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RESEARCH ARTICLE

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Functional redundancy of seasonal vitamin B_{12} biosynthesis pathways in coastal marine microbial communities

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Abstract

Vitamin B_{12} (cobalamin) is a major cofactor required by most marine microbes, but only produced by a few prokaryotes in the ocean, which is globally B_{12}-depleted. Despite the ecological importance of B_{12}, the seasonality of B_{12} metabolisms and the organisms involved in its synthesis in the ocean remain poorly known. Here we use metagenomics to assess the monthly dynamics of B_{12}-related pathways and the functional diversity of associated microbial communities in the coastal NW Mediterranean Sea over 7 years. We show that genes related to potential B_{12} metabolisms were characterized by an annual succession of different organisms carrying distinct production pathways. During the most productive winter months, archaea (*Nitrosopumilus* and *Nitrosopelagicus*) were the main contributors to B_{12} synthesis potential through the anaerobic pathway (*cbi* genes). In turn, *Alphaproteobacteria* (HIMB11, UBA8309, *Puniceispirillum*) contributed to B_{12} synthesis potential in spring and summer through the aerobic pathway (*cob* genes). Cyanobacteria could produce pseudo-cobalamin from spring to autumn. Finally, we show that during years with environmental perturbations, the organisms usually carrying B_{12} synthesis genes were replaced by others having the same gene, thus maintaining the potential for B_{12} production. Such ecological insurance could contribute to the long-term functional resilience of marine microbial communities exposed to contrasting inter-annual environmental conditions.

INTRODUCTION

Marine planktonic communities strongly influence global biogeochemical cycles (Falkowski et al., 2008), and it is now well established that their spatiotemporal dynamics are, in part, driven by the physico-chemical properties of the surrounding environment (Auladell et al., 2022; Fuhrman et al., 2006; Galand et al., 2010; Lambert et al., 2019). However, biotic interactions also shape the structure and the functions of microbial communities (Ahlgren et al., 2019; Chow et al., 2014; Frischkorn et al., 2018; Han et al., 2022; Joglar et al., 2021; Lima-Mendez et al., 2015; Nemergut et al., 2013; Strom, 2008). Prokaryotic and eukaryotic microorganisms continuously interact by exchanging beneficial metabolites such as nutrients, growth factors, or vitamins (Kouzuma & Watanabe, 2015; Seymour et al., 2017). Among vitamins, vitamin B_{12} (cobalamin) appears as a key factor controlling the growth of photosynthetic planktonic communities as demonstrated experimentally (Bertrand et al., 2007; Koch et al., 2011, 2013; Panzeca et al., 2006; Sañudo-Wilhelmy

et al., 2006). Genomic and culture-based approaches suggest, however, that most eukaryotes are auxotrophs toward B_{12} including more than a half of phytoplankton species, compared to about 25% and 8% for thiamin (vitamin B_1) and biotin (vitamin B_7), respectively (Croft et al., 2005; Sañudo-Wilhelmy et al., 2014). This high incidence of B_{12} auxotrophy is also observed within many marine bacterial lineages, including ubiquitous prokaryotes that are highly abundant in the global ocean, such as members of the SAR11 clade (Gómez-Consarnau et al., 2018; Sañudo-Wilhelmy et al., 2014; Shelton et al., 2019).

Although many microorganisms have an absolute requirement of B_{12}-related coenzymes, vitamin B_{12} is a scarce micronutrient in the ocean. Several studies have shown that most oceanic and coastal areas of the Pacific Ocean, the Atlantic Ocean, and the Mediterranean Sea are largely B_{12}-depleted (picomolar concentrations) and that their bioavailability varies with depth and seasons (Barber-Lluch et al., 2021; Bonnet et al., 2013; Fiala, 1982; Joglar et al., 2021; Koch et al., 2012; Sañudo-Wilhelmy et al., 2012; Suffridge et al., 2018). Auxotrophic organisms thus rely mainly on B_{12} de novo biosynthesis, and co-culture experiments have notably shown that some marine bacteria can satisfy the vitamin requirement of auxotrophic organisms via close mutualistic interactions (Cooper et al., 2019; Croft et al., 2005, 2006; Grant et al., 2014; Kazamia et al., 2012). However, B_{12} de novo biosynthesis is restricted to certain prokaryotes including some heterotrophic bacteria (essentially *Proteobacteria*), chemoautotrophic archaea, and phototrophic cyanobacteria (Doxey et al., 2015; Gómez-Consarnau et al., 2018; Sañudo-Wilhelmy et al., 2014; Shelton et al., 2019). More precisely, targeted characterizations of B_{12} biosynthesis genes in the Ross Sea identified members of *Gammaproteobacteria* (i.e., *Oceanospirillales*) as putative B_{12} synthesizers in the Southern Ocean surface waters (Bertrand et al., 2011, 2015). Metagenomics studies also suggest that *Thaumarchaeota* may have an important role in B_{12} biosynthesis in the deeper ocean and the Arctic (Doxey et al., 2015; Heal et al., 2017). They were, however, not the main synthesizers during a marine dinoflagellate bloom, during which *Alphaproteobacteria* and *Gammaproteobacteria* (*Oceanospirillaceae*) produced B_{12} (Zhou et al., 2020). A metatranscriptomic study carried out in the Atlantic Ocean quarterly, over 1 year, more precisely identified members of *Rhodobacterales* as the main potential B_{12} producers (Gómez-Consarnau et al., 2018). Even though several vitamin B_{12} producers have been identified punctually at different geographic locations, the extent of their diversity and dynamics over time remains to be explored.

B_{12} biosynthesis is a complex metabolic process involving about 20 genes and 30 enzymatic steps (Balabanova et al., 2021; Fang et al., 2017; Martens et al., 2002; Raux et al., 2000) (Figure 1). Within bacteria, B_{12} can be produced through two different ways, called the aerobic (*cob* genes) and anaerobic (*cbi* genes) pathways (Balabanova et al., 2021; Sañudo-Wilhelmy et al., 2014; Shelton et al., 2019), while in archaea, only the anaerobic one is used (Doxey et al., 2015; Fang et al., 2017) (Figure 1A). In contrast to bacteria and archaea, the abundant and globally distributed marine cyanobacteria *Synechococcus* and *Prochlorococcus* rather produce pseudo-cobalamin (named pseudo-B_{12}) (Helliwell et al., 2016) (Figure 1B), an analog of cobalamin thought to support lower growth yields in many eukaryotic algae (Guillard, 1968; Heal et al., 2017; Helliwell et al., 2016; Provasoli & Carlucci, 1974). Algal and bacterial pseudo-B_{12} remodelers can, however, remodel pseudo-B_{12} into vitamin B_{12} in an energy-cost-effective way through the *cobC-STU* genes (Heal et al., 2017; Helliwell et al., 2016; Ma et al., 2020) (Figure 1B). The remodelling of pseudo-B_{12} requires exogenous DMB (5,6-dimethyl benzimidazole), suggesting that DMB bioavailability in seawater could also impact B_{12} cycling in the ocean, and influence the composition of microbial communities (Wienhausen et al., 2022).

Despite their ecological importance, vitamin B_{12} producers and the pathways involved remain poorly studied in marine microbial communities, and their seasonality over several years is not known. The overall aim of this study was to identify the B_{12} production pathways present in the coastal NW Mediterranean Sea and test if they show reproducible seasonal patterns. We conducted a 7-year monthly metagenomic time series survey at the SOLA station to monitor the dynamics of genes associated with vitamin B_{12} metabolisms (biosynthesis, salvage, and remodelling) and to identify the key taxonomic groups involved in these processes throughout the year. We further verify if environmental perturbations disrupt the vitamin B_{12} seasonality.

EXPERIMENTAL PROCEDURES

Seawater sampling, environmental parameters monitoring, and DNA extraction and sequencing

Surface seawater (3 m depth) was sampled at the Banyuls Bay microbial observatory (SOLA; 42°31′N, 03°11′ E; Northwestern Mediterranean Sea, France) from January 2009 to December 2015. Water sampling, filtration, DNA extraction, and monitoring of environmental parameters have been described elsewhere (Lambert et al., 2019).

DNA samples taken between 2009 and 2011 were shotgun sequenced using Illumina NovaSeq6000 (2×150 bp) at the Centre Nacional d'Analisi

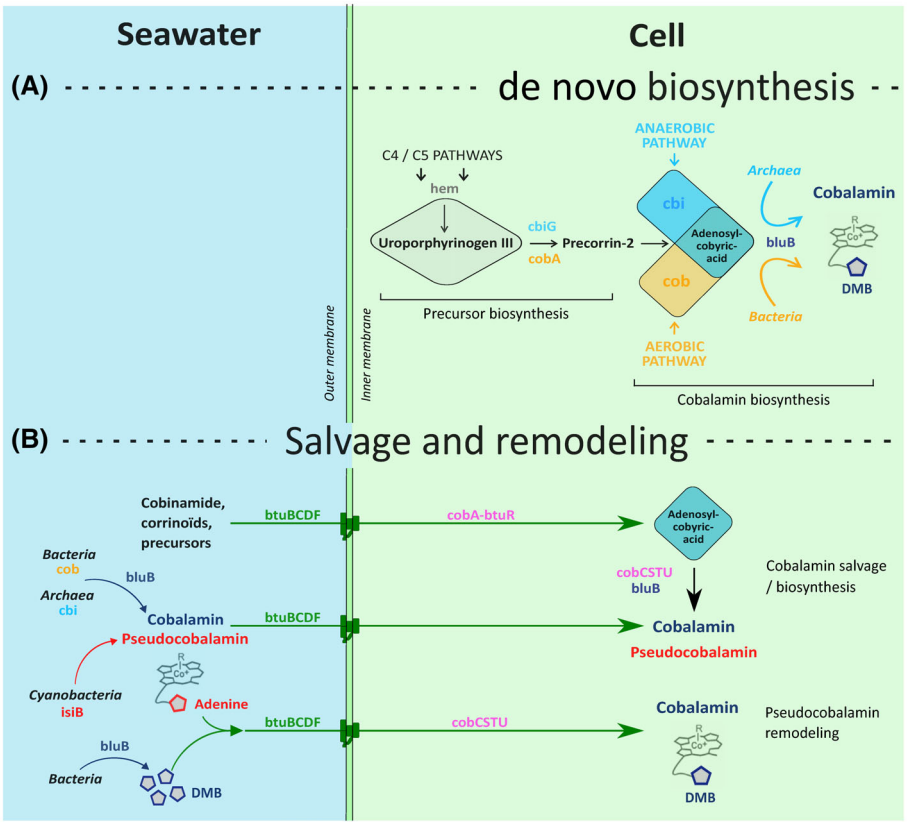

FIGURE 1 Schematic overview of vitamin B_{12} de novo biosynthesis, salvage, and remodelling pathways (Balabanova et al., 2021; Fang et al., 2017; Zhou et al., 2021). (A) Vitamin B_{12} de novo biosynthesis occurs in the cell (right part; green) and requires the prior generation of the precursor uroporphyrinogen III via the C4 and C5 pathways, which involve the *hem* genes (grey). Depending on the organisms, *cobA* or *cysG* genes' activities catalyse the synthesis of precorrin-2 from uroporphyrinogen III, which can be metabolized in vitamin B_{12} in two distinct ways: via the aerobic pathway requiring the *cob* genes present in bacteria, or via the anaerobic pathway relying on the *cbi* genes in archaea (coloured in orange and blue, respectively). Both pathways converge towards adenosylcobyric acid, which is finally converted into vitamin B_{12}. Archaea and bacteria produce cobalamin, a form of vitamin B_{12} that has a dimethylbenzimidazole (DMB) molecule as a lower axial ligand, which is produced through the activity of the gene *bluB* (coloured in dark blue), and then fused to the corring ring to form cobalamin. (B) Molecules structurally close to vitamin B_{12} (e.g., cobinamide and other corrinoids) can be directly scavenged from the seawater (left part; blue) through specific transporters such as *btuBCDF* in bacteria (coloured in green). After translocation, these molecules are metabolized in the cell by specific enzymes that ensure the generation of adenosylcobyric-acid, such as ATP:co(I)rrinoid adenosyltransferases (ACATS) encoded by *cobA-btuR* gene (coloured in pink). Adenosylcobyric acid will integrate the biosynthesis pathway and will be transformed into vitamin B_{12} by several enzymes encoded by the *cobCSTU* genes (coloured in pink). Cyanobacteria produce pseudo-cobalamin (i.e., pseudo-B_{12}) which has an adenine in the lower axial ligand and is not bioactive for most marine microorganisms. The flavodoxin gene *isiB* (coloured in red) is an orthologue of the cobalt reductase *fldA* found in some B_{12}-producing bacteria and thus is supposed to be involved in pseudo-B_{12} biosynthesis in cyanobacteria (Walworth et al., 2018). Salvaged pseudo-B_{12} can be remodelled intracellularly to vitamin B_{12} by some microalgae and bacteria that possess *cobCSTU* genes (e.g., *Pavlova* and *Vibrio*) (Helliwell et al., 2016; Ma et al., 2020). In seawater, pseudo-B_{12} remodelling requires DMB bioavailability.

Genomica (CNAG), while DNA samples taken between 2012 and 2015 were sequenced using Illumina HiSeq2500 (2 × 100 bp) (Galand et al., 2018). The raw sequences were deposited in the NCBI Sequence Read Archive under accession number PRJEB66489 and PRJEB26919.

Functional and taxonomic identification of prokaryotic sequences

Metagenomic reads were trimmed with CUTADAPT v1.16 (Martin, 2011), removing adapters and low-quality regions. Clean reads were individually assembled with MEGAHIT (D. Li et al., 2015). Genes were predicted with Prodigal v2.6.3 and MetaGeneMark v3.38 (Hyatt et al., 2010; Zhu et al., 2010). To remove redundancy, genes were clustered at 95% identity and 90% coverage with Linclust v10 (Steinegger & Söding, 2018). A gene catalogue was generated, and predicted genes were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (v.2017) with a maximum BLASTP E-value cutoff of 0.1 and a minimum percent coverage of 80%. Gene abundances per sample were estimated by backmapping metagenome reads to the catalogue using BWA (Burrows-Wheeler Alignment tool) (Li & Durbin, 2009). The number of counts per gene was

calculated with HTSeq (Anders et al., 2015) and subsequently normalized within samples by gene length and among samples by a set of 10 single-copy core genes (K06942, K01889, K01887, K01875, K01883, K01869, K01873, K01409, K03106 and K03110) (Salazar et al., 2019). As a result, gene and functional abundance tables were generated. Taxonomic affiliations of the functional genes were analysed with the Genome Taxonomy Database (GTDB) using MMseqs2 and considering the last common ancestor (LCA). KEGGs involved in B_{12} biosynthesis were identified by an exhaustive search of key markers in the literature (Balabanova et al., 2021; Fang et al., 2017; Zhou et al., 2021) (Table S1). The catalogue of prokaryotic genes associated with all the B_{12}-related KEGGs used in this study is accessible online in the Zenodo repository.

Ostreococcus tauri cobalamin bioassay

The Mediterranean strain *Ostreococcus tauri* (RCC745) cultivated in the laboratory was used as a model organism to evaluate the bioavailability of vitamin B_{12} in SOLA seawaters collected at different times of the year between 10/09/2020 and 10/09/2021. *O. tauri* is a marine picoeukaryotic phytoplankton that is auxotroph to vitamin B_1 and B_{12} and has already been used to develop a vitamin B_1 bioassay (Paerl et al., 2017). Cells from a fresh culture maintained for 6 days in B_{12}-free artificial seawater (ASW), with Streptomycin (50 μg.mL^{-1}) and Penicillin (100 μg.mL^{-1}), were inoculated in a deep well microplate (Nunc) at 5000 cells.mL^{-1} in 1.8 mL of SOLA 0.22 μm filtered seawater supplemented with B_{12}-free Keller enrichment medium and 1 nM of vitamin B_1 (Sigma-Aldrich). Cyanocobalamin (Sigma-Aldrich) was diluted in MiliQ water at 1 mg.mL^{-1} concentrations, aliquoted, and stored at −20°C until use.

First, the B_{12} requirement of *O. tauri* was assessed in cultures performed in deep well microplates inoculated at 5000 cells.mL^{-1} in 1.8 mL of B_{12}-free ASW, complemented with various concentrations of cyanocobalamin ranging between 0 and 100 pM. Above 10 pM of cyanocobalamin, significant cell growth was observed, and the maximal cell concentrations were reached at 25 pM of cyanocobalamin (Figure S2). In the bioassay, 0 to 25 pM of B_{12} were added to evaluate B_{12} bioavailability in SOLA seawaters at different times of the year.

All cultures were incubated in triplicate for 6 days under constant light conditions (35 μmol.quanta.m^{-2}.s^{-1}). After 6 days, 66 μL of cultures were fixed with 2 μL of ultrapure glutaraldehyde (Sigma-Aldrich) for 15 min at 4°C. Cell concentrations were determined by flow cytometry using an Accuri C6 flow cytometer (Beckman Coulter).

Of the 31 bioassays performed with seawater collected at SOLA between 10/09/2020 and 21/09/2021, 9 were not conclusive since a high mortality rate was observed even upon B_{12} amendment, suggesting that inhibitory compounds or viruses were present in these samples. The failed samples were collected on the following dates: 27/10/2020, 3/11/2020, 24/11/2020, 1/12/2020, 8/12/2020, 15/12/2020, 16/03/21, 30/03/2021 and 06/04/2021.

Statistics

The Lomb Scargle periodogram (LSP) was used to determine if annual rhythmic patterns were present in B_{12}-related KEGGs, or genes. Rhythmic means that a gene reoccurs every year at the same time of the year. The LSP (Lomb, 1976; Scargle, 1982) was successfully adapted by biologists to detect periodic signals in unevenly sampled time series (Lambert et al., 2019; Longobardi et al., 2022; Ruf, 1999). The computing of the 'peak normalized power' (PNmax) of LSP was accomplished via the 'lomb' package in the 'R' software (v.4.0.3). KEGGs or genes were considered as rhythmic when they had a PNmax index ≥10 with a significant p value ($p < 0.05$) as previously reported (Lambert et al., 2019), and are considered as annual if their period was 365 ± 10 days. To estimate the time of the year of maximal abundance, we determined for each year and each rhythmic pattern the week of the year with the highest number of sequences (Lambert et al., 2019). Then, we selected over the entire time series the week that most often showed the highest number of sequences (named 'phase').

NMDS and PERMANOVA (Permutational multivariate analysis of variance) were based on Bray–Curtis dissimilarity between samples and computed with the 'adonis' (permutations = 999, $p \leq 0.001$) and the 'metaMDS' functions, respectively implemented in the package vegan (Oksanen et al., 2022). Environmental variables of SOLA time series were fitted onto the ordination space using the 'envfit' function (permutations = 999), and covariance ellipses, that highlight seasonal clustering of samples, were plotted with the 'veganCovEllipse' function. Differences between predefined groups were tested with a two-way crossed analysis of similarities with the 'anosim' function (*Anosim*, permutations = 999, $p \leq 0.001$) (Clarke, 1993; Clarke & Warwick, 2001). The correlation between environmental variables (Euclidean distances) and community dissimilarity (Bray-Curtis) was assessed using Mantel tests ($r \geq 0.5$, $p \leq 0.001$). All ordinations were plotted using the R package 'ggplot2', and the seasons considered were winter (December to February), spring (March to May), summer (June to August), and autumn (September to November). Despite a $\log_{10}$ transformation, the abundance data

associated with the KEGG *metE* did not validate the conditions necessary to perform parametric tests (Shapiro–Wilk test, Bartlett test; $p < 0.05$), so non-parametric Kruskal–Wallis tests were performed.

RESULTS

Environmental conditions and cobalamin bioavailability in seawater at SOLA

Between 2009 and 2015, surface seawater showed seasonal variations in temperatures that varied from a minimum of 8.5°C in winter to a maximum of 24.3°C in summer. Nutrients (NO_2^-, NO_3^- and PO_4^{3-}) and chlorophyll-a concentrations were higher in winter and late winter, respectively (Figure S1). Salinity values dropped during short periods in December 2011, March 2013, and January 2014, and corresponded with increases in nitrate and phosphate levels.

To evaluate the bioavailability of B_{12} in seawater at SOLA, we used a B_{12} bioassay based on the model organism *Ostreococcus tauri* that was previously used in vitamin B_1 bioassays (Paerl et al., 2017). Assessing the requirement for B_{12} of our model species, maximal cell concentrations were reached at 25 pM cyanocobalamin (Figure S2).

B_{12}-limited cells incubated in SOLA-filtered seawater collected at different times of the year and enriched with nitrate, phosphate, trace metals, and vitamin B1, exhibited different growth patterns (Figure 2A). *O. tauri* grew without B_{12} addition in most water samples between April and November. Inversely, no cell growth was observed in the absence of B_{12} in most winter water samples. B_{12} amendment restored, however, growth or improved cell survival in a dose-dependent

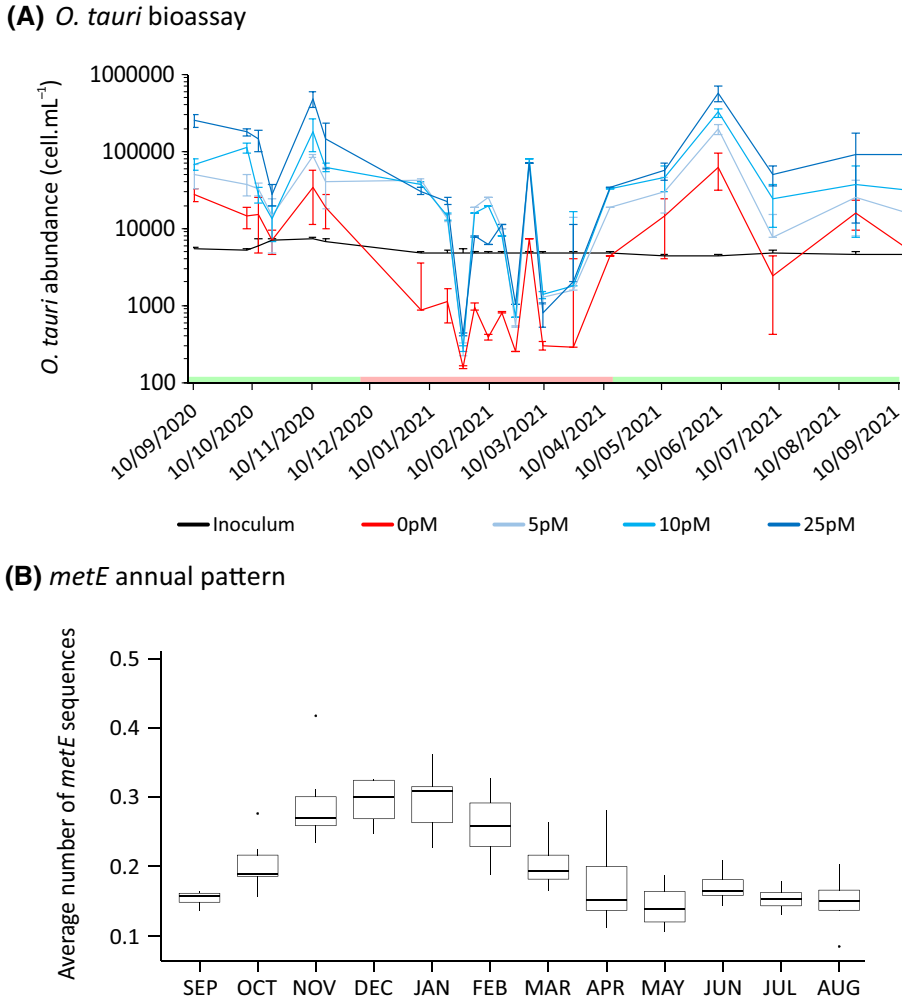

FIGURE 2 (A) Bioassay of *Ostreococcus tauri* RCC745 incubated in SOLA water collected from September 2020 to September 2021 and supplemented with increasing vitamin B_{12} concentrations (0–25 pM). The inoculum represents the cell abundance (cell.mL^{-1} ± SD, $n = 3$) of the inoculate, and the periods coloured in green indicate the growth of *O. tauri* (i.e., B_{12} availability in seawater) while those coloured in red indicate a decline of *O. tauri* cell numbers (i.e., B_{12} limitation). (B) Boxplot showing the average relative abundance of *metE* (number of sequences, averaged by month over 7 years between 2009 and 2015) used as an indicator of vitamin B_{12} bioavailability in SOLA seawater. *Met*E catalyses the methionine biosynthesis in a vitamin B_{12}-independent way.

manner in most winter samples confirming that B_{12} was limiting at the time. The annual pattern of the KEGG *metE*, which encodes the cobalamin-independent methionine synthase was used to detect potential B_{12} limitations (Figure 2B). *MetE* catalyses methionine biosynthesis in a cobalamin-independent way but is about 50 times less efficient than the cobalamin dependent methionine synthase, encoded by *metH*, which is used when cobalamin is bioavailable (Gonzalez et al., 1992; Helliwell et al., 2011; Matthews et al., 2003). In our 7-year metagenomics time series, *metE* showed significant differences in abundances between seasons (Kruskal–Wallis, $p < 0.0001$; Dunn $p < 0.01$), being more abundant from October to March, than in summer (Figure 2B). Together, Figure 2A, B suggest that B_{12} is limiting in SOLA seawater in winter and spring.

Annual patterns of cobalamin metabolisms over 7 years at SOLA

Over 7 years of monthly sampling at the SOLA station in Banyuls-sur-Mer, we identified 82 KEGGs related to B_{12} metabolisms (catabolism, production, salvage, and remodelling) (Figure 1, Table S1) that corresponded to 474,725 genes (Figure 3A). Among them, 30 were significantly rhythmic with a period of 365 ± 10 days as determined by the Lomb Scargle periodogram (LSP) method (PNmax ≥10, p values <0.05) (Table S1). These rhythmic KEGGs comprised 68.2% of the genes associated with the 82 B_{12}-related KEGGs, and 68.6% of the sequences (Figure 3A).

A nMDS analysis based on all 82 B_{12}-related KEGGs, fitted to SOLA environmental variables (Figure S1), showed that samples grouped significantly according to seasons (anosim, $r = 0.393$, $p = 0.001$) (Figure 3B). A nMDS focused on the 30 rhythmic KEGGs showed a stronger grouping of the samples according to seasons (anosim, $r = 0.459$, $p = 0.001$) (Figure 3C), while a nMDS based on the non-rhythmic ones showed a weaker seasonal grouping (anosim, $r = 0.165$, $p = 0.001$) (Figure 3D). Environmental parameters had a stronger correlation to rhythmic KEGGs composition (Mantel, $r = 0.4767$, $p = 0.001$) than to non-rhythmic KEGGs composition (Mantel, $r = 0.2724$, $p = 0.001$) (Figure 3C, D).

When considering the genes corresponding to the B_{12}-related KEGGs, the nMDS showed that there was also a strong seasonal pattern for all genes (anosim, $r = 0.568$, $p = 0.001$) (Figure 3E), and for the ones related to the rhythmic KEGGs (anosim, $r = 0.564$; $p = 0.001$) (Figure 3F) but also for the genes from the non-rhythmic KEGGs (anosim, $r = 0.572$; $p = 0.001$) (Figure 3G). There was a stronger correlation between samples and environmental variables for the gene-based analysis than for the KEGG-based ones (Figure 3B–G). Environmental parameters were similarly strongly correlated to gene composition for the whole gene dataset (Mantel $r = 0.5680$, $p = 0.001$). Summer samples were correlated to temperature and day length, whereas nutrients, dissolved oxygen concentrations, and salinity rather shaped winter and fall communities (adonis, $p = 0.001$) (Figure 3B–G, Figure S1).

Seasonality of B_{12} and pseudo-B_{12} biosynthesis, salvage, and remodelling pathways

Among the 30 rhythmic KEGGs related to vitamin B_{12} metabolisms (PNmax ≥10), we selected the 13 KEGGs specifically involved in B_{12} or pseudo-B_{12} biosynthesis, salvage, and remodelling pathways (Figure 1, Table S1). Overall, we observed that KEGGs related to the anaerobic pathway were present in winter, while the aerobic pathway was predominant in summer, concomitantly with the salvage and the remodelling pathways (Figure 4A). More precisely, KEGGs markers of the anaerobic pathway (*bluB*, *cbiE*, *cbiT* and *cobY*) had peaks of occurrence in February (Figure 4B, C). Rhythmic KEGGs involved in B_{12} de novo biosynthesis via the aerobic pathway were more abundant between May and September (Figure 4B), with maximum occurrences varying from May for *cobA*, June for *cobZ*, *cobF*, and *cobG*, and August for *cobW* and *cobK-cbiJ* (Figure 4B, C). Rhythmic KEGGs involved in the salvage pathway (*cobA_btuR*) and in pseudo-B_{12} remodelling (*cobC*) peaked in June and August, respectively (Figure 4B, C). The only potential marker of pseudo-B_{12} production by cyanobacteria, the KEGG *isiB*, had maximum abundance in December (Figure 4B, C).

Distinct organisms are involved in seasonal vitamin B_{12} and pseudo-B_{12} biosynthesis

We next focused on the taxonomic diversity of the genes coding for proteins involved in vitamin B_{12} biosynthesis (Figure S3A). A rhythmicity analysis (LSP) performed individually on the 23,045 genes associated with the 13 rhythmic KEGGs related to B_{12} and pseudo-B_{12} biosynthesis, salvage, and remodelling identified 698 annual rhythmic genes, representing 3% of biosynthesis genes and 29% of the sequences (Figure S3A). These rhythmic genes were annotated taxonomically, and most of them were assigned to the domain of *Bacteria*, accounting for about 80% of the sequences, followed by cyanobacteria and archaea

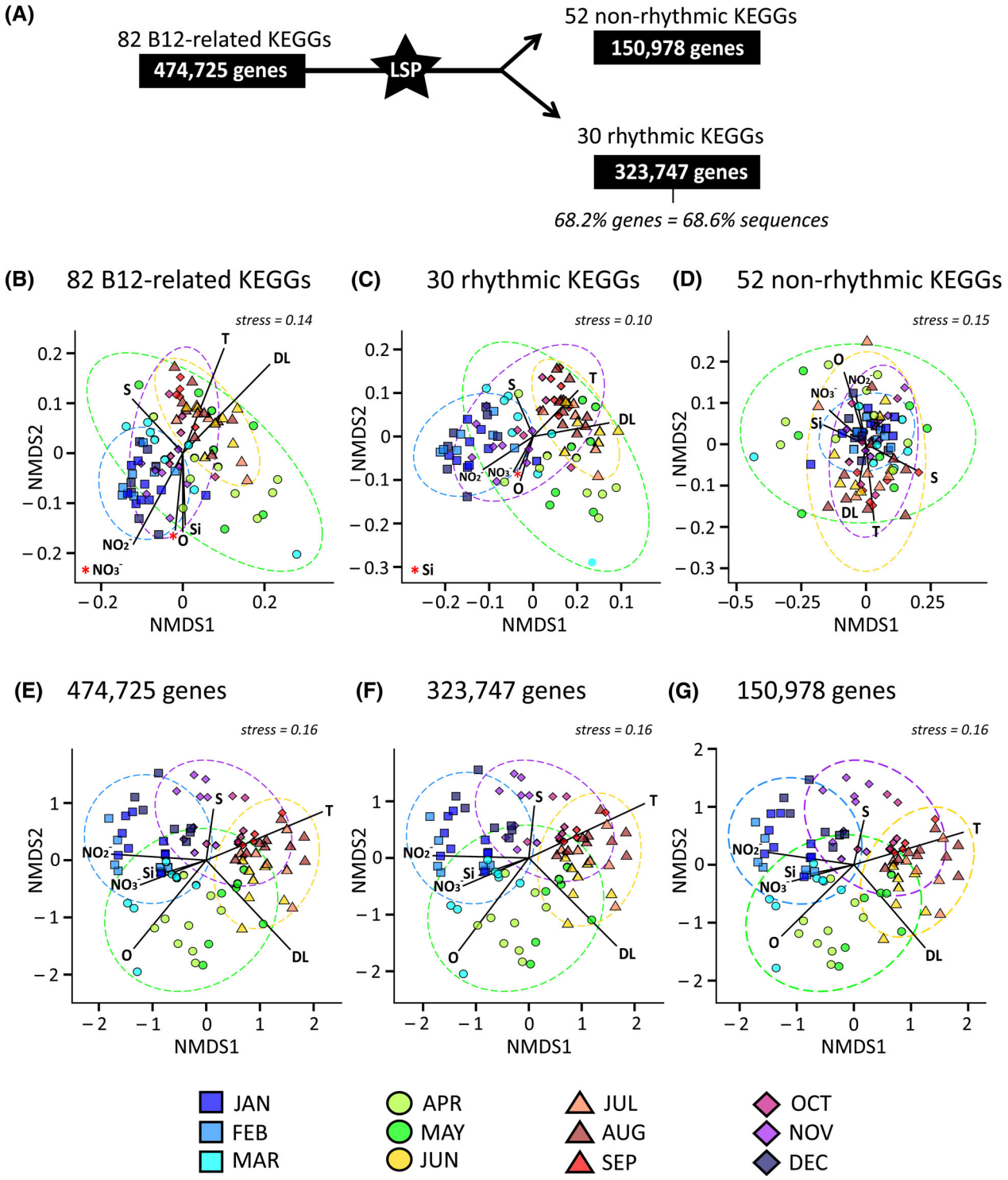

FIGURE 3 Seasonality of KEGGs related to potential B_{12} metabolisms and their associated genes. (A) Schematic representation of the rhythmicity analysis and the number of KEGGs and genes involved. (B) NMDS analysis based on all KEGGs related to vitamin B_{12} metabolisms, (C) the 30 rhythmic KEGGs, and (D) the 52 nonrhythmic KEGGS. (E) NDMS analysis based on the genes corresponding to all the KEGGs, (F) the rhythmic ones and (G) the non-rhythmic ones. Covariance ellipses group the samples by seasons: winter (blue), spring (green), summer (red) and autumn (purple). Environmental variables that significantly impact the sample's structure are fitted onto the nMDS. DL, day length; NO_2^-, nitrite; NO_3^-, nitrate; O, dissolved oxygen; S, salinity; Si, silicate ($Si(OH)_4$); T, temperature.

(Figure S3A). An nMDS analysis of these 13 marker KEGGs and their corresponding 698 rhythmic genes showed marked seasonal patterns (anosim $r = 0.716$, $p = 0.001$) and a strong influence of environmental variables, notably at the gene level (Mantel $r = 0.6879$, $p = 0.001$) (Figure S3B, C).

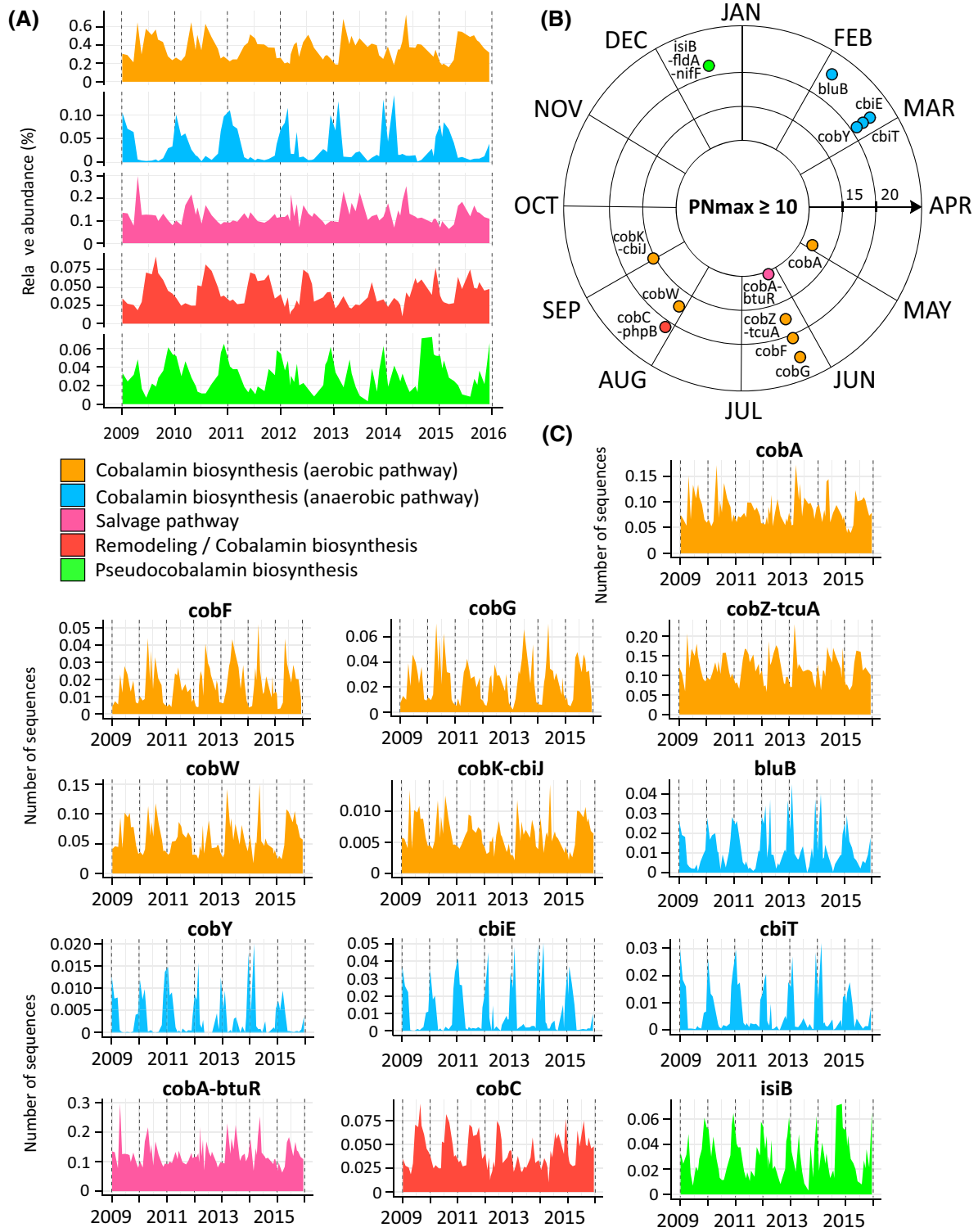

FIGURE 4 Seasonal patterns of the 13 rhythmic KEGGs related to vitamin B_{12} and pseudo-B_{12} biosynthesis, salvage, and remodelling pathways at the SOLA station for 7 years. (A) Relative abundance (%) of the KEGGs summed by pathway: aerobic and anaerobic biosynthesis pathways (orange and blue, respectively), salvage pathway (pink), pseudo-B_{12} remodelling and cobalamin biosynthesis (red), and pseudo-B_{12} biosynthesis (green). (B) The phase of the KEGGs during a year and strength of their rhythmicity (PNmax). The PNmax scale is indicated by a bold arrow on the right branch of the radar plot. (C) Individual abundance patterns of the 13 rhythmic KEGGS over 7 years (number of sequences).

Among archaea, the most abundant genes were affiliated with members of the *Nitrosopumilus* and *Nitrosopelagicus* genera, which belong to the phylum *Thaumarchaeota* (Figure 5A–C). Both genera were represented by 49 unique genes that accounted for about 94% of the archaeal sequences, which are

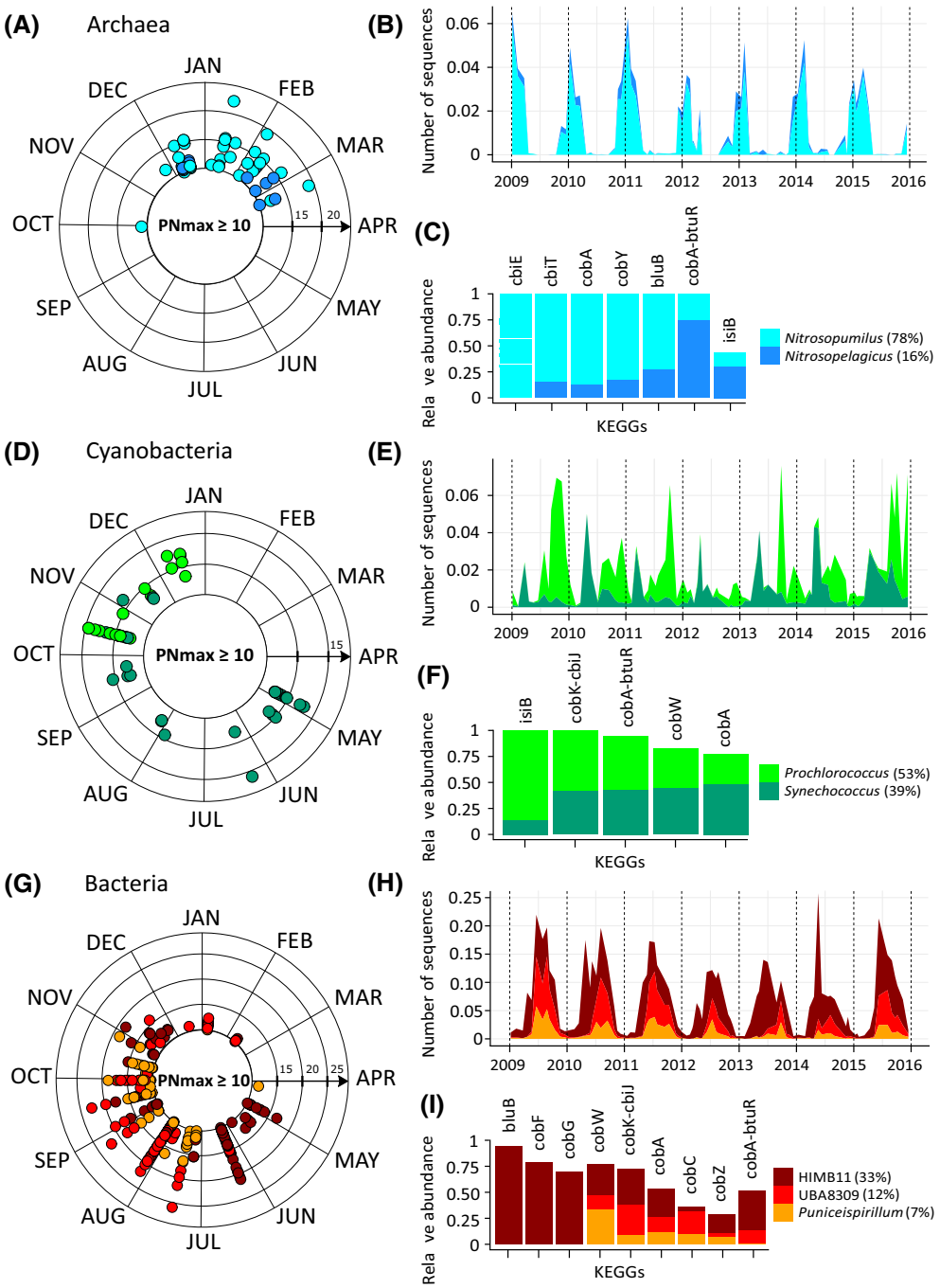

FIGURE 5 Seasonal pattern and metabolic potential of the taxonomical groups involved in vitamin B_{12} or pseudo-B_{12} production during 7 years at SOLA. For the archaeal (A, B and C), cyanobacterial (D, E and F), and bacterial genes (G, H and I) which are coloured by genus, the phase and the PNmax are indicated on the radar plots (left side). The PNmax scale is indicated by a bold arrow on the right branch of the radar plot. The relative abundance of the genes summed by genus and their dynamics over 7 years is shown on the density plots (top right side), and the proportion of sequences represented by these genes per KEGG is shown on the bar plots (bottom right side). The relative abundance of each genus in their respective dataset is indicated in brackets.

dominated by *Nitrosopumilus* (78% of archaeal sequences) (Figure 5C). *Nitrosopumilus* and *Nitrosopelagicus* had overlapping occurrence patterns in winter, with peaks of abundance observed between December and March (Figure 5A, B). All KEGGs associated with the anaerobic biosynthesis pathway were present in these archaeal sequences, as well as *bluB*, *cobA-btuR* for the salvage pathway, and *isiB* (Figure 5C). According to our data, rhythmic archaea from the *Nitrosopelagicus* genus do not seem to possess the KEGG *cbiE* (Figure 5C). Archaeal B_{12} biosynthesis genes sequences (i.e., *cbiE* and *cbiT* sequences) were

between 3 up to 9 times more abundant than bacterial B_{12} biosynthesis genes in winter (i.e., *cobG* and *cobF* sequences) (Figure S4A, B).

Among *Cyanobacteria*, the most abundant genera detected were *Prochlorococcus* (53%) and *Synechococcus* (39%), represented by 54 unique genes which account for about 92% of cyanobacterial sequences (Figure 5D–F). Genes affiliated with *Prochlorococcus* were present in autumn between October and December, while those affiliated with *Synechococcus* were present between May and November displaying a wider annual distribution (Figure 5D, E). Both genera had the same biosynthesis potential towards pseudo-B_{12} and B_{12} synthesis or salvage, with five marker KEGGs present in various proportions: *isiB*, *cobK-cbiJ*, *cobW*, *cobA* and *cobA-btuR* (Figure 5F).

For other bacteria, the most abundant genera for B_{12} metabolisms were all *Alphaproteobacteria* that belong to the genus HIMB11, which is part of the pelagic *Roseobacter* cluster (PRC), and two genera that belonged to the SAR116 clade, *Puniceispirillum* and UBA8309 (Figure 5G–I). These genera represented 268 unique genes, which accounted for about 51% of bacterial sequences (Figure 5I). Alone, the genus HIMB11 accounted for more than a third of bacterial sequences (Figure 5I). The different species within these bacterial genera had maximal abundances between May and November, with overlapping occurrence patterns during this period (Figure 5G, H). All KEGGs associated with the aerobic biosynthesis pathway were present in these rhythmic bacterial sequences, as well as *bluB* in HIMB11 and *cobA-btuR* for the salvage pathway in HIMB11 and UBA8309 (Figure 5I).

The genera HIMB11 and *Puniceispirillum* were dominated by a single gene belonging to a species that represents more than 80% of the sequences of their respective genera (i.e., HIMB11 *sp001642945* and *Puniceispirillum marinum*) (Figure S5A–D). The five most abundant phylotypes among these dominant species accounted for more than 40% of their associated sequences, with some distinct individual seasonal patterns (Figure S5A–D). The species UBA8309 *sp002457765* accounted for about 54% of the sequences related to the genus UBA8309, but other species were also abundant (Figure S5E, F), and the five most abundant phylotypes accounted for about 60% of the sequences (Figure S5E, F).

Interannual variability of vitamin B_{12} producers

We investigated the interannual variability of vitamin B_{12} metabolisms in SOLA by focusing on the 22,347 non-rhythmic genes (PNmax <10) involved in B_{12} biosynthesis, which represented 71% of the sequences

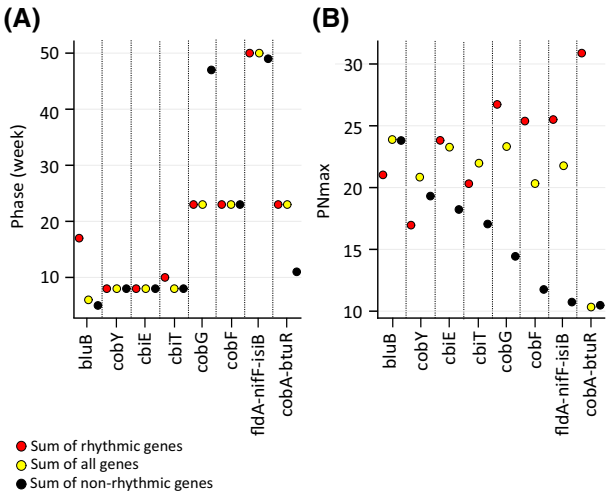

FIGURE 6 (A) Week of the year during which maximal peak of abundance is observed (i.e., phase) and (B) strength of rhythmicity (i.e., PNmax) for the eight KEGGs calculated after summing all gene abundances, only rhythmic genes abundances, or only non-rhythmic genes abundances. We summed together the abundances of all the genes annotated to the same KEGG, considering either the rhythmic genes only, the non-rhythmic genes only, or all the genes. We then performed a rhythmicity analysis (LSP) on each sum to compare the values of phases and PNmax obtained.

(Figure S3A, Figure S6A). We identified the KEGGs they belonged to and recalculated the KEGG rhythmicity by adding one non-rhythmic gene at the time from the most abundant to the rarest (Figure S6A). We hypothesized that non-rhythmic genes, which together produced an annual rhythmic KEGG (PNmax ≥10), included genes that replaced each other from year to year.

Eight out of our 13 marker KEGGs got an annual rhythm when the abundance of their related non-rhythmic genes was summed (Figure S6B). The phase of five of them (*cobY*, *cbiE*, *cbiT*, *cobF* and *isiB*) was like the one initially determined when summing all the genes, or when considering only the rhythmic genes (Figure 6A). For the other KEGGs (*bluB*, *cobG* and *cobA-btuR*), the phase varied depending on the type of genes summed (Figure 6A). The strength of rhythmicity (PNmax) was usually higher for the KEGGs based on all summed genes or summed rhythmic genes only, than for summed non-rhythmic ones (Figure 6B), excepting for *bluB* and *cobY*, for which lower PNmax values were obtained when summing the rhythmic genes.

We also identified the non-rhythmic genes that were needed to get a rhythmic KEGG (i.e., reaching the PNmax threshold of 10) (Figure 7; Figure S7). Among the KEGGs involved in the anaerobic pathway in winter, the *cbiE* rhythm was recovered when the archaea *N. catalina—cbiE_2* replaced *N. catalina—cbiE_1* during winter 2011, 2012 and 2013 (Figure 7A). For *cbiT*, the rhythm was recovered when *N. catalina—cbiE_2*

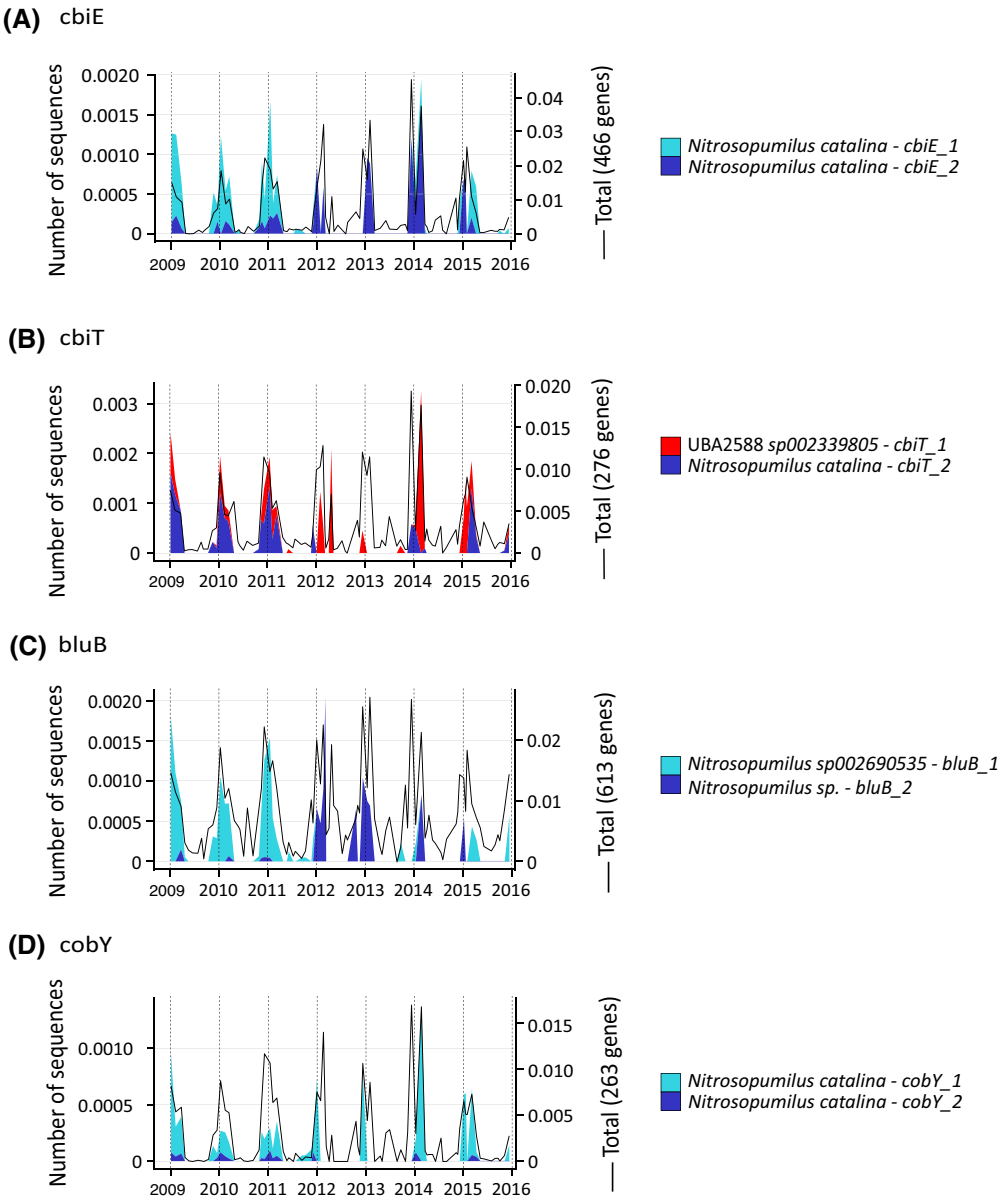

FIGURE 7 Analysis of non-rhythmic genes associated with four vitamin B_{12}-biosynthesis marker KEGGs involved in the anaerobic pathway (A: *cbiE*, B: *cbiT*, C: *bluB* and D: *cobY*). Density plots show the relative abundance (number of sequences) of the first two abundant and non-rhythmic genes (PNmax <10) sufficient to recover a rhythm at the KEGG level (PNmax ≥10). The black line shows the sum of all non-rhythmic genes of the respective KEGG.

was replaced by the bacteria UBA2588 *sp002339805—cbiT*_1 (Figure 7B). For *bluB* and *cobY*, we also observed a switch between two *Nitrosopumilus* members, belonging to the same or different species for *cobY* and *bluB*, respectively (Figure 7C, D).

For the KEGGs involved in the aerobic biosynthesis pathway (i.e., *cobF* and *cobG*) and in the salvage pathway (i.e., *cobA-btuR*) in summer, the rhythmicity threshold was reached after summing more than two nonrhythmic genes (Figure S7). *CobF* and *cobG* recovered a rhythm when adding the three and eight most abundant nonrhythmic genes, which represent 16% and 8% of the sequences, respectively (Figure S7A, B). For both KEGGs, the genes involved in the recovered rhythm were all affiliated to the genus HIMB11 represented by four species: HIMB11 *sp002684375*; HIMB11 *sp001642945*; HIMB11 *sp003486095*, and HIMB11 *sp000472185* (Figure S7A, B). For *cobA-btuR*, the 6 non-rhythmic genes sufficient to get a rhythm at the KEGG level were members of the *Alphaproteobacteria* belonging to *Rhodobacteraceae* (i.e., *Amylibacter sp900197625* and *Planktomarina temperata*) and *Gammaproteobacteria* (i.e., HTCC2207 *sp002469845* and HTCC2207 *sp002334915*) (Figure S7C).

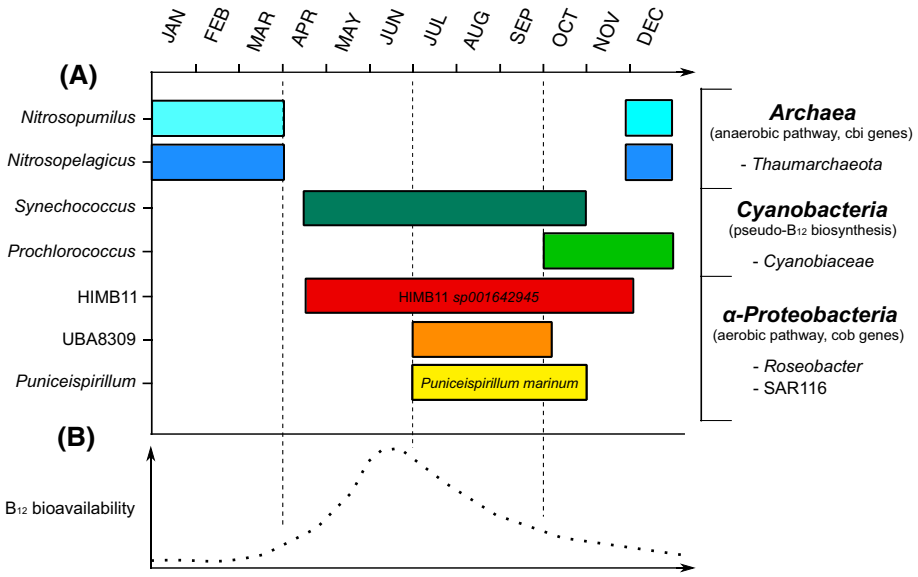

FIGURE 8 General overview of the seasonality of vitamin B_{12} and pseudo-B_{12} potential producers and related pathways. (A) Seasonality of potential vitamin B_{12} producers identified at the genus level and at the species level for HIMB11 and *Puniceispirillum* (the metabolic pathway used for B_{12} biosynthesis is also mentioned). (B) Schematic overview of B_{12} bioavailability in SOLA seawater over a year inferred from *Ostreococcus tauri* bioassay and *metE* relative abundance (Figure 3).

DISCUSSION

Our long-term metagenomic time series revealed that potential vitamin B_{12} production in the coastal NW Mediterranean was the result of a seasonal succession of different pathways throughout the year. This succession was characterized by the production of B_{12} through the archaeal anaerobic pathway in winter, of pseudo-B_{12} in spring and winter by cyanobacteria, and then, from spring to autumn, bacterial B_{12} synthesis through the aerobic pathway (Figure 8A).

This 7-year metagenomic study represents the first description of the succession of distinct B_{12} metabolic pathways performed by phylogenetically distant organisms over the years.

Archaea appeared to be the main potential B_{12} producers in winter. They belonged to the phylum *Thaumarchaeota* (i.e., *Nitrosopumilus* and *Nitrosopelagicus* genera) and could use the anaerobic pathway to produce B_{12} through the genes *bluB*, *cbiE*, *cbiT*, and *cobY* (Figure 8A). Marine *Thaumarchaeota*, such as *Nitrosopumilus*, have been shown earlier to possess B_{12} synthesis genes and were thus hypothesized to be a source of B_{12} in the water column (Doxey et al., 2015; Qin et al., 2017; Santoro et al., 2015) and to play a critical role in the deeper ocean (Heal et al., 2017). *Thaumarchaeota* are, however, known to be much less abundant than bacteria in surface waters, representing only 1%–10% of the prokaryotes (Church et al., 2003; Karner et al., 2001; Massana et al., 2000), so their importance could be limited in the upper water column. Our observations show, nevertheless, that archaeal B_{12} production genes were up to 9 times more abundant than the bacterial ones in winter in the coastal NW-Mediterranean site of SOLA. *Thaumarchaeota*, which are more abundant in winter planktonic communities in temperate seas (Doxey et al., 2015; Galand et al., 2010; Lambert et al., 2019; Pereira et al., 2021), thus certainly play a seasonally important role in B_{12} cycling in these areas. Remarkably, *Thaumarchaeota* were present at SOLA when B_{12} had the lowest bioavailability, which could indicate that their activity was not sufficient to enrich the water with B_{12}. Phytoplankton cells could, however, directly use archaeal-derived B_{12} for growth based on functional complementarity towards essential metabolite production and/or regeneration, that is, remineralization of inorganic nutrients and B_{12} production versus algal production of carbon- or sulphur-rich organic matter and vitamin B_1 as previously reported for bacteria (Durham et al., 2014; Geng & Belas, 2010; Giovannoni, 2012; Luo & Moran, 2014). The high demand for B_{12} by auxotrophic phytoplankton blooms (*Mamiellophyceae* and Dinoflagellates) observed in winter at SOLA could explain why B_{12} bioavailability in water is lowest during this period (Figure 8B). Since the isoforms of cobalamin are highly light sensitive, and their lifetime in seawater is very short (Heal et al., 2014; Martens et al., 2002), the balance between B_{12} release from prokaryotes and its consumption by other organisms remains hard to estimate (Villegas-Mendoza et al., 2019).

The cyanobacteria *Synechococcus* were the potential producers of pseudo-B_{12} from spring to autumn, relayed by *Prochlorococcus* in winter (Figure 8A). *Synechococcus* and *Prochlorococcus* are known to bloom at different times of the year in the coastal NW

Sea (Schauer et al., 2003; Sommaruga et al., 2005) as they are adapted to different ecological niches linked to their sensitivity to sunlight (Sommaruga et al., 2005). Our data suggest that the succession of these known pseudo-B_{12} producers (Bonnet et al., 2010; Doxey et al., 2015; Helliwell et al., 2016) provides a continuous input of pseudo-B12 over several months. Pseudo-B_{12} is, however, less bioavailable than B_{12} to several microalgae (Heal et al., 2017; Helliwell et al., 2016). If DMB is provided, pseudo-B_{12} could nevertheless be scavenged and/or remodelled by specific microorganisms that possess the *cobA-btuR* (i.e., archaea, bacteria, cyanobacteria) and *cobC* genes (i.e., bacteria) (Heal et al., 2017; Helliwell et al., 2016), which are more present in summer. Because bacteria and archaea both can produce DMB (*bluB* gene), cyanobacterial pseudo-B_{12} could be remodelled in summer and winter using the DMB produced successively by bacteria and archaea.

Shortly after archaea and partly concomitantly with cyanobacteria, other bacteria potentially produce B_{12} via the aerobic pathway (*cobF*, *cobG*, *cobZ_tcuA*, *cobA*, *cobW* and *cobK-cbiJ* genes) (Figure 8A). Co-occurrences between pseudo-B_{12} producing cyanobacteria and other prokaryotic producers (i.e., bacteria and archaea) suggest that pseudo-B_{12} and B_{12} could coexist in the water column at certain times of the year. Although both have distinct microbial sources and support different enzymatic demands, the coexistence between B_{12} and pseudo-B_{12} has been observed in the North Pacific subtropical gyre (Heal et al., 2017).

Seasonal bacterial B_{12} production from spring to autumn was potentially performed by members of the genera HIMB11 (*Roseobacter* clade) and *Puniceispirillum* (SAR116 clade). HIMB11 had maximal abundance between May and November and could thus be an important player in the B_{12} cycle at SOLA over several months. Most members of the marine *Roseobacter* lineage have earlier been described as B_{12} producers (Newton et al., 2010), but to date, HIMB11 has been only known for its potential role in carbon and sulphur cycles (Durham et al., 2014, 2015), and its importance in B_{12} production has not yet been highlighted. However, HIMB11 belongs to the Pelagic *Roseobacter* Cluster (PRC), whose members have the potential to produce B_{12} and other vitamins like B_2 (riboflavin), and B_6 (pyridoxine), but lack genes for the synthesis of vitamins B_1 and B_7 (Billerbeck et al., 2016).

Similarly, a phylogenomic analysis of all available SAR116 genomes showed that SAR116 members also carried the necessary genes for the biosynthesis of vitamins B_2, B_5 (pantothenate), B_6, B_9 (folate) and B_{12} (Grote et al., 2011; Roda-Garcia et al., 2021). Co-culture approaches have earlier shown potential interactions based on B_{12} requirements between *Roseobacter* members and blooming auxotrophic microalgae, including *Prorocentrum* (Wang et al., 2014), *Thalassiosira* (Durham et al., 2015, 2017) and *Ostreococcus* (Cooper et al., 2019). Interestingly, HIMB11 (*Roseobacter*) and *Puniceispirillum* (SAR116) both possess the DMSP (dimethylsulfoniopropionate) degradation pathway (Varaljay et al., 2012), suggesting that this chemical attractant could be a key metabolite in the regulation of SAR116- and *Roseobacter*-phytoplankton interactions. There might be adaptive associations between *Roseobacter* and phytoplankton strains based on the nature of exudates produced by algal species (Hahnke et al., 2013; Teeling et al., 2012). Genes encoding for the type IV secretion system (T4SS) or involved in motility and chemotaxis have notably been found in B12-producing strains of *Roseobacter* (Luo & Moran, 2014; Newton et al., 2010), and they may be important traits for B_{12}-based microbial symbiosis between auxotrophic microalgae and B_{12} providers (Luo & Moran, 2014; Newton et al., 2010; Wang et al., 2014).

An additional argument for the importance of the association between *Nitrosopumilus*, *Nitrosopelagicus*, HIMB11 and *Puniceispirillum* with phytoplankton lies in recent studies based on co-occurrence networks that showed associations between archaeal or bacterial potential B_{12} producers (e.g., *Thaumarchaeota*, *Rhodobacteraceae* and *Puniceispirillaceae*), and auxotrophic microalgae (e.g., *Micromonas*, *Bathycoccus* and *Ostreococcus*) (Joglar et al., 2021; Krabberød et al., 2022; Lambert et al., 2021). A winter interaction hub associated the picoeukaryote *Bathycoccus* with a potential B_{12} producer belonging to *Rhodobacteraceae*, in addition to archaea, which also interacts with other B_{12} auxotrophs such as dinoflagellates, diatoms, and picoeukaryotes (Krabberød et al., 2022). These findings and ours together suggest an important role of *Puniceispirillaceae* and specific members of *Rhodobacteraceae* in providing vitamin B_{12} for phytoplankton blooms. These B_{12} producers could be seen as 'core microbes' that would be essential to the seasonal functioning of the marine ecosystem (Krabberød et al., 2022).

We observed that during the 7 years of the study, B_{12}-related pathways always re-occurred, but that for some years, the genes involved were carried by different microorganisms. In some cases, the organisms that replaced each other belonged to the same species (*Nitrosopumilus catalina*). These microorganisms could thus be considered as different ecotypes that occupy different ecological niches. In other instances, the change occurred between different species. In both cases, a switch between organisms would indicate changes in environmental niches from time to time. Such changes did occur for 3 years when perturbations changed the environment conditions, and thus possibly the microbial niches at SOLA. Strong rains combined with storms brought lower salinity and higher nutrient loads to the coastal site. We hypothesize that environmental disturbances prevented certain species or

ecotypes from thriving and that they were replaced by other organisms, more or less phylogenetically close, with different or more extensive ecological niches.

The changes between microorganisms allowed the maintenance of metabolic functions at the community level. The gene-level redundancy between phylotypes could thus stimulate the long-term functional resilience of microbial ecosystems exposed to contrasted inter-annual environmental conditions (Awasthi et al., 2014). Similarly, in the human gut microbiome or the soil, functional redundancy between specific taxa maintains stability under disturbances (Li et al., 2021; Moya & Ferrer, 2016). For that process to take place, a large diversity of microorganisms carrying similar genes is needed. If diversity is reduced, the ecological insurance (Awasthi et al., 2014; Yachi & Loreau, 1999) will be lost and the functioning of the ecosystem will be impaired.

CONCLUSION

Our monthly 7-year metagenomic time series at the SOLA station uncovered a seasonal succession of distinct prokaryotic producers that use different biosynthesis pathways to produce vitamin B12 de novo. These seasonal microorganisms are essential for the functioning of this coastal marine ecosystem, notably *Roseobacter*, and the SAR116-clade in summer, and *Thaumarchaeota* in winter, when SOLA seawater is B_{12}-limited. The fact that some vitamin B_{12} producers were replaced by others carrying the same functions during contrasted years indicates the existence of functional redundancy at a gene level in several B_{12}-related functions. Environmental perturbations can thus strongly impact biodiversity patterns without perturbing microbial functions. This study contributes to our understanding of the long-term functional resilience of marine microbial communities, especially in coastal ecosystems where they could be exposed to contrasting inter-annual environmental conditions.

AUTHOR CONTRIBUTIONS

Maxime Beauvais: Writing – original draft; writing – review and editing; investigation; funding acquisition; visualization; methodology. **Philippe Schatt:** Resources; methodology. **Lidia Montiel:** Data curation; methodology. **Ramiro Logares:** Data curation; methodology. **Pierre E. Galand:** Supervision; conceptualization; investigation; writing – review and editing; validation; methodology; funding acquisition. **François-Yves Bouget:** Conceptualization; investigation; writing – review and editing; validation; supervision; methodology; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The catalogue of prokaryotic genes associated with all the B_{12}-related KEGGs used in this study is accessible online in the Zenodo repository: 10.5281/zenodo.7409517. The raw sequences were deposited in the NCBI Sequence Read Archive under accession number PRJEB66489: https://www.ncbi.nlm.nih.gov/sra/PRJEB66489 and PRJEB26919.

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