# A New Analytical Method for Direct Measurement of Particulate and Dissolved B-vitamins and Their Congeners in Seawater

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We have developed a new method to directly measure the concentration of the particulate and dissolved pools of B-vitamins in seawater. B-vitamins are coenzymes required for life, yet many organisms cannot synthesize these compounds *de novo* and must scavenge them from the environment. It has been shown that B-vitamins can control the marine microbial community's structure and activity; however, the actual B-vitamin requirements/quotas for marine microbes are not well studied due to the lack of analytical protocols. This method will enable the study of B-vitamin cellular quotas as well as their environmental cycling. With this method, we can also simultaneously determine the biochemical congeners of vitamins B<sub>1</sub> and B<sub>12</sub>, as well as vitamins B<sub>2</sub>, B<sub>6</sub>, B<sub>7</sub>, and the amino acid methionine in both the particulate and dissolved pool, using liquid chromatography-mass spectrometry after a chemical extraction (particulate) or resin preconcentration (dissolved). Particulate and dissolved B-vitamin concentrations were simultaneously determined from a microbial community in the Atlantic Ocean. Particulate B-vitamin concentrations in the Atlantic ranged from 0.01 pM (cyano-B<sub>12</sub>) to 46.4 pM (thiamin monophosphate, a B<sub>1</sub> congener) while dissolved B-vitamin concentrations ranged from 0.07 pM (adenosyl-B<sub>12</sub>) to 679.4 pM (B<sub>7</sub>). The application of this technique in different marine systems has the potential to shed new light on previously unknown biochemical processes occurring within the oceanic microbial community, and the resulting roles B-vitamins play in regulating global biogeochemical cycles.

**Keywords:** B-vitamins, LC/MS, Biological Oceanography, Phytoplankton, Bacterioplankton

## INTRODUCTION

B-vitamins are chemically diverse, water soluble coenzymes that are essential for all forms of life (Berg et al., 2007). In the ocean there is a complex relationship between organisms that are capable of *de novo* B-vitamin synthesis and those that require these compounds, or their precursors, but are unable to synthesize them (Sañudo-Wilhelmy et al., 2014; Paerl et al., 2015). Despite the known B-vitamin requirements of many climate-relevant eukaryotic phytoplankton, including species of diatoms, dinoflagellates, and coccolithophores (Croft et al., 2006; Tang et al., 2010; Sañudo-Wilhelmy et al., 2014), dissolved concentrations of these compounds are remarkably low, ranging from undetectable to hundreds of picomoles per liter of water (Suárez-Suárez et al., 2011; Sañudo-Wilhelmy et al., 2012; Heal et al., 2014). It has been shown that these concentrations are in some cases too low to support maximal microbial production in many regions of the world ocean, and

that addition of B-vitamins can cause dramatic shifts in the microbial community structure and production (Panzeca et al., 2006; Sañudo-Wilhelmy et al., 2006; Gobler et al., 2007; Koch et al., 2011; Bertrand and Allen, 2012; Bertrand et al., 2012, 2013). While the importance of B-vitamins to phytoplankton has been well known for decades (Droop, 1962; Carlucci and Silberna, 1969; Provasoli and Carlucci, 1974; Pinto et al., 2002), never before have the particulate B-vitamins from the marine microbial community and dissolved B-vitamins from the surrounding water been simultaneously and directly measured. The lack of a technique to directly measure the particulate pool of B-vitamins, coupled with insufficient existing methods to measure multiple biochemical forms of some B-vitamins (congeners) in the dissolved pool, has hindered our understanding of their role in shaping marine microbial community composition as well as the processes controlling B-vitamin cycling between the dissolved and particulate pools in the ocean.

We have developed a method to directly determine the concentrations of particulate B-vitamins in marine systems. In addition, we describe the modification of an existing dissolved B-vitamin method to include important biochemical congeners of the B-vitamins (Suárez-Suárez et al., 2011; Sañudo-Wilhelmy et al., 2012; Heal et al., 2014). The utilization of our new analytical protocol will allow for a better understanding of B-vitamin distributions in the ocean, and the roles they play in mediating global primary productivity and microbial species succession. The main focus of our method is to measure multiple biochemically relevant congeners of vitamins B<sub>1</sub> and B<sub>12</sub> which exhibit significant levels of auxotrophy (obligate requirement) among eukaryotic phytoplankton (Croft et al., 2006; Tang et al., 2010; Sañudo-Wilhelmy et al., 2014). This method quantifies three forms of thiamin: unphosphorylated thiamin (B<sub>1</sub> hereafter), thiamin monophosphate (TMP), thiamin pyrophosphate (TPP); a thiamin precursor, the pyrimidine moiety (4-amino-2-methyl-5-pyrimidinyl methanol, HMP); biotin (B<sub>7</sub>); and four forms of cobalamin (B<sub>12</sub>), adenosylcobalamin (AB<sub>12</sub>), cyanocobalamin (CB<sub>12</sub>), hydroxycobalamin (HB<sub>12</sub>), and methylcobalamin (MB<sub>12</sub>). In addition to the primary target analytes listed above, this method can be used to quantify riboflavin (B<sub>2</sub>), pyridoxal (B<sub>6</sub>), and the amino acid methionine (Met). Taken together, these analytes represent different active forms and precursors of an important subset of B-vitamins, which have essential biochemical roles in central and secondary metabolic pathways across all domains of life (Begley et al., 1999; Brown, 2005; Waldrop et al., 2012). This method simultaneously measures the particulate and dissolved pools of chemically and functionally diverse B-vitamins that are commonly required by marine microbes, thus enabling novel insights into the ways that B-vitamins impact marine microbial community structure and production.

## METHODS

### Culture Sample Collection, Processing, and Preservation

Cultures of *Synechococcus* strain CC9311 and *Vibrio* strain AND4 were used to optimize the particulate extraction protocol.

CC9311 was grown in vitamin free SN media under 24 h illumination (100  $\mu$ E) at 20°C (Waterbury et al., 1986). AND4 was grown in Zobell media at room temperature (Gómez-Consarnau et al., 2010). Cultures were grown under axenic conditions however we did not formally test for axenicity at the end of the experiment since these cultures were only used for a method development and not for a physiological study. Cells were collected in late exponential/early stationary growth phase via gentle vacuum filtration onto 25 mm diameter, 0.2  $\mu$ m pore size Supor filters (Pall Biosciences). Ten milliliters of cell culture was filtered. Cellular density was assessed using direct enumeration with epifluorescence microscopy (Hobbie et al., 1977; Waterbury et al., 1986). Filters were placed in 2 ml cryovials (Nalgene) and immediately frozen and stored at -80°C until particulate vitamin quantification.

### Environmental Sample Collection, Processing, and Preservation

Environmental samples used for verification of both the particulate and dissolved methods were collected from two locations: the San Pedro Ocean Time series (SPOT) station (33°33'N, 118°24'W, **Figure S1A**) off the coast of Southern California in March 2015, and a station in the Eastern Atlantic Ocean (33°1'16"N, 12°14'28"W, **Figure S1B**) in May of 2014. Samples were collected from 3 m at SPOT and from four depths in the Atlantic: 3 m, 50 m, 85m (deep chlorophyll maximum), and 200 m. All samples were filtered using a peristaltic filtration system. The peristaltic filtration rig was cleaned between samples with methanol to remove any residual B-vitamins. Prior to use, the rig was rinsed thoroughly with Milli-Q water followed by sample seawater (Sañudo-Wilhelmy et al., 2012). Particulate samples were screened through a 10  $\mu$ m mesh and filtered onto 0.2  $\mu$ m Supor filters. After filtration was completed, the filters were frozen and stored as described for the cultured samples. Particulate organic carbon (POC) samples were collected in parallel with environmental particulate B-vitamin samples in order to allow normalization of the particulate B-vitamin concentrations. They were filtered on precombusted GF/F filters under low vacuum pressure, desiccated at 60°C, and analyzed using a CHN elemental analyzer (Knap et al., 1996). The filtrate resulting from the particulate fraction filtration was collected for the dissolved B-vitamin analysis in 1L amber HDPE bottles and stored at -20°C until analysis (Okbami and Sañudo-Wilhelmy, 2004, 2005; Sañudo-Wilhelmy et al., 2012).

### Particulate B-Vitamin Extraction

Frozen filters with samples collected from both cultures and the environmental samples were aseptically transferred from the cryovials into acid-washed, methanol-rinsed, 15 ml heavy-walled polypropylene conical centrifuge tubes, containing 4 ml of cold lysis solution with 2 ml of 0.5 mm zirconia/silica beads (Biospec). The lysis solution consisted of 5% methanol, 95% LC/MS-grade water solution adjusted to pH 3.5 using hydrochloric acid. The ratio of extraction solution to beads was 2:1 v/v solution:beads. The exact volume of the lysis solution was determined using an analytical balance. Cells were lysed via bead beating by

vortexing on high for 5 min followed by 1 min in an ice bath to ensure that the temperature of the solution did not exceed 30°C. This process was repeated 6 times, for a total of 30 min of bead beating to achieve complete cellular lysis, which was determined by epifluorescence microscopy. Following cell lysis, the solution was incubated for 30 min in the dark at 30°C to extract the target analytes from the cellular matrix. Organic-aqueous liquid phase extractions (LPE) were used to remove hydrophobic cellular components from the cellular lysate, while simultaneously purifying the hydrophilic target analytes. Chloroform (1:1 v/v) was added to each tube, and the tubes were vigorously shaken for 3 min causing the hydrophobic cellular components to partition into the chloroform. The tubes were then centrifuged at 5000 rpm for 10 min to achieve complete phase separation, and to pelletize large cellular debris (Mashego et al., 2007; Roberts et al., 2012). The upper aqueous phase containing the B-vitamins was transferred to a new tube, and the chloroform extraction process was repeated twice. Following the liquid phase extraction, samples were adjusted to pH 6.5 by adding dilute NaOH. Sample volume was assessed before and after addition of NaOH using an analytical balance in order to correct concentrations for the added volume. Samples were centrifuged for 10 min at 5000 rpm and then syringe push filtered through methanol-rinsed 0.2  $\mu\text{m}$  Minisart RC4 matrix filters (Sartorius Stedim Biotech).

## Dissolved Sample Preconcentration

Samples for dissolved vitamin analysis were preconcentrated as it has been previously described (Okbami and Sañudo-Wilhelmy, 2004, 2005; Sañudo-Wilhelmy et al., 2012). Briefly, preconcentration columns were prepared by pouring 1:1 MeOH:C<sub>18</sub> resin [HF Bondesil (Agilent Technologies)] slurry into Poly-Prep Columns (Biorad). The resin was allowed to settle, and the excess MeOH was drained leaving 7 ml of resin. Thawed samples were adjusted to pH 6.5 using dilute HCl and passed over the preconcentration column at 1 ml per min. Samples were then adjusted to pH 2.0 using dilute HCl and were passed over the preconcentration column a second time. The residual salt was rinsed off the resin using 30 ml of LC/MS grade water. The target analytes were then eluted from the resin using 12 ml of LC/MS grade methanol into methanol-rinsed 15 ml conical centrifuge tubes. Eluted samples were then further concentrated via evaporation in a nitrogen dryer using compressed N<sub>2</sub> gas, in the dark, at room temperature. Gas pressure was initially set at 5PSI and was slowly increased to 20PSI until 300  $\mu\text{l}$  of sample remained. The samples were then stored at -20°C until LC/MS analysis which occurred within 24 h. Prior to LC/MS analysis samples were adjusted to pH 6.5 using 10  $\mu\text{l}$  of dilute NaOH. Care was taken to measure the mass of the sample before and after addition of the base and used to correct the final concentrations. Two different pH papers were used to assess pH after the final mass had been recorded. This is possible because the initial pH of each sample is known (e.g., pH 2.0 after preconcentration, pH 3.5 for the particulate samples) so the amount of base to be added can be calculated. Samples were centrifuged and push filtered as described above. Previous tests confirm that no vitamins adsorb onto the Minisart filters used for push filtering.

## LC/MS Analysis

Quantification of B<sub>1</sub>, TMP, TPP, HMP, B<sub>2</sub>, B<sub>6</sub>, B<sub>7</sub>, AB<sub>12</sub>, CB<sub>12</sub>, HB<sub>12</sub>, MB<sub>12</sub>, and Met was carried out with a Thermo TSQ Quantum Access electrospray ionization triple quadrupole mass spectrometer, coupled to a Thermo Accela High Speed Liquid Chromatography (LC/MS) system. The LC system used a stable-bond C<sub>18</sub> reversed-phase column (Discovery HS C<sub>18</sub> 10 cm  $\times$  2.1 mm, 5  $\mu\text{m}$  column, Supelco Analytical) with a 100  $\mu\text{L}$  sample loop. In order to increase the sensitivity and precision of our analysis, the LC/MS was run in full-loop mode (100  $\mu\text{l}$ /injection). The computer software Xcalibur and LCQuan™ (Thermo Scientific) were used for data acquisition and analysis. A 12-min gradient flow was used with mobile phases of methanol (MeOH) and LC/MS grade water, both buffered to pH 4 with 0.5% acetic acid. The flow rate was set at 230  $\mu\text{l}/\text{min}$  throughout the run, with a gradient starting at 93% LC/MS water: 7% MeOH for 2 min, changing to 100% MeOH by 7 min, and continuing at 100% MeOH until 9 min and returning to initial conditions until the gradient completes at 12 min. All peaks were identified using standards dissolved in LC/MS grade water; retention times are listed in **Table 1** and depicted in **Figure S2**. The mass spectrometer was run in Selected Reaction Monitoring (SRM) mode with positive polarity with a well time of 100 ms per transition. The resolution of the mass filters used for quadrupoles 1 and 2 were 0.7 and 0.1  $m/z$ , respectively. The ESI spray voltage was 4000V, sheath gas (N<sub>2</sub>) pressure was 30PSI, auxiliary gas (Ar) pressure was 3PSI, capillary temperature was 269°C, and the collision pressure was 2.1torr. The  $m/z$  of the analytes and their respective product ions, as well as the analyte specific collision energy and T-lens settings are listed in **Table 1**. The instrumental limits of detection (LOD; **Table 2**) were calculated as three times the standard deviation of the procedural blank (if no signal was detectable in the analytical blank, then they were calculated as three times the standard deviation of the lowest detectable standard). The resulting data was analyzed using the software environment R, and all figures were created using the Ggplot2 software package for R (Wickham, 2009; R Core Team, 2015).

## RESULTS

### Particulate Method Validation

The particulate B-vitamin extraction method was initially optimized using cultures of *Synechococcus* str. CC9311 and exclusively focusing on vitamins B<sub>1</sub>, B<sub>7</sub>, and CB<sub>12</sub>. These analytes were chosen due to the widespread auxotrophy for these compounds in marine microbial systems (Sañudo-Wilhelmy et al., 2014). As a starting point we used the vitamin extraction protocol described by Carlucci and Bowes (1972) (100°C incubation for 1 h in seawater adjusted to pH 3.5), however this protocol resulted in vitamin degradation as our spike recoveries were below 10%. In order to increase those recoveries, extractions were conducted with iteratively milder (both temperature and pH) conditions until it was found that bead beating in a lysis solution composed of 5% LC/MS grade methanol and 95% LC/MS grade water adjusted to pH 3.5 did not degrade any of our analytes, while also still achieving complete cellular lysis (determined by epifluorescence microscopy). This resulted in

**TABLE 1 | Analytical LC/MS parameters for all measured analytes.**

Analyte	SRM parent-product m/z	Collision energies (V)	T-lens	Column retention time (min)	Relative retention time (min)	IS used	RF
Thiamin	265 - 81, 122, 144	15, 15, 14	70	1.4	0	B1	1.00
Thiamin monophosphate	345 - 122, 126, 224	17, 29, 35	86	1.4	0	B1	0.49
Thiamin pyrophosphate	425 - 122, 126, 304	21, 34, 16	78	1.7	0.3	B1	3.94
Pyrimidine moiety	139 - 122, 81, 54	11, 18, 31	60	1.7	0.3	B1	2.04
Riboflavin	377 - 172, 197, 242	34, 37, 22	87	8.0	0	B2	1.00
Biotin	245 - 97, 123, 227	30, 26, 13	87	7.4	-0.6	B2	0.03
Adenosylcobalamin	791 - 147, 665	38, 20	98	7.7	-0.3	B2	0.61
Cyanocobalamin	678 - 147, 359, 997	40, 26, 40	91	7.4	-0.6	B2	0.02
Hydroxycobalamin	665 - 147, 359, 636	33, 17, 14	91	7.5	-0.5	B2	0.07
Methylcobalamin	673 - 147, 359, 665	47, 17, 19	92	7.9	-0.1	B2	0.31
Methionine	150 - 56, 61, 104	10	74	2.3	0.9	B1	0.96
Thiamin IS	269 - 81, 122, 144	10	42	1.4			
Riboflavin IS	383 - 174, 202, 249	35, 36, 22	176	8.0			

Relative retention time is the time the peak appears relative to the internal standard used for quantification (e.g., the TPP peak is 0.3 min after the thiamin internal standard peak).

**TABLE 2 | Analyte spike recoveries and instrumental limits of detection (L.O.D.).**

Analyte	% Particulate spike recovery	% Dissolved spike recovery	L.O.D. (nM)
Thiamin	98.6 ± 10.4	99.9 ± 16.2	5.6
Thiamin monophosphate	99.1 ± 7.6	89.7 ± 19.3	20.7
Thiamin pyrophosphate	94.1 ± 30.9	109.9 ± 36.9	149.7
Pyrimidine moiety	94.2 ± 7.1	83.9 ± 32.4	32.1
Riboflavin	100.9 ± 1.8	NA	3.6
Pyridoxal	96.9 ± 8.3	NA	9.9
Biotin	92.4 ± 10.1	92.6 ± 38	5.8
Adenosylcobalamin	99.1 ± 4.7	100.6 ± 16.4	6.1
Cyanocobalamin	99.1 ± 7.2	96.0 ± 18.5	1.3
Hydroxycobalamin	91.8 ± 7.6	109.7 ± 27.1	2.5
Methylcobalamin	94.5 ± 14.2	91.1 ± 37.5	3.4
Methionine	88.6 ± 15.2	NA	7.2

The L.O.D. was calculated as three times the standard deviation of the blank, and when no signal was present in the blank, three times the standard deviation of the lowest detectable concentration of standards was used. Procedural limits of detection depend on the preconcentration volume, extraction volume, or the volume of sample filtered, which vary between each sample. They range from 3 to 6 orders of magnitude lower than the instrumental L.O.D.

greater than 90% spike recoveries for our three targeted analytes (Table 2). Initially, a second extraction of the filter was conducted to ensure complete extraction, and no signal was observed on this subsequent extraction. Based on the high spike recoveries obtained for the targeted analytes, our analytical scope was then successfully expanded to include all analytes listed in Table 2. In addition to those analytes, we attempted to analyze a chemical form of thiazole (C<sub>3</sub>H<sub>3</sub>NS), a B<sub>1</sub> precursor, however our spike recoveries were only 60%, and a signal was only occasionally observed within the particulate pool. It is unclear if our method could be used to measure pseudocobalamins (Tanioka et al.,

2009; Helliwell et al., 2016) and other vitamin precursors such as the B<sub>1</sub> precursor 4-methyl-5-thiazoleethanol (Paerl et al., 2015). We focused on analytes that had standards readily commercially available at the time of method development, and limited our analytical scope to preserve analytical sensitivity and precision. Future studies, potentially focusing solely on one class of analytes (e.g., cobalamins and pseudocobalamins) will be needed to evaluate these, and other, B-vitamin congeners and precursors with sufficient sensitivity and precision. However, the high recoveries of the added analytes shown in Table 2 indicate that this extraction method can likely be expanded to a wide range of chemically diverse metabolites.

When *Synechococcus* biomass was initially extracted and analyzed, it became apparent that sample matrix suppression was occurring as evidenced by low recoveries, poor peak shape, and a substantial increase in LC/MS background noise. Both liquid phase extractions (LPE) and solid phase extractions (SPE) were tested to reduce the analytical signal to noise ratio by purifying our analytes of interest from the total cellular extract. The SPE used C<sub>18</sub> resin, and resulted in full recoveries of our analytes, but did not satisfactorily reduce the background noise. The LPE using chloroform (1:1 v/v) did not degrade our analytes, and no vitamins were observed to partition into the chloroform. The liquid phase extraction effectively reduced the matrix suppression problem, allowing for clear quantification of our target analytes.

To our knowledge, there is no standard reference material for particulate B-vitamins in bacterioplankton and/or phytoplankton. Therefore, full particulate B-vitamin extraction must be inferred from determining that filters and media used do not have a B-vitamin blank, observing complete cellular lysis, determining the effectiveness of our lysis solution in separating B-vitamins from the cellular matrix, and testing that our extraction procedure does not degrade B-vitamins. No B-vitamin blank was observed on the Supor filters, and no B-vitamins from the media used were observed to adsorb

onto the filters. Complete cellular lysis was observed via epifluorescence microscopy. Our lysis solution is similar to previously published particulate extraction solutions that have been determined to result in full extraction of metabolites from the cellular matrix (Roberts et al., 2012). We tested the relative accuracy and precision of our extraction procedure by adding known concentrations of each vitamin congener to samples containing filters with a known amount of *Synechococcus* CC9311 cells, and by extracting as described above. A parallel sample with only CC9311 filtered cells was also analyzed. Standard recoveries were calculated by subtracting the observed CC9311 vitamin concentration from the vitamins observed in the standards + CC9311 sample. These values were compared to extractions of known concentrations of vitamin standards without CC9311 cells, and no significant differences in recovery efficiency were observed, indicating that the addition of cellular organic compounds does not interfere with our analysis. Spike recoveries (listed in Table 2) were >88% for all analytes.

### Dissolved Method Validation

In order to fully complement the newly developed particulate B-vitamin method described above, we expanded the previously established dissolved B-vitamin method to include TMP, TPP, HMP, AB<sub>12</sub>, HB<sub>12</sub>, and MB<sub>12</sub>. In order to test the recoveries of these additional analytes, known concentrations of standards were added to vitamin free seawater and were preconcentrated using C<sub>18</sub> resin at two pHs (6.5 and 2.0) as it has been described previously (Okbami and Sañudo-Wilhelmy, 2004, 2005; Sañudo-Wilhelmy et al., 2012). After LC/MS analysis, recoveries were determined to be >83% for all newly added analytes (Table 2). We attempted to include the B<sub>1</sub> precursor thiazole in the dissolved method, however we observed that it did not successfully bind to our preconcentration resin.

### Internal Standard

Two internal standards (IS) were used for sample quantification. Stable isotopically labeled B<sub>1</sub> (<sup>13</sup>C, <sup>15</sup>N, mass shift +4, Sigma-Aldrich) was used to quantify aqueous phase eluting analytes (B<sub>1</sub>, TMP, TPP, HMP, MET), and stable isotopically labeled B<sub>2</sub> (<sup>13</sup>C, <sup>15</sup>N, mass shift +6, Sigma-Aldrich) was used to quantify organic phase eluting analytes (B<sub>2</sub>, B<sub>6</sub>, B<sub>7</sub>, AB<sub>12</sub>, CB<sub>12</sub>, HB<sub>12</sub>, MB<sub>12</sub>). The relationship between each analyte and the IS used for quantification was empirically determined by calculating a response factor (RF) for each of these molecules (Table 1, Equations 1, 2 in Supplementary Material) (Bohn and Walczyk, 2004). Quantification using the IS and RF values was validated by analyzing known concentrations of standards dissolved in LC/MS-grade water, and comparing concentrations calculated using the IS/RF versus external calibration curves. During sample analysis external calibration curves containing both target analytes and internal standards were run daily and used to verify the RF and to correct for inter-day instrument variability/drift. IS intraday variability ranged between 6–25% and 4–14% for B<sub>1</sub>-IS and B<sub>2</sub>-IS respectively; interday variability was 15 and 9% for B<sub>1</sub>-IS and B<sub>2</sub>-IS respectively. The IS increased our

analytical precision, and allowed for the correction of matrix suppression effects which are known to be common (Heal et al., 2014).

## DISCUSSION

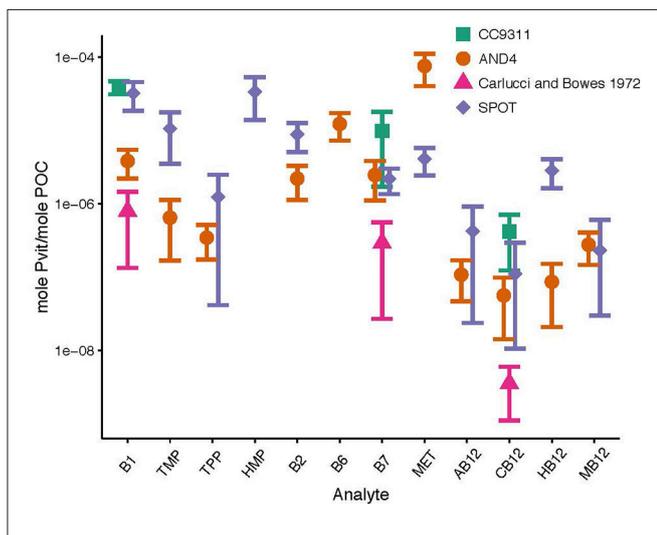
Particulate B-vitamins were extracted from cultures of two species of marine bacteria of differing metabolic lifestyles: *Synechococcus* strain CC9311 (photoautotroph), *Vibrio* strain AND4 (heterotroph). The normalized concentration (mole analyte mole POC<sup>-1</sup>) observed in CC9311 for B<sub>1</sub>, B<sub>7</sub>, and CB<sub>12</sub> were  $3.9 \times 10^{-5} \pm 7.8 \times 10^{-6}$ ,  $9.9 \times 10^{-6} \pm 8.1 \times 10^{-6}$  and  $4.2 \times 10^{-7} \pm 2.9 \times 10^{-7}$ , respectively (Table 3, Figure 1). The concentrations of these compounds in AND4 were lower than those measured in CC9311, with the differences of the means of B<sub>1</sub>, B<sub>7</sub>, and CB<sub>12</sub> concentrations equaling  $3.5 \times 10^{-5}$ ,  $7.4 \times 10^{-6}$ , and  $3.6 \times 10^{-7}$ , respectively (Table 3, Figure 1). Recent research has suggested that cyanobacteria, including CC9311, solely have the ability to synthesize and utilize pseudocobalamins, which potentially makes our observed presence of CB<sub>12</sub> within CC9311 anomalous (Tanioka et al., 2009; Helliwell et al., 2016). However, we do not believe that these findings run contrary to our observations as our cultures were not tested for axenicity at the endpoint of our experiment, and contamination with heterotrophic bacteria could have occurred. In addition, the ability of CC9311 to remodel pseudocobalamin is not well understood. We hypothesize that the observed differences in the particulate concentration of each vitamin among species, which can span several orders of magnitude, could potentially be associated with differing metabolic rates or B-vitamin requirements between the species. B-vitamins are intrinsically linked with metabolic processes, so a more metabolically active organism may require higher concentrations of particulate B-vitamins (Banerjee, 1997; Begley et al., 1999; Waldrop et al., 2012; Sañudo-Wilhelmy et al., 2014). However, we did not assess growth rate or end-point axenicity in our cultures, so we cannot definitively test this hypothesis using our data. Further studies using cultures and tightly constrained growth rates are necessary to elucidate particulate B-vitamin requirements.

In order to evaluate the particulate pool of B-vitamins in environmental samples, we collected seawater from two contrasting ocean systems. Sampling occurred at SPOT and at an oceanic station in the subtropical Eastern Atlantic Ocean (see Experimental Sample Collection section). The observed particulate concentrations (pico mole vitamin per liter of seawater, pM) at 3 m at SPOT were  $481 \pm 204$  B<sub>1</sub>,  $156 \pm 103$  TMP,  $17.6 \pm 16.8$  TPP,  $40.8 \pm 14.8$  HMP,  $129 \pm 45.7$  B<sub>2</sub>,  $31.9 \pm 9.87$  B<sub>7</sub>,  $5.82 \pm 6.53$  AB<sub>12</sub>,  $1.54 \pm 2.46$  CB<sub>12</sub>,  $40.8 \pm 14.8$  HB<sub>12</sub>,  $3.14 \pm 5.02$  MB<sub>12</sub>, and  $62.1 \pm 30.1$  Met. At the Atlantic Station, particulate concentrations (pM) in the mixed layer ranged from 3.53–44.4 B<sub>1</sub>, 5.64–46.5 TMP, 1.13–7.85 TPP, 5.91–45.56 HMP, 1.68–8.55 B<sub>7</sub>, 0.52–4.53 AB<sub>12</sub>, undetectable–1.21 CB<sub>12</sub>, 1.21–9.11 HB<sub>12</sub>, 0.19–2.46 MB<sub>12</sub>, and 1.97–16.11 Met (Figure 2, Table 4). SPOT was more eutrophic during our sampling with a mean observed POC concentration of 16.4 μM, whereas POC was observed to range between 1 and 4.5 μM throughout the mixed layer at the

**TABLE 3 | Particulate B-vitamin concentrations obtained from cultures and environmental samples collected at SPOT.**

	<i>Vibrio</i> str. AND4		<i>Synechococcus</i> str. CC9311		SPOT surface	
	Mean	SD	Mean	SD	Mean	SD
Thiamin	3.83E-06	1.63E-06	3.90E-05	7.81E-06	3.23E-05	1.37E-05
Thiamin monophosphate	6.47E-07	4.79E-07			1.06E-05	7.09E-06
Thiamin pyrophosphate	3.45E-07	1.72E-07			1.24E-06	1.26E-06
Pyrimidine moiety					3.37E-05	1.98E-05
Riboflavin	2.21E-06	1.08E-06			8.84E-06	3.74E-06
Pyridoxal	1.23E-05	5.01E-06				
Biotin	2.47E-06	1.36E-06	9.89E-06	8.18E-06	2.19E-06	8.32E-07
Adenosylcobalamin	1.08E-07	6.15E-08			4.24E-07	4.94E-07
Cyanocobalamin	5.62E-08	4.20E-08	4.18E-07	2.95E-07	1.11E-07	1.84E-07
Hydroxycobalamin	8.62E-08	6.54E-08			2.84E-06	1.21E-06
Methylcobalamin	2.77E-07	1.30E-07			2.30E-07	3.76E-07
Methionine	7.59E-05	3.56E-05			4.11E-06	1.68E-06

Values were normalized to POC (mole particulate vitamin/mole POC). Blank cells indicate that the analyte was not included in the LC/MS analysis. Conversion factors of 20fgC/cell and 200fgC/cell were used for AND4 and CC9311 respectively. The POC concentration was measured at SPOT (16.4 μM).

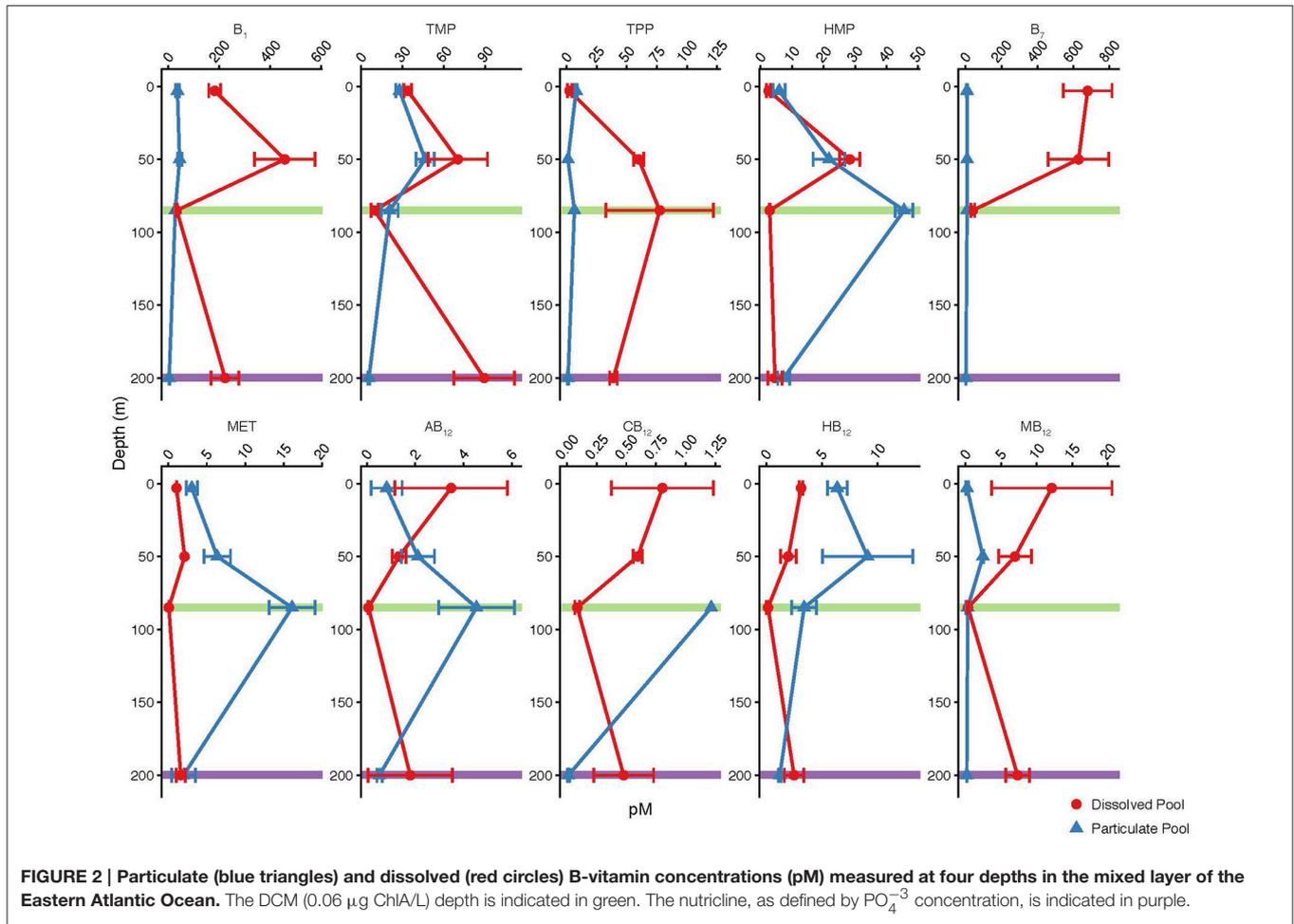


**FIGURE 1 | Particulate B-vitamin concentrations from cultures and the environment.** Samples were analyzed from cultures of *Synechococcus* str. CC9311 (green squares), cultures of *Vibrio* str. AND4 (orange circles), cultures of marine eukaryotic phytoplankton from Carlucci and Bowes (1972) (pink triangles), and samples from the surface ocean at the San Pedro Ocean Timeseries (purple diamonds). All concentrations were normalized to cellular carbon. Conversion factors of 20fgC/cell and 200fgC/cell were used for AND4 and CC9311, respectively. POC was measured at SPOT (16.4 μM).

Atlantic station (data not shown). Normalization using the POC data allowed us to take into account the density of biomass at each location. The normalized particulate B-vitamin stoichiometric molar ratios (mole particulate vitamin: mole particulate organic carbon) observed at SPOT (3 m) in March 2015 were:  $3.2 \times 10^{-5} \pm 1.4 \times 10^{-5}$  B<sub>1</sub>:  $1.1 \times 10^{-5} \pm 7.1 \times 10^{-6}$  TMP:  $1.2 \times 10^{-6} \pm 1.3 \times 10^{-6}$  TPP:  $3.4 \times 10^{-5} \pm 1.9 \times 10^{-5}$  HMP:  $8.8 \times 10^{-6} \pm 3.7 \times 10^{-6}$  B<sub>2</sub>:  $2.2 \times 10^{-6} \pm 8.3 \times 10^{-7}$  B<sub>7</sub>:  $4.2 \times 10^{-7} \pm 4.9 \times 10^{-7}$  AB<sub>12</sub>:  $1.1 \times 10^{-7} \pm 1.8 \times 10^{-7}$  CB<sub>12</sub>: 2.8

$\times 10^{-6} \pm 1.2 \times 10^{-6}$  HB<sub>12</sub>:  $2.3 \times 10^{-7} \pm 3.7 \times 10^{-7}$  MB<sub>12</sub>:  $4.1 \times 10^{-6} \pm 1.7 \times 10^{-6}$  Met: 1POC (Table 3, Figure 1). The observed particulate B-vitamin to POC molar ratios observed at 3 m in the Atlantic were  $8.1 \times 10^{-6} \pm 1.6 \times 10^{-6}$  B<sub>1</sub>:  $6.2 \times 10^{-6} \pm 5.9 \times 10^{-7}$  TMP:  $1.8 \times 10^{-6} \pm 2.3 \times 10^{-7}$  TPP:  $1.3 \times 10^{-6} \pm 4.2 \times 10^{-7}$  HMP:  $4.2 \times 10^{-6} \pm 3.3 \times 10^{-7}$  B<sub>2</sub>:  $1.8 \times 10^{-6} \pm 6.7 \times 10^{-7}$  B<sub>7</sub>:  $1.8 \times 10^{-7} \pm 1.4 \times 10^{-7}$  AB<sub>12</sub>:  $1.4 \times 10^{-6} \pm 2.0 \times 10^{-7}$  HB<sub>12</sub>:  $5.2 \times 10^{-8} \pm 3.8 \times 10^{-8}$  MB<sub>12</sub>:  $6.8 \times 10^{-7} \pm 1.6 \times 10^{-7}$  Met: 1POC. The observed particulate vitamin concentrations (pM), and the POC normalized values (mole particulate vitamin: mole POC) at 3 m in the Atlantic were lower by as much as two orders of magnitude than those observed at 3 m at SPOT for each analyte, likely because the Atlantic station was much more oligotrophic than SPOT. It is important to note that since a prefilter was used for sample collection, the particulate B-vitamin concentrations measured at our study sites only represent the bulk community ranging from 0.2 to 10 μm, which likely excludes large eukaryotic phytoplankton. The observed concentrations of particulate B-vitamins in a bulk community measurement are a function of both the size of the community (biomass standing stock), and potentially the growth rate of the individual organisms in the community. We hypothesize that the relatively oligotrophic conditions in the Atlantic (compared to the upwelling dominated community at SPOT) should result in lower microbial growth rates, and therefore likely lower particulate B-vitamin requirements.

Stoichiometric estimations showed that the B-vitamin concentrations measured in both cultures and field-collected samples were, on average, between 1 and 2 orders of magnitude higher than the values reported from the phytoplankton cultures analyzed by Carlucci and Bowes (1972) (Figure 1). We believe that this difference could either be a result of the lack of sensitivity of the bioassay used to determine vitamin concentration in their study and/or the substantial B-vitamin degradation that we observed under their original high temperature and acidity particulate extraction conditions (see Results section).



Similarly, our measured particulate B-vitamin concentrations (normalized to POC) are, on average, an order of magnitude higher than published vitamin B<sub>1</sub> and B<sub>12</sub> calculated cellular quotas based on uptake rates (Droop, 2007; Tang et al., 2010; Paerl et al., 2015). Cellular quotas of B-vitamins (pmole B-vitamin cell<sup>-1</sup>) for individual strains of eukaryotic phytoplankton have been previously estimated using differential growth rates in varying concentrations of B-vitamins (Croft et al., 2005; Droop, 2007; Tang et al., 2010; Paerl et al., 2015). These published per cell values were normalized to C values using published conversion factors: 20fgC/cell bacteria, 200fgC/cell cyanobacteria, and 138pgC/cell microplankton (Waterbury et al., 1986; Caron et al., 1999). The vitamin per carbon values from the published uptake studies (obtained using the above conversion factors) were compared to the mean vitamin per carbon values from 3 m at SPOT and all depths at the Atlantic Station. The mean total thiamin (B<sub>1</sub>, TMP, TPP combined) and total cobalamin (all congeners combined) concentrations (mole B-vitamin mole POC<sup>-1</sup>) measured at SPOT and at the Atlantic station are  $2.3 \times 10^{-5} \pm 6.8 \times 10^{-6}$  and  $2.2 \times 10^{-6} \pm 7.7 \times 10^{-7}$ , respectively (Tables 3, 4). In comparison, the mean estimated cellular quotas for thiamin and cobalamin obtained from Tang et al. (2010) and Paerl et al. (2015) are

$6.3 \times 10^{-7} \pm 8.7 \times 10^{-7}$  and  $2.4 \times 10^{-8} \pm 5.9 \times 10^{-8}$  (mole B-vitamin mole POC<sup>-1</sup>), respectively. The 1–3 orders of magnitude difference between the calculated cellular quotas (based on vitamin uptake rates), and our observed particulate concentrations seems to point to the possibility that complex environmental samples consisting of mixed communities may contain individual members with higher B-vitamin quotas than those previously studied in monocultures. Furthermore, these data also suggest that B-vitamin uptake could be a poor indicator for particulate B-vitamin concentrations in environmental samples as the cellular quotas of organisms that have B-vitamin synthesis capacity are not constrained by B-vitamin uptake rates. Future studies coupling B-vitamin uptake rates and direct particulate measurements will be necessary to fully understand these relationships.

The dissolved pool of B-vitamins was measured at the Atlantic Station in conjunction with the particulate pool. The observed concentration range (all in pM) of B-vitamins in the dissolved pool are: 33.4–457.1 B<sub>1</sub>, 9.8–89.2 TMP, 2.5–77.3 TPP, 2.5–28.3 HMP, 40.1–679.4 B<sub>7</sub>, 0.07–3.5 AB<sub>12</sub>, 0.09–0.8 CB<sub>12</sub>, 0.2–3.1 HB<sub>12</sub>, 0.4–12.1 MB<sub>12</sub>, and 0.1–2.1 Met (Table 4, Figure 2). These concentrations were decoupled from inorganic nutrient concentrations (e.g.,  $\text{PO}_4^{-3}$ ). Vitamins in the dissolved

**TABLE 4 | Dissolved (A) and particulate (B) B-vitamin concentrations (pM) determined at a station in the Eastern Atlantic Ocean (33°1'16''N, 12°14'28''W) in May of 2014.**

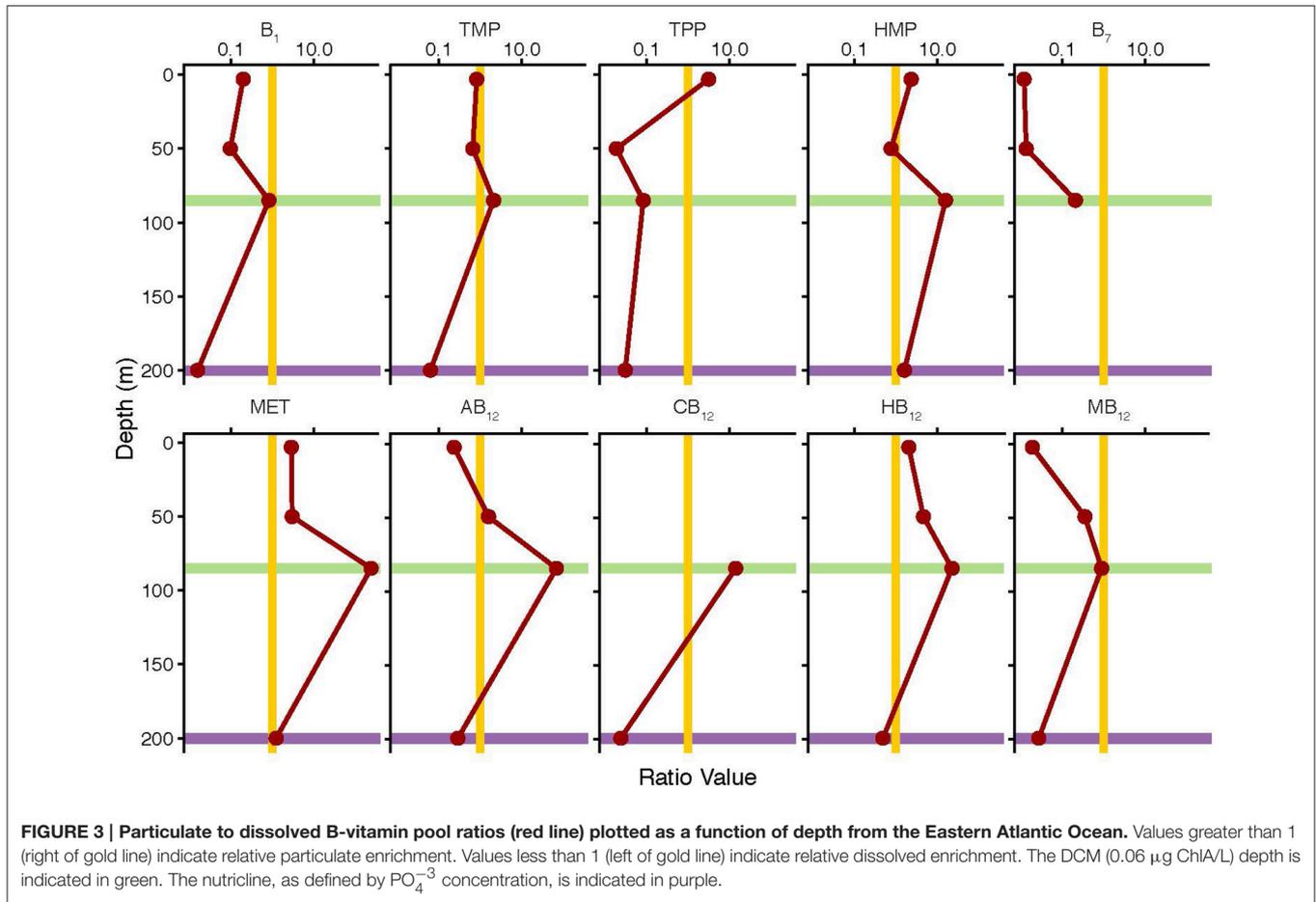
	3 m		50 m		85 m		200 m	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<b>(A) Dissolved Fraction, pM</b>								
Thiamin	182	23.2	457	118	33.4	1.67	223	54.3
Thiamin monophosphate	33.9	2.74	70.1	21.5	9.83	2.62	89.2	21.9
Thiamin pyrophosphate	2.51	1.91	59.9	4.18	77.3	44.7	38.9	3.11
Pyrimidine moiety	2.53	0.64	28.3	3.19	2.90	0.11	4.56	2.27
Biotin	679	136	629	168	40.1	10.5		
Adenosylcobalamin	3.49	2.34	1.33	0.28	0.07	0.01	1.80	1.75
Cyanocobalamin	0.80	0.43	0.59	0.04	0.09	0.02	0.48	0.25
Hydroxycobalamin	3.13	0.15	1.97	0.71	0.15	0.13	2.49	0.87
Methylcobalamin	12.1	8.46	6.97	2.30	0.37	0.13	7.33	1.68
Methionine	1.07	0.02	2.12		0.07	0.01	1.61	0.58
<b>(B) Particulate Fraction, pM</b>								
Thiamin	36.0	7.19	44.4	8.36	27.6	1.97	3.54	1.24
Thiamin monophosphate	27.9	2.62	46.5	6.59	20.7	6.11	5.64	0.60
Thiamin pyrophosphate	7.85	1.01	1.13		6.46		1.20	0.30
Pyrimidine moiety	5.91	1.90	21.7	5.05	45.6	2.79	7.31	1.87
Biotin	8.22	3.00	8.56	2.42	8.35	1.32	1.68	0.29
Adenosylcobalamin	0.82	0.64	2.11	0.69	4.54	1.57	0.52	0.11
Cyanocobalamin					1.22		0.01	0.01
Hydroxycobalamin	6.38	0.89	9.11	4.07	3.39	1.10	1.21	0.13
Methylcobalamin	0.23	0.17	2.46	0.12	0.33	0.27	0.20	0.02
Methionine	3.06	0.71	6.36	1.73	16.1	3.00	1.97	1.55

pool are sourced from biogenic processes, therefore areas that contain the most biochemically active organisms, and likely the highest particulate B-vitamin concentrations, have the potential to produce the highest dissolved vitamin concentrations. The relationship between the dissolved and particulate pools varies between analyte and sample depth, which likely indicates rapid scavenging and release of these valuable compounds.

In order to evaluate the relative enrichment of either the particulate or the dissolved pool of B-vitamins in the environment, ratios of particulate to dissolved concentration were calculated (Figure 3, Table 5). Values greater than one indicate relative particulate pool enrichment, while values less than one indicate a relative enrichment in the dissolved pool. Vitamins B<sub>1</sub>, B<sub>7</sub>, and MB<sub>12</sub> were found to be enriched exclusively in the dissolved pool with ratios ranging from 0.02 to 0.83, 0.01–0.21, and 0.02–0.89 respectively (Figure 3, Table 5). Only the amino acid methionine was found to be always relatively enriched in the particulate pool with ratio values ranging from 1.23 to 239 (Figure 3, Table 5). Ratio values for all analytes peaked at the DCM and decreased at the nutricline (Figure 3, Table 5). The maximum concentration of biomass at the DCM is likely the cause of the relative enrichment of particulate B-vitamins at this depth (Figure 3, Table 5). Increased biological activity at the DCM likely creates this relative particulate vitamin enrichment through the increase of the rates of two processes: de novo B-vitamin synthesis and B-vitamin uptake from the dissolved pool.

The relative enrichment in the dissolved vitamins pool at the nutricline (200 m) is likely a result of remineralization of sinking particles, coupled with B-vitamin degradation within dead or dying cells. Future research will be required to robustly define the relationship between these two pools.

In summary, we have developed a direct method for the determination of particulate and dissolved B-vitamins in the ocean. The particulate protocol involves a cellular extraction from filtered cells, followed by a liquid phase extraction to remove organic molecules that could interfere with the analyses. The dissolved method uses a C<sub>18</sub> resin to preconcentrate B-vitamins from seawater. Analysis for both methods is carried out using a gradient elution using a C<sub>18</sub> column, followed by direct detection using a LC/MS. This new analytical protocol is used to quantify vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, B<sub>7</sub>, B<sub>12</sub>, and the amino acid methionine as well as their chemical congeners. This method is sensitive and accurate, and enables simultaneous direct quantification of particulate and dissolved B-vitamins in the ocean. These techniques were successfully applied to cultured marine bacteria and environmental samples from an upwelling-dominated eutrophic coastal region off the coast of California, and an oligotrophic oceanic region in the Atlantic Ocean. These techniques represent a much needed methodological expansion which will enable important future studies to determine the roles that B-vitamins play in determining the structure and productivity of marine microbial communities.



**TABLE 5 | Ratios between particulate and dissolved pools of B-vitamins in the Eastern Atlantic Ocean.**

	Intracellular pool: Dissolved pool			
	3 m	50 m	85 m	200 m
Thiamin	0.2	0.1	0.83	0.02
Thiamin monophosphate	0.82	0.66	2.1	0.06
Thiamin pyrophosphate	3.12	0.02	0.08	0.03
Pyrimidine moiety	2.33	0.77	15.7	1.6
Biotin	0.01	0.01	0.21	
Adenosylcobalamin	0.24	1.59	68.4	0.29
Cyanocobalamin			14.1	0.02
Hydroxycobalamin	2.04	4.61	22.5	0.49
Methylcobalamin	0.02	0.35	0.89	0.03
Methionine	2.86	3	239	1.23

Values greater than one indicate particulate pool dominance, values less than one indicate dissolved pool dominance. Blank cells indicate that either the particulate or the dissolved pools were not detectable. DCM is at 85 meters depth.

## AUTHOR CONTRIBUTIONS

The methods described in this paper were developed by CS with support and guidance from LC and SS in laboratory space

provided by SS. All experiments were conducted by CS. The paper was written by CS. The paper was reviewed by LC and SS. Funding for this work was provided by grants from NSF (#1435666) to SS.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmars.2017.00011/full#supplementary-material>

**Figure S1 | Map of environmental sampling locations. (A)** The San Pedro Ocean Timeseries (SPOT) is located within the Southern California Bight (33°33'N, 118°24'W). **(B)** The Atlantic Station was located north of the Canary Islands and west of Morocco (33°1'16"N, 12°14'28"W).

**Figure S2 | Example LC/MS Chromatographs for each analyte.** Retention time ranges from 0 to 12 min. Peak height is reported as percent relative intensity for each analyte (e.g., the highest peak within the specific *m/z* filter is set to 100%).

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- Equation 1 | Response Factor (RF) is used to determine the relationship between known concentrations of the Internal Standard ( $C_{IS}$ ) and known concentrations of target analyte standards ( $C_{vitstd}$ ), with the observed instrumental response (curve area) of the Internal Standard ( $A_{IS}$ ) and the target analyte standard ( $A_{vitstd}$ ).
- Equation 2 | The concentration of the target analyte in a sample ( $C_{vitsam}$ ) is determined by adding a known concentration of the internal standard to the sample ( $C_{IS}$ ), and then using the RF (determined in Equation 1) to correct for the differing instrumental response between the internal standard ( $A_{IS}$ ) and the target analyte within the sample ( $A_{vitsam}$ ).
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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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