Diel Rhythms of Marine Picoplanktonic Communities Assessed by Comparative Metaproteomics

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Research

Keywords: Diel cycle, Metaproteomics, Picoplankton Communities, Marine Microbial Ecology

Posted Date: February 11th, 2020

DOI: https://doi.org/10.21203/rs.2.23020/v1

Diel Rhythms of Marine Picoplanktonic Communities Assessed by Comparative Metaproteomics

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Abstract

Background

Diel cycle is of enormous biological importance in that it imposes temporal structure on ecosystem productivity. In the world oceans, microorganisms form complex communities that carry out about half of photosynthesis and the bulk of life-sustaining nutrient cycling. Within these natural microbial assemblages, photoautotrophs, such as *Cyanobacteria*, display diel rhythmicity in gene expression. To what extent autotrophs and heterotrophs are impacted by light and dark oscillations and how this collectively influences community structure and functionality remains poorly documented. In this study, we compared eight day/night metaproteome profiles of *Cyanobacteria* and both free-living and attached bacterial fractions from picoplanktonic communities sampled over two consecutive days from the surface north-west Mediterranean Sea.

Results

Our results showed similar taxonomic structure in both free-living and particle-attached bacteria, dominated by *Alphaproteobacteria* and *Gammaproteobacteria*. Temporal rhythmicity in protein expression was observed in both *Synechococcales* and *Rhodobacterales* in light-dependent processes such as photosynthesis or UV-stress response. Other biological processes, such as phosphorus or amino acid metabolisms, were also found to cycle in phototrophs. In contrast, proteins from the ubiquitous *Pelagibacterales* remained stable independently of the day/night oscillations.

Conclusion

This work integrated for the first time diel comparative metaproteomics on both free-living and particle attached bacterial fractions in coastal oligotrophic environment. Our findings demonstrated a taxa-specific response to diel cycle with a more controlled protein

regulation for phototrophs. This study provided additional evidences that timekeeping mechanisms might be widespread among bacteria, broadening our knowledge on diel microbial assemblage dynamics.

Key words

Diel cycle, Metaproteomics, Picoplankton Communities, Marine Microbial Ecology

Background

Microorganisms in marine ecosystems are extremely diverse, dominate biomass and play key roles in biogeochemical processes [1, 2]. Picoplankton (i.e. the microorganisms of a size ranging between 0.2 - 2 µm) carries out up to the half of the world ocean's primary production and the bulk of life-sustaining nutrient cycling [3]. Marine picoplanktonic communities are composed of both free-living and particle-attached microorganisms, which can be structurally and metabolically different [4]. The 24-hours oscillation of solar radiation reaching the Earth's surface temporally structures biological events, activities and physiological processes across all kingdoms of life [5]. Sea surface picoplanktonic communities showed diel oscillations for metabolites consumption [6, 7], viral infection [8], DNA/protein synthesis and dissolved organic carbon (DOC) distribution [9]. Diel variation in abundance, activity and structure were reported in free-living and particles-attached microorganisms [10]. To what extent picoplankton communities are collectively entrained by day and night cycles, how this influences their population structure, regulates their physiologies, and impinges on species interactions are questions of immediate urgency.

Circadian rhythms consist of diel cycling biological processes governed by endogenous clock. Circadian clocks use external variable clues such as light, temperature and/or redox cycles to scale to the environment and regulate patterns of genetic expression throughout the day [11]. The model organism for bacterial circadian clock, *Synechococcus elongatus*, clock-

68 regulates the expression of numerus genes via a core oscillator composed of three principal
69 proteins (KaiA, KaiB and KaiC) [12, 13]. The existence of diel rhythmicity was reported in
70 other bacteria such as the purple bacteria *Rhodospirillum rubrum* [14] and *Rhodobacter*
71 *sphaeroides* [15]. *Kai* genes and their homologs have been reported in various prokaryotic
72 groups. While *kai*A gene was identified in *Cyanobacteria* only, *Kai*B genes also occurred in
73 *Proteobacteria* and *Kai*C genes in *Proteobacteria*, *Thermotogae* and *Chloroflexi* [16]. This
74 suggest that endogenous temporal programs might exist in other numerous bacteria [17].

75 The development of omics approaches has advanced the understanding of temporal
76 dynamics in marine microbial assemblages. Environmental transcriptomics revealed day and
77 night patterns in metabolic activity of naturally occurring picoplankton communities over 24h
78 period [18, 19, 20]. Diel transcriptional rhythms were also observed over three consecutive
79 days in marine oligotrophic bacterial community, demonstrating that temporal regulation of
80 gene expression is likely to occur in both autotrophs and heterotrophs microorganisms [21].
81 Metaproteomics allows the characterization of the final product of the gene (i.e. proteins) and
82 therefore helps to better understand of community functioning [22]. Our study is the first to
83 assess the metaproteome dynamics under day/night cycles of picoplanktonic communities. We
84 thus compared day and night metaproteomes of *Cyanobacteria* and both free-living (>0.2µm)
85 and particle-attached (>0.8µm) bacterial fractions sampled over two consecutive days at the
86 surface of north-western (NW) Mediterranean Sea. The resulting eight metaproteomes were
87 quantitatively and qualitatively compared, allowing us to assess the protein regulation under
88 diel variations.

89

90

Results

Features of sampling site

Sampling was performed in summer (June 2014) in the NW Mediterranean Sea. The average temperature and salinity, measured in June, were as follows: 18.7 ± 0.7 °C and 37.8 ± 0.1 psu respectively (Supplementary Information 1). pH was stable over the month with an average of 8.26 ± 0.04. Nutrients concentration averaged 0.03 ± 0.01 µM NH_4^+, 0.05 ± 0.03 µM NO_3^-, 0.01 ± 0.001 µM NO_2^-, 0.02 ± 0.01 µM PO_4^{3-} and 0.75 ± 0.09 µM $Si(OH)_4$.

Metagenomic analysis revealed that *Proteobacteria* was the main contributor phylum with 66.9% of the total detected bacterial reads, followed by *Bacteroidetes* (15.5%) and *Cyanobacteria* (12.2%) (Table 1). *Alphaproteobacteria* was the most represented class (47.3%), followed by *Gammaproteobacteria* (17.8%), *Flavobacteriia* (14.3%) and unclassified *Cyanobacteria* (12.3%) (Table 1). At order level, abundant *Pelagibacterales* reads were detected (28.9%), followed by *Flavobacteriales* (16.5%) and to a lesser extent, *Rickettsiales* (11.0%), *Oceanospirillales* (8.9%), *Rhodobacterales* (7.2%) and *Cellvibrionales* (6.1%) (Figure 1).

Free-living *versus* particle-attached bacteria: contrasting diel regulation of their metaproteomes

In this study, the total number of identified proteins was stable within each filter fraction (Supplementary Information 2). More proteins from free-living bacteria were identified in comparison to the particle-attached ones, with an average of 529 ± 67 and 194 ± 31 identified proteins for the 0.2 and 0.8µm pore-size filters respectively. The proportion of annotated proteins decreased with lowering taxonomic hierarchy in all samples. The total number of identified proteins was 47.9 ± 4 and 65.6 ± 1.5 at order level and 62.0 ± 2.8 and 55.2 ± 1.1 at functional level for 0.2 and 0.8µm pore-size filters respectively.

115 The four metaproteomes (Day 1, Day 2, Night 1, Night 2) from the 0.2µm pore-size
116 filters were largely dominated by *Proteobacteria* (avg. 90.9 ± 1.1%) (Table 1). At class level,
117 *Alphaproteobacteria* (avg. 69.6 ± 2.1%), *Gammaproteobacteria* (avg. 22.4 ± 1.2%) and
118 *Flavobacteriia* (avg. 4.8 ± 0.3%) were found to be the most represented (Table 1). Regarding
119 the 0.8µm pore-size filters, *Proteobacteria* were the most abundant (avg. 32.7 ± 2.7%). Classes
120 were mainly represented by *Alphaproteobacteria* (avg. 20.3 ± 1.5%) and
121 *Gammaproteobacteria* (avg. 10.9 ± 1.1%). *Cyanobacteria* were found on both 0.2 and 0.8µm
122 pore-size filters with an abundance of 1.4 ± 0.8% and 62.0 ± 2.7% respectively (Table 1).
123 Overall, phylum and class structures were stable over day and night periods in both 0.2 and
124 0.8µm fractions (Tables 1). On the contrary, more diel fluctuations in protein abundance were
125 observed at order level (Figure 1). Within the free-living bacteria, *Pelagibacterales* were more
126 represented at night, while *Rhodobacterales* and *Sphingomonadales* were found in higher
127 proportion at day. The particle-attached *Bacteriovoracales*, *Pseudomonadales* and *Rhizobiales*
128 were more represented at day and *Alteromonadales* and *Flavobacteriales* at night. The
129 *Synechococcales*, the most abundant cyanobacterial order, were more abundant at night.

130 Metaproteomic analysis revealed that housekeeping-related proteins dominated both
131 free-living and particle attached bacterial fractions (Table 2). Proteins involved in
132 transcription/translation, protein folding, or transport processes were abundant with the 60 kDa
133 chaperonin being the most represented (free-living bacteria: avg. 31.3 ± 1.7%, particle-
134 attached: avg. 23.0 ± 3.1%). The 50S ribosomal protein (avg. 13.1 ± 1.3%), as well as the DNA-
135 binding protein HU (avg. 7.4 ± 0.2%), the elongation factor proteins (avg. 6.2 ± 0.9%), the
136 amino-acid ABC transporter-binding protein (avg. 5.9 ± 0.3%) and the 10 kDa chaperonin (avg.
137 5.4 ± 0.2%) were exclusively detected in free-living bacterial metaproteomes. The ATP
138 synthase proteins (avg. 15.0 ± 3.1%), the DNA-directed RNA polymerase (avg. 8.6 ± 2.9%),
139 the elongation factor proteins (avg. 8.2 ± 1.3%) and the 50S ribosomal protein (avg. 6.4 ± 3.1%)

greatly contributed to the particle-attached bacterial metaproteomes. The phosphate-binding protein (avg. 30.1 ± 9.1%) was, in average, the most abundant protein characterized in *Cyanobacteria*, followed by the 60 kDa chaperonin (avg. 15.0 ± 2.3%), the elongation factor (avg. 12.4 ± 3.1%) and the ATP synthase (avg. 12.2 ± 3.5%).

Protein expression patterns, at the order level, were visualized using heatmaps (Figure 2). The taxonomic and functional clusters of the free-living bacterial fraction were stable in both day and night conditions (Figure 2a). *Pelagibacterales* always clustered apart from other taxa, except in Day 2 where it grouped with *Rhodobacterales*. Protein folding-related proteins formed a distinct functional cluster in all samples except in Night 2, where they clustered with proteins involved in translation. Taxonomic and functional patterns in particle-attached bacteria varied more across the metaproteomes (Figure 2b). During day, *Rhizobiales* and *Rhodobacterales* clustered apart. In Night 1, *Pelagibacterales* behaved similarly as the latter, while in Night 2, *Rhizobiales* only clustered apart from all other taxa. Proteins involved in protein folding and respiration processes formed a distinct functional cluster in Day 1 and Night 1. Protein folding-related proteins clustered apart from other proteins in Day 2 and Night 2.

Diel protein expression of the most abundant taxa: *Synechococcales*, *Rhodobacterales* and *Pelagibacterales*

Multiple biological processes were found to be periodically impacted by day and night cycle in *Synechococcales* (Figure 3a). Proteins involved in carbohydrate, nitrogen and phosphorus metabolisms and photosynthesis processes were systematically more represented at day, while proteins involved in translation, protein folding, and respiration processes were predominant at night. As represented in Figure 4a, several *Synechococcales* proteins were found to be exclusively characterized during daytime (yellow boxes) or night time (black boxes) or consistently more abundant at day (sun symbol) or at night (moon symbol). Light-dependent proteins included the light harvesting proteins, allophycocyanin, phycocyanin,

phycobiliprotein and phycoerythrin, as well as the protein FtsZ, involved in cell division process, and the Leu/Ile/Val-binding transport protein. At night, the 60 kDa and DnaK chaperonins were consistently more abundant (Figure 4a). Interestingly, proteins involved in carbohydrate metabolism showed contrasting diel expression. Glycolysis and pentose phosphate pathway were characterized by proteins exclusively detected at either day or night time, suggesting that energy production pathways were consistent over the course of the day.

Rhodobacterales proteins were detected on both free-living and particle-attached fractions (Figure 1). By grouping both protein fractions, strong diel variations were observed in *Rhodobacterales* proteomes (Figure 3b). Two oxidoreductases, catalase-peroxidase and superoxide dismutase [Fe], both involved in oxidative stress response, were specific to day time, suggesting immediate response of *Rhodobacterales* to light stress (Figure 4b). Similarly, the expression of the protein folding protein 10 kDA chaperonin, was consistently more abundant at day (Figure 4b). Sunlight was also found to favor chemotaxis, as the chemotactic signal transduction system substrate-binding protein BasB was consistently more expressed in day samples (Figures 3b, 4b). Cell motility and respiration showed diurnal changes in *Rhodobacterales* (Figures 3b, 4b). On the contrary, amino acid and phosphorus transporters were observed at both day and night times (Figure 4b). Similarly, proteins involved in transcription/translation processes, such as the ribosome-recycling factor and the ribosomal protein S12 methylthiotransferase RimO as well as viral protein and integration host factor subunit alpha were non-rhythmically detected at either day or night (Figure 4b).

On the contrary to phototrophs, *Pelagibacterales* proteins characterized in both free-living and particle-attached combined fractions seemed less consistently regulated and therefore no major diurnal change was observed between the day and night conditions (Figure 3c). *Pelagibacterales* expressed several transporters (Figure 4c). While the expression of sugar transporters was specific to daytime, amino-acid transporters were not impacted by diel

rhythms (Figure 4c). Similarly, proteins involved in amino acid biosynthesis were characterized during both day and night times. Indeed, the arogenate dehydratase and glutamine amidotransferase MTH_191 were specific to night sample, while the 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase was specific to day sample (Figure 4c). Proteins involved in ATP production were detected at any time, during day or night, suggesting that *Pelagibacterales* rely on continuous energy supply (Figures 3c, 4c) with the synthesis of proteins involved in glycolysis and pyruvate metabolism (glyceraldehyde-3-phosphate dehydrogenase, phosphate dikinase and the succinate-CoA) (Figure 4c).

Discussion

Marine oligotrophic waters present significant challenges for metaproteomics study as protein extraction is hampered by the low bacterial biomass, which requires to filter important volume of water [23, 24]. *In situ* physicochemical measurement confirmed the oligotrophic environmental conditions in which the studied picoplanktonic communities were sampled (Supplementary Information 1), therefore large volumes of water (60L/sample) were sequentially filtered onto both 0.8 and 0.2µm pore-size filters. A combined protein search database allowed us to maximize the number of protein identification [25]. Protein inference issue, commonly encountered in metaproteomics, was overcome in this study by using taxonomic and functional consensus protein annotation [26]. The total number of proteins identified per sample was found to be consistent with previous metaproteomics studies conducted in marine oligotrophic surface waters [27, 28, 29, 30] (Supplementary Information 2). The number of proteins identified within attached bacterial fraction was significantly lower than in the free-living fraction (Supplementary information 2). In NW Mediterranean Sea, free-living bacteria are generally more abundant in summer under oligotrophic conditions and contribute the most to total bacterial activity [10] as attached-bacteria rely on the availability of particulate organic carbon sources [31].

Based on relative protein abundance, the structure of the community was dominated by *Proteobacteria* followed by *Cyanobacteria* and *Bacteroidetes*, which was consistent with metagenome distribution (Table 1). These taxa were previously reported as numerically important in eastern Mediterranean Sea surface water [32] and in other marine oligotrophic environments [29, 30, 33, 34]. Interestingly, taxonomic similarity at phylum and class levels was observed between particles-associated and free-living bacteria with *Alphaproteobacteria* and *Gammaproteobacteria* dominating both fractions (Table 1). This suggested interconnections between both reservoirs as previously observed in taxonomic distribution within microbial assemblages of Mediterranean Sea [10, 35, 36].

This day/night metaproteomics study provided valuable insights into temporal rhythmicity of gene expression in surface oligotrophic picoplankton communities. At order level, protein content of the free-living bacterial fraction was found to be more stable over day and night periods than in the particle-attached fraction (Figure 2). This can be explained by the nature of particles present in the water column at the time of sampling, which influence particle-attached microbial activity and distribution [37]. Looking at specific taxa, *Synechococcales* showed strong diel variations in protein abundance (Figure 1). Interestingly, diel patterns were also observed in the purple photosynthetic bacteria *Rhodobacterales*. Even though the current data would not allow to conclude on circadian rhythms, they demonstrated diel taxa-specific regulation of total protein expression (i.e. 0.2 and 0.8µm pore-size fractions combined) (Figures 3 and 4). In *Synechococcales*, the cell division protein FtsZ was observed in day sample only, similar with observation in field population, where cell division occurs during the day [38]. Proteins involved in light-mediated processes such as photosynthesis were characterized during daytime in *Synechococcales* supporting previous (meta)-transcriptomic studies (Figures 3 and 4) [18, 21].

239 Our results suggested that mechanisms involved in light-damage repair might be
240 preferably expressed at either day or night-time in phototrophs. Interestingly, the 60 kDa and
241 DnaK chaperonins were found to be more abundant at night in *Synechococcales*, which was
242 consistent with the circadian rhythm of dnaK-reporting bioluminescent *Synechococcus* strain,
243 in which *dnaK* expression was peaking at night [39]. The 10 kDa chaperonin and the oxidative
244 stress response involved proteins were systematically more abundant at day in
245 *Rhodobacterales* (Figures 4). In contrast, no diel regulation in chaperonin expression was
246 observed in *Pelagibacterales* and DNA replication/repair and oxidative stress response
247 involved proteins were expressed during both day and night-time (Figure 3). In the euphotic
248 layer, bacteria are exposed to potentially harmful UV radiation, damaging both proteins and
249 DNA. Chaperonins were shown to be abundant in marine environment (Table 2) [28, 29 40],
250 since they are ubiquitous and vital as their main function is to prevent protein misfolding [41].
251 Chaperonins are essential for coping with UV-induced protein damage and maintaining proper
252 protein function [42]. Because of UV absorbing compounds, phototrophs benefit a better
253 protection against photolesions in DNA than heterotrophs such as *Pelagibacterales*, in which
254 proteins involved in DNA repair system represented a significant part of their proteomes.

255 Numerous amino acid ($5.9 \pm 0.3\%$) and phosphate-binding ($30.1 \pm 9.1\%$) proteins were
256 characterized within free-living bacteria and *Cyanobacteria* respectively (Table 2), suggesting
257 an adaptation to oligotrophic environment, where a strong competition for limiting nutrients
258 such as nitrogen or phosphorus was reported [43]. Interestingly, no such transporters were
259 identified in the attached-bacterial fraction (Table 2), which could suggest less environmental
260 pressure for nutrient transporter expression in the microenvironment formed on particles
261 sinking through the water column. Previous studies reported diel periodicity in bacterial
262 activity sampled from oligotrophic surface waters [6, 9, 44]. Here, proteins involved in
263 phosphorus or amino acid metabolisms, mainly represented by transporters, were more

abundant at day in *Synechococcales* and *Rhodobacterales* respectively, while respiration-related proteins were more abundant at night (Figure 3). During daytime, when photosynthesis takes place, phototrophic organisms must compete for N and P sources with heterotrophs [6], which could lead to an overexpression of transporters. In *Rhodobacterales*, proteins involved in chemotaxis and amino acid (i.e. octopine) transporters were expressed and characterized during the day (Figures 3 and 4). Chemotaxis proteins are critical for nutrient competition, suggesting that *Rhodobacterales* have evolved strategies using both movements towards nutrients and efficient carbon/nitrogen uptake system during the day.

Pelagibacterales was observed in higher abundance at night (Figure 1) and showed contrasting diel patterns compared to phototrophs (Figure 3). Regulation of protein expression was more likely sample dependent rather than governed by a day/night cycle (Figure 3c). Unlike phototrophic organisms, respiration in *Pelagibacterales* was not especially enhanced in the dark phase (Figure 3c). Moreover, the relative stability in carbohydrate metabolism (Figure 3c) and the detection of proteins involved in glycolysis, pyruvate metabolism and electron chain transfer at both day and night periods (Figure 4c), might suggest that energy production in *Pelagibacterales* is not controlled by diel fluctuation. In all samples, numerous transporters were detected at both day and night periods (Figure 4c). *Pelagibacterales* are non-motile heterotrophs that rely on constitutive expression of transporters for efficient nutrient scavenging [43]. By consistently expressing their proteins during the day and night time, *Pelagibacterales* would prevent from an energetically costly diel protein turnover [45]. Regulation might also take place at transcript level as evidenced by Ottesen and colleagues [21].

Conclusion

Picoplankton communities are key actors in surface marine environment, where diel fluctuation of solar radiation imposes daily temporal structure. Assessing the impact of day and night cycle on microbial assemblages is essential to better understand this complex ecosystem. This work compared diel metaproteome dynamics of free-living and particles-attached picoplanktonic fractions within coastal oligotrophic environment. Our study was conducted over two consecutive days, going one step further than previous metaproteomic efforts and allowing a better understanding of cyclic regulation of protein expression. Despite the overall stability of the community proteome profile, our results showed diel taxa-specific variation of protein expression with stronger regulation in phototrophs than in heterotrophs. The observation of diel regulations in other phototrophic taxa (*Rhodobacterales*) than *Cyanobacteria* reinforced evidences that timekeeping mechanisms might be widespread in Bacteria, raising new questions in marine microbial ecology and evolution. Therefore, studying the *in situ* diel variations using multi-diel omics investigations will undoubtedly broaden our knowledge on microbial assemblage dynamics and provide key elements for understanding taxa-specific diel functioning.

Methods

Water Sampling

Seawater samples were collected in summer (June 2014) at the SOLA station, located 500 m offshore of Banyuls-sur-mer, in the NW Mediterranean Sea (42° 49' N, 3° 15' W). Samples were collected over two days on a two samples per day basis (one at dusk and one at down). Each sample consisted of 60 liters of sea surface water, pre-filtered at 5 µm and subsequently sequentially filtered through 0.8 and 0.2 µm pore-sized filters (polyethersulfone membrane filters, PES, 142 mm, Millipore). The eight filters were flash frozen into liquid nitrogen

before storage at – 80 °C. The physicochemical parameters were provided by the Service d'Observation en Milieu Littoral (SOMLIT). Temperature, salinity and nutrient (NH_4^+, NO_3^-, NO_2^-, PO_4^{3-} and $Si(OH)_4$) concentrations were measured in the sampling site (3 m depth) over the month of June.

Protein isolation

A combination of different physical (sonication/freeze–thaw) and chemical (urea/thiourea containing buffers, acetone precipitation) extraction techniques were used on the filtered seawater samples to maximize the recovery of protein extracts from the filters. The filters were removed from their storage buffer and cut into quarters using aseptic procedures. The filters were suspended in a lysis buffer containing 8 M Urea / 2 M Thiourea, 10 mM HEPES, and 10 mM dithiothreitol. Filters were subjected to five freeze–thaw cycles in liquid N2 to release cells from the membrane. Cells were mechanically broken by sonication on ice (5 cycles of 1 min with tubes on ice, amplitude 40 %, 0.5 pulse rate) and subsequently centrifuged at 16 000 g at 4 °C for 15 min. To remove particles that did not pellet during the centrifugation step, we filtered the protein suspension through a 0.22 mm syringe filter and transferred into a 3 kDa cutoff Amicon Ultra-15 filter unit (Millipore) for protein concentration. Proteins were precipitated with cold acetone overnight at −80 °C, with an acetone/aqueous protein solution ratio of 4:1. Total protein concentration was determined by a Bradford assay, using the Bio-Rad Protein Assay kit (Bio-Rad, Hertfordshire, UK) according to manufacturer's instructions, with bovine γ-globulin as a protein standard. Protein samples were reduced with 25 mM dithiothreitol (DTT) at 56 °C for 30 min and alkylated with 50 mM iodoacetamide at room temperature for 30 min. For gel-free liquid chromatography tandem mass spectrometry analysis, a trypsic digestion (sequencing grade modified trypsin, Promega) was performed overnight at 37 °C, with an enzyme/substrate ratio of 1:25.

Liquid chromatography tandem mass spectrometry analysis

Purified peptides from digested protein samples were identified using a label-free strategy on an UHPLC-HRMS platform composed of an eksigent 2D liquid chromatograph and an AB SCIEX Triple TOF 5 600. Peptides were separated on a 25 cm C18 column (Acclaim pepmap 100, 3 µm, Dionex) by a linear acetonitrile (ACN) gradient [5–35 % (v/v), in 15 or 120 min] in water containing 0.1 % (v/v) formic acid at a flow rate of 300 nL min-1. Mass spectra (MS) were acquired across 400–1,500 m/z in high-resolution mode (resolution > 35 000) with 500 ms accumulation time. Six microliters of each fraction were loaded onto a pre-column (C18 Trap, 300 µm i.d.×5 mm, Dionex) using the Ultimate 3000 system delivering a flow rate of 20 µL/min loading solvent (5 % (v/v) acetonitrile (ACN), 0.025 % (v/v) TFA). After a 10 min desalting step, the pre-column was switched online with the analytical column (75 µm i.d.×15 cm PepMap C18, Dionex) equilibrated in 96 % solvent A (0.1 % (v/v) formic acid in HPLC-grade water) and 4 % solvent B (80 % (v/v) ACN, 0.1 % (v/v) formic acid in HPLC-grade water). Peptides were eluted from the pre-column to the analytical column and then to the mass spectrometer with a gradient from 4-57 % solvent B for 50 min and 57-90 % solvent B for 10 min at a flow rate of 0.2 µL min−1 delivered by the Ultimate pump. Positive ions were generated by electrospray and the instrument was operated in a data-dependent acquisition mode described as follows: MS scan range: 300 – 1 500 m/z, maximum accumulation time: 200 ms, ICC target: 200 000. The top 4 most intense ions in the MS scan were selected for MS/MS in dynamic exclusion mode: ultrascan, absolute threshold: 75 000, relative threshold: 1 %, excluded after spectrum count: 1, exclusion duration: 0.3 min, averaged spectra: 5, and ICC target: 200 000. Metaproteomic data were submitted to iProx [46] (Project ID: IPX0002008000).

Databases creation and protein identification

Protein searches were performed with ProteinPilot (ProteinPilot Software 5.0.1; Revision: 4895; Paragon Algorithm: 5.0.1.0.4874; AB SCIEX, Framingham, MA) (Matrix Science, London, UK; v. 2.2). Paragon searches 34 were conducted using LC MS/MS Triple TOF 5600 System instrument settings. Other parameters used for the search were as follows: Sample Type: Identification, Cys alkylation: Iodoacetamide, Digestion: Trypsin, ID Focus: Biological Modifications and Amino acid substitutions, Search effort: Thorough ID, Detected Protein Threshold [Unused ProtScore (Conf)] >: 0.05 (10.0%).

Three DBs were created using the same metagenome (Project number: ERP009703, Ocean Sampling Day 2014, sample: OSD14_2014_06_2m_NPL022, run ID: ERR771073) and were generated with mPies v. 0.9 [26]. The three DBs were: (i) a non-assembled metagenome-derived DB (NAM-DB), (ii) an assembled metagenome-derived DB (AM-DB) and (iii) a taxonomy-derived DB (TAX-DB). Protein search was performed for each sample against the three DBs. Subsequently to each search, each DB was restricted to the protein sequences identified in the first-round search. The resulting DBs were merged and redundant protein sequences were removed, leading to a unique combined DB per sample. Finally, protein search was performed against combined DB and the identified proteins were used for downstream analysis. A FDR threshold of 1%, calculated at the protein level was used for each protein searches. Proteins identified with one single peptide were validated by manual inspection of the MS/MS spectra, ensuring that a series of at least five consecutive sequence-specific b-and y-type ions was observed.

Protein annotation and downstream analyses

Identified proteins were annotated using mPies [26]. The mPies tool used Diamonds [47] to align each identified protein sequences against the non-redundant NCBI DB and the UniProt DB (Swiss-Prot) respectively and retrieved up to 20 best hits based on alignment score.

For taxonomic annotation, mPies returned the last common ancestor (LCA) among the best NCIBI hits via MEGAN (bit score >80) [48]. For functional annotation, mPies returned the most frequent protein name, with a consensus tolerance threshold above 80% of similarity amongst the 20 best UniProt hits. Proteins annotated with a score below this threshold were manually validated. Metaproteome comparison was done using the total relative abundance of peptide detected within identical taxon or function. Taxa and functions displaying a total relative abundance below 1 or 2% in all samples were gathered into "Other" category in tables and figures. The heatmaps (Figure 2) were generated with R v. 3.6.0 [49] and the R package ComplexHeatmap v. 2.1.0 [50].

Declarations

Availability of data and materials

The metaproteomic data are available from iProx [47] (Project ID: IPX0002008000). The physicochemical data are available from SOMLIT on request. The metagenomic data are available from EBI (Project number: ERP009703, Ocean Sampling Day 2014, sample: OSD14_2014_06_2m_NPL022, run ID: ERR771073).

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Royal Society, UK (RG160594), the "Belgian Fund for Scientific Research (Grand equipment - F.R.S - FNRS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Augustin Géron is the recipient of a 50/50 match funding scholarship between the University of Stirling (Scotland, UK) and the University of Mons (Belgium).

Author's contributions

SMS conceived the study, performed water sampling, protein extraction and mass spectrometry analysis. AG and JW analyzed all data and prepared the figures. AG wrote the manuscript. SMS, RW and PL contributed resources. All authors proofread the manuscript and approved the final version.

Acknowledgements

The authors acknowledge the use of de.NBI cloud and the support by the High Performance and Cloud Computing Group at the Zentrum für Datenverarbeitung of the University of Tübingen and the Federal Ministry of Education and Research (BMBF) through grant no 031 A535A. Authors would like to kindly thank Wade Jeffrey and Sapna Chitlapilly Dass for helping SMS with the water sampling during the optimization steps of this comparative metaproteomics study.

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Tables

Table 1: Comparison of the microbial community structure. Metagenomic data consisted in relative abundance of reads of small subunit rRNA observed over the OSD14 sampling effort. Metaproteomic data consisted in relative abundance of proteins detected in each metaproteome. (This table should be placed at the end of the section "Features of sampling site").

	Metagenome	**Metaproteome**						
	0.2µm size-fraction	**0.2µm size-fraction**				**0.8µm size-fraction**			
	OSD June 2014	Day 1	Night 1	Day 2	Night 2	Day 1	Night 1	Day 2	Night 2
Phylum									
Proteobacteria	66,9	90,1	93,5	88,2	91,8	38	36,6	27,8	28,5
Bacteroidetes	15,5	6,6	5	6,1	5,7	1,9	4,7	3,7	3,9
Cyanobacteria	12,2	1	0,3	3,6	0,5	57,4	57,3	67,5	65,9
Rhodothermaeota	1,8	1	0,1	0,8	1	0	0	0	0
Planctomycetes	0,1	0	0	0	0	1,4	0,4	0,2	0,5
Other (<1%)	0,5	1,3	1	1,3	1	1,4	0,9	0,8	1,2
Class									
Alphaproteobacteria	47,3	69,5	75,3	65	68,5	21,9	23,5	18,8	17,1
Gammaproteobacteria	17,8	22,7	18,8	23,8	24,2	13,5	11,3	8,3	10,3
Flavobacteriia	14,3	5,2	4,3	4,2	5,4	0	1,2	0,2	1,5
Unclassified *Cyanobacteria*	12,3	1,1	0,3	3,8	0,6	56,7	56,9	65,6	62,6
Bacteroidia	0,1	0,3	0,7	1	0,3	0,9	3,3	3,6	2
Cryptophyta	0,0	0	0	0	0	1,4	0,7	1,6	2,4
Deltaproteobacteria	0,0	0,1	0	0	0	0,5	0,9	0,4	0,6
Oligoflexia	0,0	0	0,1	0	0	1,4	0	0,4	0,6
Planctomycetia	0,0	0	0	0	0	1,4	0,5	0,2	0,5
Other (<1%)	2,9	1,1	0,5	2,1	1,1	2,3	1,6	0,8	2,4

Table 2: Comparison of the microbial functions. Values represent the total peptide relative abundance of function detected in each sample in free-living bacteria, particle-attached bacteria and *Cyanobacteria*. (This table should be placed after the third paragraph of the section "Free-living versus particle-attached bacteria: contrasting diel regulation of their metaproteomes").

	Free-living bacteria				**Particle-attached bacteria**				***Cyanobacteria***			
	Day 1	Night 1	Day 2	Night 2	Day1	Night 1	Day 2	Night 2	Day 1	Night 1	Day 2	Night 2
10 kDa chaperonin	5,2	6,0	5,5	5,0	2,3	5,6	4,7	3,7	0	3,1	1,9	8,7
30S ribosomal protein	3,2	2,1	2,6	3,2	0	0	0	0	0	1,6	0,6	0,7
50S ribosomal protein	12,5	9,7	14,4	16,0	1,1	15,5	3,5	5,3	0	7,8	5	6,7
60 kDa chaperonin	33,3	34,1	31,6	26,4	16,1	20,6	25	30,5	8,4	18,6	17,6	15,4
Aconitate hydratase B	0,2	0,0	0,0	0,0	0	0	3,5	0	0	0	0	0
Amino-acid ABC transporter-binding protein	6,1	6,0	5,1	6,5	0	0	0	0	0	0	0	0
ATP synthase	3,3	3,0	3,6	3,0	13,8	7,7	22,7	15,6	4,8	11,6	10,7	21,5
Chaperone protein DnaK	3,3	3,3	3,3	4,2	8	4,7	3,5	3,3	2,4	0	0	3,4
Cysteine synthase	0	0	0	0	0	0	0	0	0	0,8	0,6	2
DNA-binding protein HU	7,2	7,2	7,1	8,1	0	0	0	0	0	0	0	0
DNA-directed RNA polymerase	0,5	0,6	0,6	0,9	13,8	12,9	1,7	6,2	0	0	0,6	1,3
Elongation factor	5,3	4,7	5,9	8,9	6,9	6,9	7	11,9	8,4	14,7	6,3	20,1
Flagellin	4,0	5,6	4,5	5,1	5,7	3,9	4,1	3,3	0	0	0	0
Fructose-1,6-bisphosphatase	0	0	0	0	0	3,4	0	0	0	0	0	0
Glutamine synthetase	2,4	2,5	2,7	1,8	2,3	2,1	0,6	0,4	2,4	1,6	1,9	1,3
Glyceraldehyde-3-phosphate dehydrogenase	0,2	0,1	0,2	0,1	1,1	1,3	6,4	7,4	2,4	1,6	2,5	2
Glycine--tRNA ligase	0	0	0	0	2,3	0,9	0,6	1,6	0	0	0	0
Histone-like protein	0,2	0,2	0,1	0,1	14,9	5,2	7,6	2,5	0	0	0	0
Isocitrate dehydrogenase [NADP]	0	0	0	0	0	0,9	1,7	4,1	0	0	0	0
Molybdopterin molybdenumtransferase	0	0	0	0	2,3	0	1,2	0,8	0	0	0	0
Phosphate-binding protein	0,4	0,5	0,2	0,1	0	0	0	0	54,2	32,6	21,4	12,1
Phycoerythrin	0	0	0	0	0	0	0	0	6	0	12,6	0,7
Ribosomal protein S12 methylthiotransferase RimO	0	0	0	0	0	2,1	0	0	0	0	0	0
Rubrerythrin	1,5	2,6	1,2	1,4	0	0	0	0	0	0	0	0
Tubulin	0	0	0	0	2,3	4,7	5,2	0,4	0	0	0	0
Other (<1%)	11,3	11,9	11,3	9,3	7,1	1,6	1,0	3,0	11,0	6,0	18,3	4,1

Figure captions

Figure 1: Comparison of the microbial community structure at order level. Metagenomic data consisted in total relative abundance of reads of small subunit rRNA observed over the OSD14 sampling effort. Metaproteomic data consisted in total relative peptide abundance in each metaproteome.

Figure 2: Heatmaps of the taxonomic (top clusters) and the functional (right clusters) linkages for (**a**) free-living bacteria and (**b**) particle attached bacteria. Clusters were determined using complete linkage hierarchical clustering and Euclidean distance metric.

Figure 3: Comparison of the total relative peptide abundance in functions identified in *Synechococcales* (**a**) and free-living and particle-attached *Rhodobacterales* (**b**) and *Pelagibacterales* (**c**). The presence of a sun or moon symbol means that the protein was periodically more abundant at day or night respectively.

Figure 4: Cellular representation of protein expression over day and night periods in (**a**) *Synechococcales* and (**b**) free-living and particle-attached *Pelagibacterales* (blue tag) and *Rhodobacterales* (red tag). The presence of a sun or moon symbol means that the protein was periodically more abundant at day or night respectively. Colored yellow and black boxes meant that the protein was specific to day or night respectively.

Additional files

Supplementary information 1: Supplementary_information1.xlsx. The physicochemical parameters measured by the Service d'Observation en Milieu Littoral (SOMLIT).

Supplementary information 2: Supplementary_information2.xlsx. Taxonomic and functional protein annotation. Comparison of the proportion of proteins for which a consensus annotation was found in each metaproteome.

Figures

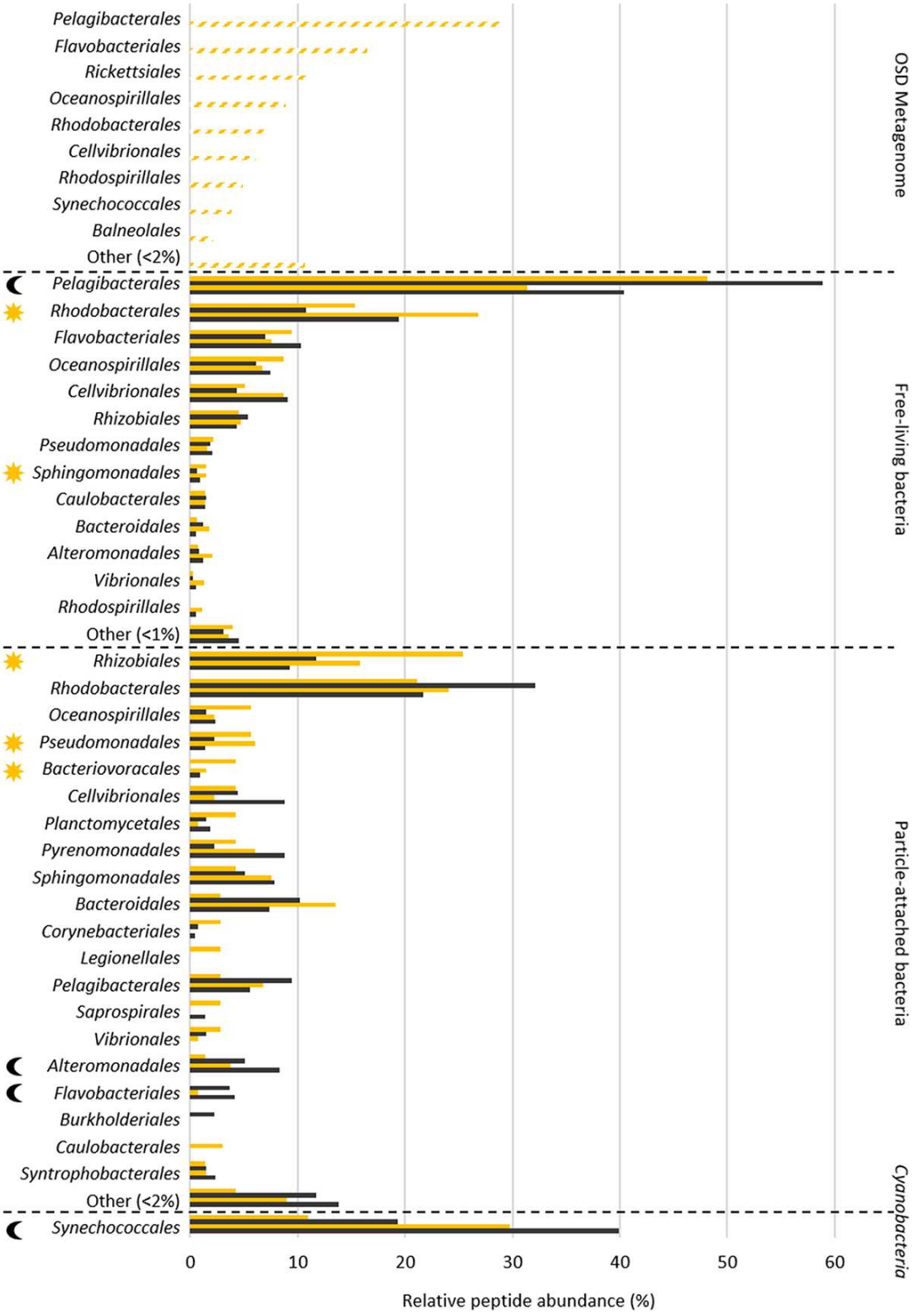

Figure 1

Comparison of the microbial community structure at order level. Metagenomic data consisted in total relative abundance of reads of small subunit rRNA observed over the OSD14 sampling effort. Metaproteomic data consisted in total relative peptide abundance in each metaproteome.

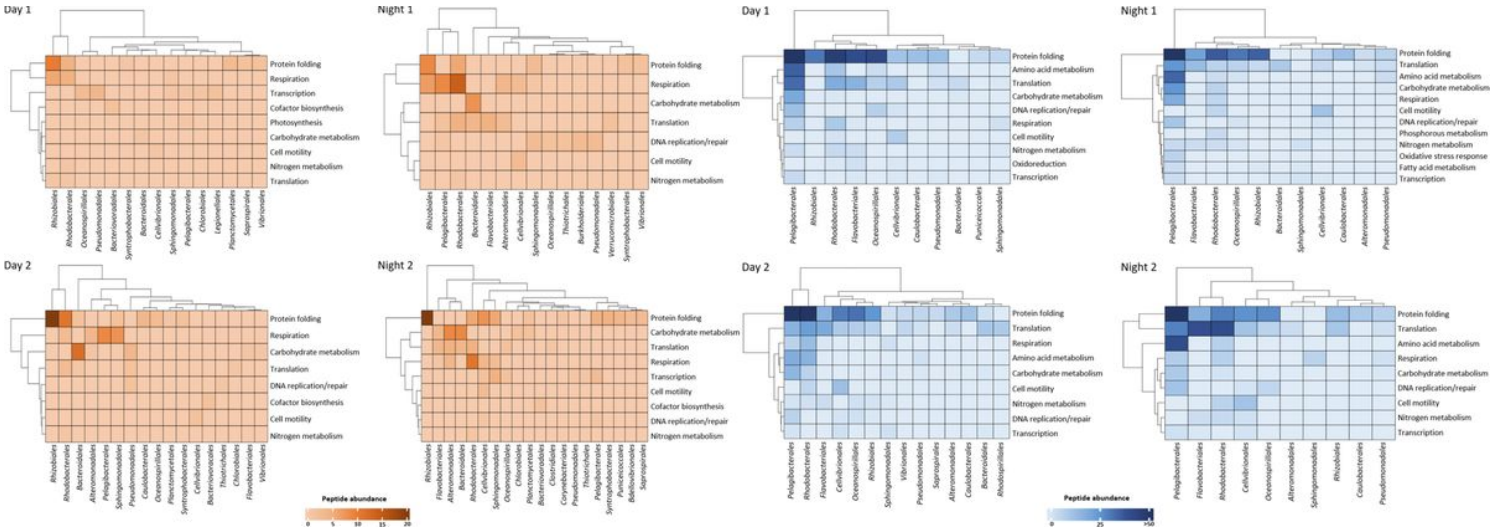

Figure 2

Heatmaps of the taxonomic (top clusters) and the functional (right clusters) linkages for (a) free-living bacteria and (b) particle attached bacteria. Clusters were determined using complete linkage hierarchical clustering and Euclidean distance metric.

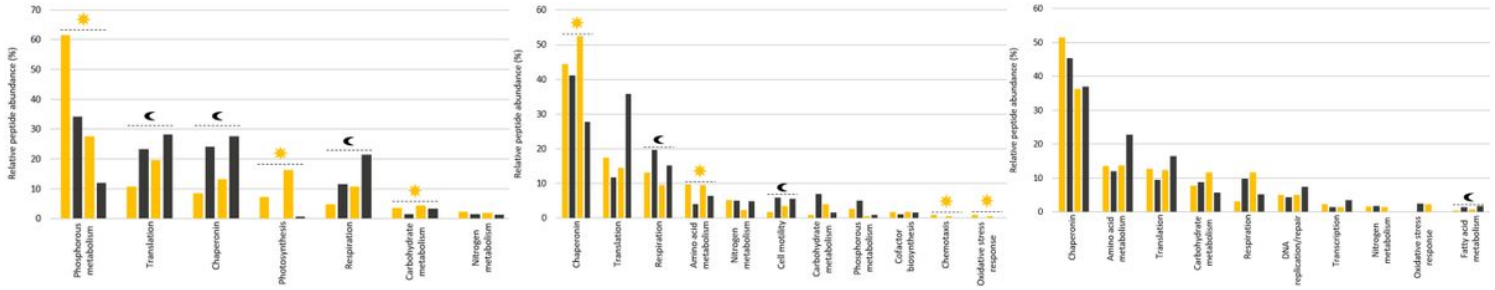

Figure 3

Cellular representation of protein expression over day and night periods in (a) Synechococcales and (b) free-living and particle-attached Pelagibacterales (blue tag) and Rhodobacterales (red tag). The presence of a sun or moon symbol means that the protein was periodically more abundant at day or night respectively. Colored yellow and black boxes meant that the protein was specific to day or night respectively.

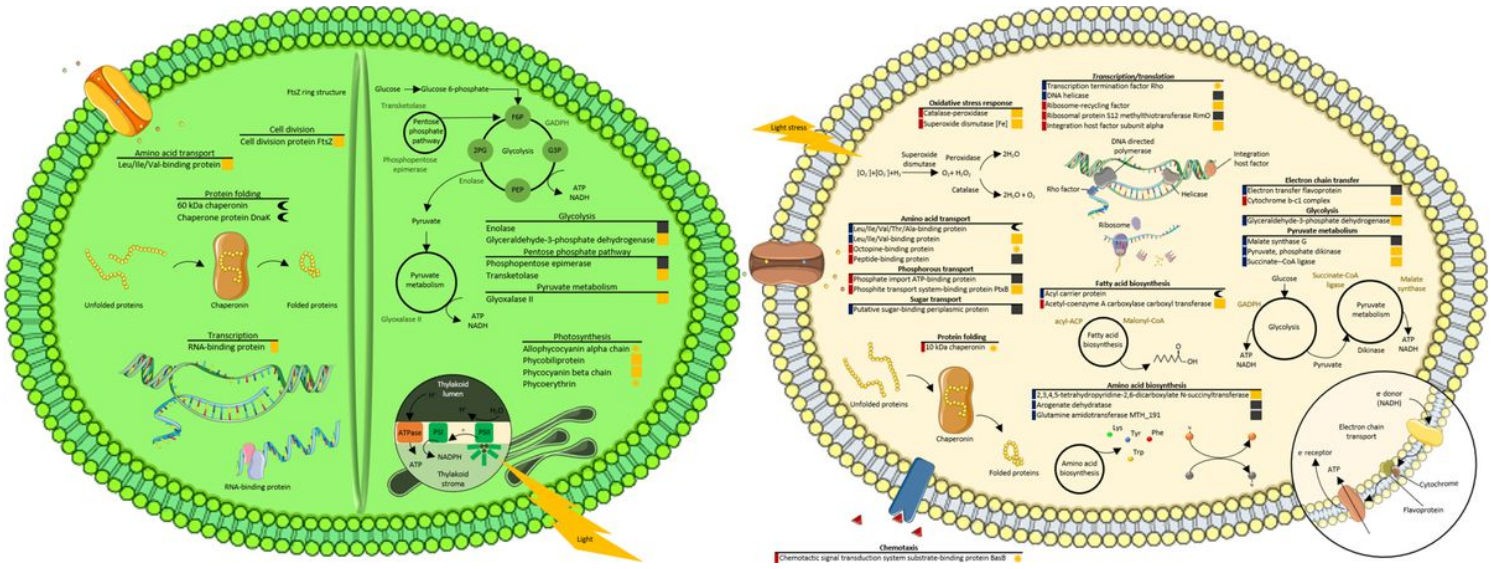

Figure 4

Cellular representation of protein expression over day and night periods in (a) Synechococcales and (b) free-living and particle-attached Pelagibacterales (blue tag) and Rhodobacterales (red tag). The presence of a sun or moon symbol means that the protein was periodically more abundant at day or night respectively. Colored yellow and black boxes meant that the protein was specific to day or night respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table2.xlsx
- Table1.xlsx