Mosaic patterns of B-vitamin synthesis and utilization in a natural marine microbial community

Laura Gómez-Consarnau (iD),[1,2]* Rohan Sachdeva,[1]
Scott M. Gifford,[3] Lynda S. Cutter,[1]
Jed A. Fuhrman,[1]
Sergio A. Sañudo-Wilhelmy[1,4] and
Mary Ann Moran (iD)[5]

[1]Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA.
[2]Departamento de Oceanografía Biológica, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Ensenada, 22860, Mexico.
[3]Department of Marine Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.
[4]Department of Earth Sciences, University of Southern California, Los Angeles, CA 90089, USA.
[5]Department of Marine Sciences, University of Georgia, Athens, GA 30602, USA.

Summary

Aquatic environments contain large communities of microorganisms whose synergistic interactions mediate the cycling of major and trace nutrients, including vitamins. B-vitamins are essential coenzymes that many organisms cannot synthesize. Thus, their exchange among *de novo* synthesizers and auxotrophs is expected to play an important role in the microbial consortia and explain some of the temporal and spatial changes observed in diversity. In this study, we analyzed metatranscriptomes of a natural marine microbial community, diel sampled quarterly over one year to try to identify the potential major B-vitamin synthesizers and consumers. Transcriptomic data showed that the best-represented taxa dominated the expression of synthesis genes for some B-vitamins but lacked transcripts for others. For instance, Rhodobacterales dominated the expression of vitamin-B_{12} synthesis, but not of vitamin-B_7, whose synthesis transcripts were mainly represented by Flavobacteria. In contrast, bacterial groups that constituted less than 4% of the community (e.g., Verrucomicrobia) accounted for most of the vitamin-B_1 synthesis transcripts. Furthermore, ambient vitamin-B_1 concentrations were higher in samples collected during the day, and were positively correlated with chlorophyll-*a* concentrations. Our analysis supports the hypothesis that the mosaic of metabolic interdependencies through B-vitamin synthesis and exchange are key processes that contribute to shaping microbial communities in nature.

Introduction

Molecular studies show that marine microbial systems are extremely diverse, containing thousands of different taxa that combined sustain ecosystem functioning (Sogin *et al.*, 2006). In general, while a few species numerically dominate a particular microbial community, they are complemented by a large number of less abundant species whose specific function is not well understood (Pedrós-Alió, 2012). For example, similar to the Hutchinson's paradox of the plankton (Hutchinson, 1961), it is unclear why the marine heterotrophic bacterioplankton community is so diverse at certain locations if all share the same niche and ecological functions (Moran, 2008). Ultimately recognizing the connectivity among the species that compose the heterotrophic bacterioplankton community is essential to understand the fate of up to half of the marine primary production that is routed through the microbial loop (Azam *et al.*, 1983). Field and laboratory studies indicate that resource allocation from algal-derived organic matter can explain some marine bacterioplankton successions and the dominance of certain microbial taxa capable of distinct decomposition pathways (McCarren *et al.*, 2010; Teeling *et al.*, 2012). However, these mechanisms cannot explain the large background heterotrophic community that exists under non-bloom conditions during most of the year. Therefore, the bacterial connectivity and diversity observed in microbial communities could reflect complex interdependencies associated with external metabolites such as the B-vitamins, as observed in mixed culture experiments (Croft *et al.*, 2005).

B-vitamins are the most versatile and ancient coenzymes (Monteverde *et al.*, 2017). They catalyze a wide spectrum of critical metabolic reactions, such as the

tricarboxylic acid (TCA) and Calvin cycles (thiamin; vitamin-B_1; VB_1), carbon fixation via the reverse TCA cycle (biotin; vitamin-B_7; VB_7) or the synthesis of methionine (cobalamin; vitamin-B_{12}, VB_{12}) (Sañudo-Wilhelmy *et al.*, 2014). Vitamin B_6 (pyridoxine, VB_6) seems to be remarkably important as it catalyzes almost 2% of all prokaryotic functions (Percudani and Peracchi, 2003) and yet, has rarely been studied in marine systems (Sañudo-Wilhelmy *et al.*, 2012). Despite their relevance, B-vitamins auxotrophy is widespread among marine eukaryotes (Croft *et al.*, 2006; Tang *et al.*, 2010; Paerl *et al.*, 2015) and may influence the taxonomic composition of phytoplankton communities as well as the rates of primary production and carbon fixation (Panzeca *et al.*, 2006; Sañudo-Wilhelmy *et al.*, 2006; Koch *et al.*, 2011). For decades, it was assumed that marine prokaryotes were the source of exogenous B-vitamins for marine phytoplankton (Kurata, 1986; Croft *et al.*, 2005). However, the extensive genomic information that is now available suggests that not all bacteria and archaea are *de novo* synthesizers (LeBlanc *et al.* 2011; Sañudo-Wilhelmy *et al.*, 2014). For example, the highly streamlined *Candidatus* Pelagibacter ubique lacks the biosynthetic pathways for VB_1, VB_5 and VB_7, and can reach concentrations of 10^9 cells ml^{-1} only when these vitamins are present, regardless of how much C, N or P is available (Carini *et al.*, 2013). This example alone suggests that exogenous sources of B-vitamins (dissolved and/or particulate) must be available within the water column. Experimental data further indicate that "metabolic cooperation" via vitamin exchange does occur in co-cultures of particle associated bacteria and phytoplankton (Raes and Bork, 2008; Croft *et al.*, 2005; Wagner-Döbler *et al.*, 2010; Kazamia *et al.*, 2012). Furthermore, dissolved vitamins are also released to the environment through processes that do not involve direct microbial interactions, as VB_{12} excretion during cell division has been observed in pure cultures of cyanobacteria (Bonnet *et al.*, 2010). Regardless of whether or not B-vitamin auxotrophs rely on direct or indirect microbial exchange to obtain these metabolites, the identification of the potential vitamin producers and consumers in a natural microbial community is key to better understand the processes sustaining the ecosystem. Recent work of Heal *et al.* (2017) studied the potential VB_{12} interdependencies among producers and consumers in the North Pacific Ocean. However, these ectocrine interactions have not been examined for other B-vitamins in any other marine environment.

In this study, we used metatranscriptomics to identify the transcriptional investment of different taxa in B-vitamin synthesis and utilization for VB_1, VB_6, VB_7 and VB_{12} within a natural marine microbial community. Our data show that some of the best-represented taxa dominated the expression of synthesis genes for some, but not all B-vitamins, suggesting the need for metabolite exchange among

microbial groups. The concentration of dissolved VB_1 and VB_6 in this environment followed diel oscillations, with higher levels during the day compared to night. These environmental diel fluctuations of B-vitamins could be related to their metabolic function as protectants against oxidative stress and potentially constitute a mechanism of vitamin supply to the auxotrophic members of the community.

Material and methods

Sample collection

Samples for metatranscriptomics were collected quarterly (2008: August 6–7, November 5–7; 2009: February 15–17, May 13–15, August 12–14) at Marsh Landing, Sapelo Island, Georgia, USA (31°25'4.08 N, 81°17'43.26 W) as part of the Sapelo Island Microbial Observatory program (http://simco.uga.edu). Sampling occurred at four consecutive high tides resulting in two consecutive pairs of day–night samples per season. Additional samples were collected for specific environmental measurements including chlorophyll-*a* and dissolved B-vitamins. RNA from the water samples was extracted as previously described (Poretsky *et al.*, 2009; Gifford *et al.*, 2011; 2013; 2014). Briefly, 6–8 l were directly filtered for 11–14 min from a depth of 1 m through a 3-µm pore-size prefilter to exclude larger microbial fractions (Capsule Pleated Versapor Membrane; Pall Life Sciences, Ann Arbor, MI, USA) and 0.22-µm pore-size filter (Supor polyethersulfone; Pall Life Sciences). The 0.22-µm filter was immediately flash frozen in liquid nitrogen until RNA processing and sequencing.

RNA processing and sequencing

Samples were processed as earlier described (Gifford *et al.*, 2011; 2013; 2014). Total RNA was extracted using a RNAEasy kit (Qiagen) and DNA was removed using a Turbo DNA-free kit (Applied Biosystems, Austin, TX, USA). mRNA was enriched via enzymatic rRNA reduction using Epicentre's mRNAOnly isolation kit (Madison, WI, USA) and subsequently with MICROBExpress and MICROBEnrich rRNA (Applied Biosystems) subtractive kits. Enriched mRNA samples were linearly amplified using the MessageAmp II-Bacteria kit (Applied Biosystems) and double stranded cDNA synthesized with Promega's Universal RioboClone cDNA synthesis system and random primers. cDNA synthesis reactions were cleaned up with a QIAquick PCR purification kit (Qiagen). Samples were sheared and size-selected to ∼300 bp and sequenced with an Illumina GAIIX to obtain 150 × 150 bp paired end reads.

Bioinformatics pipeline

Reads with an average quality score ≤ 20 and a length < 100 bp were removed and overlapping paired end reads from each fragment were assembled using SHERA (Rodrigue *et al.*, 2010) with a score >0.5 (Gifford *et al.*, 2013; 2014). Sequences were combined from all samples, subsampled to 25 000 reads and searched against the SILVA small and large subunit databases using BLASTn (Altschul *et al.*, 1997) with

bit score $\geq$ 50 to create a more compact database for further rRNA gene filtering. The total reads from all metatranscriptomes were then filtered using BLASTn against the compact database. Putative non-rRNA sequences were then searched against the National Center for Biotechnology Information's (NCBI; http://www.ncbi.nlm.nih.gov) RefSeq protein database (version 43, September 2010) using BLASTx with a bit score cutoff $\geq$ 50 to identify protein encoding sequences. Functional annotation and taxonomy were assigned based on the top scoring hit to RefSeq. RefSeq counts for each sample were randomly subsampled (McKinney, 2010; van Rossum, 2010) to the total number of non-rRNA reads of the smallest library (1 332 199 reads) to account for variation in sample sequencing effort.

Annotation of vitamin related transcripts

Vitamin related transcripts for the active forms of VB_1 (thiamin monophosphate; TMP or thiamin pyrophosphate; TPP), VB_6 (pyridoxal 5'-phosphate), VB_7 and VB_{12} (methylcobalamin, adenosylcobalamin, hydroxocobalamin) were identified by text-based query for either the EC number, gene or enzyme name in the retrieved RefSeq functional annotations. The specific vitamin synthesis genes and vitamin dependent enzymes are listed in Supporting Information Table S1 and all vitamin-related hits are included in Supporting Information Table S2. For each of the vitamins (VB_1, VB_6, VB_7, VB_{12}), vitamin dependent enzymes were identified using the ExPASy database (http://www.expasy.org) and are defined as the metabolic reactions that require a specific B-vitamin as co-enzyme. Vitamin synthesis functional annotations are those enzymes that belong to the synthesis pathway of a specific B-vitamin, and were determined using the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/; Supporting Information Table S2). For VB_7 synthesis pathway, the genes fabI, fabB, fabG and fabZ were not included in our analysis, as they are also part of the fatty acid biosynthesis pathways and are present in most microorganisms including vitamin-B_7 auxotrophs. Therefore, including those genes would produce false positives. Sequences were also classified by sequence similarity to a list of vitamin synthesis/dependence genes in order to verify that RefSeq description annotation precisely represents counts of vitamin synthesis and dependence. Sequences for synthesis and dependence of each vitamin were retrieved from the RefSeq protein database. All sequences were searched against separate BLAST databases for synthesis and dependence of each vitamin (e.g., VB_6 dependence and VB_6 synthesis). Only matches with a bit score $\geq$ 40 and that do not have a higher scoring non-vitamin RefSeq match were retained. This strategy produced slightly underestimated counts of vitamin synthesis/dependence classifications ($86 \pm 4.2\%$; Supporting Information Table S5), but did not affect the overall pattern of vitamin usage.

Identification of taxa involved in B-vitamin synthesis and utilization

Linear modelling robust to outliers was employed to identify taxa that disproportionately contributed to vitamin synthesis or dependence gene expression above the typical expression for the whole community. Iterated re-weighted least squares (IWLS) linear regression was fit with 1×10^6 maximum iterations for outlier removal between synthesis:total expression, dependence:total expression and dependence:synthesis using the 'MASS' R-package (Venables and Ripley, 2013). Ratios near the linear regression line (low residuals) are defined as typical for the microbial community. A positive residual suggests a ratio greater than the typical ratio, whereas a negative residual is less than the typical ratio. For example, the negative Cyanobacteria dependence:synthesis residual for VB_1 suggests that Cyanobacteria are important synthesizers of VB_1. Underlying this analytical approach is the assumption that vitamin requirements scale similarly with vitamin-dependent transcripts for all taxonomic groups (i.e., a vitamin dependent transcript represents an equivalent vitamin requirement across all taxa). The synthesis:dependence regression lines of each vitamin could be influenced by the fact that particular taxa are auxotrophs for a particular vitamin (e.g., eukaryotes and VB_{12}), and by different taxa having varied numbers of enzymes that require the vitamin. For instance, there are more than 20 enzymes that require VB_{12} in prokaryotes (Neil and Marsh, 1999), and only three VB_{12} dependent enzymes in eukaryotes (Helliwell et al., 2011). Another example is VB_1 which, among other functions, is used by enzymes in the Calvin cycle of photoautotrophs and this cycle will be absent in heterotrophic microorganisms. Finally, as in any other metatranscriptomic analysis, our results could not account for any post-transcriptional regulation.

Chlorophyll-a quantification

Chlorophyll a analysis was done as described in Parsons et al. (1984). Seawater samples of 250 ml were filtered onto GFF membranes (Whatman plc, Maidstone, United Kingdom), placed in conical tubes filled with 50 ml of 90% acetone and stored at $-20°C$ until analysis. Sample fluorescence was measured by sonicating the samples for 30 s, then adding 3 ml to a 5 cm quartz cuvette and measuring on a Turner fluorometer. Estimates of phaeo-pigments were obtained by acidifying the same sample with 10% HCl and rerunning on the fluorometer. Sample chlorophyll-a concentrations were then calculated based on comparison to a standard curve of fluorescence from chlorophyll-a standards.

Quantification of dissolved B-vitamins in situ

The concentration of dissolved B-vitamins was determined using HPLC-MS after preconcentration of 500 ml of 0.2 μm-filtered seawater. Dissolved samples were preconcentrated by passing the sample over a C18 resin at two pHs (6.5 and 2.0), followed by elution with 12 ml of methanol. A nitrogen (N_2) dryer was used to evaporate the samples to about 250 μl. Quantification of dissolved B-vitamins, VB_1 as thiamin hydrochloride ($C_{12}H_{17}ClN_4OS*HCl$), VB_6 as pyridoxine hydrochloride ($C_8H_{11}NO_3*HCl$), VB_7 as biotin ($C_{10}H_{16}N_2O_3S$) and VB_{12} as cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), was conducted using a Thermo Scientific Quantum Access electrospray ionization triple quadrupole mass spectrometer, coupled to a Thermo Scientific Accela High Speed Liquid Chromatography

(LC/MS) system. The LC system used a stable-bond C18 reversed-phase column (Discovery HS C18 10 cm × 2.1 mm, 5 μm column, Supelco Analytical), with a methanol:water gradient program. A full description of the analytical protocol for the vitamins quantification including all of the MS operating conditions have been reported elsewhere (Sañudo-Wilhelmy et al., 2012). Although our analytical protocol is able to measure a suite of B-vitamins, the small volume of the water samples did only allow the determination of vitamins found at relatively high concentrations such as thiamin and pyridoxine (both measured against standards prepared in their hydrochloride forms). The concentrations of dissolved VB$_7$ and VB$_{12}$ were below our detection limit in all samples (< 0.1 pM).

Results and discussion

Microbial community composition

Taxonomic diversity of metabolically active bacteria through the different seasons was estimated using the total number of transcripts recruited for each microbial group (Gifford et al., 2014). Total community expression was represented by bacterial taxa that are typically found in marine coastal ecosystems (Gifford et al., 2013; 2014), including Alphaproteobacteria (Rhodobacterales, relative expression 11%–18%; SAR11, 1%–14%; SAR116, 3%–7%), Flavobacteria (2%–11%), Gammaproteobacteria (unclassified, 6%–11%) and Betaproteobacteria (3%–7%) (Fig. 1 and Supporting Information Table S3). Eukaryotic

mRNAs were the second-most expressed transcripts detected after Rhodobacterales in this 3.0-micron pre-filtered community (on average ~11% of reads; range 4%–38%), and were particularly important during periods of high chlorophyll-a (Chl-a) (Summer '08, Fall '08 and Spring '09, accounting for 18%–38% of the total mRNA library). The microbial picoeukaryotic taxa with the highest expression in this 0.2 to 3.0 μm plankton size fraction were *Micromonas* (20%–44% of eukaryotic sequences), *Ostreococcus* (20%–39%) and *Chlorella*-like (5%–36%; Table 2, Supporting Information Table S3). The relative increase in expression of certain bacterioplankton groups during the Chl-a peaks (e.g., Flavobacteria during the phytoplankton bloom of Winter '09) is consistent with similar trends repeatedly reported in the literature (Kirchman, 2002; Teeling et al., 2012). However, the relative contribution of the major microbial community taxa to total community transcripts remained relatively stable during our year-long study (e.g., SAR11, SAR116, Rhodobacterales, Betaproteobacteria; Fig. 1, Supporting Information Table S3). Archaeal transcripts reached 7.5%–10.5% of all transcripts during summer 08 and were also high but to a lesser extent in summer '09 (2%–4% of all transcripts), while they were low in all other seasons (Hollibaugh et al., 2011; 2014). Other taxonomic groups such as Cyanobacteria and Verrucomicrobia were also present throughout the year although comprising less than 4% of the prokaryotic expression (Fig. 1, Supporting Information Table S3).
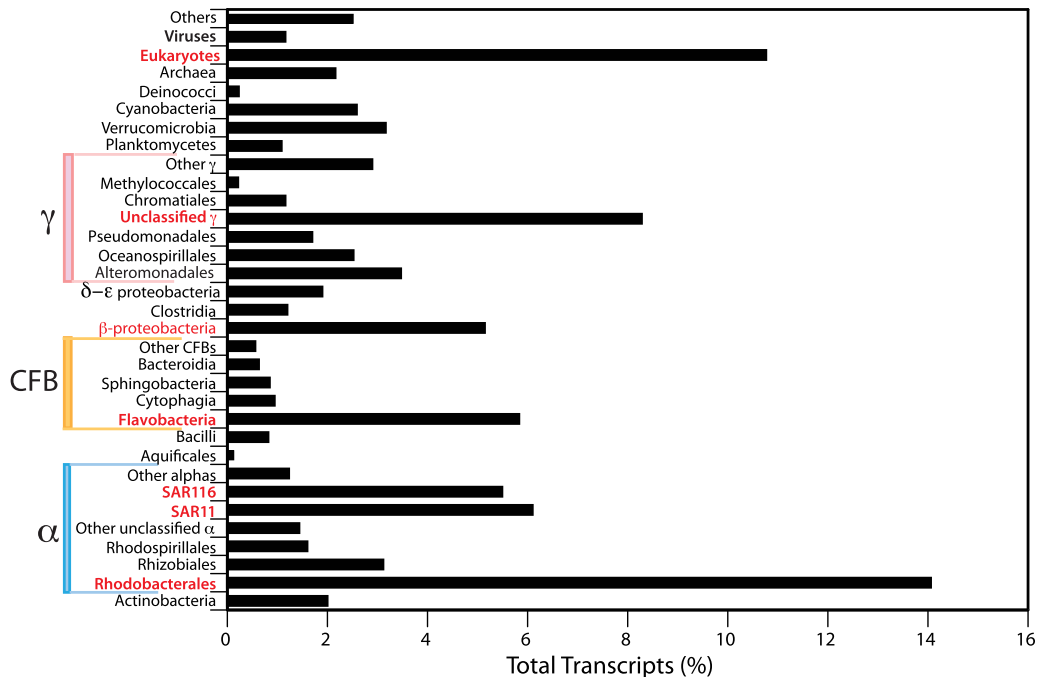

Fig. 1. Percentage of the total transcripts detected in 16 southeastern United States metatranscriptomes, classified by taxon. Red font indicates the best-represented taxonomic groups as determined from mRNA relative abundance. The symbols α, β, γ, δ and ε refer to the different proteobacteria classes. CFB refer to the Cytophaga-Flavobacterium-Bacteroides (CFB) phylum.

Expression of vitamin synthesis and vitamin-dependent metabolisms

The correlation between gene expression and the actual associated metabolic process in the environment is not always easy to discern (Gifford *et al.*, 2011). It is currently known that the ratio between mRNA and protein can be quite variable and may not quantitatively reflect the immediate cell response to environmental changes (Taniguchi *et al.*, 2010). The inability to predict protein levels from mRNA could be explained by the long half-life of proteins compared to mRNA, in addition to variable post-transcriptional modifications and translation efficiencies among other factors (Moran *et al.*, 2013). Nevertheless, metatranscriptomics is a powerful tool to identify patterns that can be used to find the potential organisms involved in different environmental processes. To try to understand B-vitamin synthesis and utilization in this coastal environment, we quantified the total number of annotatable transcripts involved in the synthesis of VB_1, VB_6, VB_7 and $VB_{12,}$ as well as their vitamin-dependent enzymes (Table 1, Supporting Information Table S1). This semi-quantitative approach allows the evaluation of the overall transcript investment in B-vitamin synthesis and dependent functions within metatranscriptomes. On the synthesis side, the highest number of transcripts was found for VB_6 synthesis (0.05% of the community transcriptome), followed by VB_{12}, VB_1 and VB_7 (Table 1). On the dependence side, the majority of vitamin-dependent functions detected in the microbial community were ascribed to VB_1, with 101 501 reads (0.78% of total reads; Table 1), consistent with the importance of VB_1-dependent enzymes in central metabolism (Supporting Information Table S1). VB_6 had the second highest number of vitamin-dependence transcripts (0.69% of all reads), followed by VB_7 and VB_{12}. Differences in the number of synthesis or dependence transcripts between the vitamins is likely to reflect variations in the complexity of their biosynthesis pathways, the number of reactions that require them, the times they can be reused, and the efficiency of their cellular salvage pathways.

Table 1. Total number of B-vitamin synthesis and dependence transcripts found in our study.

Vitamin	N. Transcripts	
	Synthesis	Dependent
VB_1	4921 (0.04%)	101 501 (0.78%)
VB_6	6856 (0.05%)	89 474 (0.69%)
VB_7	2540 (0.02%)	36 364 (0.28%)
VB_{12}	5546 (0.04%)	17 592 (0.14%)

Values in parentheses are the percentages of the B-vitamin reads in the dataset.

Identification of B-vitamin synthesizers within the microbial community

We further analyzed the taxonomic distribution of vitamin synthesis and utilization transcripts among the members of the microbial community (Fig. 2, Supporting Information Tables S3 and S2). Figure 2 shows the percent of community transcripts for vitamin synthesis or dependence that is attributed to a taxon. Some of the best-represented taxa dominated the expression of several vitamin synthesis pathways. Rhodobacterales, the most transcriptionally abundant bacterial group identified in this study, was potentially the major producer of VB_{12}, accounting for almost 50% of all the environmental VB_{12} synthesis transcripts in some samples (VB_{12} synthesis:total transcript ratio had a residual of 27% from the community-wide vitamin regression line; Fig. 2A, Supporting Information Table S4). This is in contrast to what was reported from incubation experiments in the Southern Ocean, in which Oceanospirillaceae appeared to be the major VB_{12} synthesizers (Bertrand *et al.*, 2015). Members of proteobacteria, which include Rhodobacterales and Oceanospirillales, have previously been identified as globally important VB_{12} synthesizers in marine surface waters (Doxey *et al.* 2015). While being the main VB_{12} synthesizers in our study, Rhodobacterales VB_{12} dependence transcript abundance was also high, resulting in a VB_{12} dependence:synthesis transcript ratio typical for this community (VB_{12} dependence:synthesis ratio had a residual of less than 2% from the community-wide regression line; Fig. 2C). For other B-vitamins, though, Rhodobacterales vitamin-synthesis transcripts were clearly below the typical dependence:synthesis transcript relationship. For VB_1 and VB_6, only 7% and 12% of the synthesis transcripts belonged to Rhodobacterales, while they were responsible for a twofold higher percentage of the vitamin-dependent gene transcripts (dependence:synthesis residuals of 9% and 10% respectively; Fig. 2, Supporting Information Table S4). Their dependence:synthesis transcript relationship for VB_7 was even more unbalanced with 2% of the community synthesis genes compared to 20% of the community requirement (synthesis-dependence residuals of 18%; Fig. 2C, Supporting Information Table S3 and S4). These field-based results are consistent with genomes from isolated marine strains showing that while almost all of the cultured Rhodobacterales can synthesize vitamin-B_{12}, only 50% and 70% can produce VB_1 and VB_7 respectively (Sañudo-Wilhelmy *et al.*, 2014). Figure 3 further shows the percentage of mRNA sequences that each phylogenetic group invested in B-vitamin synthesis and dependent processes. This analysis allows to qualitatively distinguish to what extent the different taxa rely on B-vitamin metabolism compared to the average community. Similarly to the total transcript analysis (Fig. 2), Rhodobacterales invested a
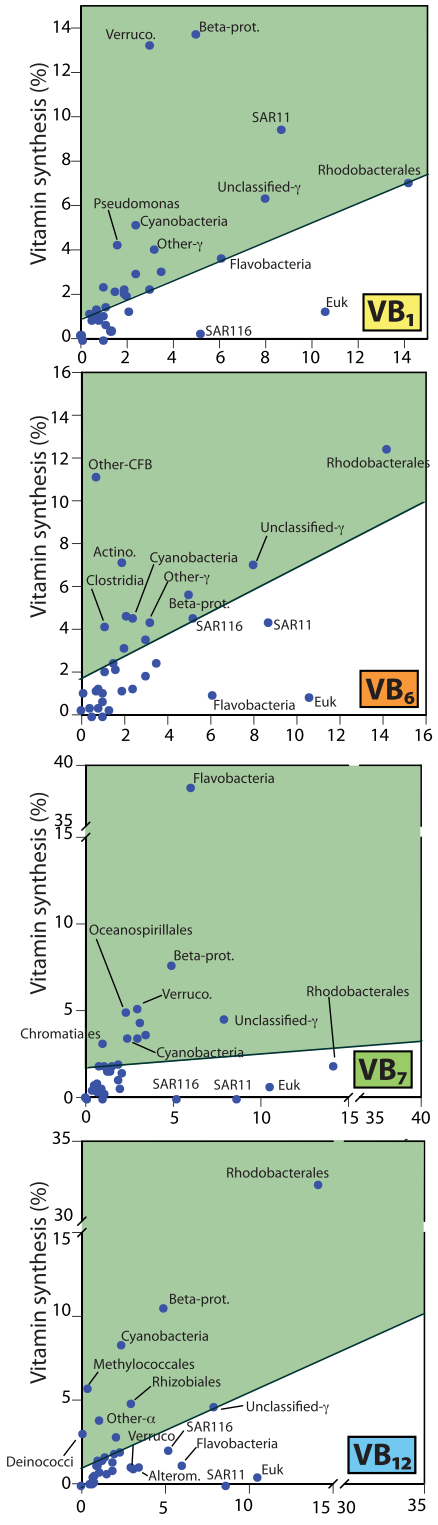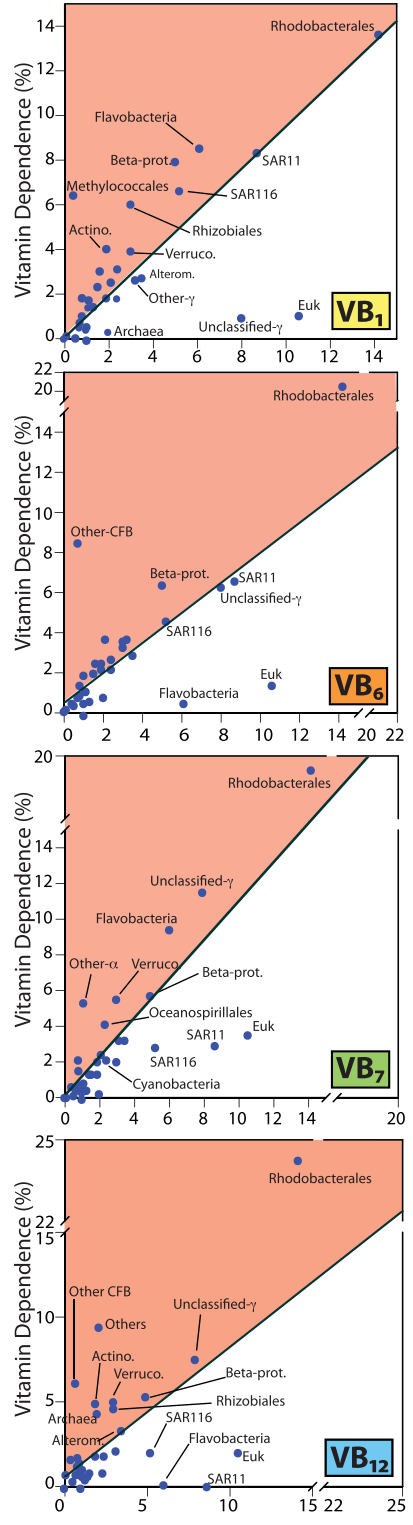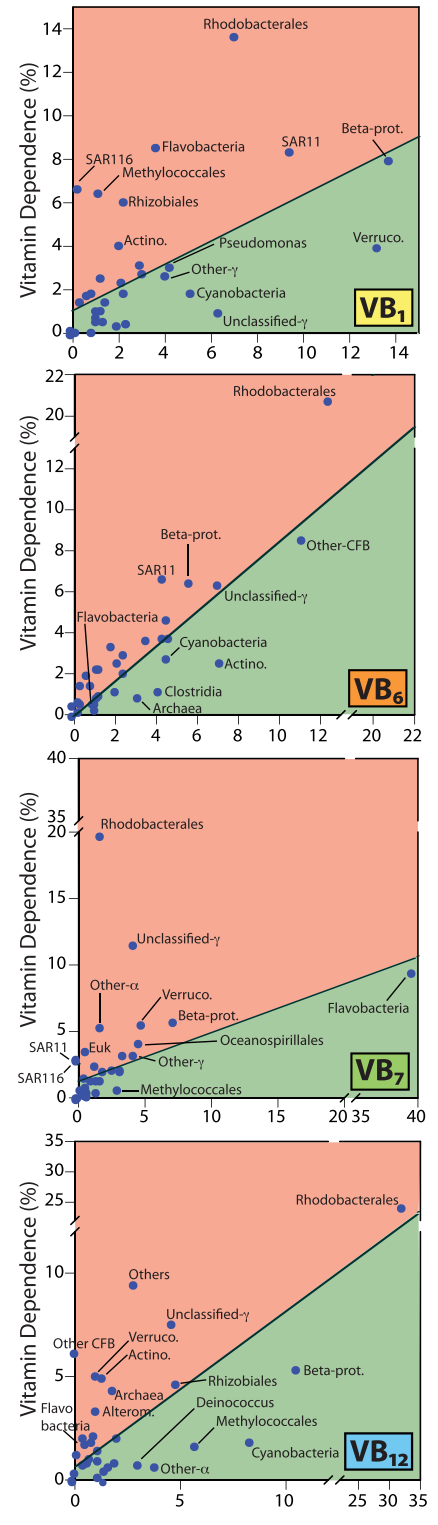
Fig. 2. B-vitamin synthesis and dependence transcripts in different microbial taxa.
A. Percentage of B-vitamin synthesis transcripts and
B. Percentage of vitamin dependent transcripts compared to the total transcript abundance for each taxonomic group.
C. Percentage of B-vitamin synthesis transcripts vs. percentage of B-vitamin dependence transcripts. Green areas show the microbial groups with a higher percentage of the community vitamin synthesis transcripts compared to total transcript abundance (A) or the percentage of their vitamin dependent gene transcripts (C). Red areas show the microbial groups with a higher percentage of vitamin-dependent transcripts as compared to total transcript abundance (B) or B-vitamin synthesis transcripts (C). It is assumed that the microbial groups on the regression line have a neutral impact in the community, as vitamin synthesis processes are relatively well balanced with their requirements. Regression lines are determined by outlier removal with IWLS (10^6 maximum iterations, Supporting Information Table S4).

Phylogenetic group	Percentage of transcripts invested per taxon							
	VB1 syn	VB1 dep	VB6 syn	VB6 dep	VB7 syn	VB7 dep	VB12 syn	VB12 dep
Actinobacteria	0.039	1.571	0.189	0.881	0.011	0.198	0.029	0.344
Rhodobacterales	0.019	0.746	0.046	1.000	0.003	0.386	0.097	0.230
Rhizobiales	0.028	1.541	0.062	0.826	0.022	0.185	0.065	0.197
Rhodospirillales	0.055	1.201	0.086	0.927	0.020	0.260	0.047	0.059
Other unclassified Alphas*	0.010	0.833	0.010	0.357	0.023	0.104	0.045	0.052
SAR11	0.041	0.745	0.026	0.527	0.000	0.097	0.000	0.002
SAR116	0.002	0.983	0.046	0.608	0.000	0.150	0.017	0.053
Other alphas	0.047	0.996	0.094	0.692	0.031	1.243	0.134	0.083
Aquificales	0.059	0.429	0.112	1.268	0.018	0.270	0.012	0.023
Bacilli	0.063	0.595	0.074	0.783	0.008	0.241	0.025	0.127
Flavobacteria	0.023	1.085	0.009	0.063	0.122	0.430	0.009	0.003
Sphingobacteria	0.044	1.614	0.073	0.764	0.039	0.660	0.010	0.167
Cytophagia	0.036	0.934	0.020	1.148	0.010	0.492	0.027	0.274
Bacteroidia	0.050	0.120	0.001	0.565	0.025	0.073	0.007	0.080
Other bacteroidetes	0.050	0.757	0.741	7.381	0.021	0.170	0.004	1.003
Betaproteobacteria	0.103	1.233	0.060	0.891	0.030	0.322	0.088	0.146
Clostridia	0.020	1.166	0.180	0.675	0.005	0.200	0.028	0.117
Delta-Epsilon proteobacteria	0.043	0.722	0.033	0.797	0.020	0.286	0.020	0.129
Alteromonadales	0.033	0.615	0.037	0.569	0.020	0.259	0.013	0.127
Oceanospirillales	0.046	1.011	0.028	0.640	0.040	0.477	0.034	0.048
Pseudomonadales	0.099	1.471	0.071	1.092	0.022	0.235	0.017	0.071
Unclassified gammas	0.011	0.098	0.046	0.545	0.011	0.402	0.025	0.128
Methylococcales	0.085	0.779	0.069	2.620	0.120	0.386	0.097	0.255
Chromatiales	0.216	4.532	0.020	0.377	0.009	0.099	0.218	0.204
Other gammas	0.047	0.659	0.072	0.813	0.027	0.279	0.016	0.090
Planctomycetes	0.040	0.419	0.056	0.399	0.010	0.210	0.050	0.196
Verrucomicrobia	0.163	1.011	0.032	0.749	0.033	0.505	0.015	0.225
Cyanobacteria	0.077	0.572	0.095	0.759	0.027	0.241	0.147	0.101
Deinococci	0.007	0.756	0.266	0.795	0.004	0.156	0.596	0.475
Archaea	0.035	0.162	0.080	0.280	0.006	0.040	0.038	0.283
Eukaryotes	0.005	0.080	0.005	0.095	0.001	0.094	0.002	0.027
Viruses	0.000	0.034	0.000	0.021	0.000	0.000	0.057	0.000
Other bacteria	0.021	0.829	0.102	1.081	0.012	0.291	0.050	0.540
Average community	**0.038**	**0.780**	**0.053**	**0.687**	**0.020**	**0.279**	**0.043**	**0.135**

Fig. 3. Percentage of transcripts invested in B-vitamin metabolism (B-vitamin synthesis or dependence) for each phylogenetic group. Heatmap colors designate the level of transcriptome investment compared to the average microbial community. White background represents the average percent investment, while green are above and orange are below average respectively. *Unclassified alphaproteobacteria outside the SAR11 group.

remarkably small percentage of their transcripts in VB_7 synthesis, and to a lesser extent, in the production of VB_6, and VB_1. In contrast, the percentage of transcripts invested in VB_{12} synthesis and dependent processes by Rhodobacterales was close to the community average (Fig. 3). This suggests that in order to maintain their high level of total expression in this microbial ecosystem (Fig. 1), the Rhodobacterales may rely on other external vitamin sources (e.g., VB_7 produced by other microbes).

Flavobacteria were also well represented and dominated the expression of VB_7 synthesis genes, contributing as much as 64% of the total community synthesis transcripts for this vitamin (38% on average; Fig. 2A, Supporting Information Table S3 and S4). Moreover, the VB_7 requirements for this group were also higher than typical (Fig. 2), suggesting a potentially high VB_7 synthesis, usage and, thus, turnover for this group. It is currently known that different members of Flavobacteria, even close species of the same genus, have different vitamin-B_1 synthesis capabilities (Gómez-Consarnau *et al.*, 2016). However, as a group compared to the community average, Flavobacteria expressed a higher percentage of VB_1 dependence transcripts compared to synthesis (4% compared to 9% on average respectively; Fig. 2B and C, Supporting Information Table S3 and S4). VB_{12} synthesis gene transcripts were low in Flavobacteria (1% of the community). Furthermore, the expression of genes for VB_{12} dependent functions was almost absent (0.1% on average), suggesting a low requirement for this vitamin. This is consistent with the observation that none of the whole genome sequenced Flavobacteria available have a complete pathway for VB_{12} synthesis (Sañudo-Wilhelmy *et al.*, 2014). Notably, the percentage of VB_7 transcript synthesis was higher in this group than in any other taxon (0.12% of all Flavobacteria transcripts; Fig. 3). The potentially significant VB_7 contribution from Flavobacteria to the bacterial community and its VB_1 dependence are also consistent with whole genome results showing that about 60% of the sequenced cultures of that group produce VB_7 but only 25% can synthesize VB_1 and none can make VB_{12} (Sañudo-Wilhelmy *et al.*, 2014). However, our analysis showed that neither of the two Flavobacteria reference genomes that recruited the most transcripts in our study, Flavobacteria strains MS024-2A and MS024-3C, have the complete pathway to synthesize VB_7 *de novo* (Supporting Information Table S3). These organisms only partially expressed the VB_7 synthesis pathway and would potentially require the combined enzymatic activity of other bacteria to be able to obtain VB_7. Alternatively, it is possible that recruitment to Flavobacteria reference genomes split transcripts from a single population into different genome bins. Nevertheless, our data suggests that Flavobacteria, whether they have the complete gene set for VB_7 or not, potentially occupy an important niche for its production in this community.

Members of the SAR11 and SAR116 clades were also well represented in our samples (9% and 5% on average; Fig. 1, Supporting Information Table S3), and they are both auxotrophic for VB_1 and VB_7. However, these two groups differ in their VB12 synthesis potential. *Candidatus* Puniceispirillum marinum IMCC1322, the SAR116 genome recruiting the greatest number of reads in our samples, can synthesize VB12 while SAR11 members neither synthesize nor have requirements for this vitamin (Giovannoni *et al.*, 2005) (Fig. 2, Supporting Information Table S3). The percentage of community transcripts for both VB_{12} synthesis and dependence for SAR116 was the same (2%), suggesting that their VB_{12} synthesis could meet their requirements (Supporting Information Table S3). SAR11 and SAR116 genomes have incomplete pathways for the synthesis of VB_1 and can only produce one of the two moieties that form the thiamin molecule (4-methyl-5-(2-phosphoethyl)-thiazole, THZ) (Carini *et al.*, 2014). SAR11 VB_1 synthesis genes accounted for 10% of all the community transcripts while the VB_1 dependent functions were about 8% (Fig. 2). However, they would need to acquire the other VB_1 moiety (4-amino-5-hydroxymethyl-2-methylpyrimidine, HMP) from the environment. In contrast to SAR11, SAR116 VB_1 synthesis transcripts were nearly absent (0.3%) while their VB_1-requiring transcripts made up to 7% of the community (VB_1 dependence:synthesis residuals of 6%; Fig. 2C, Supporting Information Table S3), suggesting a strong dependence on exogenous sources of VB_1. In line with the total community transcriptomic data analysis (Fig. 2), the percentage of SAR11 and SAR116 transcripts invested in B-vitamin metabolism was low, only being slightly above the average community for VB_1 synthesis in SAR11 and dependence in SAR116 (Fig. 3).

Although the small eukaryotes (< 3 μm) accounted for up to 38% of the total transcripts (Fig. 1, Supporting Information Table S3), their B-vitamin synthesis genes always remained under 6% of the community B-vitamin synthesis transcripts (Fig. 2A, Supporting Information Table S3). Furthermore, the eukaryotic B-vitamin dependent gene transcripts remained under 11%, suggesting that their B-vitamin requirements were also relatively low (Fig. 2B, Supporting Information Table S3). This observation was consistent with the percent of eukaryotic transcripts invested in B-vitamin metabolism, which remained below the community average for all the vitamins (Fig. 3). This was unexpected since eukaryotic phytoplankton have been considered major vitamin-dependent organisms of the microbial plankton (Croft *et al.*, 2005; Tang *et al.*, 2010). However, complex regulatory processes in response to iron limitation (Cohen *et al.*, 2017) or riboswitch controls (McRose *et al.*, 2014) may impact the expression of vitamin-related genes, as observed in

eukaryotic alga. Another important consideration is that some eukaryotic phytoplankton could synthesize some vitamins using a non-canonical pathway. For instance, *Emiliania Huxleyi* can grow using either a complete VB_1 molecule or only one of the VB_1 – forming moieties (HMP) while lacking the genes that encode for the other moiety (HET-P; 4-methyl-5-b-hydroxyethylthiazole adenosine diphosphate), suggesting an alternative uncharacterized VB_1 synthesis pathway (McRose *et al.*, 2014). In contrast, picoeukaryotic algae (e.g., *Micromonas* and *Ostreococcus*) can utilize VB_1 but not the individual moieties that form the vitamin (McRose *et al.*, 2014; Paerl *et al.*, 2015). Instead these picoeukaryotic phytoplankton are able to meet their VB_1 requirements using a still chemically uncharacterized HET-P precursor together with HMP (Paerl *et al.*, 2017). Finally, larger eukaryotic phytoplankton groups (> 3 μm) were not included in this analysis and may have a more skewed ratio of synthesis to dependence transcripts.

In contrast to eukaryotic phytoplankton, betaproteobacterial B-vitamin synthesis genes were high for VB_1, VB_{12} (average: 14% and 10% respectively) and for VB_7 (8%; Fig. 2; Supporting Information Table S3). Furthermore, their vitamin dependence genes generally accounted for a similar percent of the community (close to the typical regression lines, Fig. 2), suggesting that this bacterial group was very active at producing and utilizing these B-vitamins. Consistent with this observation, the percentage of total betaproteobacterial transcripts invested in B-vitamin metabolism appeared to always be above the community average (Fig. 3). However, there were significant differences in the number of VB_{12} synthesis and dependence transcripts within Betaproteobacteria (Supporting Information Table S2). While the abundant betaproteobacterial genomes dominated the VB_{12} requirement transcripts (e.g., *Methylovorus glucosetrophus* SIP3–4, beta proteobacterium KB13 or Methylophilales bacterium HTCC2181), the recruited reads for VB_{12} synthesis belonged to different Betaproteobacteria (e.g., *Ralstonia solanacearum* PSI07 and *Chromobacterium violaceum* ATCC 12472), which appeared to be much less abundant in this environment (Supporting Information Table S2).

Overall, our data suggests that the vitamin synthesis and utilization potential is compartmentalized among the different members of the microbial community (Figs 1–3). Some microbial taxa representing a small percent of community transcripts appeared to be nonetheless important synthesizers of some B-vitamins (Fig. 2A, Supporting Information Table S3), such as Verrucomicrobia with VB_1 ($<$ 4% of total transcripts but 13% of VB_1 synthesis transcripts; dependence:synthesis residual of -5%), Actinobacteria with VB_6 (2% of total transcripts but 7% synthesis transcripts; dependence:synthesis residual of -4%), Cyanobacteria with VB_{12} (3% of total transcripts but 9% of synthesis transcripts; dependence:synthesis residual of -5%) and Methylococcales with VB_{12} (1% of total transcripts but 6% of synthesis transcripts; dependence:synthesis residual of -3%). Thus, less abundant members of the bacterial assemblage might also be important vitamin producers in the community, and therefore, play larger ecological roles than their abundance would suggest. Our data suggest that this coastal marine microbial community is composed of organisms with distinct transcript contributions to vitamin synthesis and utilization. These expression patterns are certainly complex and may be the result of a combination of diverse regulatory processes that are specific to some taxonomical groups (e.g., eukaryotic phytoplankton; McRose *et al.*, 2014) and are difficult to discern at the community level. Future controlled laboratory studies using specific members of the microbial community will be needed to remove any constraints in our field results.

Seasonal and circadian regulation of B-vitamins gene expression and their potential impact on water-column dissolved vitamin concentrations

We hypothesized that the ambient dissolved vitamin concentrations measured in the water column should reflect, to some extent, a balance between the synthesis and utilization by the microbial community. During our study, the concentrations of dissolved VB_7 and VB_{12} were the lowest of all, and below our detection limits. These low levels of VB_7 and VB_{12} might suggest a very tight synchronization between synthesis and utilization that may not allow any build-up of these two vitamins in this coastal environment. This hypothetical tight synthesis-uptake coupling, however, was not observed at the level of gene expression, as the dependence:synthesis transcripts for VB_{12} and VB_7 varied over time with no clear temporal patterns (Fig. 4). In this study, we only quantified the cyanocobalamin form of VB_{12} and we cannot rule out the presence of the other upper (methyl-, adenosyl- and hydroxo-) and lower (pseudo-) axial ligand forms of this vitamin (Banerjee, 1997; Suárez-Suárez *et al.*, 2011; Helliwell *et al.*, 2016; Heal *et al.*, 2017; Suffridge *et al.*, 2017). It is still unclear how the availability of the different chemical forms relates to gene expression and further research will need to address this question.

Dissolved VB_1 ranged from 0.1 to 5 pM and followed diel oscillations, with consistently higher levels during the day compared to night within each season (Fig. 4). However, as for VB_7 and VB_{12}, the VB_1 dependence:synthesis transcripts did not show any clear diel or seasonal dynamics (Fig. 4). We attributed the higher dissolved VB_1 concentration during the day to bacterioplankton VB_1 production or excretion, as most of the synthesis transcripts belonged to heterotrophic bacteria rather than to small phytoplankton. (Fig. 2A, Supporting Information Table S3). Nonetheless, we cannot rule out the impact of the large phytoplankton
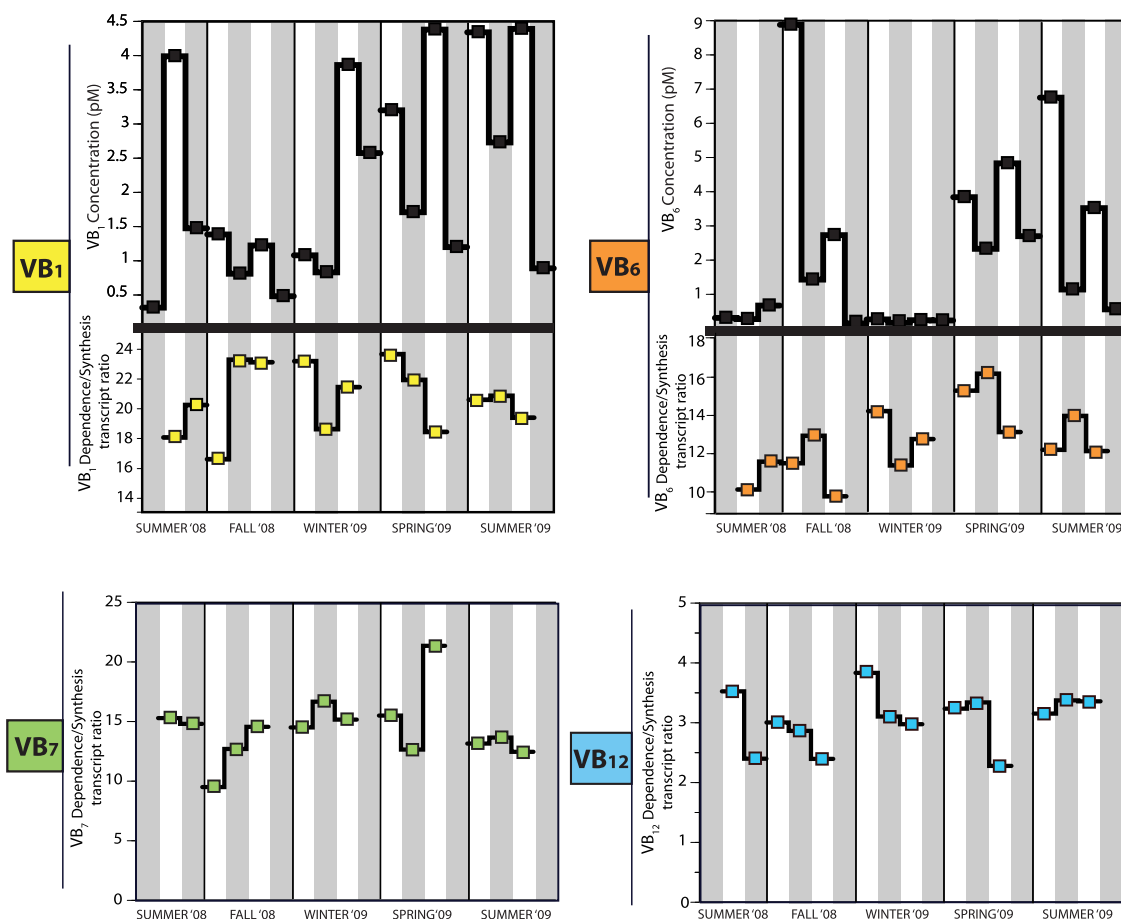
Fig. 4. Environmental concentrations of dissolved VB_1 and VB_6 compared to vitamin dependence:synthesis transcript ratios. Concentrations of dissolved VB_7 and VB_{12} were below our detection limit and only vitamin dependence:synthesis transcript ratios are shown. Grey and white background denotes night and day samples, respectively. Specific concentrations and times of sampling can be found in Supporting Information Table S6.

(> 3 μm) on vitamin levels as they were not included in our samples. One explanation for the overall higher VB_1 concentrations in the day samples could be its function as photo-protectant to oxidative stress during light exposure, as shown for *Escherichia coli* and *Arabidopsis thaliana* (Jung and Kim, 2003; Tunc-Ozdemir *et al.*, 2009). Notably, Gifford *et al.* (2014) showed that the activity of Rhodobacterales (inferred by the expression of ribosomal proteins) in the same metatranscriptomes was also influenced by diel cycles. These similar patterns could indicate a relationship between the activity of Rhodobacterales and the observed higher concentrations of dissolved VB_1 found in the day samples, as most Rhodobacterales are auxotrophic for this vitamin or at least for one of its moieties (Table 2). However, the matter of VB_1 availability is further complicated by the fact that several microbial groups can only meet their biological demands with the uptake of VB_1 precursors instead of the complete VB_1 molecule (Carini *et al.*, 2014). Even though we did not quantify the different moieties of this vitamin, knowing the standing stock of VB_1

is relevant, as some of its decomposition products could be used for cellular growth by diverse phytoplankton groups (Gutowska *et al.*, 2017). Future studies will need to address the effect of VB_1 moieties and pathway intermediates in natural communities as recently established in culture growth studies (Gutowska *et al.*, 2017; Paerl *et al.*, 2017).

The levels of dissolved VB_6 ranged from 0.1 to 8.9 pM and were also higher in the day samples, except for winter '09 when all VB_6 concentrations were below our detection limit (< 0.1 pM; Fig. 4). Similar to VB_1, the higher VB_6 concentrations during the day could be explained by its function as antioxidant during light exposure (Bilski *et al.*, 2000; Mooney *et al.*, 2009). In fact, the antioxidant properties of VB_6 against oxidative stress even exceed those of vitamins C and E (Ehrenshaft *et al.*, 1999). Furthermore, this coenzyme is particularly important in amino acid metabolism (e.g., amino acid synthesis and transaminations; Hayashi, 1995), which may be more relevant during the night (Cuhel *et al.*, 1984; Poretsky *et al.*, 2009; Gifford

Table 2. Presence of B-vitamin synthesis pathways for the 30 most abundant genomes (according to total transcripts) found in the Sapelo island metatranscriptome.

			Total hits	VB1-HMP ($ThiC/thi5$)	VB1-HET-P ($thiG/thi4$)	VB6 ($pdxJ$)	VB7 ($bioB$)	VB12 *
Bacteria	Alphaproteobacteria	Unclassified Alphaproteobacteria						
		Candidatus Puniceispirillum marinum IMCC1322	610 543	×	✓	✓	×	✓
		Candidatus Pelagibacter sp. HTCC7211	543 968	×	✓	✓	×	×
		Candidatus Pelagibacter ubique HTCC1002	188 171	×	✓	✓	×	×
		Candidatus Pelagibacter ubique HTCC1062	122 266	×	✓	✓	×	×
		Candidatus Pelagibacter sp. IMCC9063	68 417	×	✓	✓	×	×
	Rhodobacterales	alpha proteobacterium BAL199	132 928	✓	✓	✓	×	✓
		Roseobacter sp. AzwK-3b	69 182	×	✓	✓	×	✓
		Silicibacter lacuscaerulensis ITI-1157	60 964	×	✓	✓	×	✓
		Dinoroseobacter shibae DFL 12	51 176	✓	✓	✓	×	✓
		Rhodobacteraceae bacterium HTCC2083	76 242	×	×	✓	✓	×
		Rhodobacterales bacterium HTCC2255	61 449	×	×	✓	×	×
		Rhodobacteraceae bacterium HTCC2150	53 528	×	×	✓	×	×
	Gammaproteobacteria	Unclassified Gammaproteobacteria						
		marine gamma proteobacterium HTCC2080	188 652	×	×	✓	×	×
		gamma proteobacterium HTCC2207	126 002	×	×	✓	×	×
		gamma proteobacterium IMCC3088	108 019	×	×	✓	×	×
		marine gamma proteobacterium HTCC2143	92 583	×	×	✓	✓	×
		marine gamma proteobacterium HTCC2148	80 092	×	×	✓	×	×
		gamma proteobacterium NOR51-B	116 949	×	×	✓	×	×
		gamma proteobacterium NOR5-3	61 805	×	×	✓	×	×
	Betaproteobacteria	Methylophilales						
		Methylophilales bacterium HTCC2181	66 512	✓	✓	✓	✓	×
		Unclassified Betaproteobacteria						
		beta proteobacterium KB13	71 029	✓	✓	✓	✓	×
	Bacteroidetes	Flavobacteria						
		Flavobacteria bacterium MS024-2A	126 000	×	×	✓	×	×
		Flavobacteria bacterium MS024-3C	65 893	×	×	✓	×	×
	Verrucomicrobia	Opitutae						
		Coraliomargarita akajimensis DSM 45221	97 641	✓	✓	✓	✓	×
		Verrucomicrobiae						
		Pedosphaera parvula Ellin514	75 244	✓	✓	✓	✓	×
		Verrucomicrobiae bacterium DG1235	62 508	×	×	✓	×	×
Archaea	Nitrosopumilales	Nitrosopumilaceae						
		Nitrosopumilus maritimus SCM1	164 627	✓	✓	✓	✓	✓
Eukaryota	Chlorophyta							
		Micromonas sp. RCC299	139 279	×	×	✓	✓	×
		Ostreococcus tauri	103 422	×	×	✓	✓	×
		Chlorella variabilis	63 212	✓	✓	✓	✓	×

Vitamin-B₁ synthesis was determined using the *thiC-thi5* and *thiG-thi4* genes for the production of the 4-amino-5-hydroxymethylpyrimidine (HMP) and 4-methyl-(β-hydroxyethyl) thiazole (THZ) moieties respectively; Vitamin-B₆ synthesis was determined by the presence of the *pdxJ* gene. Vitamin-B₇ synthesis was determined using the *bioB* biotin synthase gene. To account for possible missannotations of single genes on the vitamin-B₁₂ synthesis pathway, it was considered present when a genome contained more than 75% of the VB₁₂ *de novo* synthesis genes of orthologous groups (COGs) retrieved from the Integrated Microbial Genomes database (http://img.jgi.doe.gov) (COG0007, COG0310, COG0368, COG1010, COG1270, COG1429, COG1492, COG1797, COG1903, COG2073, COG2082, COG2087, COG2099, COG2109, COG2241, COG2242 and COG2243), as previously reported by Sañudo-Wilhelmy et al. (2014).
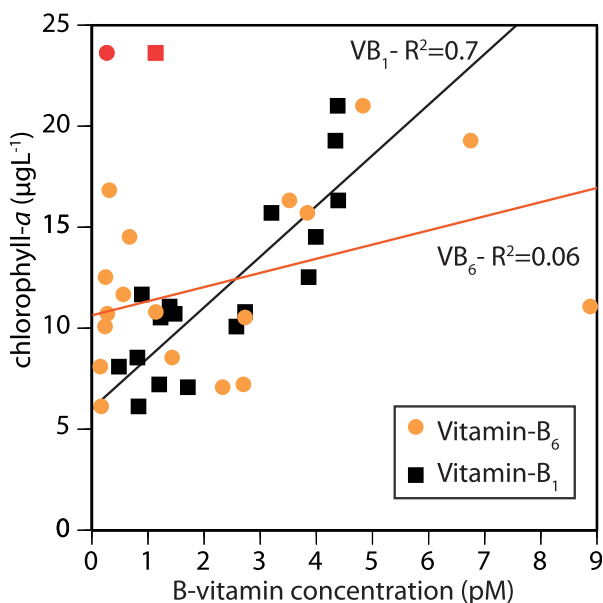
Fig. 5. Correlation between total chlorophyll-*a* and dissolved VB$_1$ and VB$_6$ concentrations. The red data points are considered outliers and were excluded from the statistical analysis; those correspond to the sample collected in winter '09.

et al., 2014). Yet, we did not observe diel oscillations in VB$_6$ dependence:synthesis transcript ratios. This is in contrast to a metatranscriptomic study in the South Pacific, in which the expression of VB$_6$ synthesis genes was higher during the night compared to the day (Poretsky *et al.*, 2009). Additional studies analyzing intracellular B-vitamin concentrations will be necessary to identify their circadian fluctuations in relation to the internal cellular processes over time.

The observed higher dissolved B-vitamin concentrations during sunlight exposure could have important consequences for the functioning of the marine microbial community. An obvious one is that vitamin auxotrophs that take advantage of this source would end up following the same circadian rhythm as the vitamin producers. Ottesen *et al.* (2014) reported a tight diel synchronization between the metatranscriptomes of the cyanobacteria *Prochlorococcus* and heterotrophic bacterioplankton *in situ*, suggesting an interaction (direct or indirect) between these organisms (Armbrust, 2014; Ottesen *et al.*, 2014). In our study, dissolved VB$_1$ concentrations were positively correlated to Chl-*a* throughout the yearlong dataset (Fig. 5). Although a direct cause–effect relationship between the two parameters has not been demonstrated, this vitamin is involved in carbon fixation during the Calvin cycle (Sañudo-Wilhelmy *et al.*, 2014; Monteverde *et al.*, 2017). Because the VB$_1$ dependent transcripts of eukaryotic picophytoplankton were low, the correlation between dissolved VB$_1$ and total Chl-*a* may involve the larger phytoplankton

community not sampled by our size-fractionation scheme ($0.2 > 3 \ \mu$m). Further studies will be needed to evaluate the cause of the observed VB$_1$/Chl-*a* relationship and confirm the identity of the key organisms responsible for this interdependence. In contrast to the strong correlation between VB$_1$ and Chl-*a*, we did not find any relationship between the concentrations of dissolved VB$_6$ and phytoplankton biomass (Fig. 5).

Although the auxotrophy for VB$_1$, VB$_7$ and VB$_{12}$ has been recognized in marine microbes for decades (Provasoli, 1963; Provasoli and Carlucci, 1974; Croft *et al.*, 2005; Sañudo-Wilhelmy *et al.*, 2014), VB$_6$ dependence had not yet been considered. Indeed, VB$_6$ auxotrophy is likely to be minor as the VB$_6$ synthesis pathway is widespread in free-living microorganisms and plants and more than a hundred enzymatic reactions are catalyzed by this coenzyme (Hayashi, 1995). Nonetheless, our results suggest that some members of the microbial community express more VB$_6$ synthesis transcripts than others in relation to their total transcript contribution to the community and their vitamin requirement transcripts (Fig. 2). Moreover, if the excess of VB$_6$ is released to the environment on daily basis, it is possible that at least some taxa have adapted to take advantage of this source to reduce their vitamin synthesis requirements. Finally, because the inferred vitamin metabolism in this coastal community involves bacterial groups that are cosmopolitan, our findings could potentially be extrapolated to other marine environments.

Acknowledgements

Author contributions

LG-C and MM designed the study. SG collected and processed field samples. LC and SS-W analyzed B-vitamin concentrations. LG-C, RS, SG, LC, JF, SS-W, MM analyzed data and contributed to the writing of the paper.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of B-vitamin synthesis and B-vitamin dependent functions used in the metatranscriptome analysis. Vitamin dependent functions were identified using the ExPASy database (http://www.expasy.org) and vitamin synthesis genes were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/). In addition to the listed enzymes, the reads annotated as

'vitamin synthesis' or 'vitamin dependent/requiring' included in Table S2 and Table S3 were also included.

Table S2. List of all B-vitamin synthesis and B-vitamin dependent function reads identified in the metatranscriptomics dataset. In addition to the enzymes listed in table S1, reads annotated as 'vitamin synthesis' or 'vitamin dependent/requiring' were also included.

Table S3. Summary of the B-vitamin synthesis and dependent gene transcript abundance for the best-represented taxonomic groups in each individual sample.

Table S4. Percentages of B-vitamin synthesis and dependence transcripts in different microbial taxa and regression lines shown in Fig. 2.

Table S5. Comparison of annotation methods for vitamin synthesis and dependence transcripts. To verify that RefSeq description annotation accurately represents counts of vitamin synthesis and dependence, reads were also classified by sequence similarity to a list of vitamin synthesis and dependence genes. Reference sequences for synthesis and dependence genes of each vitamin were retrieved from the RefSeq protein database. Metagenomic reads were searched against separate BLAST databases for the synthesis and dependence genes associated with each vitamin. Only matches with a bit score $>= 40$ and that did not have a higher scoring non-vitamin RefSeq match were retained.

Table S6. Environmental concentrations of Chlorophyll-a, vitamin B_1 and B_6 and specific sampling times.