environmental microbiology

Environmental Microbiology (2014) **16**(9), 2953–2965 doi:10.1111/1462-2920.12278

Marked seasonality of aerobic anoxygenic phototrophic bacteria in the coastal NW Mediterranean Sea as revealed by cell abundance, pigment concentration and pyrosequencing of *pufM* gene

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Summary

The abundance and diversity of aerobic anoxygenic phototrophs (AAPs) were studied for a year cycle at the Blanes Bay Microbial Observatory (NW Mediterranean) and their potential links to an array of environmental variables were explored. Cell numbers were low in winter and peaked in summer, showing a marked seasonality that positively correlated with day length and light at the surface. Bacteriochlorophyll *a* concentration, their light-harvesting pigment, was only detected between April and October, and pigment cell quota showed large variations during this period. Pyrosequencing analysis of the *pufM* gene revealed that the most abundant operational taxonomic units (OTUs) were affiliated to phylogroup K (*Gammaproteobacteria*) and uncultured phylogroup C, although they were outnumbered by alphaproteobacterial OTUs in spring. Overall, richness was higher in winter than in summer, showing an opposite trend to abundance and day length. Clustering of samples by multivariate analyses showed a clear seasonality that suggests a succession of different AAP subpopulations over time. Temperature, chlorophyll *a* and day length were the environmental drivers that best explained the distribution of AAP assemblages. These results indicate that AAP bacteria are highly dynamic and undergo seasonal variations in diversity and abundance mostly dictated by environmental conditions as exemplified by light availability.

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Introduction

Aerobic anoxygenic phototrophs (AAPs) are photoheterotrophic organisms that require organic substrates for their metabolism and growth, but can derive a portion of their energy requirements harvesting light using bacteriochlorophyll *a* (BChl *a*). These bacteria are found in diverse aquatic systems including open ocean waters (Kolber *et al*., 2001; Cottrell *et al*., 2006; Jiao *et al*., 2007), shelf and coastal environments (Masín *et al*., 2006; Zhang and Jiao, 2007; Cottrell *et al*., 2010), coastal lagoons (Lamy *et al*., 2011), freshwater lakes (Salka *et al*., 2011; Masín *et al*., 2012) and rivers (Ruiz-González *et al*., 2013). Initially, it was hypothesized that AAPs would be abundant in nutrient-poor environments because of their potential ecological advantage over strict heterotrophs (Kolber *et al*., 2000), but recent results contradict this hypothesis because AAPs have been found to thrive better in more eutrophic environments (Hojerová *et al*., 2011). Genomic and experimental evidences have shown that AAP bacteria are metabolically diverse (Fuchs *et al*., 2007; Koblízek *et al*., 2010; 2011), can grow on a wide range of carbon sources from low molecular-weight to complex molecules (Koblízek *et al*., 2003; Biebl *et al*., 2005), can metabolize organic carbon with high efficiency (Hauruseu and Koblízek, 2012) and exhibit high growth rates (Koblízek *et al*., 2007; Ferrera *et al*., 2011). Nevertheless, AAP cells typically account for less than 5% of the total prokaryotic community regardless of the trophic status of the environment (Schwalbach and Fuhrman, 2005; Cottrell *et al*., 2010; Hojerová *et al*., 2011; Ritchie and Johnson, 2012). Factors such as association to particles, temperature, light attenuation, nutrient limitation or vulnerability to predation have been identified as factors that influence the abundance of AAP bacteria, but their role is still not well understood.

The concentration of their light-harvesting pigment (BChl *a*) in the sea is, in general, low compared with that of chlorophyll *a* (Chl *a*), in particular in oligotrophic waters.

However, the cell quota of AAP cells can be significant and comparable with the cell quota of picocyanobacteria (Cottrell *et al*., 2006; 2010). It is known that the BChl *a* quota of AAPs in culture can largely vary in response to light (Koblízek *et al*., 2003). However, the available field data do not show a consistent correlation between BChl *a* and light availability (Cottrell *et al*., 2006; 2010; Jiao *et al*., 2010; Lamy *et al*., 2011), reflecting that light is not the sole controlling factor of pigment synthesis. Association to particles (Cottrell *et al*., 2010) or intracellular redox status (Spring and Riedel, 2013) can also influence BChl *a* concentration and cell quota.

The AAPs are phylogenetically diverse, consisting of several groups distributed across the *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. Yutin and colleagues (2007) established the classification of AAPs into 12 different phylogroups (named A–L) based both on the phylogeny of the *pufM* gene and the structure of the *puf* operon using the Global Ocean Sampling (GOS) expedition metagenomic data. This study followed by others (Salka *et al*., 2008; Cottrell and Kirchman, 2009; Cottrell *et al*., 2010; Lehours *et al*., 2010) showed that the phylogenetic structure of AAP communities is highly variable, with different prevalent phylogroups depending on location. For example, brackish and freshwater environments are typically dominated by betaproteobacterial clades. In the oligotrophic open ocean waters from the Atlantic and Pacific Oceans, AAP communities are dominated by phylogroups A and B, both lacking cultured representatives. Contrarily, AAP communities in the Mediterranean seem to be dominated by phylogroup K (*Gammaproteobacteria*) during summer (Lehours *et al*., 2010). The *Roseobacter*-related phylogroup G (*Alphaproteobacteria*) appears to be the most ubiquitous one across different environments and is more abundant in mesotrophic to eutrophic environments. In addition to these geographical differences, variability in community structure has also been observed according to depth (Lehours *et al*., 2010). These observations point out that the genetic diversity of AAP bacteria is likely structured in relation to certain environmental variables such as light and nutrient gradients.

While in the last decade we have generated knowledge on the spatial distribution of this functional group in aquatic environments, we still have a poor understanding of the factors that affect their seasonality. Differences were observed between seasons in the Arctic Ocean (Cottrell and Kirchman, 2009), the East China Sea (Zhang and Jiao, 2007) and off the coast of Southern California (Schwalbach and Fuhrman, 2005). Recently, Lamy and colleagues (2011) reported large variations in the abundance of AAP bacteria in a Mediterranean coastal lagoon over a 10 month study. However, this study did not examine variations on the structure of AAP communities. Yet, a precise understanding of how abiotic and biotic factors affect microbial populations would help develop an ecological framework for modelling community dynamics across environmental gradients.

For this purpose, we studied the seasonal dynamics of AAP bacteria in a coastal oligotrophic environment in the Northwestern Mediterranean (Blanes Bay Microbial Observatory, BBMO) and tried to decipher the main environmental factors that control the abundance and diversity of this functional group. The AAP community was studied over a complete year period (14 months). Variability was addressed in terms of abundance (cell counts and pigment concentration) and diversity (pyrosequencing of the *pufM* gene), and the links to an array of environmental data were explored.

Results

Environmental setting

Surface water temperature at the BBMO from January 2010 to February 2011 ranged from 12.3 (17 February 2010) to 24.4°C (14 September 2010, Table S1). Total Chl *a* was below 2 $\mu g\ l^{-1}$ with lower values measured, in general, during the warmer months (June to September, Fig. S1). The maximal Chl *a* concentration was measured in March and the minimal value in September. Total Chl *a* followed a similar trend than Chl *a* in the size fraction less than 3 μm ($P < 0.05$) (Fig. S1). Inorganic nutrient concentrations were higher in winter (Table S1) except for ammonium that underwent large variations throughout the year and showed a maximum peak in May.

Abundance of total prokaryotes, Synechococcus *and AAP bacteria*

Abundance of total prokaryotes in surface waters ranged between 2.34×10^5 and 1.00×10^6 cells ml^{-1}, with maxima in May and November (Fig. 1A). Abundance of *Synechococcus*, the most abundant cyanobacteria in the study site, did not follow the same trend than total prokaryotes, and numbers were highest in spring and lowest in winter (Fig. 1A). No significant correlation was found between these two groups and Chl *a* ($P > 0.05$). AAP abundance ranged between 1.12×10^3 and 5.02×10^4 cells ml^{-1}, resulting in 0.2–6.3% of total prokaryotes (Fig. 1B). Abundance of AAP bacteria was low from November to March and presented higher numbers from April to October, showing a marked seasonality both in absolute numbers (cells ml^{-1}) and in relative abundance (% of total prokaryotes). Although BChl *a* analyses were carried out in samples collected throughout the entire study period, it could only be detected between April and October when AAP abundances were maximal (Fig. 1C). Both the

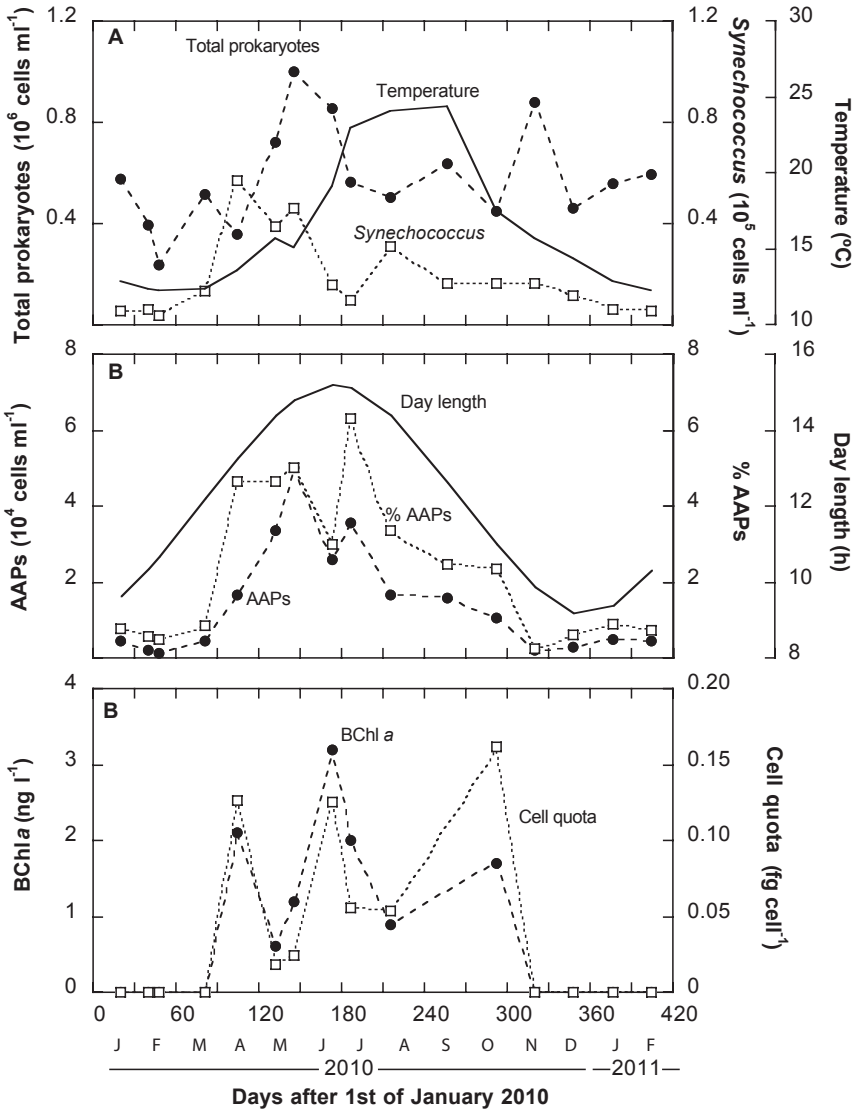

Fig. 1. Abundance of (A) total prokaryotes and *Synechococcus*, (B) total and relative abundance (percentage to total prokaryotes) of AAP bacteria and (C) concentration of BChl *a* and cell quota measured through the study period (January 2010–February 2011) at the Blanes Bay Microbial Observatory. The seasonal changes in (A) temperature and (B) day length are also shown.

BChl *a* cell quota (BChl *a* per cell) and total pigment concentration varied much more than AAP bacterial abundance (Fig. 1C). As shown previously (Jiao *et al.*, 2010), the proportion of BChl *a* to total pigments (BChl *a* + Chl *a*), which changed from 0.2% to 0.6%, increased at lower Chl *a* concentrations (Fig. S2A).

The abundances of AAP bacteria were not significantly correlated with total prokaryotes and *Synechococcus* [false discovery rate (FDR)-corrected $P > 0.05$]. Absolute and relative AAP abundances were correlated ($P < 0.05$) with day length and light at the surface measured as photosynthetically active radiation (PAR). In addition, the percentage of AAPs was inversely correlated with nitrite and Chl *a* (< 3 μm fraction). BChl *a* concentration was also correlated with day length ($R = 0.74$, $P = 0.017$) and inversely with nitrite ($R = -0.73$, $P = 0.019$). No significant correlation was found between any indicator of AAP abundance and temperature.

To further understand the influence of the physicochemical variables on the dynamics of AAP bacteria, we performed a distance-based redundancy analysis (dbRDA), which ordinates the samples based on biological data while constraining the ordination by a set of environmental variables. The four variables measured in relation to AAP bacteria (total and relative abundance, BChl *a* concentration and pigment cell quota) were used as biological data, and 10 variables (temperature, Secchi depth, day length, total and < 3 μm Chl *a* concentration, and concentration of PO_4, NH_4, NO_2, NO_3 and Si) were used as environmental data. Light at the surface was excluded from this analysis because of missing data points. Likewise, two sampling points (14 September

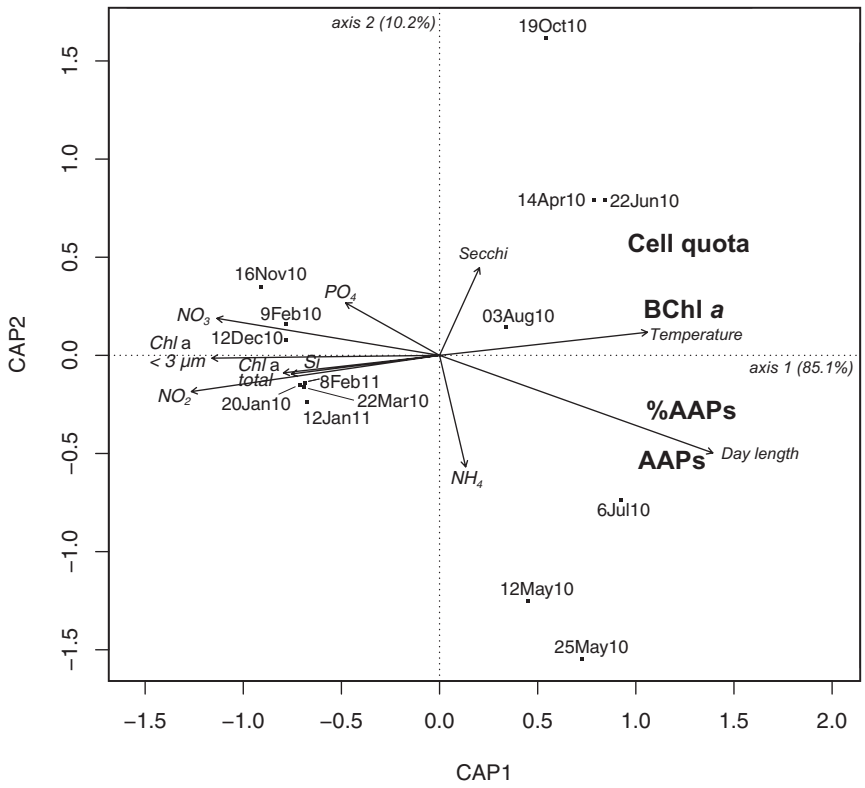

Fig. 2. Distance-based redundancy analysis of biological data (shown in bold; total and relative abundance of AAP, BChl *a* concentration and BChl *a* cell quota) and environmental data as explanatory variables (shown in arrows; temperature, Secchi depth, day length, total and < 3 µm Chl *a* concentration, and the concentration of PO_4, NH_4, NO_2, NO_3 and Si). Samples are identified by sampling date (see Table S1).

2010 and 17 February 2010) were excluded because of missing data in either the biological or the environmental variables. The results of the analysis indicated that 96.4% of the variability in AAP dynamics could be explained by these environmental variables (axis 1: 85.1%, axis 2: 10.2%). The dbRDA plot (Fig. 2) shows a clear separation along the first axis between samples from late fall to early spring (low abundance) and those from late spring and early fall (high abundance), indicating that the measured variables largely explained the marked seasonality observed. Late fall to early spring samples were associated to higher concentrations of NO_2, NO_3 and Chl *a* while day length and temperature were the main variables influencing late spring and summer samples. The axis 2 was mainly defined by Secchi depth and ammonium.

Diversity of AAP bacteria

Twelve samples, one representative of each month of the year (Table S1), were selected for pyrosequencing analysis of the *pufM* gene. A total of 113.422 high-quality sequences were obtained, with an average of 9.452 sequences per sample (minimum 3598, maximum 22767). Curated sequences were clustered into 82 different operational taxonomic units (OTUs; 12–36 per sample) using a 94% cut-off, which had previously been shown to be optimal to group related phylotypes of the *pufM* gene (Zeng *et al*., 2007; Lehours *et al*., 2010). From these, only six OTUs were present in all samples, but they represented ~ 75% of total reads. The majority of *pufM* OTUs retrieved belonged to 8 of the 12 phylogroups previously identified in the GOS metagenomic survey, named phylogroups A–L (Yutin *et al*., 2007). Taking into account the whole dataset, the most abundant phylogroup (~ 69% of total reads, 33–86% per sample) was by far phylogroup K, which contains cultured representatives of the gammaproteobacterial AAP bacteria such as *Congregibacter litoralis* KT71 (Fuchs *et al*., 2007). Among the 17 OTUs affiliated to phylogroup K, three of them were present throughout the entire year (i.e. OTU-48, -70 and -71), and four of them (i.e. OTU-34, -54, -62 and -81) were detected only in a single sample. The second most abundant group was phylogroup C (~ 13% of total reads), which contains no cultured representatives. Interestingly, this group was represented by a unique OTU (OTU-52) that was present in all samples. *Roseobacter*-like group G (~ 6%) and *Rhodobacter*-like groups E (~ 5%) and F (~ 3%) were also detected and showed high intergroup diversity. Phylogroup H represented ~ 1%, and phylogroups I and J were rarely detected (< 1%) (Fig. 3). None of the sequences recovered were affiliated to phylogroups A, B, D and L, and only 2% of the reads remained unassigned to any of the previously defined phylogroups.

Nearly saturation of rarefaction curves revealed that most of the present diversity (measured as Chao1 richness) was retrieved from the samples (Fig. S3). Overall,

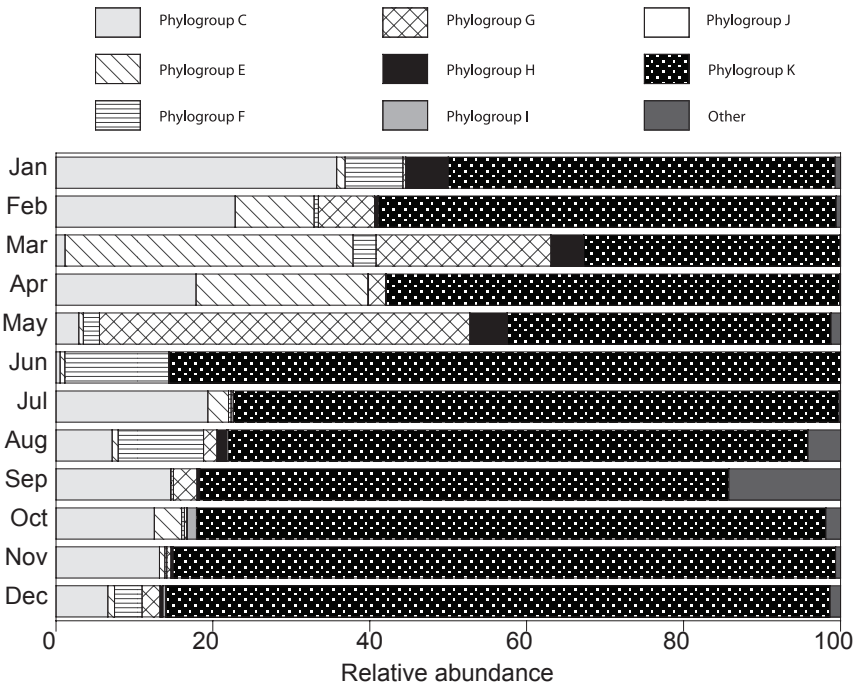

Fig. 3. Percentage of the relative contribution of each phylogroup to the total diversity of AAP community through the year studied at the Blanes Bay Microbial Observatory.

samples were more diverse in the colder months than in summer, showing an opposite trend to abundance. Chao1 values showed a significant inverse correlation with day length ($R^2 = 0.44$, $P = 0.02$). Furthermore, some interesting differences were observed in the contribution of the different phylogroups through the year (Fig. 3). For example, despite being abundant all year, the contribution of phylogroup K was considerably higher in summer and fall than during the rest of the year. Phylogroup C, which was on average the second most abundant group, was more abundant in winter and in contrast was < 1% in June. The *Rhodobacter*-like phylogroup E and *Roseobacter*-like phylogroup G, both with relatively low abundances during most of the year, peaked in late winter and spring. In fact, a single OTU affiliated to the *Roseobacter*-like bacteria made up to 47% of the total sequences in May. Likewise, phylogroup G was on average 23% from February to April whereas the rest of the year was rarely present (~ 1%). Intriguingly, an OTU that could not be affiliated to any of the previous phylogroups appeared in late summer, being over 12% of total reads. Blast analyses showed that this OTU is closely related to the clone PROSOPE-8, which forms a separated clade between groups D and E, which was retrieved in summer from the Mediterranean by Lehours and colleagues (2010). Comparing the abundances of the different phylogroups, we observed an inverse correlation between phylogroup K and phylogroups G ($R^2 = 0.47$, $P = 0.013$) and H ($R^2 = 0.61$, $P = 0.002$). Visualization of Bray–Curtis dissimilarities between samples using nMDS (non-metrical multidimensional scaling) plots revealed that, in general, each sample was most similar to a sample adjacent in time. This clustering according to time point suggests that there is likely a succession in AAP community composition over time (Fig. S4).

Distribution of AAP assemblages in relation to environmental variables

To evaluate whether community composition was modulated by environmental variables, the OTU-based community dissimilarity matrix was compared with the distance matrix constructed with the 11 environmental variables listed in Table S1 by using Spearman rank correlations. Mantel tests confirmed the presence of a significant relationship between the two matrices ($R = 0.34$, $P = 0.016$). To test which combination of environmental variables correlated best with the community assemblages observed, we used the BIOENV method using Spearman correlations (Clarke and Ainsworth, 1993). The environmental variables that better explained the distribution of AAPs at the OTU level were temperature, Chl *a* and day length. A subsequent Mantel test including only these environmental parameters confirmed that indeed the overall pattern in OTU distribution was significantly correlated ($R = 0.61$, $P = 0.001$) with this subset of variables.

Additionally, a dbRDA was performed to further explore to which extend the abiotic factors influenced temporal variability in community composition (Fig. 4). The dbRDA revealed that 91.3% of the OTU variability was explained by 10 variables (light at the surface was excluded because of missing data points). The first two axes explained up to

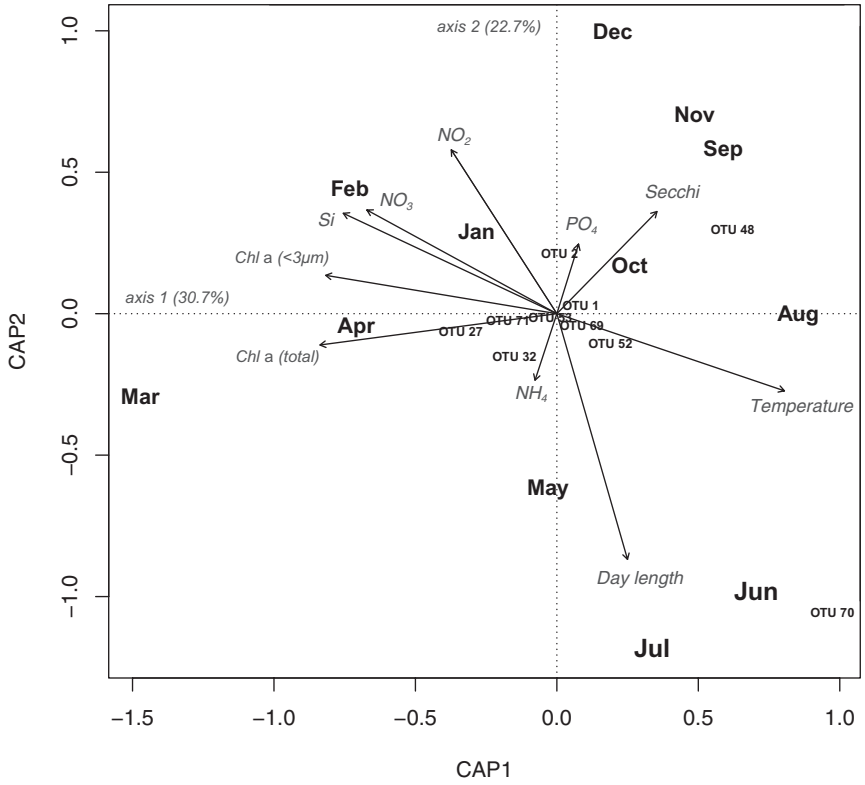

Fig. 4. Distance-based redundancy analysis between community composition at the OTU level and environmental data in the different samples identified by sampling month. Explanatory variables are shown in arrows (temperature, Secchi depth, day length, total and < 3 µm Chl *a* concentration, and the concentration of PO_4, NH_4, NO_2, NO_3 and Si). Analyses are based on the relative abundance of all OTUs but only most abundant OTUs (> 1% relative abundance) are shown to improve visualization.

30.7% (axis 1) and 22.7% (axis 2) of the variation and separated samples according to the sampling time; the first axis separated January to May samples from June to December, whereas the second axis separated spring and summer samples from fall and winter. Summer samples were associated with higher temperatures and longer day length whereas winter samples were placed in the opposite direction. March and April samples were associated with higher Chl *a* concentrations. Higher concentrations of nitrite, nitrate and silicate were associated to AAP communities in winter (Jan and Feb). Ammonia and phosphate had less influence than the rest of the measured variables and explained variability only in certain periods (ammonium in May and phosphate towards late fall and early winter). Plotting the most abundant OTUs (relative abundance > 1% of total pyrosequencing reads) in the dbRDA showed that they were clearly associated to different environmental variables (Fig. 4). The strongest influence was observed on OTU-70 associated to day length and OTU-48 associated to Secchi depth, both belonging to the *Gammaproteobacteria*. OTU-2, also *Gammaproteobacteria*-like, was associated to phosphate whereas OTU-52 (phylogroup C) was mainly influenced by temperature. Among the *Alphaproteobacteria*, *Roseobacter*-like OTU-32 seemed to be associated to ammonium concentrations and *Rhodobacter*-like OTU-27 to Chl *a* concentration.

Discussion

Marine microbial communities are dynamic both in phylogenetic and functional composition. Seasonal variations in the phylogenetic composition of bacterioplankton communities have been observed in different oceanic regions (Morris *et al*., 2005; Carlson *et al*., 2009; Eiler *et al*., 2009; Gilbert *et al*., 2009; 2012; Andersson *et al*., 2010; Galand *et al*., 2010; Malmstrom *et al*., 2010; Fortunato *et al*., 2012). Likewise, similar changes in specific phytoplankton populations have been related to environmental factors such as salinity or inorganic nutrients (e.g. DuRand *et al*., 2001; Fehling *et al*., 2012), but much less is known about other functional microbial groups. Yet, the availability of high-resolution data of microbial communities and their dynamics is critical to accurately modelling microbial activity in a biogeochemical framework. For this reason, we investigated the seasonality of the AAP bacteria in terms of abundance and population dynamics by cell enumeration, pigment concentration and pyrosequencing of the *pufM* gene at the BBMO in the NW Mediterranean during a year cycle.

Seasonal dynamics of AAP abundance

AAP bacteria were present in all samples, but their abundance changed considerably throughout the year (from 1.12×10^3 to 5.02×10^4 cells ml^{-1}). Despite this temporal

variability, these values are within the range observed in other marine environments (Schwalbach and Fuhrman, 2005; Cottrell *et al*., 2006; Zhang and Jiao, 2007; Hojerová *et al*., 2011; Ritchie and Johnson, 2012). AAP bacteria showed a different seasonal pattern than total prokaryotes and *Synechococcus* with minimum numbers in winter and maximum in summer. The relative contribution of AAP bacteria to total prokaryotic abundance ranged from less than 1% to ~ 6%, reaching higher values in summer. Data previously obtained in September 2007 and June and July 2009 from the same Mediterranean station (Ferrera *et al*., 2011; Hojerová *et al*., 2011) indicated that AAP bacteria represented 4–7% of total prokaryotic community in agreement with our current results. Seasonal variations in the abundance of AAP bacteria had been previously reported by Cottrell and Kirchman (2009) in Arctic waters. These authors observed an increase in AAP bacterial abundance from winter to summer, despite the fact that no differences in their relative abundance were found. Likewise, higher numbers of AAPs were observed in late spring and summer in the coastal East China Sea (Zhang and Jiao, 2007), the south Baltic Sea (Masín *et al*., 2006) and in a Mediterranean coastal lagoon (Lamy *et al*., 2011). However, no detailed information existed from their seasonal dynamics in the oligotrophic ocean. Our results together with previous observations indicate that marine AAP bacteria are highly dynamic and confirm that, in general, are more abundant in summer regardless of the trophy of the environment.

Light and nutrient availability, temperature and Chl *a* had been suggested as possible regulating factors on AAP abundance (Masín *et al*., 2006; Hojerová *et al*., 2011; Lamy *et al*., 2011). In our study, dbRDA analysis showed that day length was the main explanatory factor for the temporal distribution of AAP bacteria. Nitrite, nitrate, Chl *a* and temperature were also contributing to a certain degree. Likewise, pairwise correlations showed a positive correlation between day length and AAP abundance (absolute and relative values) as well as BChl *a* concentration, thus confirming that light availability strongly influences AAP bacteria in the coastal oligotrophic ocean. After the discovery of AAP bacteria by Kolber and colleagues (2000), the working hypothesis was that the photoheterotrophic metabolism of AAP would provide an important competitive advantage in oligotrophic environments under nutrient and specially carbon-limited conditions. However, this initial hypothesis was not exempt of controversy after the publication of contradictory data on the links between AAP abundance and the trophic status of the environment (Schwalbach and Fuhrman, 2005; Lami *et al*., 2007; Zhang and Jiao, 2007). Hojerová and colleagues (2011) found that, in the Mediterranean Sea, summer AAP communities developed better in environments of higher trophy. Contrarily, at a temporal scale, we found a negative trend between AAP abundance and Chl *a* (Fig. S2B) and inorganic nutrients. Furthermore, higher numbers of AAP bacteria and pigment content were measured in summer when dissolved organic carbon (DOC) accumulates annually and becomes chemically and structurally more complex in this coastal station (Vila-Reixach *et al*., 2012; Romera-Castillo *et al*., 2013). This observation may indicate that AAP bacteria rely on phototrophy as auxiliary energy source both when labile and/or refractory DOC are available, and that in spite of lower inorganic nutrient availability, summer conditions are clearly favourable for the growth of AAP bacteria. Light has indeed been shown to enhance their organic carbon utilization efficiency and growth in culture (Hauruseu and Koblízek, 2012). Furthermore, it has also been hypothesized that the additional energy gained could serve to enhance inorganic nutrient acquisition (Masín *et al*., 2008). Thus, in the Mediterranena Sea, AAPs might be more competitive over other heterotrophs in summer when DOC is available and nutrients are scarce.

Recently, Kirchman and Hanson (2013) estimated energy yields and costs for photoheterotrophic bacteria. For an AAP cell possessing an average number of 1700 photosynthetic units (PSU), net energy gain via phototrophy exceeds maintenance cost only when light intensity is greater than 400 μmol photons m^{-2} s^{-1}. This threshold value was clearly attained at Blanes Bay during late spring and summer (average PAR was 919 μmol photons m^{-2} s^{-1}) when the average number of PSU per AAP cell was approximately 2300 according to our BChl *a* data. This latter value fairly agrees with those previously reported for coastal Mediterranean regions as compiled by Kirchman and Hanson (2013). The failure to detect BChl *a* in winter samples and the low PAR at surface waters during winter months (170 μmol photons m^{-2} s^{-1} on average) suggest that AAP bacteria could not overcome energy limitations during the winter season and that net growth is probably impossible because of bioenergetic constraints.

Seasonal dynamics of AAP diversity

Diversity analyses of the *pufM* gene revealed that the annual diversity (12 samples) found at this coastal station is larger than that found in 29 summer samples from several stations across the Mediterranean (Lehours *et al*., 2010). However, this increase is likely due to the application for the first time of massive parallel sequencing to the *pufM* gene analysis as compared with the cloning and fingerprinting approaches previously used. In the last years, polymerase chain reaction (PCR)-based pyrosequencing has successfully been applied to investigate the diversity of microbial populations based on ribosomal RNA genes (e.g. Sogin *et al*., 2006; Kirchman *et al*.,

2010; Pommier *et al*., 2010), but diversity studies based on functional genes are scarce (Kip *et al*., 2011; Sánchez *et al*., 2013). Rarefaction analyses showed that our sequencing depth was sufficient to capture most, if not all, the *pufM* gene diversity in the analysed samples.

Most of the sequences obtained belonged to phylogroups previously defined by Yutin and colleagues (2007). Marine AAPs typically belong to different phylogenetic groups distributed across the *Alphaproteobacteria* and the *Gammaproteobacteria*. The vast majority of our sequences were related to phylogroup K, which contains cultured gammaproteobacterial AAP members of the NOR5/OM60 clade (Fuchs *et al*., 2007). A previous study showed that Mediterranean summer AAP communities were dominated by *Gammaproteobacteria* (Lehours *et al*., 2010). Our results confirm that this group is certainly the most abundant in summer but that it also constitutes a large fraction of the AAP community throughout all seasons. Although we cannot rule out potential PCR- and primer-related biases in the relative quantification of the different phylogroups (Polz and Cavanaugh, 1998), we have also observed the dominance of gammaproteobacterial AAP bacteria in BBMO using a different primer set (I. Ferrera, unpubl. data), and other authors have reported the dominance of different groups in distinct oceanic regions (Waidner and Kirchman, 2008; Cottrell and Kirchman, 2009; Lehours *et al*., 2010). Additionally, we observed a high diversity within phylogroup K, which included 17 different phylotypes that showed substantial variations in abundance through seasons. Lehours and colleagues (2010) found that certain gammaproteobacterial OTUs dominated at Mediterranean mesotrophic and eutrophic stations whereas others prevailed in oligotrophic stations. The temporal dynamics of these phylotypes could likewise be explained by different adaptations to trophic conditions or by different capabilities to persist in their habitat in winter, when environmental conditions (low temperature and light, short day length) seem to be unfavourable for the growth of AAP bacteria.

Despite the prevalence of *Gammaproteobacteria*, we did observe some intriguing differences in the contribution of the different groups through the year (Fig. 3). Interestingly, alphaproteobacterial groups E, F and G closely related to the *Rhodobacteraceae* outnumbered the gammaproteobacterial clade in spring, when chlorophyll and nutrients were higher. Large abundances of *Roseobacter/Rhodobacter* have been observed previously associated to high-nutrient conditions (Brown *et al*., 2005) or phytoplankton blooms (González *et al*., 2000), including assessments of bacterial diversity at the BBMO (Alonso-Sáez *et al*., 2007; Ruiz-González *et al*., 2012). Furthermore, the second most abundant phylogroup we found, i.e. phylogroup C, peaked in winter (Fig. 3). This seasonal dynamics may explain why this group was hardly found in the previous study carried out across the Mediterranean during the summer season (Lehours *et al*., 2010). Interestingly, we observed a correlation between the relative abundance of phylogroup C and BChl *a* concentration (Fig. S5). A recent report showed that the strategies of pigment production and regulation differ between AAP members of the *Alphaproteobacteria* and of the *Gammaproteobacteria* (Spring and Riedel, 2013). Because Group C lacks cultured representatives, no information is available on the regulation of pigment synthesis in members of this group. However, our observation may be an indication of higher contribution of phylogroup C to community pigment production as result of a different regulation mechanism.

Additionally, we explored the potential interactions between the different groups of AAPs by correlating their abundances, and we found a significant inverse correlation between phylogroup K and phylogroups G and H. Genome sequencing and strain characterization of AAP isolates have revealed differences in the metabolic capabilities and physiological properties of different strains (e.g. Koblízek *et al*., 2003; 2010; 2011; Fuchs *et al*., 2007; Spring and Riedel, 2013). These data together with our observation may suggest that intergroup competition between different AAPs exists and/or that different phylogroups posses diverse strategies to adapt to a changing environment.

A certain degree of variability in AAP diversity between summer and winter samples had previously been observed in the Arctic Ocean (Cottrell and Kirchman, 2009). However, the present study represents the first attempt to accurately capture the seasonal dynamics of AAP diversity by including an entire year of sampling and by the novelty of applying pyrosequencing to the *pufM* gene to trace the diversity of abundant and rare AAP phylotypes. The clustering of samples in nMDS plots according to sampling time suggests a succession of AAP subpopulations over time as it has previously been shown for bacterial community assemblages (Fuhrman *et al*., 2006; Andersson *et al*., 2010; Gilbert *et al*., 2012). Previous studies have shown a differential distribution of AAP bacterial communities in spatial gradients, with some assemblages being more adapted to oligotrophic and others to eutrophic environments (Jiao *et al*., 2007; Yutin *et al*., 2007; Lehours *et al*., 2010). Chl *a*, nitrate and PAR have been pointed out as possible factors explaining these spatial patterns of AAP diversity (Jiao *et al*., 2007; Lehours *et al*., 2010). However, the environmental factors driving the temporal dynamics of different AAP assemblages remained largely unexplored. The dbRDA analysis revealed that 91.3% of the variance in OTUs was explained by 10 environmental variables. Thus, despite being subjected to high grazing pressure (Koblízek *et al*., 2007; Ferrera *et al*., 2011), physical variables and nutrient con-

centration modulated AAP assemblages to a large extent. Spearman rank correlations confirmed that temperature, Chl *a* concentration and day length explained most of the diversity patterns observed at the OTU level. Furthermore, the redundancy analysis showed that the influence of abiotic factors on the most abundant OTUs is variable, an observation exemplified by the effect of light availability on some gammaproteobacterial members (OTU-48 and -70) (Fig. 4). Despite the clear seasonality observed in abundance, the differential effects of the abiotic factors to different phylogenetic groups may indicate that not all phylogroups of AAPs are functionally and ecologically equal.

A further interesting observation was the significant inverse correlation between AAP community richness (Chao1 indicator) and day length. In spatial gradients, a decreasing trend in AAP diversity along increasing Chl *a* concentration gradient was observed in the Pacific, Atlantic and Indian Oceans (Jiao *et al*., 2007) whereas a positive correlation with PAR was found in a longitudinal gradient in the Mediterranean (Lehours *et al*., 2010). Recently, a thorough seasonal study of microbial diversity carried out for 6 years by Gilbert and colleagues (2012) in the Western English Channel showed that day length explained most of the variability in bacterial OTU richness and that alpha-diversity follows a cyclic variation pattern, with values peaking in winter. Our results suggest that this trend may also occur for particular functional groups, and interestingly, the patterns of diversity of AAP communities seem to be opposite to their patterns of abundance.

Concluding remarks

The results presented here show that the AAP bacteria constitute a highly dynamic functional group. The positive correlation between AAP abundance and day length and PAR values supports the idea that summer conditions are optimal for the growth of these photoheterotrophs. Application for the first time of high-throughput parallel sequencing revealed a *pufM* gene diversity larger than previously reported and confirmed that Mediterranean communities are dominated by gammaproteobacterial AAPs, yet striking differences were observed in the relative contribution of the different groups through the year. Statistical analyses showed that AAP bacteria undergo a clear seasonality in response to environmental conditions. AAPs are known to be subjected to high grazing pressure and thus top-down controlling factors may contribute to this seasonal pattern; however, in this study we focused on the bottom-up factors that contribute the most to the seasonality of AAP populations. We found out that temperature, Chl *a*, nutrient concentration and light availability are the primary abiotic drivers affecting AAP bacterial assemblages. Overall, our data support the hypothesis that photoheterotrophs may rely on light to gain advantage over strict heterotrophs at least in late spring and summer. Because AAPs are metabolically active organisms and contribute largely to bacterial production in the euphotic zone of the oceans (Kolber *et al*., 2001), our data provide a framework for modelling their dynamics and ecological role in relation to seasonality of the marine carbon cycling.

Experimental procedures

Sample collection and basic data

Surface waters were collected from the BBMO (41°40'N, 2°48'E), which is a shallow (~ 20 m) coastal site about 1 km offshore on the Mediterranean coast, approximately 70 km north of Barcelona, Spain. Samples were collected monthly from January 2010 to February 2011 except in February and May 2010, when samples were collected twice a month (Table S1). Samples were sieved through a 200 μm mesh and transported to the laboratory within 2 h. Water temperature was measured *in situ* with a conductivity, temperature and depth probe, and light penetration/transparency was estimated by using a Secchi disk. Underwater profiles of PAR at the sampling site were measured with a multichannel filter radiometer (PUV-2500; Biospherical Instruments, San Diego, CA, USA). The concentration of inorganic nutrients was determined spectrophotometrically by using an Alliance Evolution II autoanalyzer according to standard procedures (Grasshoff *et al*., 1983). Chl *a* concentration was measured from acetone extracts by fluorometry from the total fraction (< 200 μm) and the fraction less than 3 μm. Abundances of cyanobacteria were measured by flow cytometry as described elsewhere (e.g. Alonso-Sáez *et al*., 2007).

Enumeration of total prokaryotes and AAP bacteria by epifluorescence microscopy

Subsamples were fixed with 2% formaldehyde and filtered on a 0.7 μm polycarbonate filter. Cells were stained with 4'6-diamidino-2-phenylindole (DAPI) and counted by using an Olympus BX51TF fluorescence microscope (Olympus, Barcelona, Spain) as described previously (Masín *et al*., 2006). Briefly, three fluorescence images were captured for each frame. First, total DAPI-stained bacteria were recorded in the blue part of the spectrum; Chl *a* autofluorescence was then recorded in the red part of the spectrum; and finally, both BChl *a*- and Chl *a*-containing organisms were recorded in the infrared part of the spectrum (> 850 nm). For each sample, 8–10 frames (400–600 DAPI-stained cells) were recorded and analysed semi-manually with the Cell F software (Olympus) to distinguish among heterotrophic bacteria, picocyanobacteria and AAP bacteria. To obtain net AAP bacterial counts, the contribution of Chl *a*-containing organisms to the infrared image was subtracted.

BChl a *concentration*

Two litres of water were filtered onto two 47 mm GF/F filters of 0.7 μm pore nominal size (Whatman, Barcelona, Spain) set in

sandwich to increase cell retention, and immediately frozen in liquid nitrogen and stored at −80°C until processed. All sample manipulations were carried out at room temperature and under dim light to prevent photo-oxidation of labile pigments. The filters were thawed and cut into small pieces using sterile scissors. Pigments were extracted in Ac : Met (7:2 vol : vol, Scharlau HPLC Grade, Eppendorf Ibérica S.L.U., San Sebastián de los Reyes, Spain) after a mild sonication for 30 s at 4°C using a B-Braun Labsonic 2000 disruptor (B.Braun Melsungen AG, Melsungen, Germany). Extracts were stored overnight at −30°C and then clarified by centrifugation at 10 000 r.p.m. for 15 min in a benchtop centrifuge (Eppendorf 5415-D, Eppendorf Ibérica S.L.U.). Pigment extracts were analysed by reversed-phase high-performance liquid chromatography according to Borrego and Garcia-Gil (1994). Chromatograms were recorded at 771 nm, and BChl *a* peak areas were translated to concentration after proper calibration of the system using a BChl *a* standard and the appropriate extinction coefficient (Porra, 2006).

Biomass collection and DNA extraction

About 10 l of surface seawater was sequentially filtered through a 3 μm pore-size polycarbonate filter (Poretics, GE Osmotics, Delft, the Netherlands) and a 0.2 μm Sterivex filter (Millipore, Madrid, Spain) using a peristaltic pump. The Sterivex units were filled with 1.8 ml of lysis buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0 and 0.75 M sucrose) and kept at −80°C until extraction was performed. Sterivex units were treated with lysozyme, proteinase K and sodium dodecyl sulphate, and the nucleic acids were extracted with phenol and concentrated in an Amicon 100 (Millipore), as described in Massana and colleagues (1997). DNA was quantified spectrophotometrically (Nanodrop, Thermo Scientific, Madrid, Spain), and a subsample was sent to pyrosequencing.

Sequence generation and processing

Pyrosequencing was performed by the Research and Testing Laboratory (Lubbock, TX, USA; http://www.researchandtesting.com/) using the bTEFAP method by 454 GL FLX technology as described previously (Dowd *et al.*, 2008). Partial amplification of the *pufM* gene (~ 245 bp fragments) was done by using the primer set pufMF forward (5′-TACGGSAACCTGTWCTAC-3′, Béjà *et al.*, 2002) and pufWAW reverse (5′-AYNGCRAACCACCANGCCCA-3′, Yutin *et al.*, 2005). The generated pyrosequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME; Caporaso *et al.* 2010) pipeline. QIIME takes all sequences from a pyrosequencing run and assigns sample IDs using a mapping file and the barcode assigned to each sample. After sample IDs were assigned, a step of sequence filtration was performed before denoising. Sequences were removed from the subsequent analyses if they were shorter than 100 bp, had an average quality score < 25 calculated in sliding windows of 50 bp, contained primer mismatches or had an uncorrectable barcode. The remaining sequences were run through Denoiser implemented in QIIME to reduce the impact of pyrosequencing errors (Reeder and Knight, 2010). Curated sequences were then grouped into OTUs or phylotypes using UCLUST (Edgar, 2010), with a minimum identity of 94%. A representative sequence from each phylotype was chosen by selecting the most abundant sequence within that particular phylotype and was used for taxonomic identification. A custom database based on the sequences reported by Yutin and colleagues (2007) and Lehours and colleagues (2010) was used for taxonomic identification, which was performed by using local Blast in QIIME. The Chao1 richness estimator and the rarefaction curves were also computed in QIIME. The OTU tables were rarefied at a sequence depth of 3500 because this was the lower number of sequences per sample obtained in the time series. Rarefaction curves were based on 1000 randomizations. A summary of the information regarding the sequence dataset can be found in Table S2. Sequence data have been deposited in the MG-RAST public database (http://metagenomics.anl.gov/) under ID 4527380.3.

Statistical analyses

To assess links between response and explanatory variables, we performed linear regressions and pairwise correlations among abundance, diversity data and physicochemical variables (Pearson's correlation coefficient) using the JMP software (SAS Institute, Cary, NC, USA). Variables were log-transformed when necessary. The results were thresholded at $P < 0.05$ and FDR-corrected for multiple comparisons (Pike, 2011). For community composition analyses, a dissimilarity matrix (Bray–Curtis) was constructed based on the relative abundance (square root transformed) of each OTU and visualized using nMDS. Mantel tests were conducted to find correlations between this matrix and a matrix of environmental variables (Euclidean distance matrix). The null hypothesis (H_o) of 'no relationship between matrices' was tested applying Spearman rank's correlation coefficient and 999 permutations. The environmental parameters included in the analysis were temperature, Secchi depth, day length, light at the surface measured as PAR, Chl *a* concentration (total and < 3 μm fraction) and the concentration of PO_4, NH_4, NO_2, NO_3 and Si. To investigate which combination of environmental variables were best related to AAP bacterial community assemblages, we used the BIOENV procedure (Clarke and Ainsworth, 1993), which finds the best subset of environmental variables so that its Euclidean distances have maximum rank correlation with community dissimilarity. Additionally, the ordination of the AAP abundance (total and relative abundance, BChl *a* concentration and pigment cell quota) and AAP diversity (OTU distribution) in relation to environmental data was examined by means of multivariate analyses using transformed data (log for environmental data and square root transformation for biological data). AAP abundance data were standardized by subtracting its mean and dividing by its standard deviation. We used dbRDA (Legendre and Anderson, 1999) in R (Vegan package) to find the environmental predictors that best explained the patterns of abundance and diversity of AAPs over time (Oksanen *et al.*, 2013).

Acknowledgements

We thank the several people involved in the BBMO for sampling and measuring the environmental variables and especially Vanessa Balagué for extracting the DNA. We are particularly grateful to Matthew T. Cottrell for preliminary train-

ing in *pufM* analyses and assistance with taxonomic identification of sequences. David L. Kirchman and Ramiro Logares-Haurie are acknowledged for additional support. This research was funded by projects ECOBAF (CTM2010-10462-E/MAR), SUMMER (CTM2008-03309/MAR) and STORM (CTM2009-09352/MAR) funded by the former Spanish Ministry of Science and Innovation.

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Supporting information

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

Fig. S1. Seasonal evolution of the concentration of Chl *a* (total and < 3 μm size fraction) measured at the Blanes Bay Microbial Observatory.

Fig. S2. (A) Molar ratio (in %) of BChl *a* to BChl *a* + Chl *a* and (B) absolute abundance of aerobic anoxygenic phototrophs (AAPs) bacteria plotted against the concentration of Chl *a*. BChl: Bacteriochlorophyll; Chl: Chlorophyll.

Fig. S3. Rarefaction curves of *pufM* OTUs delineated at 94% cut-off. The limit of rarefaction depth was defined at 3500 sequences, which was the lowest sample depth obtained in the time series at Blanes Bay Microbial Observatory.

Fig. S4. Non-metrical multidimensional (nMDS) analysis based on the OTU distribution in the monthly samples. The position of samples reflects how different AAP bacterial assemblages are from each other based on their distance in a two-dimensional plot. Distance is derived from Bray–Curtis similarity coefficients calculated from the square root transformed relative abundance of each OTU.

Fig. S5. Relative abundance of phylogroup C plotted against the concentration of BChl *a*.

Table S1. Values of physicochemical variables measured at the Blanes Bay Microbial Observatory during the study period.

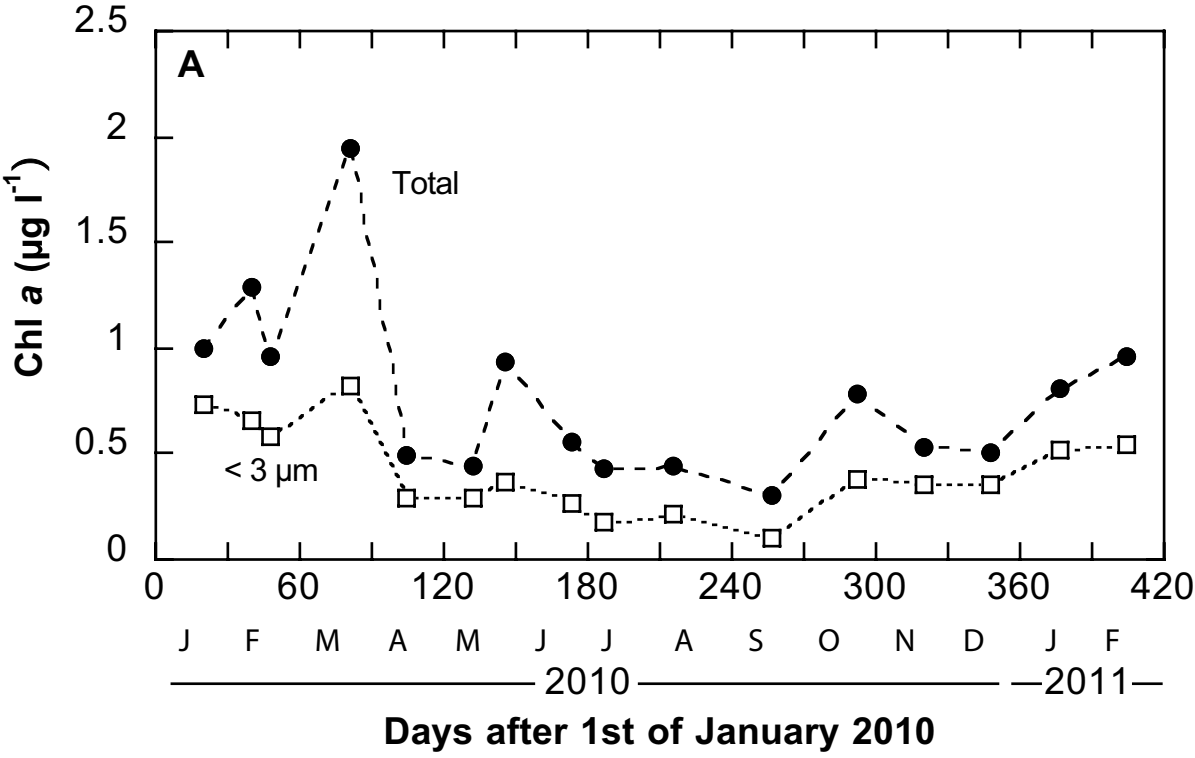
Table S1. Values of physicochemical variables measured at the Blanes Bay Microbial Observatory during the study period.

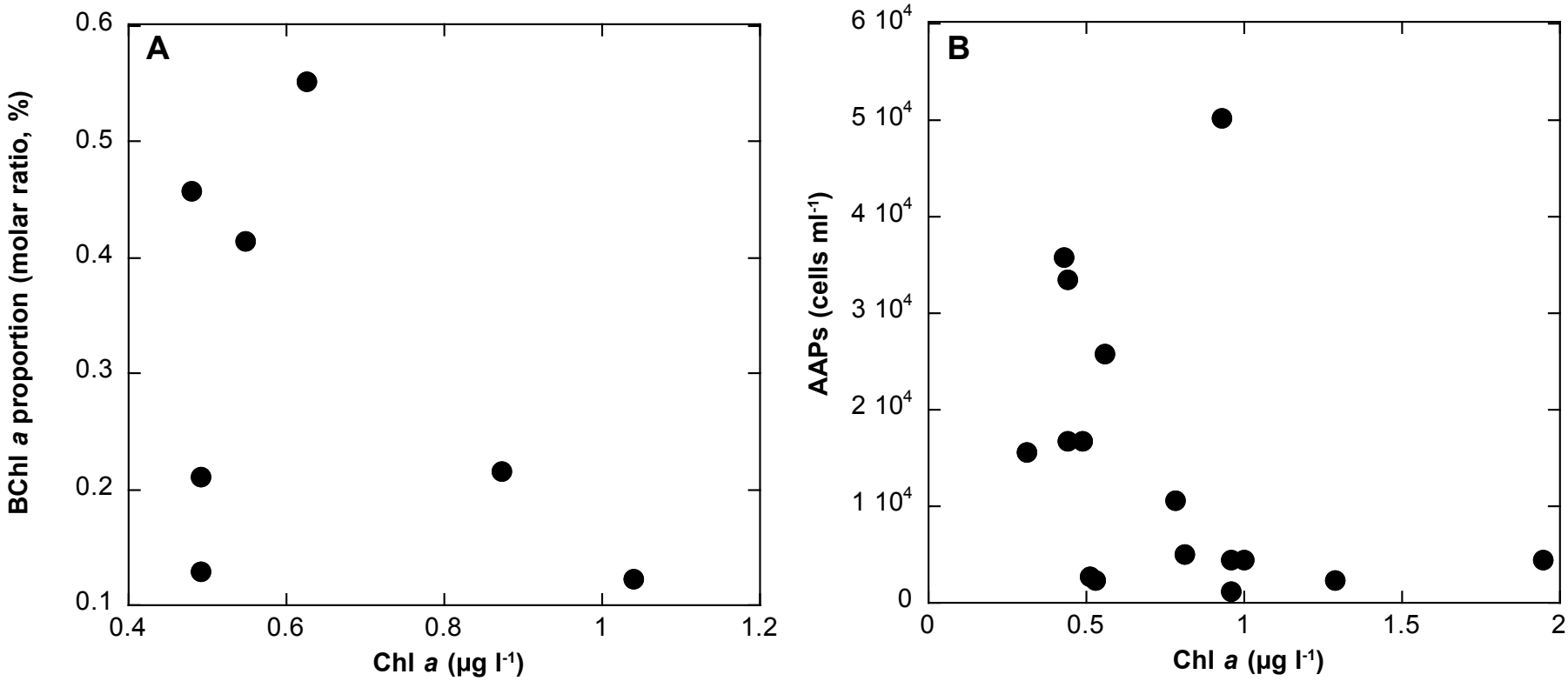
Date of sampling	Daylength (h)	Temp. (ºC)	Secchi (m)	Chl *a* ($\mu g\ l^{-1}$)	Chl *a* < 3 µm ($\mu g\ l^{-1}$)	PAR[b] (µmol photons $m^{-2}\ s^{-1}$)	$[PO_4]$ µM	$[NH_4]$ µM	$[NO_2]$ µM	$[NO_3]$ µM	[Si] µM
20 Jan 2010[a]	9.6	12.9	16.0	1.00	0.73	294	0.11	0.07	0.23	2.79	1.90
09 Feb 2010	10.3	12.5	5.0	1.29	0.66	38	0.47	0.10	0.16	2.53	1.70
17 Feb 2010[a]	10.7	12.3	10.0	0.96	0.58	110	0.11	0.45	0.24	nd	2.49
22 Mar 2010[a]	12.2	12.4	9.0	1.95	0.82	250	0.13	0.15	0.11	1.50	1.57
14 Apr 2010[a]	13.3	13.7	15.0	0.49	0.29	747	0.11	0.14	0.08	1.06	1.82
12 May 2010	14.4	15.7	10.0	0.44	0.29	nd	0.10	0.04	0.06	0.49	1.60
25 May 2010[a]	14.8	15.2	8.0	0.93	0.37	nd	0.09	1.15	0.14	0.43	1.56
22 Jun 2010[a]	15.2	19.2	15.0	0.56	0.27	nd	0.11	0.27	0.06	0.51	0.81
06 Jul 2010[a]	15.1	23.1	10.0	0.43	0.18	2240	0.12	0.06	0.01	0.08	0.30
03 Aug 2010[a]	14.4	24.1	15.0	0.44	0.21	646	0.15	0.12	0.07	0.21	0.78
14 Sep 2010[a]	12.6	24.4	22.0	0.31	0.10	1560	0.12	0.20	0.03	0.13	0.62
19 Oct 2010[a]	11.0	17.6	11.0	0.78	0.38	100	0.14	0.23	0.06	0.57	1.33
16 Nov 2010[a]	9.9	15.8	8.0	0.53	0.35	222	0.08	0.14	0.15	0.42	0.87
14 Dec 2010[a]	9.2	14.5	18.0	0.51	0.36	nd	0.18	0.12	0.25	1.46	1.52
12 Jan 2011	9.4	12.9	12.5	0.81	0.52	nd	0.11	0.62	0.28	1.54	2.14
08 Feb 2011	10.3	12.4	13.5	0.96	0.54	nd	0.11	0.56	0.24	1.41	2.03

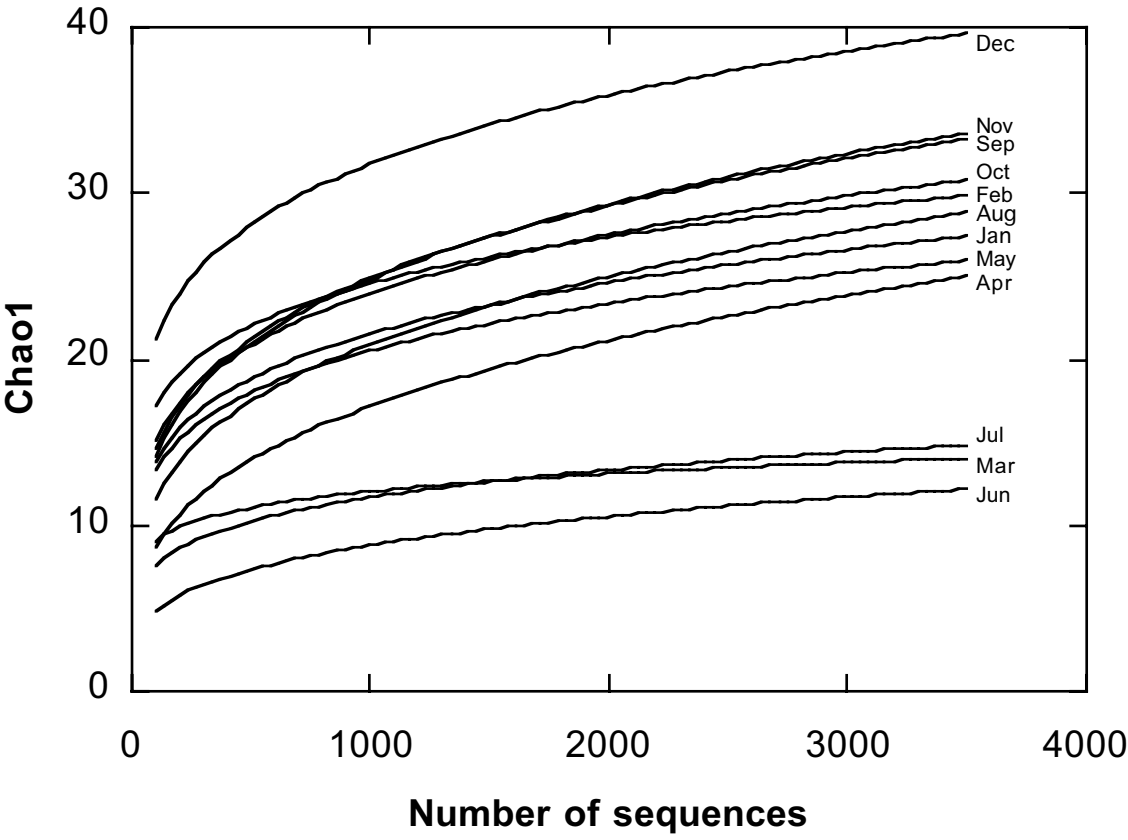
[a] Samples selected for pyrosequencing of *pufM* gene

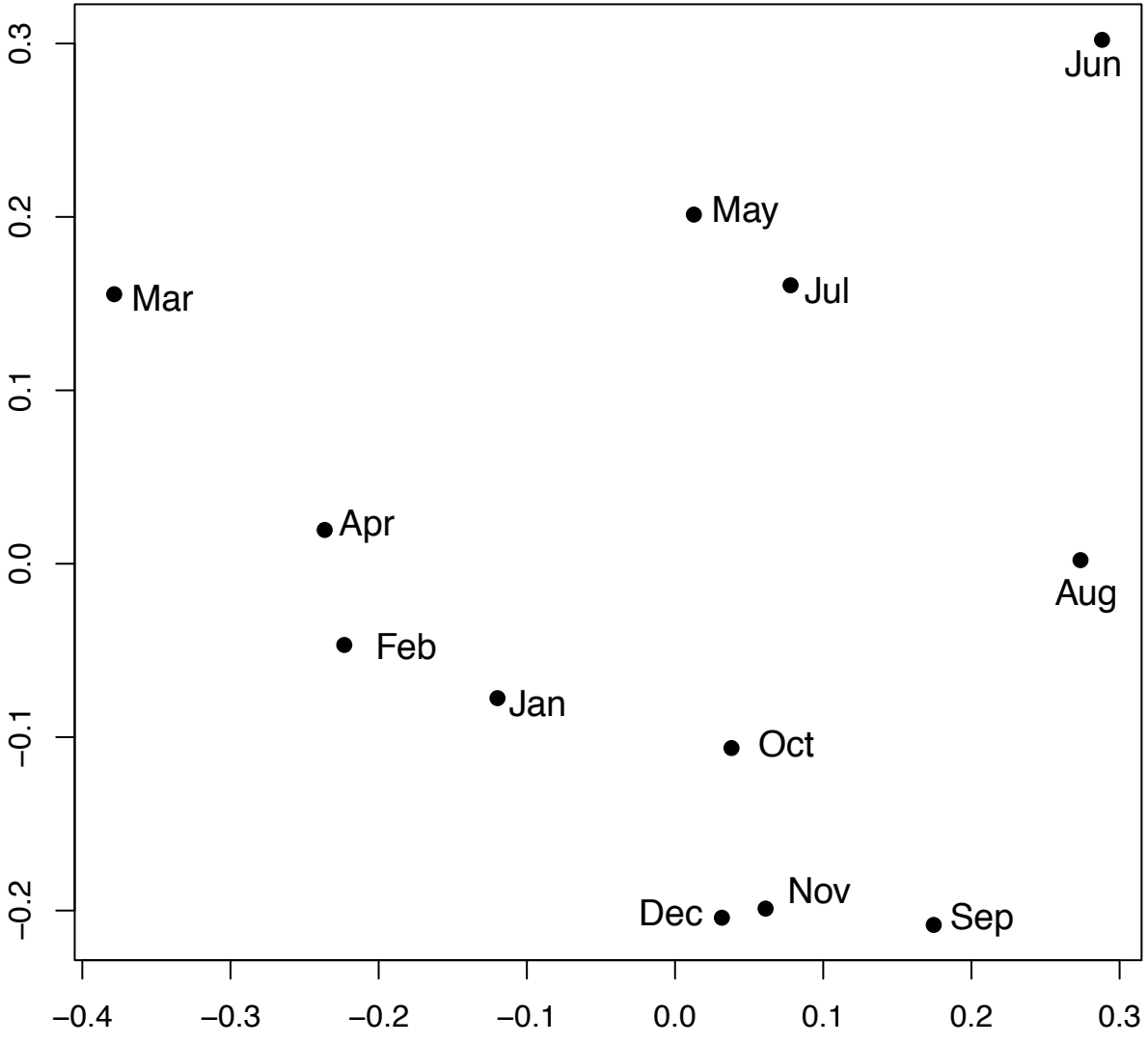
[b] PAR: photosynthetically active radiation measured at the surface at the time of sampling

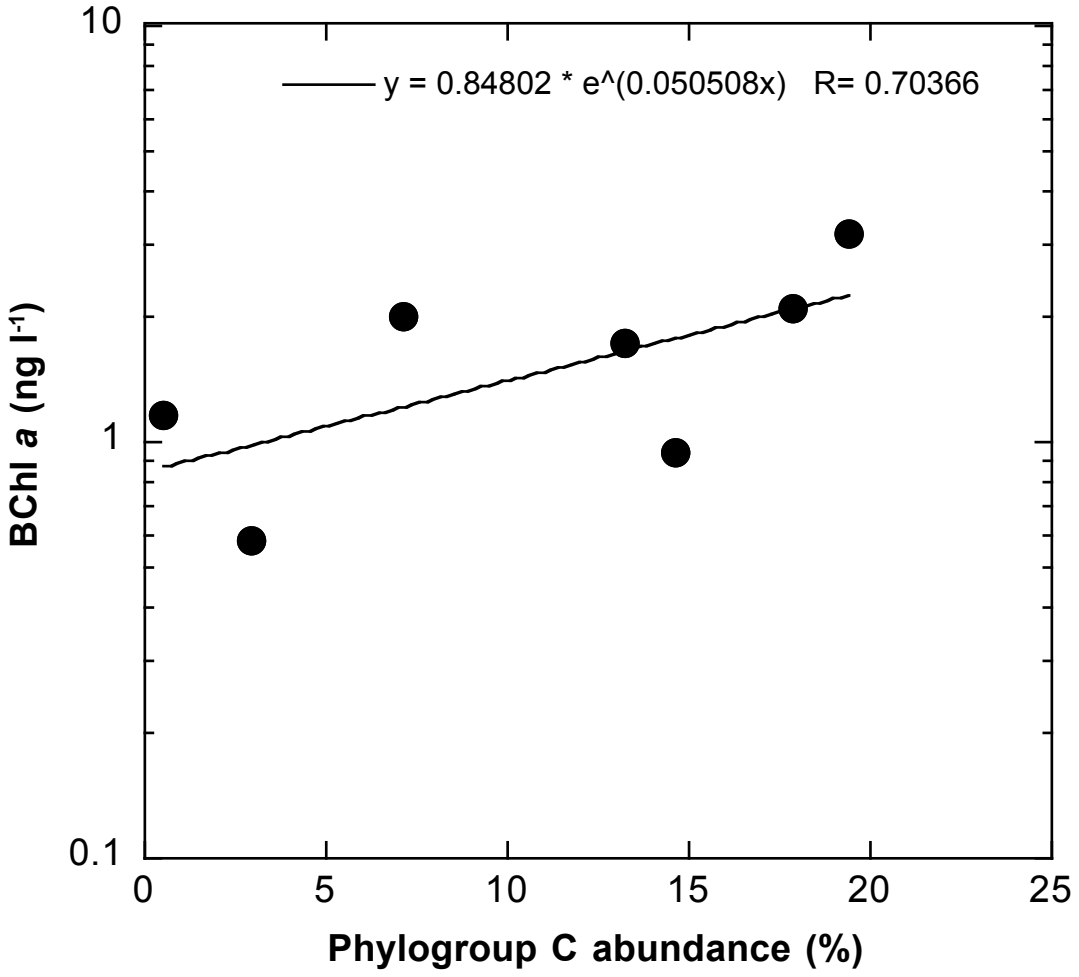
nd: non determined


A
Total
< 3 µm
Chl a (µg l-1)
2.5
2
1.5
1
0.5
0
0
60
120
180
240
300
360
420
J F M A M J J A S O N D J F
2010
2011
Days after 1st of January 2010


A
BChl a proportion (molar ratio, %)
0.6
0.5
0.4
0.3
0.2
0.1
0.4
0.6
0.8
1
1.2
Chl a (µg l-1)
B
AAPs (cells ml-1)
6 10^4
5 10^4
4 10^4
3 10^4
2 10^4
1 10^4
0
0
0.5
1
1.5
2
Chl a (µg l-1)


40
30
20
10
0
Chao1
0
1000
2000
3000
4000
Number of sequences
Dec
Nov
Sep
Oct
Feb
Aug
Jan
May
Apr
Jul
Mar
Jun


Jun
May
Jul
Mar
Apr
Aug
Feb
Jan
Oct
Dec
Nov
Sep
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3


y = 0.84802 * e^(0.050508x) R= 0.70366
BChl a (ng l-1)
10
1
0.1
0
5
10
15
20
25
Phylogroup C abundance (%)