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B_{12} production by marine microbial communities and *Dinoroseobacter shibae* continuous cultures under different growth and respiration rates

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ABSTRACT: *In situ* dissolved B_{12} concentration in marine ecosystems is controlled by the balance between rates of release of B_{12} by prokaryotes, uptake by prokaryotes and eukaryotes, and abiotic degradation. We used chemostats at a range of specific growth rates (μ, d^{-1}; 0.1 to 1) with natural communities of prokaryotes and monospecific cultures of a B_{12} producer, *Dinoroseobacter shibae*. We measured the dissolved B_{12} concentration produced in the culture ($B_{12\text{-d}}$), the B_{12} in the particulate fraction ($B_{12\text{-p}}$), cell concentration, respiration rate, particulate organic carbon and nitrogen (POC, PON), and the 16S amplicon composition. Total dissolved B_{12} concentrations (0.92 to 4.90 pmol l^{-1}) were comparable to those found in the surface ocean. $B_{12\text{-p}}$ concentration was 6 to 35 times higher than $B_{12\text{-d}}$. $B_{12\text{-d}}$, $B_{12\text{-p}}$, and community composition showed no relation to μ for either natural populations or *D. shibae*. The chemostats allowed calculation of the rates of production: $B_{12\text{-d}}$ (0.34 ± 0.28 pmol l^{-1} d^{-1}) and $B_{12\text{-p}}$ (5.65 ± 2.34 pmol l^{-1} d^{-1}), and the B_{12} cell quota (900 to 3300 molecules cell^{-1}). In multispecies and *D. shibae* cultures, B_{12} production rates per cell increased with respiration rates (volumetric or per cell), and with rates of cellular organic carbon and nitrogen production. Rates increased with μ, but not the concentrations of $B_{12\text{-d}}$ or of $B_{12\text{-p}}$. To understand the physiological and ecological dynamics of B_{12}, concentrations alone are insufficient since they do not provide rates, which are important in understanding the dynamics between producers and consumers.

KEY WORDS: Marine bacteria · Intracellular and dissolved B_{12} · Continuous cultures

1. INTRODUCTION

The presence and importance of organic compounds as regulators of plankton growth in the ocean has been known for almost 100 yr; one group of compounds that has been studied in detail is the group of B vitamins, specifically biotin (B_7), thiamin (B_1), and cobalamin (hereafter B_{12}). Measurements of B_{12} in the ocean have indicated sub-picomolar concentrations offshore and picomolar concentrations in coastal regions (Menzel & Spaeth 1962, Okbamichael & Sañudo-Wilhelmy 2004, Sañudo-Wilhelmy et al. 2006, Panzeca et al. 2009). Despite its low concentration in seawater, B_{12} plays a vital role in auxotrophic prokaryotic and eukaryotic organisms (Sañudo-Wilhelmy et al. 2014). Approximately half of eukaryotic phytoplankton are dependent on the B_{12} produced by bacteria and archaea (Croft et al. 2005, Sañudo-Wilhelmy et al. 2012, Helliwell 2017, Gómez-Consarnau et al. 2018). Helliwell et al. (2016) showed the rapid experimental adaptation of a B_{12}-independent algae, *Chlamydomonas reinhardtii*, to a B_{12}-dependent clone. It has been suggested that eukaryotic microorganisms that are now auxotrophic have lost the capacity to synthesize the B_{12}-independent methionine synthase (MetE) (Helliwell et al. 2011), and it is possible that a similar mechanism exists for prokaryotes. In this case, they would depend on the

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enzyme methionine synthase (MetH), which requires B_{12} as a cofactor. Laboratory studies have indicated that the concentration of dissolved B_{12} required to maintain axenic algal growth ranges from sub-picomolar and picomolar (Tang et al. 2010, Bertrand et al. 2012) to 15 and 37 pmol l^{-1} (Kazamia et al. 2012). These concentrations are comparable to those found in the ocean (Sañudo-Wilhelmy et al. 2014).

The literature indicates that the production of B_{12} is unique to prokaryotes (Warren et al. 2002, Grossman 2016). *In silico* analysis of microbial genomes suggests that the potential to synthesize B_{12} is found in different groups of bacteria and archaea (Rodionov et al. 2003, Doxey et al. 2015, Heal et al. 2017). Genetic and experimental evidence has shown that some bacterial taxa lack the de novo B_{12} biosynthetic pathway; instead, they possess salvage pathways or assimilate extracellular B_{12} precursors (Burkholder & Lewis 1968, Woodson & Escalante-Semerena 2004, Helliwell et al. 2016). Taylor & Sullivan (2008) found heterotrophic bacteria communities in the ocean that assimilate B_{12} at rates proportional to their productivity and biomass. To maintain proportionality, the ratio of B_{12} producers and consumers in bacterial communities should be preserved. However, it is not clear how the B_{12} demand of the bacterial community is supplied in the ocean, considering the low growth rates of bacterioplankton (0.05 to 0.3 d^{-1} in oligotrophic systems and coastal waters, respectively; Ducklow 2000).

Currently, it is difficult to estimate the potential for B_{12} biosynthesis in microbial communities based on their taxonomic composition (Helliwell et al. 2016, Heal et al. 2017). Bertrand et al. (2011) showed that of 45 sequenced strains of *Vibrio* genomes, only *V. splendidus* LGO32, and *Vibrio* MED222 have the potential to synthesize B_{12}. Moreover, the potential for the microbial community to produce B_{12} is not only dependent on its taxonomic composition but also on environmental conditions such as temperature, availability of dissolved organic matter (DOM), cobalt, nitrogen, and iron (Bertrand et al. 2011).

The B_{12} biosynthesis pathway is energetically expensive, involving 30 genes and up to 3% of cellular metabolism (Roth et al. 1996, Raux et al. 2000), therefore only a minority of healthy bacteria are expected to exude or release B_{12} to the sea (Provasoli 1963, Berland et al. 1976). One probable pathway for microbial producers to provide B_{12} to auxotrophic prokaryotes and eukaryotes is release by lysis (cell death, exudation, senescence, viral processes) (Wilhelm & Suttle 1999, Karl 2002, Croft et al. 2005, 2006, Droop 2007).

The dissolved B_{12} concentration in seawater results from the balance of production to consumption, including B_{12} degradation by photochemistry and other processes. There is little information available on the cellular B_{12} concentration (Sañudo-Wilhelmy et al. 2014). Recently, Suffridge et al. (2017) measured particulate and dissolved B-vitamins in cultured marine bacteria and environmental samples and observed that the concentration of dissolved and particulate vitamins varied between microbial communities without a definite pattern between these 2 pools. In the present study, our goal was to obtain information that would help to interpret the dissolved and particulate B_{12} concentrations found in natural waters. We used natural bacterial communities that should have included consumers and producers as well as monospecific cultures of a B_{12} producer, *Dinoroseobacter shibae*, to explore the coupling between dissolved and particulate B_{12} production rates, applying continuous cultures under substrate-limited growth at different slow growing rates. *D. shibae* was chosen for the mono-species culture for different reasons: its capacity for B_{12} production (Wagner-Döbler et al. 2010), and because it has been shown to be a common member of the organotrophic marine community and has the ability to supply marine phytoplankton with B_{12} in culture (Cruz-López et al. 2018).

2. MATERIALS AND METHODS

2.1. Culture preparation

All cultures were run as chemostats (Herbert et al. 1956), either monospecific (*Dinoroseobacter shibae*) or inoculated with natural coastal bacterial communities. The cultures were prepared following Cajal-Medrano & Maske (2005). Batches of 20 l of growth media were prepared from GF/F-filtered seawater aged in the dark in glass containers for at least 3 mo. The seawater was collected in the California Current. During aging, the seawater was filtered again (GF/F). At the end, the seawater was bubbled with an ozone stream for 24 h, then activated charcoal was added for 24 h, which was then removed by GF/F filtration. We could not be sure that all degraded B_{12} species were removed by the activated charcoal, therefore we measured the dissolved B_{12} concentration in the growth media (see below). Inorganic nutrients and glucose were added as an organic carbon source (NH_4Cl: 30 µM; KH_3PO_4: 5 µM; $FeCl_3$: 0.4 µM;

glucose: 20 µM). Subsequently, the growth media was acidified by bubbling with CO_2, autoclaved for 1 h at 1.5 psi (~10 kPa), cooled down to room temperature, and bubbled with sterile air to replenish O_2 back to saturation. Final growth media pH ranged from 8.1 to 8.3. All containers and tubing used for the continuous culture chemostat systems were Teflon or silicon, as described by Cajal-Medrano & Maske (2005). Before terminating and measuring each chemostat, we assured the sterility of the growth medium by inoculating it in sterile Zobell growth medium (see Fig. 1 in Cajal-Medrano & Maske 2005).

The inoculated culture was maintained as a batch culture for 24 h in the dark and then diluted at specific dilution rates between 0.1 and 1 d^{-1}; cultures were stirred slowly and run in the dark at 18°C. The steady-state of the continuous cultures was defined by a constant bacterial abundance that varied from 2.31×10^6 to 7.16×10^6 cells ml^{-1} depending on the dilution rate. Samples were collected at steady-state to measure intracellular and dissolved B_{12}, bacterial abundance and virus-like particles (VLPs), particulate organic carbon (POC), particulate organic nitrogen (PON), CO_2 production, and taxonomic composition. The steady state was defined by <10% change; it was reached after 10 h at high dilution rates and was longer at low dilution rates up to 100 h.

For each culture, a separate inoculum was collected from coastal waters, Bahía Todos Santos (31° 51′ N, 116° 40′ W) or the station Antares (31° 75′ N, 116° 95′ W) between February 2013 and September 2013. The inoculum seawater sample of 250 ml was filtered through a 0.8 µm polycarbonate filter before inoculation. Each inoculum was expected to have a different bacterial community composition. We used the dilution rate throughout to identify the experimental results. We specifically collected inocula for each experiment at different times and different locations because we sought results related to dilution rates independent of the taxonomic composition of the community.

The marine bacterium *Dinoroseobacter shibae* (from Dr. D. Bartlett, Scripps Institute of Oceanography) was inoculated from maintenance cultures growing in liquid growth medium (NH_4Cl: 30 µM; KH_3PO_4: 5 µM; $FeCl_3$: 0.4 µM; glucose: 20 µM). The continuous cultures were aseptically inoculated with 10 ml of the 0.8 µm filtered sample to the 2 l sterile chemostat vessel. Microscopic samples did not indicate contamination by phototrophic organisms at any time. The culture volume of 2 l only allowed for the collection of one sample each for POC/PON, DNA, inorganic carbon, and B_{12} analysis.

2.2. Enumerating bacteria and VLPs by microscopic examination

Bacterial cell abundance and VLPs were determined by counting randomly selected fields, using an epifluorescence microscope. A 20 ml sample of culture was fixed with buffered formaldehyde (2% final concentration). Then, 0.2 to 0.5 ml of the sample was incubated with 4′,6-diamino-2-phenylindole (DAPI; final concentration: 1 µg ml^{-1}) (Porter & Feig 1980) and immediately filtered on 0.2 µm black polycarbonate filters (Poretics). A total of 10 fields were counted for >300 cells (Kirchman 1993) using a epifluorescence microscope (Carl Zeiss) with an 100× objective, and a 175 W xenon lamp (Lambda LS, Sutter) connected through a liquid light guide.

To verify the manual count of bacteria, we used image stacks (0.2 µm focal distance between images) with a CCD camera (Clara E, Andor) and a Focus Drive Motor (Ludl Electronic Products) controlled with Micro-Manager software (v.1.3.40; Vale Lab, University of California at San Francisco). The image stacks were processed using the ImageJ program (Schneider et al. 2012). Biovolume estimates were based on the area representing bacteria as documented in the camera images using ImageJ processing.

For VLP enumeration, samples were stained with SYBR Green I (1:500 final dilution commercial stock; Molecular Probes). A 1 ml aliquot of fixed samples was filtered through a 0.02 µm pore size Anodisc filter (25 mm diameter; Whatman). Samples were then incubated in the dark for 10 min and mounted using an anti-fade solution (10% of p-phenylenediamine in a solution of equal parts of 0.05 M phosphate-buffered saline and glycerol).

2.3. Determination of POC and PON

Samples for POC and PON were obtained by filtering the culture (approximately 400 ml) and the growth medium (800 ml) using pre-combusted glass fiber filters (GF/F) (450°C, 2 h). The filters were stored frozen in scintillation vials at −40°C and dried in an oven at 60°C. POC and PON were measured with a CHN analyzer in the laboratory of Marine Science at the University of California, Santa Barbara. For part of the samples, we used a second filter after the proper sample filter to estimate the amount of organics adsorbed to the glass fibers of the filter. Because the amounts of POC and PON in the cultures were calculated from the difference of the particulate organics in the growth medium and the cul-

tures, a correction for organics adsorbed to the filter was not necessary because we assumed that both sample filters had the same quantity of organics adsorbed.

2.4. Respiration measured by CO_2 production

Bacterial respiration by CO_2 production was determined by measuring dissolved inorganic carbon (DIC). The concentration of DIC was measured using the coulometric method described by Johnson et al. (1987). Certified reference material (CRM) from Dr. Andrew Dickson's laboratory at Scripps Institution of Oceanography, University of California at San Diego was used for assessing the precision and accuracy of measurements. The reference material gave a relative difference averaging 2.2 ± 1.1 µmol kg^{-1}, with a maximum of 4 µmol kg^{-1} (0.2 % error) concerning the certified value. Bacterial respiration was calculated from the difference between the DIC concentration in culture and the sterile growth medium. This difference was multiplied by the specific dilution rate to obtain the respiration rates.

2.5. Extraction and ELISA quantification of dissolved and intracellular B_{12}

The dissolved and particulate fraction of B_{12} were separated by combusted glass fiber filter (GF/F). The low concentration of dissolved B_{12} in the filtrate was concentrated by solid phase extraction (Okbamichael & Sañudo-Wilhelmy 2004). Between 500 and 1000 ml of sample were concentrated in columns with 2 g of C18 resin (Bondesil-C18 HF; Agilent Technologies) using a sample flow rate of 1 ml min^{-1}. The column was then extracted with 5 ml methanol (≥99.9 %; Sigma-Aldrich #34860). To prepare the samples for the ELISA immunoassay, the methanol was evaporated in a water bath at 60°C, applying a gentle vacuum for 6 min; the dried samples were re-suspended in distilled water (2 ml final volume) (Zhu et al. 2011) to be used for the ELISA assay. The extraction efficiency was tested using the B_{12} standard of the ELISA kit, by processing the standard the same way as the samples including solid phase extraction, methanol evaporation including heating, and taking up in water. The extraction efficiency was 84.9 % with a coefficient of variation of 17.2 %. Separately, we tested the effect of heating B_{12} to 60°C for 6 to 15 min in methanol under vacuum, which resulted in a loss of 11 % of B_{12}. This means that most of the loss of the extraction procedure was due to the heating in methanol under vacuum. The dissolved B_{12} data were corrected for the extraction efficiency. We measured the dissolved B_{12} in the growth media and in the cultures and defined the difference between both concentrations as dissolved B_{12} ($B_{12\text{-d}}$) because we were interested in the B_{12} production by the culture. See Table 1 for the total dissolved B_{12} concentration and $B_{12\text{-d}}$. The total dissolved B_{12} concentration refers to the measured concentration without subtracting the concentration in the pure growth medium, whereas $B_{12\text{-d}}$ refers to the concentration minus the concentration in the pure growth medium. The limited culture volume did not allow taking replicate samples for B_{12} measurements.

The particulate B_{12} fraction on the filter was extracted in 1.5 ml of NaCN buffer (1 M final concentration; pH: 4.5) to ensure B_{12} stability in the sample extract (Van Wyk & Britz 2010). The particulate B_{12} samples ($B_{12\text{-p}}$) collected on GF/F filters were homogenized with a homogenizer (Beadbeater; BioSpec) for 600 s in 2 steps to avoid heating, at 5000 oscillations min^{-1} with 0.5 mm diameter zirconia/silica beads. After homogenization, to ensure complete extraction without breaking down B_{12}, the samples were frozen (−40°C) and heated (60°C) at least 5 times with repeated treatment by ultrasound (constant frequency of 30 kHz). The extract was passed through a Sephadex column (PD-10; GE-Healthcare) to eliminate NaCN. A 1 ml sample of the eluent was used for the ELISA assay. To test the matrix effect, a *D. shibae* culture was spiked with a known amount of B_{12}. The concentration of B_{12} recovered from the spiked sample was 20 % higher than the sum of the added B_{12} and the $B_{12\text{-p}}$ measured in the *D. shibae* culture. We cannot explain this analytical error of an increase of 20 % and therefore we decided not to correct the $B_{12\text{-p}}$ concentration of the cultures.

Contrary to the $B_{12\text{-d}}$ samples, we did not routinely take samples for $B_{12\text{-p}}$ from the growth media. In preliminary experiments we found no $B_{12\text{-p}}$ in culture growth media, which we explained by the process of growth media preparation (see above) minimizing the presence of particulate B_{12}.

To obtain the B_{12} production rates (mol B_{12} l^{-1} d^{-1}), and B_{12} production rates per cell (mol B_{12} $cell^{-1}$ d^{-1}), we multiplied the respective concentrations by the specific dilution rates (µ, d^{-1}).

A sensitive, specific ELISA (Immunolab) was used to quantify B_{12} (Zhu et al. 2011). This method does not distinguish between the different B_{12} components Zhu et al. (2011) could quantitatively detect (cyanocobalamin, methylcobalamin, coenzyme B_{12}, and hy-

droxycobalamin) and compared favorably with the HPLC method. They also showed that the method does not recognize other B vitamins including thiamin (B_1), riboflavin (B_2), and pyridoxine (B_6). Zhu et al. (2011) did not test the response of the assay to pseudocobalamin (a vitamin that can serve a similar metabolic function as B_{12}); therefore, we cannot be sure that this method included the quantification of pseudocobalamin. Thus, when we refer to our data as B_{12} this includes the above-mentioned different forms of the cobalamin.

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between B_{12} concentration in the sample and the assay signal intensity. Once the protocol assay indicated by the manufacturer was completed, we ran the microplate reader and immediately conducted measurement at 450 nm. For the standard curve, the data were linearized by plotting the log of the B_{12} concentrations (standards provided by the ELISA company: 0, 0.4, 1, 4, 10, and 40 ng ml^{-1}) versus the OD, and the calibration equation was determined by regression. The value of r^2 for the different regressions of the standards varied from 0.97 to 0.99. The sample concentration was multiplied by the dilution factor ($\bar{x}$ = 1550), which gave a detection range for the samples from 0.3 to 25.8 pg ml^{-1} of the B_{12} provided by the standards. The average coefficient of variation of 15 different triplicate standards was 2%. We tested the reproducibility of the optical measurement of the plate readers by comparing 2 different microplate spectrophotometers of the same type (Multiskan GO UV/Vis; Thermo Scientific). We also tested the reproducibility of the measurements adding 10 ng ml^{-1} B_{12} standard (Immunolab) to samples. The results showed no significant difference between plate readers. We also tested the possible photodegradation of B_{12} during sample handling; we assume that there was no degradation in culture because they were kept in the dark. We found no change in B_{12} concentration after exposing the sample to laboratory illumination compared to processing the sample in the dark.

2.6. Determination of bacteria community composition

The community composition of 10 different continuous cultures was assessed using 16S rRNA amplicon sequencing. Total genomic DNA was extracted using the 'Gentra Puregene Yeast/Bact Kit' according to the manufacturer's protocol (Qiagen). Nucleic acids were sent to the Research and Testing Laboratory (Lubbock, TX) for 454-pyrosequencing analysis of bacterial communities. Primers 28F (5'-GAG TTT GAT CNT GGC TCA G-3') and 519R (5'-GTN TTA CNG CGG CKG CTG-3') were used for amplification of the variable regions V1–V3 of the bacterial 16S rRNA gene according to (La Duc et al. 2012)

Bacterial sequences were processed and analyzed using the MOTHUR software package (Schloss et al. 2009), with the 'AmpliconNoise' algorithm implemented. Previously described standard operating procedures were followed for the analysis of sequence data in this study (Schloss et al. 2011). Sequences were removed from consideration if they (1) did not contain the primer sequence, (2) contained an uncorrectable barcode, (3) were 200 nt in length, (4) had homopolymers longer than 8 nt, or (5) had a quality score of 25. Unique sequences were aligned using the 'Greengenes' reference alignment (Schloss et al. 2009, McDonald et al. 2012) and trimmed such that only regions of overlapping sequence data were considered (85% of the overlapping sequence length). Filtered sequences were assigned to samples according to their 12 nt barcode. After chimeras were removed, sequences were classified in accordance with the new 'Greengenes' training set and taxonomy (McDonald et al. 2012, Werner et al. 2012) and clustered into operational taxonomic units (OTUs) at the 0.03 level (i.e. at 97% similarity) (Hazen et al. 2010).

As explained in Maske et al. (2017), the taxonomic levels used in the data analysis were based upon the following criteria: 97% identity (<3% divergence) was applied to resolve the species level, between 95 and 97% was used to define the genus level. We reported 3 sequences results of 3 cultures in Maske et al. (2017); 7 more sequences were registered for this publication. All 10 sequences are registered in the NCBI database (www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP099306) for public access.

Richness and evenness is expressed by the Shannon diversity index (Shannon 1948), calculated as $H' = -\sum(p_i \ln p_i)$, where $p_i = n_i / N$ is the proportion of individuals found in species i, n_i is the number of individuals in species i and N is the total number of individuals in the community.

To analyze the similarity of community composition by cluster analysis, we applied a dendrogram by using a sum of dissimilarities, and the 'hclust' function and 'dendextend' package in R version 3.5 (Galili 2015, R Core Team 2018) (see Fig. 4). We used the A,c-diamide synthase gene (*cobB*) as a proxy for potential B_{12} production by taxa in our culture communities, as has

been done before for bacteria (Bertrand et al. 2011, Heal et al. 2017) and archaea (Doxey et al. 2015). We used the GeneBank database to identify the potential presence of B_{12} producers identified by the taxa in our multispecies cultures (Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/a083p251_supp1.pdf). We used the registered *cobB* gene presence as an indicator of the occurrence of a probable B_{12} producer following the general idea of Bertrand et al. (2011).

2.7. Statistical analysis

All correlations were calculated as a linear parametric regression of 2 independent variables (type II) with R software function 'lmodel2'.

3. RESULTS

3.1. $B_{12\text{-p}}$ and $B_{12\text{-d}}$ concentrations and biomass production

The B_{12} concentrations produced in both the monospecific and multispecific bacterial communities were lower in the $B_{12\text{-d}}$ fraction (0 to 2.15 pmol l^{-1}; mean: 0.81 pmol l^{-1}) than in the $B_{12\text{-p}}$ fraction of the culture (7.73 to 15.85 pmol l^{-1}; mean: 11.02 pmol l^{-1}) (Table 1), with mean values for $B_{12\text{-p}}$ being 13.6 times higher than $B_{12\text{-d}}$. The lowest $B_{12\text{-p}}$ production rate per cell was 0.4×10^{-21} mol^{-1} cell^{-1} d^{-1} corresponding to communities where μ = 0.18 and 0.26 d^{-1}; the highest $B_{12\text{-p}}$ production rate per cell was 2.7×10^{-21} mol^{-1} cell^{-1} d^{-1} for a community culture at μ = 0.79 d^{-1} (Table 1).

Because of the similarity of B_{12} intracellular concentration of multispecies and *Dinoroseobacter shibae* cultures, we decided to join the data in Fig. 1, and to calculate one set of regressions for $B_{12\text{-p}}$ molar concentration with the POC molar concentration ($B_{12\text{-p}}$ = 0.03(POC) + 8.2; R^2 = 0.54, p = 0.05), and the PON concentration ($B_{12\text{-p}}$ = 0.36(PON) + 8.1; R^2 = 0.59, p = 0.03) (Fig. 1).

The multispecies community culture (μ = 0.18 d^{-1}) showed the highest $B_{12\text{-d}}$ concentration (1.70 pmol l^{-1}), but also the lowest B_{12} production rate (1.98 pmol l^{-1} d^{-1}) (Table 1). In another culture (μ = 0.92 d^{-1}), a low $B_{12\text{-d}}$ (0.42 pmol l^{-1}) corresponded to the highest $B_{12\text{-p}}$ production rate (9.46 pmol l^{-1} d^{-1}) (Table 1). For comparison, in the cultures of *D. shibae* the lowest $B_{12\text{-p}}$ production rate was 3.51 pmol l^{-1} d^{-1} but the same culture showed a relatively high $B_{12\text{-d}}$ concentration of 1.29 pmol l^{-1} (μ = 0.27 d^{-1}; Table 1). Both the

Table 1. B_{12} in different fractions in continuous culture (multispecies and *Dinoroseobacter shibae*). $B_{12\text{-d}}$: dissolved B_{12} concentration produced in the culture; $B_{12\text{-p}}$: particulate B_{12} fraction

Continuous bacteria culture	Specific growth rate (d^{-1})	Total dissolved B_{12} concentration (pmol l^{-1})	$B_{12\text{-d}}$ concentration (pmol l^{-1})	$B_{12\text{-p}}$ concentration (pmol l^{-1})	$B_{12\text{-d}}$ production rate (pmol l^{-1} d^{-1})	$B_{12\text{-p}}$ production rate (pmol l^{-1} d^{-1})	$B_{12\text{-p}}$ cell concentration ($\times 10^9$ cells l^{-1})	$B_{12\text{-p}}$ cell quota ($\times 10^{-21}$ mol cell^{-1})	$B_{12\text{-p}}$ per biovolume (pmol µm^{-3})	$B_{12\text{-p}}$ production rate per cell ($\times 10^{-21}$ mol cell^{-1} d^{-1})
Multispecies	0.18	2.40	1.70	10.74	0.44	1.98	4.87	2.21	0.017	0.4
Multispecies	0.26	1.20	0.50	9.23	0.31	2.42	6.00	1.54	0.014	0.4
D. shibae	0.27	3.00	1.29	13.02	0.81	3.51	7.16	1.82	0.018	0.5
Multispecies	0.37	0.98	0.39	14.07	0.36	5.20	2.63	5.35	0.046	2.0
D. shibae	0.36	3.86	2.15	13.86	1.39	4.99	7.60	1.82	0.012	0.7
Multispecies	0.46	3.43	1.33	9.74	1.58	4.48	3.62	2.69	0.016	1.2
Multispecies	0.50	2.19	1.60	15.85	1.10	7.92	3.19	4.97	0.022	2.5
Multispecies	0.60	2.10	0.00	7.73	1.27	4.67	3.34	2.31	0.012	1.4
D. shibae	0.68	4.90	0.00	10.68	3.34	7.27	4.44	2.40	0.013	1.6
Multispecies	0.75	1.95	0.37	9.48	1.46	7.12	2.88	3.29	0.024	2.5
D. shibae	0.76	4.90	0.00	8.03	3.70	6.07	4.24	1.89	0.011	1.4
Multispecies	0.79	2.32	0.74	10.58	1.84	8.40	3.13	3.39	0.026	2.7
Multispecies	0.92	0.92	0.42	10.28	0.85	9.46	6.40	1.61	0.010	1.5
Mean	0.53	2.63	0.81	11.02	1.42	5.65	4.58	2.71	0.018	1.4

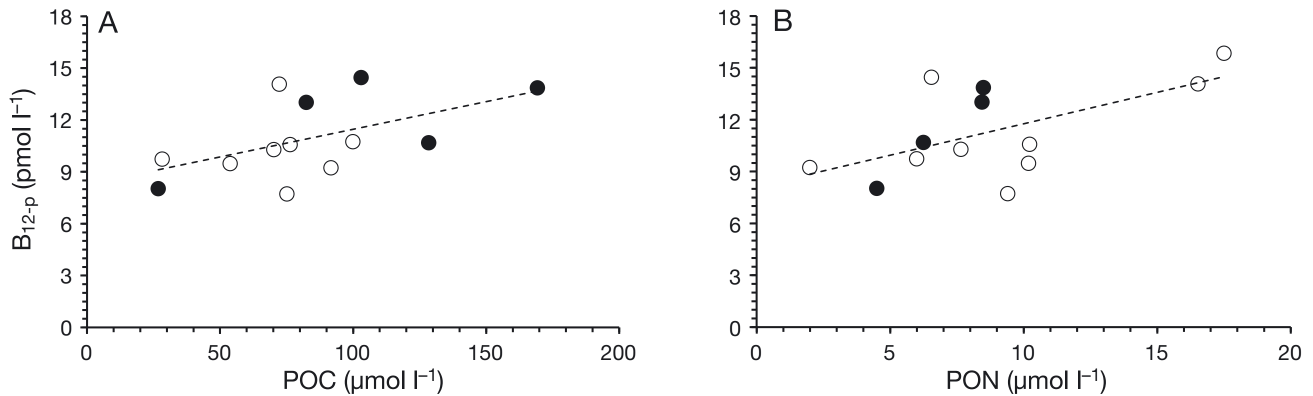

Fig. 1. Particulate B_{12} ($B_{12\text{-}p}$) versus (A) particulate organic carbon (POC) and (B) particulate organic nitrogen (PON) in multispecies (open circles) and *Dinoroseobacter shibae* cultures (filled circles). Dashed lines: (A) $B_{12\text{-}p} = 0.03(\text{POC}) + 8.2$; $R^2 = 0.54$, n = 13, p = 0.05; (B) $B_{12\text{-}p} = 0.36(\text{PON}) + 8.1$, $R^2 = 0.59$, n = 13, p = 0.03

marine bacteria communities and *D. shibae* cultures (Fig. 2) showed significant and overlapping relationships between the cellular rates of production of $B_{12\text{-}p}$ and POC (Fig. 2A) or PON (Fig. 2B).

3.2. B_{12} production and bacterial respiration

The respiration rate measured as CO_2 production in our cultures ranged from 6.4 to 132.8 µmol l^{-1} CO_2 d^{-1} and from 32.0 to 140.5 µmol l^{-1} CO_2 d^{-1} for multispecies bacterial communities and *D. shibae* monospecific cultures, respectively (Fig. 3A). On a volumetric basis, the $B_{12\text{-}p}$ production rate increased in a logarithmic fashion with the respiration rate, with a higher production rate for the multispecies bacterial community.

3.3. B_{12} production and bacterial community composition

We investigated the taxonomic differences between the bacterial communities. Pyrosequencing yielded the presence of representatives across 54 families, 238 genera, and 253 species among all 10 samples (Table S1 in Supplement 2 at www.int-res.com/articles/suppl/a083p251_supp2.xlsx). Sequences were published in the NCBI database (www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP099306). The grade of richness was different between the experiments; the diversity expressed by *H'* (Shannon 1948) ranged from 1.4 to 3.3 and the evenness defined by the Pielou index ranged from 0.35 to 0.7 (Table S2). We did not find a significant pattern of these indices with either µ or B_{12} intracellular pro-

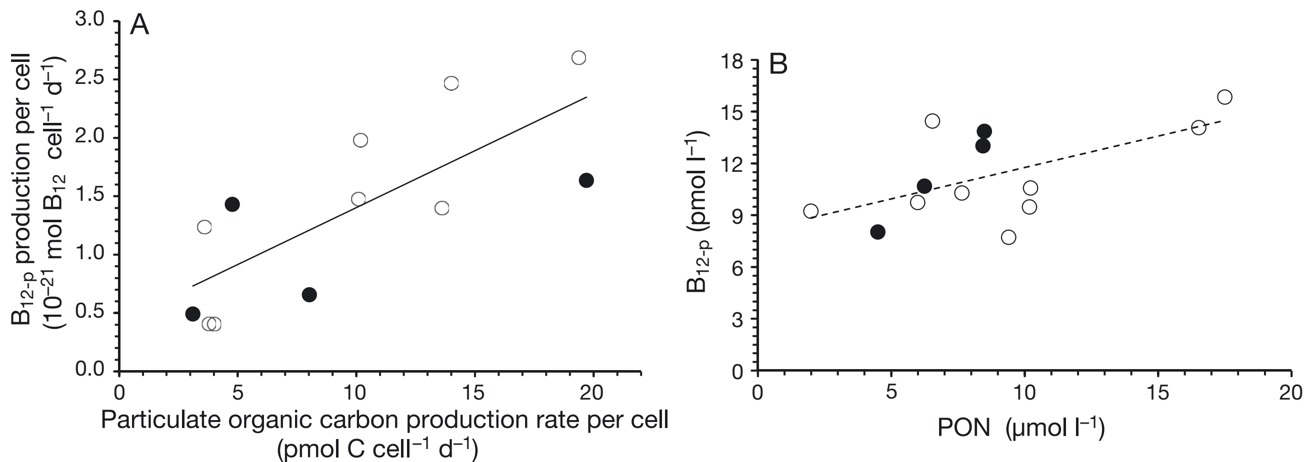

Fig. 2. (A) Particulate B_{12} ($B_{12\text{-}p}$) production rate per cell versus particulate organic carbon production rate per cell in multispecies (circles) and *Dinoroseobacter shibae* cultures (filled circles). Continuous line: $y = 0.09 \times 10^{-21}(x) + 0.4 \times 10^{-21}$; $R^2 = 0.76$, n = 12, p ≤ 0.05. (B) $B_{12\text{-}p}$ production rate per cell versus particulate organic nitrogen (PON) production rate per cell. Continuous line: $y = 0.75 \times 10^{-21}(x) + 0.5 \times 10^{-21}$; $R^2 = 0.95$, n = 13, p ≤ 0.05

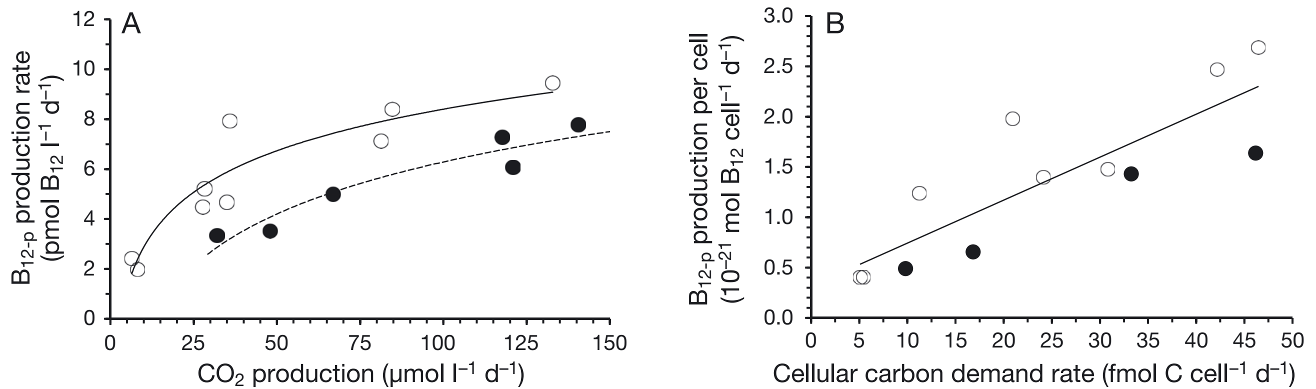

Fig. 3. (A) Particulate B_{12} ($B_{12\text{-p}}$) production rate versus CO_2 production rate in multispecies cultures (open circles; continuous line: $y = 2.4 \ln(x) - 2.66$; $R^2 = 0.87$, n = 8, $p \leq 0.05$) and *Dinoroseobacter shibae* (filled circles; dashed line: $y = 3.0 \ln(x) - 7.56$; $R^2 = 0.97$, n = 6, $p \leq 0.05$). (B) Cellular carbon demand rate and the $B_{12\text{-p}}$ production rate per cell. Continuous line: $y = 0.04 \times 10^{-21}(x) + 0.31 \times 10^{-21}$; $R^2 = 0.85$, n = 12, $p \leq 0.05$

duction rate per cell. In the cluster analysis (Fig. 4), the dendrogram of community composition indicated no relationship with µ. Fig. S1 shows the 2 dominant taxa of each culture, with the potential for B_{12} production (published *cobB* gene presence) indicated. Fig. S1 again showed no relationship between µ, the 2 dominant taxa, and the potential presence of the *cobB* gene.

3.4. VLPs

We monitored the VLPs in the cultures and found much lower concentration in relation to bacterial abundance than reported for oceanic samples. The ratio of VLPs to bacteria (VBR) was always lower than 1.4.

4. DISCUSSION

We used chemostats to investigate the response of bacteria communities and B_{12} production under different growth rates. We used chemostats because they allowed us to calculate the production rates of intracellular and dissolved B_{12} by marine prokaryotes (Table 1). To our knowledge, this information has not been previously documented before, so the data presented here would be the first estimate.

4.1. B_{12} cell quota

The B_{12} cell quota in our monospecific chemostats ranged from 1.82 to 2.40 $\times 10^{-21}$ mol cell^{-1}, and in the community cultures from 1.54 to 5.35 $\times 10^{-21}$ mol cell^{-1}

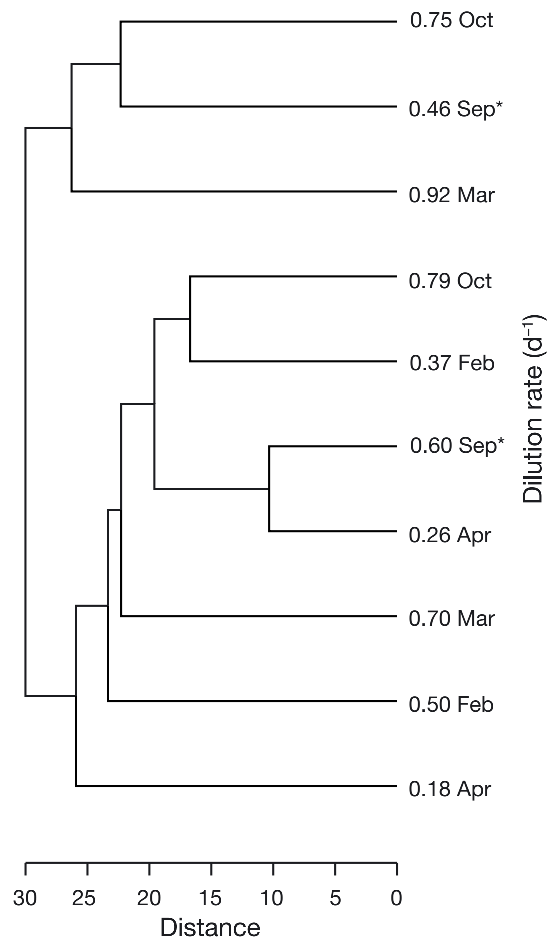

Fig. 4. Cluster analysis based on sum of dissimilarities ('hclust' function and the 'dendextend' package in R) showing groups based on taxa present in each culture. Dilution rate (d^{-1}) of each culture is indicated to the right of the dendogram. (*) inocula sampled in the Gulf of California; other inocula were collected in the California Current. Sampling month is indicated

Table 2. B_{12} or pseudocobalamin per cell. POC: particulate organic carbon; –: no information available

Sample	B_{12} (mol cell^{-1})	B_{12} or pseudo-cobalamin ($\times 10^{-9}$ mol [mol POC]$^{-1}$)	B_{12} or pseudo-cobalamin (molecules cell^{-1})	Reference
Multispecies marine bacteria	3.04×10^{-21}	160	1831	This work[a]
Dinoroseobacter shibae	1.99×10^{-21}	120	1198	This work[a]
Vibrio AND4 str	–	419	140	Suffridge et al. (2017)[b]
Synechococcus CC9311 str	–	418	1115	Suffridge et al. (2017)[b]
Bacteria	–	1–260	14–1840	Heal et al. (2017)[c]
Archaea	–	2800–11600	1360–4480	Heal et al. (2017)[c]
Synechococcus, *Prochlorococcus*	–	130–3000	1230–16600	Heal et al. (2017)[d]

[a]The number of molecules cell^{-1} was calculated using the mean values of molecular concentration cell^{-1} and POC

[b]POC was based on conversion factors: AND4, 20 fg C cell^{-1} and CC9311, 200 fg C cell^{-1}; the B_{12} concentration is the sum of adenosylcobalamin, cyanocobalamin, hydroxycobalamin, and methylcobalamin. These concentrations are not product of these cells because reportedly they do not have the biosynthetic capacity for true B_{12}

[c]Cobalamin concentration, molecules cell^{-1} are based on cell concentration

[d]Pseudocobalamin concentration, molecules cell^{-1} are based on cell concentration

(Table 1). In Table 2 we recalculated these concentrations as number of molecules per cell and compared these numbers with literature values. Our values correspond well with values given by Suffridge et al. (2017), Cruz-López et al. (2018), and Heal et al. (2017) despite differences in methods. The molar ratio of B_{12} to POC was relatively constant regardless the great difference in cell size between *Vibrio* AND4 and *Synechococcus* CC9311. In contrast, the number of molecules per cell of these 2 strains varied 8-fold, bracketing our value. In our marine bacteria community cultures, the average cell concentration was 4.58 $\times 10^9$ cells l^{-1}. Assuming 10^9 bacteria l^{-1} in the surface ocean and assuming our average cell quota, then we arrive at intracellular $B_{12\text{-p}}$ concentrations of approximately 2 pmol l^{-1} in the bacteria-size fraction of surface waters, a value not much lower than the values reported by Suffridge et al. (2017) for the total particulate fraction in eastern Atlantic surface waters. Heal et al. (2017) reported values for particulate cobalamin and pseudocobalamin of less than 0.1 pmol l^{-1} in the Pacific Ocean (Table 2). These data point to the great difference in $B_{12\text{-d}}$ and $B_{12\text{-p}}$ concentrations.

Sañudo-Wilhelmy et al. (2012) reported a wide range (0 to 30 pmol l^{-1}) of dissolved vitamin concentrations off the coast of Baja California, with some of the data showing much higher concentrations than the sum of total dissolved (Table 1) and $B_{12\text{-p}}$ in the cultures (9.83 to 18.04 pmol l^{-1}), although their data did not include all known types of cobalamin. Our consistently low concentrations were not caused by low extraction efficiency (see Section 2.5), but were possibly related to the continuous dilution of our cultures with low $B_{12\text{-d}}$ growth medium. Related to this, we observed very low concentrations of VLPs in both types of cultures, which suggest that the bacteria present in our culture vessels experienced a low rate of cellular lysis. An alternative hypothesis to explain the difference between our low $B_{12\text{-d}}$ and the higher concentrations in natural seawater could be that phytoplankton was stimulating bacterial B_{12} production through mutualistic interactions (Kazamia et al. 2012, Sañudo-Wilhelmy et al. 2014).

4.2. $B_{12\text{-p}}$ production rate

In the marine environment, many variables can contribute to the concentration of $B_{12\text{-d}}$; for example, it could be influenced by abiotic and biotic factors, such as variations in the availability of precursors (Menzel & Spaeth 1962, Panzeca et al. 2009), and the ratio of B_{12} producers to consumers (Croft et al. 2005, 2006, Bonnet et al. 2010). In seawater, phytoplankton will constitute a significant fraction of B_{12} consumers, contrary to our community cultures which we expect to have included heterotrophic auxotrophs. Understanding of the relationship between the trophic growth conditions of bacteria and the production of B_{12} could be decisive to our understanding of the agents that determine the amount of B_{12} available to the auxotrophic organisms.

In our cultures, we did not observe a clear relationship between the trophic conditions determined by the dilution rate and the $B_{12\text{-p}}$ and $B_{12\text{-d}}$ concentrations, but always the same general pattern that the $B_{12\text{-p}}$ concentrations were significantly higher than $B_{12\text{-d}}$ (Table 1). These results indicate that the major

reservoir of B_{12} is found within the cells. The standing stock POC and PON concentrations showed a weak but significant relationship with $B_{12\text{-p}}$ concentration (Fig. 1) in both the *D. shibae* cultures and the natural marine bacteria communities. This is in contrast to $B_{12\text{-d}}$ concentration, which appears to be of limited value in interpreting bacterial ecology. The $B_{12\text{-p}}$ production rate (pmol l^{-1} d^{-1}) was clearly related to the rate of POC and PON formation (Fig. 2), and to bacterial respiration (Fig. 3A). The intracellular concentration (pmol l^{-1}) was not related to growth rate, whereas the $B_{12\text{-p}}$ production rate (pmol l^{-1} d^{-1}) was related to μ (d^{-1}). We suggest that the bacteria obtain homeostasis of average intracellular B_{12} concentration in single-species producer communities or communities containing both producers and consumers.

4.3. Taxonomic composition in the cultures

The taxonomic communities present in the cultures were different in each culture and showed no relationship to season or the location where the inoculum was sampled. The prokaryote composition of the inoculum seems to have been very different in each case and was possibly determined in part by the intra-phylum relationship between bacteria and the primary producers of organic substrates, the phytoplankton. Independent of the specific taxonomic composition, and given the richness of the culture communities (between 51 and 141 taxa), we consider it likely that they included B_{12} producers and auxotrophs. The steady-state $B_{12\text{-d}}$ concentration in the cultures should then be a result of the balance of the activity of B_{12} producers and consumers in limited glucose growth media, similar to the coexistence of producers and consumers in natural communities. The dendrogram (Fig. 4) indicated no relationship between μ and community composition when all identified taxa were included in the analysis. Using only the 2 dominant taxa, we again found no pattern with μ or the presence of a registered *cobB* gene that might indicate a B_{12}-producing taxon (Fig. S1). The presence of the *cobB* gene by itself is probably not sufficient proof for the potential of a certain taxon to synthesize B_{12} (Helliwell et al. 2016).

4.4. Bacterial respiration, carbon demand, and $B_{12\text{-p}}$ production rate

Because the concentration of $B_{12\text{-d}}$ and $B_{12\text{-p}}$ showed no relationship in our cultures to μ (d^{-1}) or respiration rates (mol CO_2 l^{-1} d^{-1}), they revealed little about their physiological roles. When B_{12} production rates per cell were compared with respiration rates per cell we found significant linear relationships for multispecies and *D. shibae* cultures; in multispecies cultures (mol $B_{12\text{-p}}$ $cell^{-1}$ d^{-1} = $0.07 \times 10^{-21} + 0.54 \times 10^{-21}$ mol CO_2 $cell^{-1}$ d^{-1}; $R^2 = 0.81$), and in *D. shibae* cultures (mol $B_{12\text{-p}}$ $cell^{-1}$ d^{-1} = $0.05 \times 10^{-21} + 5.1 \times 10^{-21}$ mol CO_2 $cell^{-1}$ d^{-1}; $R^2 = 0.95$) (Fig. S2). In the multispecies cultures, the $B_{12\text{-p}}$ production rate per cell was related to the CO_2 production rate per cell, thus the slope of the relationship should depend on the fraction of $B_{12\text{-p}}$ producers in the community. This relationship (Fig. S2) is valid for our culture temperature (18°C) but can not necessarily be extrapolated to other temperatures or higher growth rates without further testing.

We assume that our community cultures included B_{12} auxotrophs, and that these auxotrophs could maintain themselves in our cultures. As in nature, the very low $B_{12\text{-d}}$ should be related to the ways B_{12} was delivered from producer to consumer (Grant et al. 2014). Since the B_{12} intracellular concentration changed very little (Table 1), approximate homeostasis was maintained between bacterial production and consumption rates by mechanisms unknown to us; but the literature suggests various possibilities. Suffridge et al. (2017) suggested that this balance might be due to rapid scavenging and release of the $B_{12\text{-d}}$ fraction. One mechanism of B_{12} release into the dissolved phase may be stochastic cell death (Wang et al. 2010), which might explain why the metabolic rates were not associated with $B_{12\text{-d}}$. The B_{12} concentrations present in the dissolved and particulate phases of our cultures (Table 1) did not change systematically with μ, suggesting that at different community metabolic rates a balance between B_{12} production and consumption was established. The differences in concentrations might be the result of the community compositions in the specific culture. The low dissolved B_{12} concentration in the natural marine bacteria community cultures may also be produced by B_{12} sequestration by high-reactivity proteins (Ludwig et al. 1996) or direct cellular exchange between B_{12} producers and consumers (Grant et al. 2014).

The significant linear increase in $B_{12\text{-p}}$ with POC and PON (Fig. 1) implies that the $B_{12\text{-p}}$ concentration is linked to cell reproduction rates, as expected for a cofactor that enhances overall metabolism; for example, DNA synthesis regulation, transmethylation, and synthesis of methionine (Sañudo-Wilhelmy et al. 2014), and in general for the rate of biomass

synthesis (see Fig. 2). The $B_{12\text{-}p}$ rate increased with PON production rate, but this increase was higher for *D. shibae* than for the multispecies cultures (Fig. 2B). The increased B_{12} production rates relative to the respiratory metabolism, measured as CO_2 production (Fig. 3A), is consistent with the above argument that B_{12} production is linked to cell reproduction rates and cellular metabolism (Wood et al. 1986, Ragsdale 1991, Stupperich 1993). These relationships (Fig. 3A) were found to be different for both culture types, contrary to the relationship of cellular B_{12} production rate versus cellular carbon demand rate (Fig. 3B), which showed little differences between culture types. The dependence of the bacterial community metabolism on B_{12}, where a large part of the community is not producing B_{12}, suggests the necessity to quantify the intracellular B_{12} for a better understanding of B_{12} as a limiting growth factor in the marine environment.

The B_{12} supply to the B_{12} auxotrophs of the bacterial community depends on the taxonomic structure of the community and its physiological status. Our chemostat experiments received inoculum sampled from different seawater sources and at different times of the year. Regardless of this, B_{12} was produced in all the experiments, suggesting that B_{12} producers were always present in our sampling sites. Our results suggest that community metabolic rates determine the production of intracellular B_{12}, whereas the concentration of dissolved B_{12} which does not respond to the metabolic rates is determined by different mechanisms that control the exchange of B_{12} between producers and consumers.

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