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Light supports cell-integrity and growth rates of taxonomically diverse coastal photoheterotrophs

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Summary

Despite the widespread distribution of proteorhodopsin (PR)-containing bacteria in the oceans, the use of light-derived energy to promote bacterial growth has only been shown in a few bacterial isolates, and there is a paucity of data describing the metabolic effects of light on environmental photoheterotrophic taxa. Here, we assessed the effects of light on the taxonomic composition, cell integrity and growth responses of microbial communities in monthly incubations between spring and autumn under different environmental conditions. The photoheterotrophs expressing PR *in situ* were dominated by *Pelagibacterales* and SAR116 in July and November, while members of *Euryarchaeota*, *Gammaproteobacteria* and *Bacteroidetes* dominated the PR expression in spring. Cell-membrane integrity decreased under dark conditions throughout most of the assessment, with maximal effects in summer, under low-nutrient conditions. A positive effect of light on growth was observed in one incubation (out of nine), coinciding with a declining phytoplankton bloom. Light-enhanced growth was found in *Gammaproteobacteria* (*Alteromonadales*) and *Bacteroidetes* (*Polaribacter* and *Tenacibaculum*). Unexpectedly, some *Pelagibacterales* also exhibited higher growth rates under light conditions. We propose that the energy harvested by PRs helps to maintain cell viability in dominant coastal photoheterotrophic oligotrophs while promoting the growth of some widespread taxa benefiting from the decline of phytoplankton blooms.

Introduction

Photoheterotrophy is the ability of some heterotrophic prokaryotes to transform solar energy into an electrochemical gradient while using organic molecules as a carbon and energy source. Photoheterotrophic microbes are widespread in marine environments (Rusch *et al*., 2007; Campbell *et al*., 2008), distributed among major phyla (de la Torre *et al*., 2003; Olson *et al*., 2018), and present in two groups: (i) aerobic anoxygenic phototrophic (AAP) bacteria (Shiba *et al*., 1979), which contain bacteriochlorophyll *a* (Bchl a); and (ii) PR-containing prokaryotes including bacteria and archaea (Béjà *et al*., 2000; de la Torre *et al*., 2003; Frigaard *et al*., 2006), which use PR proteins to generate energy. AAPs usually represent less than 10% of the total bacterial cells in marine waters, though this value is occasionally exceeded in estuarine waters (Schwalbach and Fuhrman, 2005). In contrast, PR-containing microbes are widely distributed in the oceans, comprising more than 50% of total microbial abundance in some oceanic regions such as the Sargasso Sea (Venter *et al*., 2004; Campbell *et al*., 2008) and up to 90% in coastal waters (Moran and Miller, 2007; Finkel *et al*., 2013). PR genes have been detected in the three domains of life and have a widespread taxonomic distribution (Sharma *et al*., 2006), including some of the most abundant clades in the ocean: SAR11 (Giovannoni *et al*., 2005), SAR86 (Béjà *et al*., 2000) and different members of *Flavobacteriia* (Gómez-Consarnau *et al*., 2007; González *et al*., 2008; Yoshizawa *et al*., 2012).

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Despite the great abundance of photoheterotrophy genes in the ocean, the metabolic advantages of harbouring light-harvesting mechanisms remain unclear. In the case of AAPs, light-promoted growth has been observed in the cultures of *Rhodobacteraceae* (Biebl and Wagner-Döbler, 2006), *Erythrobacter* (Yurkov and van Gemerden, 1993) and also in natural assemblages (Ferrera *et al*., 2017). It has been observed that PR-containing microbes can use light radiation to generate a transmembrane proton gradient, which can be used to synthesize ATP (Martínez *et al*., 2007) and/or to facilitate uptake of essential nutrients (Gómez-Consarnau *et al*., 2016). Different works have shown that this additional energy can be used by the cells to enhance survival, as observed for a *Vibrio* (strain AND4) and *Candidatus* Pelagibacter ubique (strain HTCC1062) isolates under low availability of carbon (Gómez-Consarnau *et al*., 2010; Steindler *et al*., 2011). It has also been shown that light can support the growth of a handful of strains predominately affiliated with Flavobacteria (e.g., *Dokdonia* strain MED134; Gómez-Consarnau *et al*., 2007, *Psychroflexus torquis* ATCC 700755; Feng *et al*., 2013, *Nonlabens marinus* S1-08; Yoshizawa *et al*., 2014, *Polaribacter* MED152; Fernández-Gómez,-2012), in addition to the Gammaproteobacterium HIMB30 (Michelou and Rappe, 2014). The mechanism behind the light-enhanced growth in these strains remains unclear, but it has shown to be related to the uptake of the vitamin B_1 in auxotrophic *Dokdonia* isolates, promoted by the PR-generated proton motive force (PMF; Gómez-Consarnau *et al*., 2016). However, the fact that light-enhanced growth was not observed in other widespread PR-containing microbial isolates, such as the *Cand*. P. ubique strain HTCC1062 (Giovannoni *et al*., 2005; Steindler *et al*., 2011) or the SAR92 strain HTCC2207 (Stingl *et al*., 2007), suggests that the energy obtained by PR is predominately used in metabolic processes which do not result in increased growth. Furthermore, the lack of experimental evidence of the effect of light on the growth of photoheterotrophs in environmental waters has led to the view that PR activity may not have any relevant impact on the biomass of natural communities of heterotrophic bacteria.

Analysing the metabolic effects of light on widespread photoheterotrophic taxa under environmental conditions is, however, challenging. Indirect effects of light, such as the increase in photosynthate availability in the incubations is the most obvious confounding factor in experiments conducted with entire microbial communities, hindering the real effect of light on photoheterotrophic bacterial biomass and activity. Seasonally changing environmental conditions, particularly resource availability, may also have an important impact on the response of dominant photoheterotrophic taxa, but has been rarely assessed in studies on photoheterotrophs in natural waters. Here, we carried out experimental incubations of natural bacterial communities during a nearly complete annual cycle from a temperate marine site in NE Atlantic coastal waters. After removing most of the autotrophic cells and incubating the remaining microbes under light and dark conditions, we: (i) identified active photoheterotrophs in this coastal system; and (ii) determined the potential effect of photoheterotrophy on the growth and physiological integrity of widespread marine bacterial taxa.

Results

Environmental setting and identification of active photoheterotrophic bacteria in situ by metatranscriptomics

Seawater was sampled monthly between April and December of 2012 at the mid-shelf station of the Southern Bay of Biscay (E2-Gijón/Xixón), which ranges from oligotrophic to mesotrophic conditions. Dissolved inorganic nitrogen concentration showed a maximum in March (4.95 μmol kg^{-1}) and decreased thereafter to a minimum in June (0.51 μmol kg^{-1}, Fig. S1). Maximum values of *in situ* chlorophyll *a* (Chl *a*) concentration were found at the beginning of May (1.41 $\mu g\ L^{-1}$) and December (1.80 $\mu g\ L^{-1}$, Fig. S1), and the minimum value was found in July (0.39 $\mu g\ L^{-1}$). Shortly after the peak of the beginning of May, Chl *a* dropped abruptly to 0.58 $\mu g\ L^{-1}$ 3 weeks later, indicative of typical post-bloom conditions in late spring at the E2-Gijón/Xixón station. Cell-specific heterotrophic bacterial production (sBP) was also analysed as a proxy of dissolved organic matter (DOM) availability. Maximum sBP was found in May (7.9×10^{-5} pmol Leu $L^{-1}\ h^{-1}\ cell^{-1}$) during post-bloom conditions, and minimum values were observed in July ($0.5\ 10^{-5}$ pmol Leu $L^{-1}\ h^{-1}\ cell^{-1}$).

A metatranscriptomic analysis conducted on samples collected in April, May, July and November during the period of study was used to identify the most active photoheterotrophs *in situ*. Transcripts of *pufM* (a marker gene for aerobic anoxygenic photosynthesis) were only found in April and July and their relative abundance in the metatranscriptomes was very low (<0.001% of transcripts); thus, these transcripts were not further analysed. By contrast, PR transcripts were highly expressed throughout the year (with up to 2.7% of total transcripts in July, Fig. 1A). The composition of the microbial communities expressing PRs showed important changes at different times of the year. For example, *Pelagibacteraceae* contributed a relatively high percentage of PR transcripts (up to 50%) in July and November but contributed only 10% of transcripts during spring (Fig. 1B). While PR

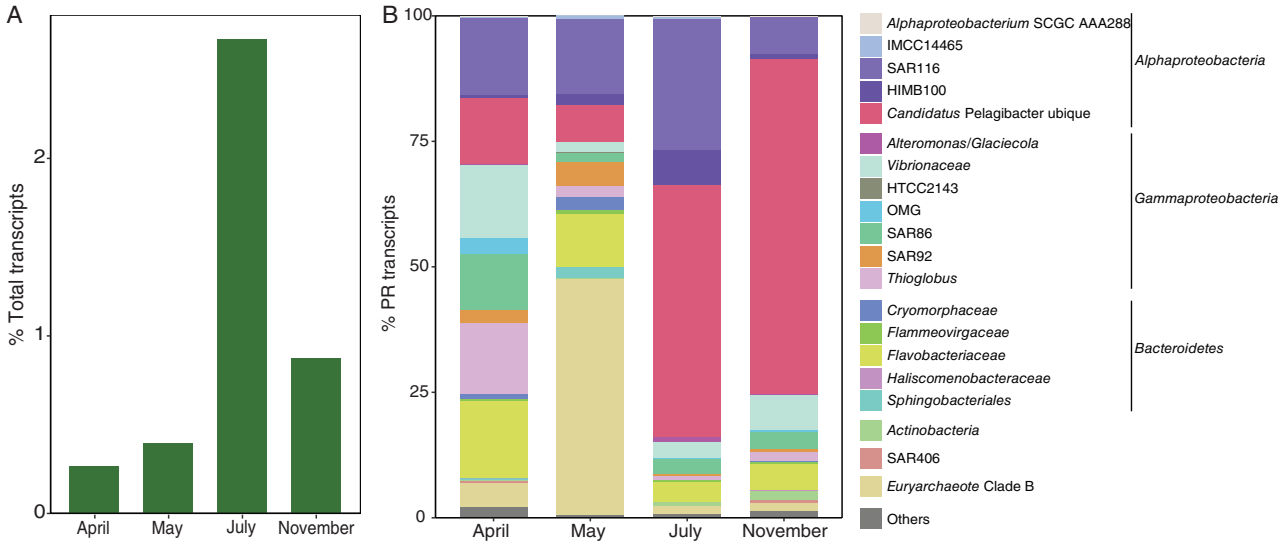

Fig 1. A. Percent of PR transcripts relative to all detected transcripts found at the *in situ* conditions of April, May, July and November. B. The relative contribution of different prokaryotic taxa to the PR transcript pool.

transcripts affiliated with *Cand*. P. ubique HTCC1062 and HIMB058 were detected in July and November, HIMB058 dominated *Pelagibacteraceae* PR expression in spring (Fig. S2). SAR116 (*Alphaproteobacteria*) PR transcripts were also abundant in summer, representing 26% of total PR transcripts in July. In April, a clear dominance of *Gammaproteobacteria* (46% of PR transcripts) was observed, while in May, *Euryarchaeota* dominated PR expression (44%). Most of the PR-containing *Gammaproteobacteria* were affiliated with SAR86 and SAR92, while *Thioglobus* were also significant contributors in April (Fig. 1B, Fig. S2). PR expression by members of the *Bacteroidetes* phylum (primarily from the family *Flavobacteriaceae*) was also noticeable, with maximum expression in spring (17% and 16% of PR transcripts in April and May respectively).

Effects of light on prokaryotic cell membrane integrity and specific growth rates across a gradient of seasonally changing environmental conditions

Seawater samples were prefiltered (using 0.8 μm pore-size filters to remove potential bacterial predators and most phytoplankton cells) and incubated either under saturating (435 ± 5 μmol photons m^{-2} s^{-1}) photosynthetically active radiation (PAR) light irradiance ('light treatment') or completely dark conditions ('dark treatment') in triplicate bottles. We found that the pre-filtration treatment removed virtually all heterotrophic nanoflagellates (HNFs) and Chl *a*, including most cyanobacterial cells (Table 1), while reducing only 8.4% (±15.5% SD) of heterotrophic bacteria on average. In particular, we removed 85.2% of picoeukaryotes and 87.7% of cyanobacteria (comprised of mostly *Synechococcus* since *Prochlorococcus* was absent from March until August, typical of this coastal system; Calvo-Díaz *et al*., 2008). In order to control the potential impact of regrowth of phototrophic organisms under light conditions during the incubations, the dynamics of cyanobacteria and autotrophic eukaryotes were analysed by flow cytometry (Figs S3 and S4). In April, the abundance of cyanobacteria remained low during the incubations (less than 1000 cells ml^{-1}), while the abundance of picoeukaryotes increased after 1 day of incubation. In May, we did not observe any growth of cyanobacteria or autotrophic eukaryotes during the entire incubations. In summer, *Synechococcus* grew after the first day of incubation under light conditions, particularly in July and September, when they reached up to 55.8×10^3 cells ml^{-1}. Phototrophic picoeukaryotes also showed slight growth in June and July under light conditions. In autumn, no significant growth of *Synechococcus* was found under light conditions, but *Prochlorococcus* abundance increased in the dark incubations, particularly in October. Notably, the increase in *Prochlorococcus* abundance under dark conditions took place only during the first day of incubation, and thereafter, their abundance remained constant until the end of the incubations. The same effect was found for *Synechococcus* in December, when they grew during the first day in the dark, but not under light conditions.

The flow cytometric analysis of cell-membrane integrity (i.e., cells with intact or damaged membranes) showed that the contribution of *Live* cells in the light treatment was significantly higher than in the dark treatment for all the analysed months (paired *t*-test, *p*-value = 0.001,

Table 1. *In situ* values for temperature and pre-filtration and post-filtration abundances of heterotrophic bacteria, heterotrophic nanoflagellates (HNF), cyanobacteria (*Synechococcus* + *Prochlorococcus*) and autotrophic picoeukaryotes and chlorophyll *a* concentration.

		April	May	June	July	August	September	October	November	December
Temp (°C)		13.3	14.2	16.4	18.7	21.2	20.8	18.3	16.28	13.3
Pre-filtration values	Bacterial abundance (10^5 cell ml^{-1})	9.4 ± 0.7	2.0 ± 0.04	4.2 ± 0.03	7.2 ± 0.2	7.6 ± 1.1	10.5 ± 0.1	3.9 ± 0.4	4.3 ± 0.02	6.3 ± 0.2
	Chl *a* (μg l^{-1})	1.41	0.59	0.44	0.14	0.40	0.69	1.01	1.26	1.80
	HNF abundance (cell ml^{-1})	407 ± 32	713 ± 55	841 ± 102	946 ± 279	616 ± 32	539 ± 11	351 ± 3	631 ± 12	515 ± 70
	Cyanobacteria (10^3 cells ml^{-1})	6.3	0.3	16.4	18.5	52.1	89.6	67.5	8.1	22.5
	Picoeukaryotes (10^3 cells ml^{-1})	27.5	1.7	7.9	3.5	5.2	6.4	5.4	15.2	15.8
Post-filtration values	Bacterial abundance (10^5 cell ml^{-1})	6.8 ± 0.4	1.8 ± 0.1	3.8 ± 0.2	7.6 ± 0.2	9.5 ± 0.5	10.1 ± 0.2	3.7 ± 0.1	4.1 ± 0.1	5.6 ± 0.2
	Chl *a* (μg l^{-1})	0.03	0.03	0.03	0.02	0.02	0.06	0.16	0.12	0.07
	HNF abundance (cell ml^{-1})	10 ± 9	n.d.	2 ± 2	11	11 ± 7	8	4 ± 1	6	6 ± 1
	Cyanobacteria (10^3 cells ml^{-1})	0.1	0.0	2.3	1.5	1.2	NA	26.1	2.2	1.5
	Picoeukaryotes (10^3 cells ml^{-1})	2.0	0.4	1.9	0.6	0.3	NA	0.2	4.2	1.5

'n.d.' indicates that no abundance was detected and NA that data are not available.

n = 9, Fig. 2A). On average, the contribution of *Live* cells to total microbial abundance was 89.8% ± 3.7% in the light treatment, dropping to a mean of 77.7% ± 8.5% in the dark, although it varied temporally. Such a difference was already noticeable during the first day of incubation (Fig. S5), when the average of *Live* cells was 89.7% ± 4.9% and 76.8% ± 9.1% in light and dark treatments respectively. The largest difference in the percentage of *Live* cells occurred in summer (on average in August, 87.8% and 65.1% for light and dark incubations respectively). In autumn, the difference was minor (92.9% vs. 90.0% in November and 93.7% vs. 89.4% in December), although still significant (*t*-test, *p*-value <0.001, n = 6 for both months).

In the same light-controlled incubations, maximum specific growth rates of the bulk microbial community were observed in spring (Fig. 2B), with a peak in May (1.59 ± 0.03 day^{-1}), while relatively low values were measured from July through December (minimum in October, 0.17 ± 0.02 day^{-1}). In May and June, we found significant differences in the growth rates between light and dark treatments (ANOVA, *p*-value <0.001), with up to two times higher bacterial growth rates in the light treatment in May. Although a significant increase in the growth rate of heterotrophic bacteria under light conditions was also observed in July, we cannot exclude the possibility that this was related to the concomitant growth of cyanobacteria in those incubations. For the rest of the year, growth rates were not significantly different in light and dark treatments except for November, when growth rates were slightly higher in the dark (*t*-test, *p*-value = 0.006, n = 6). Regarding the maximum microbial abundance reached during the incubations or carrying capacity (Huete-Stauffer *et al*., 2015), the highest differences between the light and dark treatment were also found in spring and early summer (Fig. S6).

Effects of light on microbial community composition

We analysed the variability in bacterial community composition monthly at *in situ* conditions and during some of the incubations under light and dark treatments (June, July, August and December, Fig. 3 and Fig. S7). Under *in situ* conditions, microbial communities were clearly dominated by members of *Flavobacteriaceae* (such as *Tenacibaculum*) or *Rhodobacteraceae* (*Roseobacter* clade) in April and May, and by SAR11 from June to December. The bacterial community composition changed substantially over the course of the incubations (Fig. 3B, Fig. S7). However, differences in the overall community composition between the light and dark treatment at the end of the incubation were not large (Fig. 3B, Fig. S7). The lack of replicates and the challenges associated with the analysis of relative abundance data

precludes drawing clear conclusions from the comparison of light and dark incubations based on sequencing data. However, this analysis allowed us to identify taxa from widespread taxonomic groups present *in situ* and in the seawater incubations known to harbour PR but with little or no experimental evidence of their metabolic response to light (e.g., *Polaribacter*, *Tenacibaculum* and Alteromonadales), which were subsequently targeted with specific probes (see results below).

At the beginning and the end of the incubations in May and June, when stronger effects of light on bacterial growth rates were observed (Fig. 2), we also analysed full-length bacterial 16S rRNA genes by cloning and sequencing to obtain a finer taxonomic resolution (Figs S8 and S9). Although we found few clones affiliated with *Gammaproteobacteria* at the beginning (T0) of the May incubation, 30% of the total clones were affiliated with *Alteromonadales* by the end of the incubation, some of which (15% of *Alteromonadales* clones) were closely related (>99% similarity) to two strains known to harbour the PR gene (*Alteromonas* LOR and *Alteromonas* MB-3u-76; Kopel *et al*., 2014; Tully *et al*., 2017; Fig. S8). In the June experiment, *Gammaproteobacteria* were clearly dominated by the SAR86 clade both at the initial and final timepoints of the incubation. In the phylum *Bacteroidetes*, clones related to the PR-containing flavobacterium MS024-2A (*Formosa* and *Polaribacter*) were found at the end of the light incubations in May and June. Regarding *Rhodobacteraceae*, clones related to taxa known to contain Bchl *a* were found: mainly *Loktanella*, and also *Thalassobium* or *Planktomarina temperata* (Fig. S9). In the case of *Pelagibacterales*, at the initial timepoint of the incubations in May most clones were related to the Group II (Fig. S9), while at the end of the incubations no clones were detected due to the low relative abundance of SAR11 in comparison with other groups (<1%). In June, we found clones affiliated with the SAR11 Group I at the beginning and the end of light incubations.

Effect of light on the growth rates of specific bacterial taxa

The effect of light on the growth of *Gammaproteobacteria*, *Bacteroidetes*, *Rhodobacteraceae* and SAR11 was assessed monthly from April to December by Catalysed Reporter Deposition Fluorescence *In Situ* Hybridization (CARD-FISH; Fig. 4A) in incubations that lasted between 3 and 5 days. A significant effect of light on the specific growth rates of SAR11 was found in May and June (ANCOVA, p-value <0.001). The largest difference was found in May, when phototrophic microorganisms were completely absent from the filtered incubations and SAR11 showed a specific growth rate of 1.45 day^{-1} under light conditions but no growth in the dark (Fig. 4A). Differences in growth rates between the light and dark

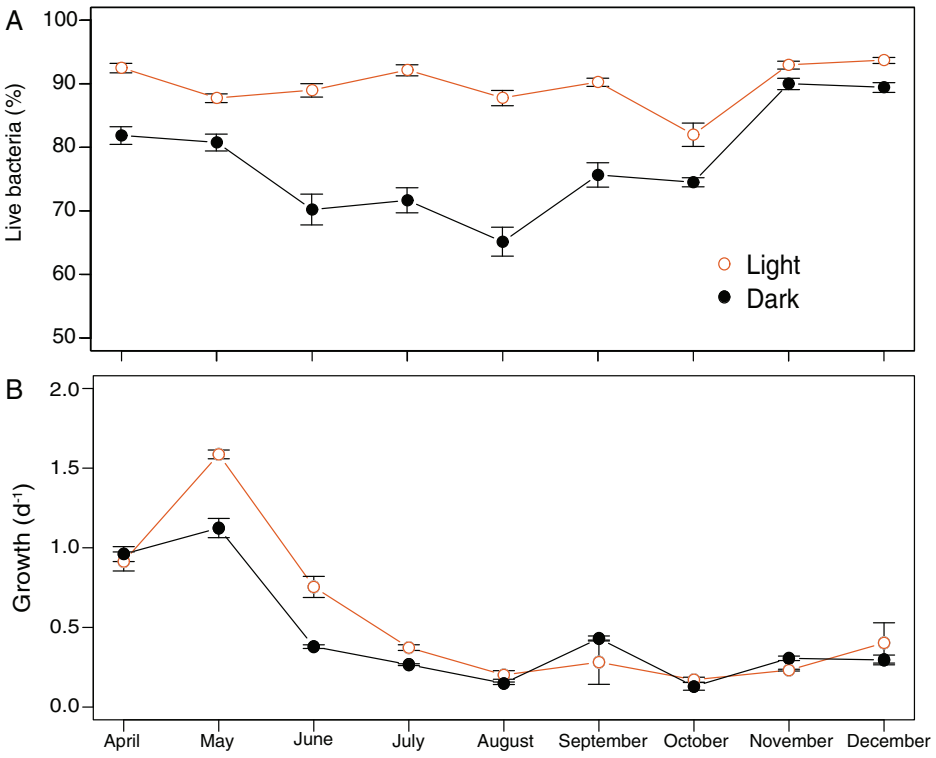

Fig 2. A. Percentage of cytometric group *Live* bacteria in light (orange) and dark (black) treatments and (B) total bacterial community growth rates for the entire bacterial community. Error bars represent SD between three replicates.

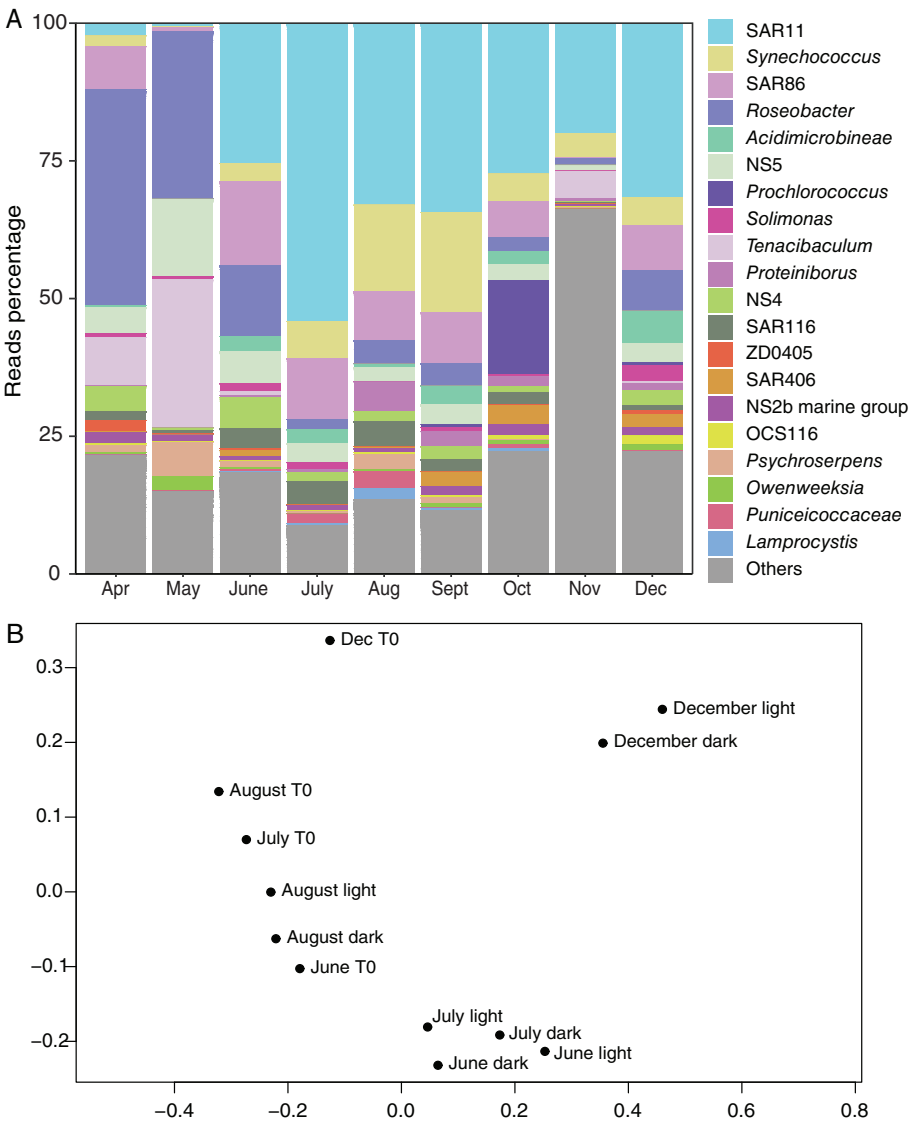

Fig 3. A. The relative abundance of top 20 bacterial OTUs at *in situ* conditions as analysed by amplicon sequencing between April and December 2012. B. Multidimensional scaling (MDS) plot of bacterial community composition obtained by amplicon sequencing for the incubations of June, July, August and December at the beginning of the incubation (T0) and the end of the light and dark treatments.

treatments for *Gammaproteobacteria* and *Bacteroidetes* were also most conspicuous in May, with significantly higher specific growth rates under light exposure (ANCOVA, p-value<0.001) representing 1.7 and 1.8-fold increases respectively, compared with the dark incubations. In June, the increase in specific growth rate for these groups was less pronounced but still significant for *Bacteroidetes* (ANCOVA, p-value<0.001).

At a more specific taxonomic level, the growth response to light exposure in *Tenacibaculum*, *Polaribacter* and *Alteromonadales* was analysed in May (Fig. 4B). These three bacterial groups contributed up to 14%, 4% and 2% of sequence reads at the initial timepoint in May respectively (Fig. 3A). *Tenacibaculum* showed three times higher growth rates in the light compared with the dark treatment (1.67 and 0.56 day^{-1} in light and dark respectively), while *Polaribacter* showed 1.6 times higher growth (1.90 and 1.10 day^{-1} in light and dark). In the case of *Alteromonadales*, the increase in growth was even more noticeable (up to 15-fold, 2.38 day^{-1} in the light vs. 0.15 day^{-1} in the dark). In general, the effects of light on the growth of *Rhodobacteraceae* were less pronounced, although rates were significantly higher in the light compared with the dark during the September incubation (ANCOVA, p-value = 0.003). On the contrary, occasional higher growth rates in the dark treatment than in the light were also observed, particularly in April for *Bacteroidetes* and *Gammaproteobacteria*.

TonB receptors in bacterial genomes and their in situ expression

Since the expression of TonB receptors has been shown to be related to the light-enhanced growth of some PR-containing flavobacteria (Gómez-Consarnau *et al*., 2016),

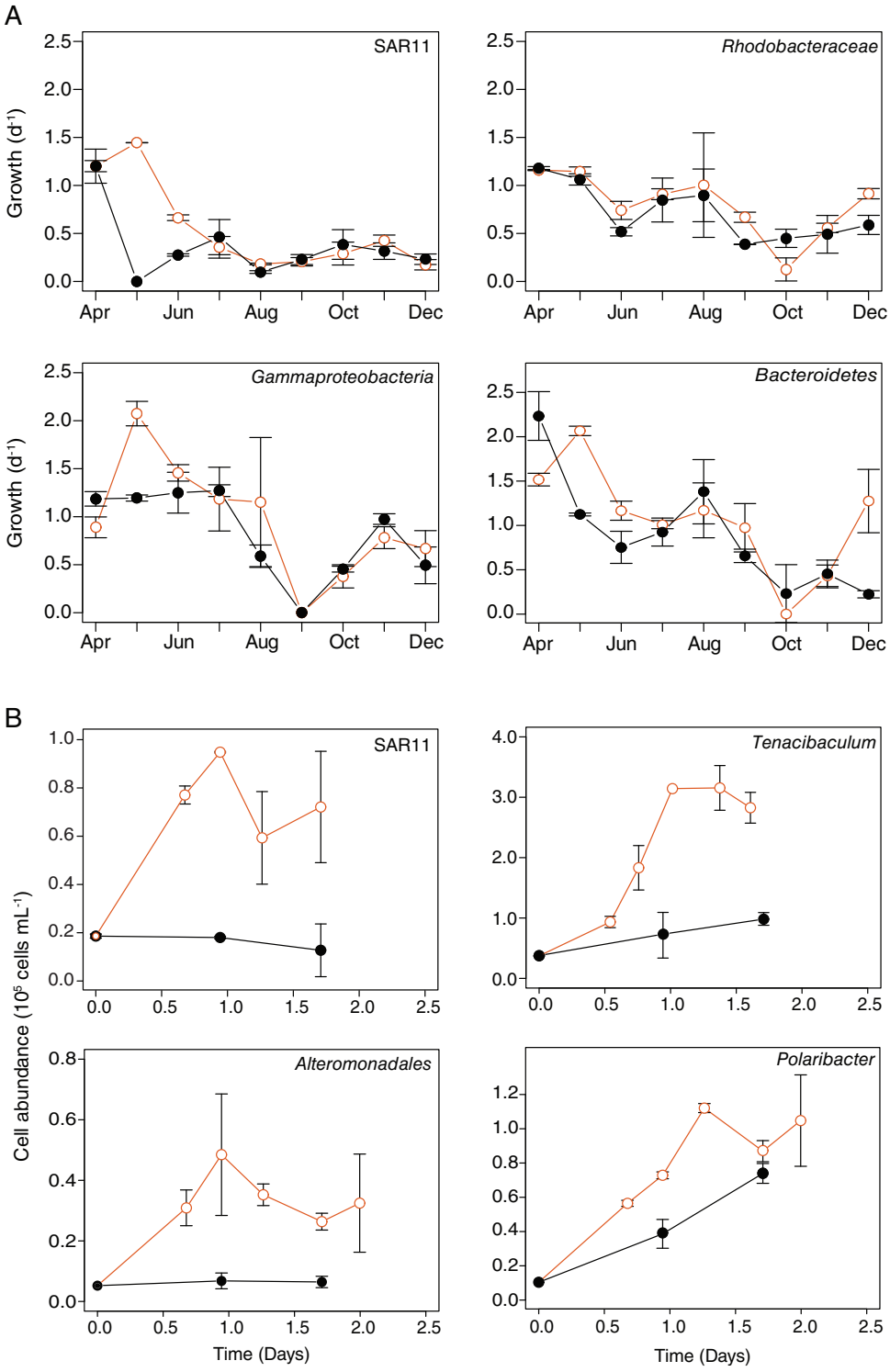

Fig 4. A. Bacterial growth rates for each phylogenetic group (SAR11, *Rhodobacteraceae*, *Gammaproteobacteria* and *Bacteroidetes*) and each monthly experiment. B. Growth curves for *Polaribacter*, *Tenacibaculum* (*Bacteroidetes*), *Alteromonadales* (*Gammaproteobacteria*) and SAR11 (*Alphaproteobacteria*) groups in the incubation of May in light (orange line) and dark (black line) treatments. Error bars represent SD between two replicates.

we addressed the potential relevance of this process in our samples. First, we carried out an analysis of TonB receptors in a comprehensive database of marine bacterial genomes (MAR database; Klemetsen *et al*., 2018), searching for three main types of TonB transporters: TonB receptors involved in the transport of

vitamin B_1; TonB involved in the transport of iron (haem groups); and the gene tandem *susC* (TonB transporter)-*susD* (substrate binding and delivery to *susC*), involved in polymer degradation and transport of degradation products. We found that TonB transporters were widely distributed among members of *Gammaproteobacteria* and *Flavobacteriia*, while they were absent from *Pelagibacter*. In particular, members of *Alteromonadales* and *Flavobacteriia*, including PR-containing genera for which a light-enhanced growth has been shown in this or previous studies (*Dokdonia*, *Tenacibaculum* and *Polaribacter*) had a high number of TonB transporters in their genomes (Fig. 5B). Predicted *susC-susD* genes were dominant in *Flavobacteriia* (~30% of total TonB transporter genes), although we also found some receptors of this type in several genomes of *Alteromonas* (Fig. 5B). A copy of the TonB receptor specific for transporting vitamin B_1 was predicted in all 72 *Flavobacteriia* and all 27 *Alteromonas* genomes (except one) in the MAR database, including those closely related to the taxa found in our incubations (e.g., *Polaribacter* sp. Hel1_33_78 or *Alteromonas* sp. MB-3u-76; results not shown). Our search also retrieved eight *Bacteroidetes* genomes not affiliated with *Flavobacteriia*: *Caldithrix abyssi* (*Calditrichaeota*) and a *Sulfurovum* genome (*Epsilonproteobacteria*). The phylogenetic reconstruction was congruent with the taxonomy, although the position of *Sulfurovum* suggests a lateral gene transfer event (Fig. S10).

Second, we analysed the *in situ* expression of these transporters in the metatranscriptomes collected at the E2-Gijón/Xixón station to assess their transcriptional activity across the seasonal gradient. We found that the TonB receptor genes analysed were highly expressed, at the same level as PRs (ranging from 0.5% to 1.3% of all transcripts). The expression was maximal in spring and declined in summer and autumn (Fig. 5A). On average, the expression of TonB receptors specific for transporting vitamin B_1 and haem receptors was lower (~0.01% of all transcripts), but the maximum expression of vitamin B_1 receptor genes were found in May.

Discussion

The high diversity of PR genes found in the surface ocean represented one of the most outstanding discoveries from the sequencing of the first marine metagenomes (Venter *et al*., 2004). Later studies have supported the view that PR-based photoheterotrophy is widespread in dominant microbial taxa in the marine photic layer (de la Torre *et al*., 2003; Campbell *et al*., 2008). Given the large number of planktonic heterotrophic prokaryotes containing this light-harvesting mechanism, their contribution to the ocean's energy flux is potentially large. However, its quantification remains challenging (DeLong and Béjà, 2010), and in general, our understanding of the metabolic and biogeochemical consequences of harbouring light-harvesting systems has grown at a much slower pace compared with our understanding of their genetic diversity. At the community level, there is evidence for light enhancing major metabolic processes such as heterotrophic bacterial production (Morán *et al*., 2001). However, results have not always been consistent (Ruiz-González *et al*., 2012) and dark conditions are still widely used for measuring biomass production rates of marine heterotrophic bacteria and archaea (Lemée *et al*., 2002; Tada *et al*., 2011), thereby neglecting the potential impact of photoheterotrophy.

Most of the studies addressing the metabolic advantages of photoheterotrophy have almost exclusively focused on bacterial cultures. The lack of experimental evidence of environmental photoheterotrophic activity is mostly due to the difficulty in differentiating a direct effect of light on the metabolism of photoheterotrophs from an indirect effect, e.g., mediated by the carbon supply by phytoplanktonic organisms. Here, we used a pre-filtration step, which was generally very effective in reducing the concentration of Chl *a* and the abundance of photosynthetic organisms in the incubations, except in autumn when cyanobacterial communities were largely comprised of the small-sized *Prochlorococcus*. Yet, in some months we observed substantial regrowth of *Synechococcus* (particularly in July and August) or picoeukaryotes (in April and November) under light conditions in the incubations. Interestingly, the opposite result was found in the case of *Prochlorococcus* from October to December, as their abundance increased under dark conditions during the first day of incubation. *Prochlorococcus* have a tightly synchronized cell cycle regulated by the photoperiod, dividing at the beginning of the dark period (Vaulot *et al*., 1995; Jacquet *et al*., 2001; Biller *et al*., 2018). Thus, we hypothesize that *Prochlorococcus* cells went through a first round of division after placing them under dark conditions, but their growth ceased afterwards. The decrease in the abundance of *Prochlorococcus* cells under light conditions could be due to a photoinhibition effect by PAR light, as also found in previous studies (Sommaruga *et al*., 2005; Mella-Flores *et al*., 2012). Higher specific growth rates of heterotrophic bacteria in the dark incubations were also occasionally detected, which could also be related to photoinhibition (Ruiz-González *et al*., 2012). A negative effect of PAR radiation on the uptake of organic compounds (amino acids) in abundant groups such as SAR11 has been experimentally tested in previous studies (Alonso-Sáez *et al*., 2006; Ruiz-González *et al*., 2012). We infer that a similar effect may have happened in April, limiting the growth rates of some bacteria

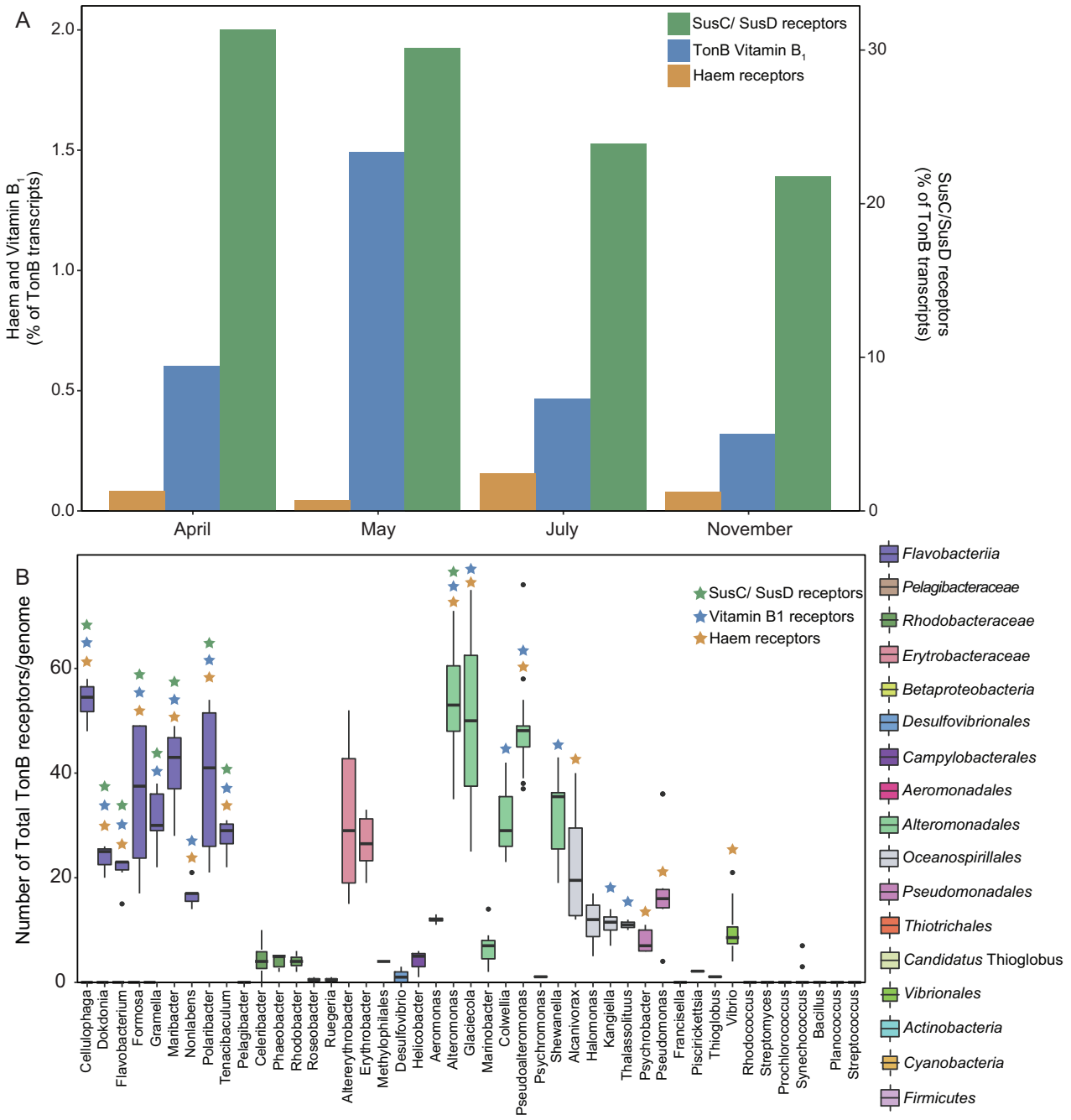

Fig 5. A. Percent of reads that target genes that encode haem and vitamin B_1 transporters (left *y*-axis) and TonB (in *susC-susD* tandem) transporter (right *y*-axis) relative to all TonB receptor transcripts found in the metatranscriptomes. B. The number of predicted TonB transporter receptors for each bacterial genus found in a set of complete genomes downloaded from the MAR database. Different colour boxes indicate taxonomical affiliation. Green, blue and yellow stars specify the presence of SusC/SusD, specific vitamin B_1 and haem TonB receptor genes in at least one of the genomes of each genus.

under light conditions. In any case, it is also remarkable that the regrowth of photosynthetic organisms in the incubations during April, summer and autumn did not substantially impact the growth rates of bacterial heterotrophic communities. More importantly, in May, when we observed the highest increase of bacterial growth rates under light conditions, cyanobacteria were not detected in the incubations (either by flow cytometry or by high-throughput sequencing) and autotrophic eukaryote abundances and Chl *a* concentrations were

negligible. The absence of autotrophic cells implies that the results obtained in that month were independent of the potential effects of photosynthetic organisms.

Although the growth rates and overall taxonomic composition were similar in both dark and light incubations for most of the analysed months, an increase in the abundance of bacteria with intact cell membranes was generally found under the light. The mean contribution of *Live* cells in the experiments conducted in the light ranged from 82% to 94%, similar to the variability found in natural communities from the same site (Morán and Calvo-Díaz,- 2009). In the dark, however, this value dropped below 80% in half of the monthly incubations, a value never found at our study site under natural conditions (Morán and Calvo-Díaz, 2009; Huete-Stauffer *et al*., 2015). While we cannot rule out that the growth of photosynthetic organisms in the incubations may have impacted this result, the increase of *Live* cells in the light treatment was also clearly observed in May, when the potential impact of photoautotrophs was negligible. Furthermore, the marked decrease of *Live* cells under dark conditions (generally within 1 day) took place before the regrowth of photosynthetic organisms (Fig. S3), supporting the view that light was sustaining cell-membrane integrity in a substantial fraction of bacterial cells. We hypothesize that the energy provided by PR may be crucial for maintaining essential metabolic processes in some marine bacteria, avoiding cell death. In support of our results, a previous study on the photoheterotrophic isolate *Vibrio* AND4 showed a positive effect of light on cell survival under oligotrophic conditions (Gómez-Consarnau *et al*., 2010), and light has been found to promote the maintenance of ATP content and substrate transport rates in starved SAR11 cells (Steindler *et al*., 2011). Remarkably, we found that the maximum effect of light on cell membrane integrity was found in incubations with seawater collected in summer, when oligotrophic conditions prevail at our study site (Calvo-Díaz and Morán, 2006; Huete-Stauffer *et al*., 2015) and SAR11 was dominant. However, further work is needed to confirm this pattern, as we could not avoid the confounding factor of cyanobacteria re-growth in summer incubations.

The specific growth rate is a key parameter to characterize the ecological success of bacterial taxa (Kirchman, 2016). Thus, the ability to use light to promote growth may represent an important competitive advantage for some photoheterotrophs. However, in previous studies, light has been found to positively impact the growth rates of only a few photoheterotrophic bacterial isolates, mainly affiliated with *Flavobacteriia* (Gómez-Consarnau *et al*., 2007; Yoshizawa *et al*., 2014). Here, we found that during the decline of the spring phytoplankton bloom, light promoted the growth of different taxa, including two ecologically relevant flavobacteria: *Polaribacter* and *Tenacibaculum*. These two taxa are typically associated with phytoplankton (Klindworth *et al*., 2014; Xing *et al*., 2015; Pearman *et al*., 2016) and contain members that harbour PR genes in their genomes (González *et al*., 2008; Yoshizawa *et al*., 2012; Pearman *et al*., 2016). Yet, evidence showing that light can promote their growth is scarce, i.e., only observed for one *Polaribacter* strain (MED152; Gómez-Consarnau *et al*., 2007), with no experimental evidence reported for *Tenacibaculum*. The fact that *Polaribacter* and *Tenacibaculum* grew better under light conditions at the time of the highest seasonal DOM bioavailability (according to the bacterial production measurements; Fig. S1) contrasts with previous isolate-based studies in which light-enhanced growth was found mostly under low or intermediate DOM concentrations (Gómez-Consarnau *et al*., 2007; Michelou *et al*., 2007; Yoshizawa *et al*., 2014). Thus, our results provide new perspectives to understand the environmental context where light can promote the growth of photoheterotrophic bacteria in coastal waters.

Members of another environmentally relevant taxon in marine waters, the *Alteromonadales* order, also grew in response to light. The role of photoheterotrophy for this model copiotroph has not been previously considered, as they are dominant members of the aphotic oceanic realm (Salazar *et al*., 2016) and typically outgrow other heterotrophic taxa in dark bottle incubations (Alonso-Sáez *et al*., 2010). Indeed, PR genes are not widely distributed among *Alteromonadales*; from a total of 123 genomes of *Alteromonadaceae* in the MAR database, we found PR-coding genes in only four strains (*Alteromonas* sp. LOR 57, MB-3u-76, MED668 and LTR 27) which have been described very recently (Kopel *et al*., 2014; Tully *et al*., 2017). Interestingly, in the light incubations in May, some of the clones affiliated with *Alteromonas* had >99% similarity with some of these PR-containing strains (i.e., *Alteromonas* LOR, LTR, MB-3u76), which was consistent with their growth-response under light conditions. Additionally, some clones were closely related to *Glaciecola*, in which PR-coding genes have been also observed (Qin *et al*., 2012; Boeuf *et al*., 2016). Although less abundant than *Alteromonas*, *Glaciecola* was also present in our incubations and can be hybridized by the ALT1413 probe. Thus, we cannot discard the possibility that some of the ALT1413-hybridized cells with light-enhanced growth were affiliated with the latter genus.

Another surprising observation from our results was the higher growth rates of some SAR11 members in May under light conditions. Previous studies have shown that light does not promote the growth of the SAR11 model strain *Cand*. P. ubique HTCC1062 (Giovannoni *et al*., 2005), although a positive effect on their survival has been observed (Steindler *et al*., 2011). *Cand*.

P. ubique HTCC1062 is a representative of the SAR11 Group I, which dominated the *Pelagibacterales* sequence reads in 8 out of 9 months of our analysis (Arandia-Gorostidi *et al*., 2017). However, in the samples taken in May, a clear shift in their composition was found, with SAR11 Group II becoming dominant (Arandia-Gorostidi *et al*., 2017). While SAR11 Group I is mostly found under oligotrophic conditions, the relative contribution of Group II usually peaks in Spring (Giovannoni, 2017), in agreement with the observations in our study. Even if the specific growth rates of *Pelagibacterales* members are generally low (Kirchman, 2016), occasional high activity of SAR11 cells has been also reported (Malmstrom *et al*., 2004). Similarly, the SAR11 growth rates we measured in May were high, comparable to members of groups such as *Rhodobacteraceae* and *Gammaproteobacteria* (Arandia-Gorostidi *et al*., 2017), but as we show in this work, only under light conditions. Thus, our results suggest that some members of SAR11 increase their metabolism under light conditions, most likely related to their photoheterotrophic ability.

In general, the fact that light promotes the growth of only a fraction of PR-bearing isolates is intriguing. One recent study showed that some *Dokdonia* isolates auxotrophic for vitamin B_1 (DSW-1^T and MED134) actively expressed a predicted TonB receptor specific for the transport of vitamin B_1 under light conditions. This result indicated that the PMF generated by PR under light conditions facilitated the incorporation of this essential growth factor, likely explaining their higher growth rates in the light (Gómez-Consarnau *et al*., 2016). Besides vitamins, TonB membrane transporters are used to take up a wide range of compounds, including iron, carbohydrates and small peptides (Tang *et al*., 2012). Additionally, uptake rates of other compounds (such as taurine) have been observed to be more active in the presence of light (Steindler *et al*., 2011), suggesting that light-enhanced metabolism can indeed be related to an increased ability to incorporate compounds by PR-containing bacteria. This high abundance of TonB transporter transcripts detected under the phytoplankton post-bloom conditions in May (with a maximum relative abundance of vitamin B_1 receptors) may suggest that taxa harbouring PRs can take advantage of the PMF generated from light to incorporate essential compounds such as vitamin B_1.

In summary, PR expression is an adaptation by some members of the bacterioplankton community to take advantage of light energy. Here, we detected that in the absence of potential confounding effects of photosynthetic organisms, the use of light promoted the growth of taxonomically diverse bacterial taxa, but only under very specific environmental conditions (i.e., a post-bloom scenario). This would reconcile our results with those of previous studies where light did not impact the taxonomic composition of bacterial communities in seawater incubations (Schwalbach *et al*., 2005). The photoheterotrophs showing light-enhanced growth were affiliated with environmentally relevant taxa (including *Tenacibaculum*, *Polaribacter* and *Alteromonadales*), which may have a key role in carbon processing under high DOM availability conditions. Some members of *Pelagibacterales* may also take advantage of the photoheterotrophic activity to promote growth, expanding the known effects of light upon this globally abundant marine bacterial taxon. Our results further suggest that light may promote the maintenance of cell integrity in a substantial fraction of coastal marine bacteria. If confirmed, the light should be regarded not only as an 'extra' energy source but also as a requirement for some photoheterotrophs to survive in the ocean.

Experimental procedures

Study area and sample collection

Seawater was collected monthly between April and December 2012 at the E2 Gijón/Xixón station in a mid-shelf area located 37 km off the coast of Spain (43.675° N, 5.578° W) in the Southern Bay of Biscay. For convenience, the two samples collected in May, on the 2nd and the 23rd, were referred to as 'April' and 'May' respectively). Surface water samples were collected at 5 m depth in 5 L Niskin bottles and pre-filtered through 0.8 μm to minimize the presence of predators and phytoplankton cells in the incubations. In addition to seawater collection, CTD (SeaBird 25) profiles were carried out in the water column of the sampling area to measure *in situ* parameters. Samples were transported to the lab within 6 h in 20 L polycarbonate bottles (Nalgene). *In situ* Chl *a* concentration was determined by filtering 200 ml of seawater on 0.2 μm pore-size polycarbonate filters. Filters were frozen at −20°C and processed within 2 weeks as described by Calvo-Díaz and Morán (2006).

RNA extraction and processing for metatranscriptomic analysis

Seawater samples for RNA extraction were collected in April, May, July and November. Seawater was filtered onto 0.2-μm pore-size polycarbonate filtered and frozen in liquid nitrogen until the arrival to the laboratory, where they were stored at −80°C. These filters were shattered with a mallet, vortexed in falcon tubes containing Power Soil beads (Mobio), and the lysate was mixed with 70% ethanol (1:1 volume). The RNA extraction was carried out with the RNeasy Mini Kit (Qiagen). RNA was treated with Turbo DNAse (Ambion) and the ribosomal RNA was depleted using the mRNA-only isolation kit (Epicentre)

and the MicrobeExpress and MicrobeEnrich kits (Ambion). The enriched mRNAs were linearly amplified using the Message Amp II-Bacteria kit (Ambion), reverse transcribed to double-stranded complementary DNA (cDNA) with the Universal Riboclone cDNA synthesis system (Promega) and purified with the QIAQuick PCR purification kit (Qiagen). The eight cDNA samples were subjected to single-end sequencing by Illumina Miseq technology.

A custom-made database contained the peptides derived from the set of complete and partial genomes from the MAR database (9407 genomes, August 2018). Only the peptides of interest (rhodopsin and TonB receptor families) were annotated based on a search using hidden Markov models (HMM) either already available in the families and superfamilies in the Protein Families Database (Pfam) v. 31.0 or TIGRFAM v. 15.0 databases or an HMM specifically designed as described in supplementary information 1. BLASTx mapped the metatranscriptome sequence reads to the peptide database. A BLASTx hit was considered valid if the bit score was equal or greater than 50.

Experimental design

A volume of 20 L of seawater was collected at E2 Gijón/Xixón station and filtered through 0.8 μm as described above. Once in the lab, three transparent 4-L polycarbonate bottles (Nalgene) were filled with 2 L of the sampling water ('light treatment'). Additionally, three polycarbonate bottles were filled with 2 L of water and wrapped with aluminium foil and black tape to keep the water under complete dark conditions ('dark treatment'). The bottles were maintained in incubators between 3 and 5 days (except in the incubation of December, which lasted 7 days) at *in situ* temperature and under saturating PAR light irradiance of 435 mmol photons m^{-2} s^{-1}, conditions mimicking the photoperiod of the sampling day. Every day during the incubations, 2–3 samples were taken to determine microbial and flagellate abundance by flow cytometry, as well as by CARD-FISH analysis.

To estimate the abundance of *Live* and *Dead* microbes, cells with intact and damaged membrane respectively, the nucleic acid double staining protocol was used (Gregori *et al*., 2001). Volumes of 400 μl for each replicate were stained with 4 μl of Sybr Green I (SG1; Molecular Probes, Eugene, OR; 10 μg ml^{-1}) and 4 μl cell-impermeant PI (Sigma Chemical) fluorescent probe (10 μg ml^{-1}) and incubated in the dark for 15 min to quantify *Live* cells (with intact membranes) and *Dead* cells (with damaged membranes). Microbial abundance was analysed with a FACSCalibur flow cytometer (Becton Dickinson) equipped with a blue (488-nm) argon laser.

HNF abundance was determined on 1% glutaraldehyde-fixed samples by flow cytometry using a FACSCalibur Flow Cytometer (Becton Dickinson; see details in Christaki *et al*., 2011). To determine the abundance of cyanobacteria and picoeukaryotes, 1.8 ml of water was sampled before and after the pre-filtration treatment. Samples were fixed in 1% paraformaldehyde and 0.05% glutaraldehyde (final concentration) and stored at −80°C until the flow cytometry analysis. Both groups were differentiated using red (FL3) and orange (FL2) fluorescence signals.

Abundance and growth of specific phylogenetic bacterial groups

To estimate the abundance of different phylogenetic bacterial groups during the incubations, duplicate samples were analysed by *CARD-FISH* (Pernthaler *et al*., 2004). The probes and formamide concentrations used were: SAR11-441R (Morris *et al*., 2002), 45% formamide for SAR11 group; Ros537 (Eilers *et al*., 2000), 55% formamide for *Rhodobacteraceae*; Gam42a (Amann *et al*., 1990) and its competitor Bet42a (Manz *et al*., 1992), 55% formamide for *Gammaproteobacteria*; CF319 (Amann *et al*., 1990), 55% formamide for *Bacteroidetes*; POL740 (Malmstrom *et al*., 2007), 35% formamide for *Polaribacter*; Alt1413 (Eilers *et al*., 2000), 60% formamide for *Alteromonas*; and a probe newly designed in this work, Ten135 (5′-aggctatcctcctgtacaag-3′) and its competitor Ten135c (5′-aggctatcctcctgtaaaag-3′), 20% formamide for *Tenacibaculum*. Hybridized samples were transferred onto slides and stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg ml^{-1}. DAPI and CARD-FISH stained bacteria were quantified with an epifluorescence microscope Leica DM5500B, a monochromatic camera Leica DFC360 FX and the ACMETool2 automatized image analysis software (Zeder *et al*., 2011).

Growth rates of bacteria were estimated for the entire microbial community, as well as for each phylogenetic group, as the slope between ln-transformed abundance against time during the exponential growth phase. Maximum abundance reached during the stationary growth phase by the bacterial community and each phylogenetic group was also calculated. Statistical differences between the growth rates of each treatment were estimated by covariance analysis (ANCOVA), determining statistical differences in the change of the bacterial abundance with time for each treatment.

DNA extraction and sequencing

Samples for DNA extraction were collected at the initial time of the incubations and when total microbe abundances reached their maximum, which was determined

by daily monitoring of bacterial abundance using flow cytometry. Samples were pre-filtered through GF/A 47 mm filters (Whatman) and biomass was collected onto 0.2 μm pore-size 47 mm polycarbonate filters (Millipore) for DNA extraction. Filters were immediately frozen at −80°C for storage before extraction. DNA was extracted using the PowerWater DNA Isolation Kit (Mobio, Carlsbad, USA) according to the manufacturer's instructions.

To determine prokaryotic community composition, DNA was amplified (see details in supplementary information 1) and analysed by pyrosequencing with a 454 FLX+ system (ROCHE software v2.8). Sequences were analysed with the Mothur platform (Schloss *et al*., 2009) as indicated in Alonso-Sáez *et al*. (2015). In addition to amplicon sequencing, clone libraries were analysed in the spring samples (May and June, *in situ* and light treatment) to obtain a finer phylogenetic resolution of the taxa growing in the incubations by sequencing the full 16S rRNA gene (see details in supplementary information 1).

Bioinformatic pipeline for TonB receptors and PR analysis

We analysed the abundance of predicted SusC/SusD and TonB receptor genes, including receptors considered specific for vitamin B_1 and for haem groups in a set of complete genomes downloaded from the MAR database (https://mmp.sfb.uit.no/databases/; 798 genomes; August 2018). See supplementary information 1 section for more information. Similarly, the abundance of PR peptides was also identified by the corresponding PFAM (PF01036) in the Protein Families Database (Pfam) v. 32.0. Further description of these analyses is provided as supplementary information 1.

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Appendix S1: Supporting Information

Appendix S2: Supporting Information