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FROM THE COVER

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Quantifying long-term recurrence in planktonic microbial eukaryotes

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Abstract

How much temporal recurrence is present in microbial assemblages is still an unanswered ecological question. Even though marked seasonal changes have been reported for whole microbial communities, less is known on the dynamics and seasonality of individual taxa. Here, we aim at understanding microbial recurrence at three different levels: community, taxonomic group and operational taxonomic units (OTUs). For that, we focused on a model microbial eukaryotic community populating a long-term marine microbial observatory using 18S rRNA gene data from two organismal size fractions: the picoplankton (0.2–3 µm) and the nanoplankton (3–20 µm). We have developed an index to quantify recurrence in particular taxa. We found that community structure oscillated systematically between two main configurations corresponding to winter and summer over the 10 years studied. A few taxonomic groups such as Mamiellophyceae or MALV-III presented clear recurrence (i.e., seasonality), whereas 13%–19% of the OTUs in both size fractions, accounting for ~40% of the relative abundance, featured recurrent dynamics. Altogether, our work links long-term whole community dynamics with that of individual OTUs and taxonomic groups, indicating that recurrent and non-recurrent changes characterize the dynamics of microbial assemblages.

KEYWORDS
community assembly, diversity, marine protists, recurrence, seasonality, temporal patterns

1 | INTRODUCTION

Microbes are key players in most ecosystems, yet we have a limited understanding of which processes determine their community structure. Ecological theory predicts that community turnover can be explained by the combined action of four main processes: *selection*, *ecological drift*, *dispersal* and *speciation* (Vellend, 2010). Determining to what extent the combination of these four processes structures the communities is a current challenge for ecologists. This conceptual framework, which derives mostly from the study of plants and animals, has recently been applied to the microbial world (Hanson, Fuhrman, Horner-Devine, & Martiny, 2012; Logares et al., 2018; Nemergut et al., 2013; Stegen et al., 2013). In prokaryotes, the analysis of community turnover along multiple spatial gradients indicates that environmental selection seems to be the most important process structuring communities (Hanson et al., 2012; Lindström & Langenheder, 2012), although there is also evidence that stochastic processes (e.g., drift and dispersal) have a role (Logares et al., 2018; Ofiteru et al., 2010). In the last years, several studies have also analysed the temporal turnover of microbial communities (Bunse & Pinhassi, 2017; Fuhrman, Cram, & Needham, 2015). Given the relevance of marine microbes for the functioning of the biosphere (Falkowski, 2012), these temporal studies are fundamental to understand their response to environmental disturbances or global change.

Microbial community turnover operates at multiple time scales (hours, days, months) in response to different biological and

non-biological forces (Fuhrman et al., 2015). Depending on the time scale considered, different ecological processes or interactions can be investigated (e.g., daily sampling allows studying physiological microbial responses, whereas weekly or monthly sampling provides a view on phytoplanton blooms and other seasonal changes). Annual cycles driven by meteorological seasons in temperate zones have clear effects on terrestrial and marine ecosystems. In phytoplankton, annual cycles in light, temperature and nutrients are known to induce biomass dynamics, generally with one or two peaks per year, although cases with unclear patterns have also been observed (Winder & Cloern, 2010). Different studies, mostly on marine or freshwater prokaryotes, also indicate that other components of the plankton are correlated with meteorological seasons (Bunse & Pinhassi, 2017; Cram et al., 2015; Fuhrman et al. 2015). These studies have detected repeatable and predictable seasonal dynamics in prokaryotic communities. Furthermore, it has been proposed that environmental variables (e.g., day length, temperature and nutrients) seem to be governing this seasonality (Bunse & Pinhassi, 2017; Cram et al., 2015; Fuhrman et al., 2006; Galand, Gutiérrez-Provecho, Massana, Gasol, & Casamayor, 2010; Gilbert et al., 2012; Grubisic et al., 2017; Shade et al., 2007).

Yet, in order to gain a full understanding of microbial dynamics, unicellular eukaryotes need to be considered (Caron, Worden, Countway, Demir, & Heidelberg, 2009) as they may show different temporal dynamics than prokaryotes due to their structural and behavioural differences. Unicellular eukaryotes have a higher morphological and behavioural complexity than prokaryotes, including the capacity to ingest other organisms (Keeling & Del Campo, 2017; Massana & Logares, 2013). So far, marine and freshwater studies found evidence of seasonality in microbial eukaryotic communities populating surface waters, while seasonality was weaker in deeper waters. However, in contrast to prokaryotes, the analysed environmental variables only explained partially protistan seasonality (Countway, Vigil, Schnetzer, Moorthi, & Caron, 2010; Genitsaris et al., 2015; Kim et al., 2014; Piredda et al., 2017; Romari & Vaulot, 2004; Simon et al., 2015), suggesting that other abiotic or biotic variables may be driving this process.

Most studies on microbial dynamics have investigated whole community patterns, which are typically driven by abundant taxa. Still, investigating the temporal behaviour of individual taxa is important as each taxa may show different dynamics, for instance responding differently to cyclic abiotic environmental variation (i.e., selection), or presenting no response. In addition, focusing on individual taxa may highlight specific dynamics, with some species exhibiting smooth temporal fluctuations, while others display rapid fluctuations. Furthermore, as most microbial communities are composed by a few abundant taxa and a large number of low-abundant ones (Logares et al., 2014; Logares, Mangot, & Massana, 2015; Pedrós-Alió, 2006, 2012), it is relevant to explore microbial dynamics (i.e., the recurrence) across the abundance spectrum. Taxa within the rare biosphere can be metabolically active (Logares et al., 2015), respond to environmental change (Campbell, Yu, Heidelberg, & Kirchman, 2011; Lindh et al., 2015; Lynch & Neufeld, 2015) and present repeatable community assembly patterns (Alonso-Sáez, Díaz-Pérez & Morán, 2015). In temporal surveys, rare taxa can be assigned to one of three categories: (a) *seasonal* taxa that were rare at the time of sampling, but which are systematically recruited to the abundant community at particular times of the year, for example, some months, (b) *opportunistic* taxa that are generally rare but become exceptionally abundant for a short time (aka *conditionally rare* taxa; Shade et al., 2014) or (c) *permanently rare* taxa that never (within the limitations of the sampling design) become abundant (Logares et al., 2015).

Here, we analyse and quantify the recurrence in the long-term community dynamics of microbial eukaryotes inhabiting an oligotrophic coastal site in the Mediterranean Sea (Blanes Bay Microbial Observatory—BBMO; Gasol et al., 2016). Microbial eukaryotes from two size fractions were sampled monthly during 10 years, and their diversity was assessed by *Illumina* sequencing the 18S rRNA gene (V4 region). Our questions were as follows: Is community composition temporally recurrent? Can we find recurrence at specific taxonomic levels? Which factors drive community changes? Do rare taxa present seasonality? We developed a metric to detect and quantify temporal recurrence in specific taxa (hereafter *Recurrence Index*), which allowed us to identify seasonality in a substantial fraction of the picoeukaryotic (0.2–3 μm) and nanoeukaryotic (3–20 μm) taxa. As environmental variables followed a yearly cycle, we hypothesized that environmental selection would be a major force driving protist community seasonality. We observed that the overall system presented annual seasonality with two main recurrent configurations and that communities showed comparable similarity through time along the 10 sampled years.

2 | MATERIALS AND METHODS

2.1 | Study site and sampling

We carried out a monthly sampling during 10 years at the Blanes Bay Microbial Observatory (BBMO) located in the northwestern Mediterranean Sea (41°40′N, 2°48′E). This is a well-studied temperate oligotrophic coastal site that has relatively little human or riverine influence (Gasol et al., 2016). Surface water was sampled about 1 km offshore over a water column of 20 m depth, from January 2004 to December 2013. Water temperature and salinity were measured in situ with a CTD. Seawater was pre-filtered through a 200-μm nylon mesh, transported to the laboratory under dim light in 20-L plastic carboys and processed within 2 hr. Samples for determination of chlorophyll *a* concentration were filtered in GF/F filters, extracted with acetone and processed by fluorometry (Yentsch & Menzel, 1963). Inorganic nutrients (NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, SiO_2) were measured spectrophotometrically using an Alliance Evolution II autoanalyser (Grasshoff, Ehrhardt, & Kremling, 1983). In statistical analyses, these variables were standardized as *z*-scores, that is, deviations of the values from the global mean.

About 6 L of the 200-μm prefiltered seawater were sequentially filtered using a peristaltic pump through a 20-μm nylon mesh, a 3-μm

pore size polycarbonate filter of 47 mm diameter (nanoplankton fraction, 3–20 μm) and a 0.2-μm pore size Sterivex unit (Millipore, Durapore) (picoplankton fraction, 0.2–3 μm). Sterivex units and the 3-μm filters were stored at −80°C. DNA extractions were performed at the end of the sampling period using a standard phenol–chloroform protocol (Schauer, Balagué, Pedrós-Alió, & Massana, 2003), with a final step of purification in Amicon units (Millipore). Nucleic acid extracts were quantified in a NanoDrop-1000 spectrophotometer (Thermo Scientific) and stored at −80°C until analysis.

2.2 | 18S rRNA gene sequencing and bioinformatic analyses

The eukaryotic universal primers TAReukFWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3 (5′-ACTTTCGTTCTTGATYRA-3′; Stoeck et al., 2010) were used to amplify the V4 region of the 18S rRNA gene (~380 bp). PCR amplification and amplicon sequencing were carried out at the Research and Testing Laboratory (http://rtlgenomics.com/) using the Illumina MiSeq platform (2 × 250 bp paired-end sequencing). Illumina reads were processed following an in-house pipeline (Logares, 2017). Operational taxonomic units (OTUs) were delineated by clustering sequences at 99% similarity using UPARSE (Edgar, 2013) as implemented in USEARCH v8.1. Only OTUs present in at least three samples were retained. Taxonomy was assigned roughly at class level by BLASTN (Altschul et al., 1997) searches of OTU representative sequences against PR^2 (Guillou et al., 2013) and two additional in-house marine protist databases (available at https://github.com/ramalok) based on a collection of Sanger sequences (Pernice, Logares, Guillou, & Massana, 2013) and 454 reads (Massana et al., 2015). Metazoan, streptophyta and nucleomorphs OTUs were removed. Two OTU tables were generated: (a) the pico-nano-eukaryotic OTU table that had 120 samples of picoeukaryotes and 89 of nanoeukaryotes (samples from May 2010 to July 2012 and from four additional dates were discarded due to suboptimal sequencing) and (b) the picoeukaryotic OTU table that had 120 samples. To enable comparisons between samples, both OTU tables were randomly subsampled to the lowest number of reads in the study to ensure an equal number of sequences per sample using *rrarefy* function in vegan (Oksanen et al., 2008). The pico-nano-eukaryotic table was subsampled to 5,898 reads per sample (total of 14,771 OTUs), while the picoeukaryotic table was subsampled to 7,553 reads per sample (13,040 OTUs). Sequences are available at the European Nucleotide Archive with Accession No. PRJEB23788 (http://www.ebi.ac.uk/ena).

2.3 | Recurrence analyses and community dynamics

We developed a *Recurrence Index* (RI) to identify taxa presenting repeatable dynamics. To calculate the RI, we first applied the ACF (autocorrelation function) for each taxa, which evaluates the correlation between observations (i.e., relative abundances of taxa) separated by different time lags (i.e., months) to find repeating patterns. Then, we sum the absolute ACF values along the complete temporal series (RF) for each taxa. Afterwards, we randomized 1,000 times the taxa abundances, to compare the RF values against a null distribution, summed the absolute randomized ACF values and calculated the mean of the null model (RF_{random}), plus its 95% confidence intervals (CI). The RI was calculated as follows: $RI = RF/RF_{random}$. Based on empirical observations and simulated data sets, a given taxon was considered recurrent if (a) its RI was above a given threshold, here 1.20 for picoeukaryotes, and 1.15 for nanoeukaryotes, and if (b) its RF was significantly higher than RF_{random} (i.e., outside the CI and within the upper 2.5% probability). The developed code to calculate the RI is available at https://github.com/CaterinaRG/RecurrenceIndex, and it is also implemented in the ecological R-toolbox EcolUtils (Salazar, 2015) to facilitate its use. Recurrent picoeukaryotic taxa were further classified to reflect how long they persisted in the temporal series according to their changes in abundance. For this, we identified for each taxon the number of months with abundances above the 10-year mean. Taxa displaying >30 months above this mean were considered "*long*"-persistent, while those displaying less than 30 months were considered "*short*"-persistent.

To investigate temporal patterns in whole community dynamics, we computed the mean β-diversity (Bray–Curtis dissimilarities) for all pairs of samples taken *n* months apart (e.g., *n* ranging from 1 to 119 months for picoeukaryotes). In order to determine whether the observed β-diversity could be generated by random community dynamics, we calculated the Raup–Crick metric (Chase, Kraft, Smith, Vellend, & Inouye, 2011) using Bray–Curtis dissimilarities (hereafter RC_{bray}) for the picoeukaryotes, following Stegen et al. (2013). RC_{bray} compares the measured β-diversity against the β-diversity that would be obtained under random community assembly. For each pair of communities, the randomization was run 999 times and included only OTUs with >500 reads over the entire data set, to prevent biases due to possible undersampling. RC_{bray} values >+0.95 or <−0.95 are interpreted as significant departures from a stochastic community assembly, pointing to selection or dispersal-related processes, while RC_{bray} values between −0.95 and +0.95 point to stochastic community assembly (Chase et al., 2011).

2.4 | Community turnover and response of single OTUs to environmental variables

Non-metric multidimensional scaling (NMDS) analyses were based on Bray–Curtis dissimilarity matrices. In NMDS, differences between predefined groups were tested with ANOSIM (ANalysis Of SIMilarity; Clarke, 1993) using 1,000 permutations. To investigate the proportion of variation in community composition explained by environmental variables we used PERMANOVA (permutational multivariate analysis of variance). We also analysed the correlation between environmental variables and community dissimilarity using partial Mantel tests (Legendre & Legendre, 1998) as well as by fitting environmental variables onto the ordination space of the NMDS using the *envfit* function in vegan. Finally, we performed an IndVal analysis (INDicator VALues; Dufrene & Legendre, 1997) to identify OTUs associated to a specific season. OTUs with

statistically significant ($p < 0.05$) IndVal values >0.5 were considered season-specific, following Logares et al. (2013). The seasons considered were as follows: winter (including all samples from January to March), spring (April–June), summer (July–September) and autumn (October–December). Analyses were performed using functions implemented in the packages vegan (Oksanen et al., 2008), pvclust (Suzuki & Shimodaira, 2006) and labdsv (Roberts, 2016) within the R Statistical environment (R Development Core Team, 2008). Detailed analyses are available at https://github.com/CaterinaRG/Blanes_Temporal_Series.

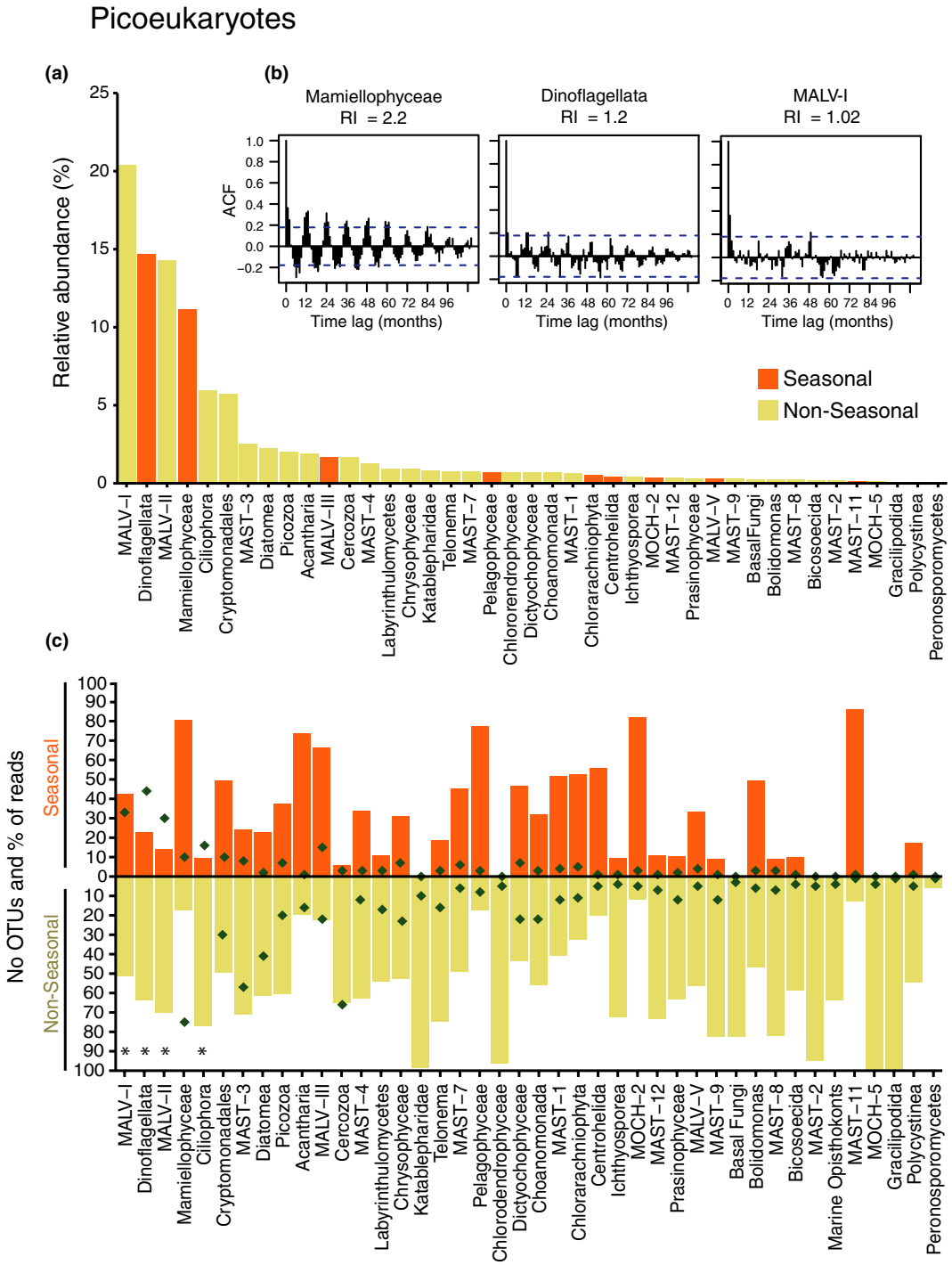

FIGURE 1 Taxonomic groups constituting the picoeukaryotic community in the BBMO and indications of their seasonality. (a) Mean relative abundances of groups accounting for >0.1% of the reads are shown. Orange bars indicate groups that exhibit seasonality. (b) Selected autocorrelation function (ACF) plots showing strong seasonality (Mamiellophyceae), moderate seasonality (Dinoflagellata) and no seasonality (MALV-I), together with their RI value. (c) Seasonal (above central line) and non-seasonal (below central line) signals for the main groups, showing the number of OTUs (dots) and the corresponding percentage of reads within the taxonomic group (bars). Only OTUs present in >10 samples were considered (* indicate more than 100 OTUs)

Correlations between individual OTUs and environmental variables of the same time point were done using extended local similarity analysis (eLSA; Ruan et al., 2006; Xia et al., 2011). The analysis used the subsampled picoeukaryotic OTU table, considering only OTUs present in at least 10 of 120 months, and nine environmental variables. eLSA was run with default normalization (a *z*-score transformation using the median and median absolute deviation) and *p*-value estimations under a mixed model that performs a random permutation test of a co-occurrence only if the theoretical *p*-values for the comparison are <0.05. Missing data for the environmental variables were interpolated linearly from adjacent months. Missing data were less than 0.9% in six variables and up to 5.8% in one case. We did not allow any time delay.

The temporal patterns of rare OTUs within the picoeukaryotic data set were analysed. OTUs with abundances per sample always <0.1% were considered permanently rare (Logares et al., 2015). To exclude the possibility that rare OTUs were aberrant sequence variants of abundant ones, we only analysed rare OTUs that had a similarity <97% with any abundant counterpart (3,095 rare OTUs were kept). We considered as temporally abundant those OTUs with a mean abundance >0.1% along the 10 years. Conditional tare taxa (i.e., opportunistic) were detected following Shade et al. (2014).

3 | RESULTS

3.1 | Recurrence at the taxonomic group level

Microbial eukaryotes present at the BBMO were very diverse, belonging to 63 taxonomic groups, most of them found both in the pico- and nanoeukaryotic fractions, but with different relative abundances in the two fractions (Figure S1). Picoeukaryotes were dominated by different alveolates (MALV-I, Dinoflagellata, MALV-II) and Mamiellophyceae, while many other groups displayed lower relative abundances (Figure 1a). Instead, nanoeukaryotes were dominated by Dinoflagellata, Diatomea and MALV-I (Figure 2a). An inspection of the dynamics of the different groups using ACF and our developed RI allowed us to identify recurrent groups following cycles of 1-year periodicity when RI >1.20 for picoeukaryotes and RI >1.15 for nanoeukaryotes (e.g., Mamiellophyceae and Dinoflagellata; Figure 1b) as well as temporally non-recurrent groups (e.g., MALV-I; Figure 1b). In picoeukaryotes, 13 of 58 tested taxonomic groups (accounting for 39% of the sequence abundance) exhibited recurrent seasonality (Figure 1a, Table S1). In particular, MALV-III and Mamiellophyceae, exhibited strong seasonality (RI >2.0), whereas the remaining 11 groups were moderately seasonal (1.2 < RI < 2.0). In nanoeukaryotes, 13 of 38 groups exhibited seasonal behaviour (representing 8% of the sequence abundance), with only MALV-III showing strong seasonality (Figure 2a, Table S2).

3.2 | Recurrence at the OTU level

Given that a recurrent behaviour at the taxonomic group level does not imply that all composing OTUs are recurrent and also that there can be recurrent OTUs in non-recurrent groups, we then explored the temporal behaviour of individual OTUs. We focused on OTUs that were present in at least 10 samples for picoeukaryotes and seven samples for nanoeukaryotes, to allow for at least one occurrence per year (accounting for 1,898 and 2,266 OTUs left, respectively, in each size fraction, representing in both cases ~90% of the sequence abundance). Based on the *Recurrence Index*, only 251 picoeukaryotic OTUs (13% of the OTUs representing 39% of the sequence abundance) were seasonal (see Figure S2 for examples of seasonal OTUs). As expected, recurrent (i.e., seasonal) groups generally contained a majority of recurrent OTUs or OTUs with higher sequence abundance than non-recurrent ones (Figure 1c). Exceptions to this trend (i.e., recurrent groups that had more non-recurrent OTUs) were Dinoflagellata, which had a RI just above the cut-off (RI = 1.23), and some low-abundance groups (e.g., MALV-V).

We also identified recurrent OTUs in groups that did not show seasonality (i.e., non-recurrent groups). In particular, Acantharia, Bolidomonas, Cryptomonadales, Dictyochophyceae, MAST-1 and MAST-10 displayed a higher sequence abundance belonging to recurrent OTUs than to non-recurrent ones (Figure 1c). Similar patterns were found in nanoeukaryotes (Figure 2b), where 423 OTUs (19% of the OTUs, accounting for 37% of the sequence abundance) were seasonal.

3.3 | Persistence in the system over time

Recurrent taxa (i.e., groups and OTUs) exhibited different strategies in terms of how long they persisted in the system based on their abundance fluctuation over time (see Methods). We defined "long-" and "short-persistence" taxa (see examples in Figure S3). Nine of the 13 recurrent picoeukaryotic groups (accounting for 99.5% of the recurrent sequence abundance) displayed long persistence, while the remaining four groups displayed short persistence (Table S1). Persistence was also analysed in the 251 recurrent picoeukaryotic OTUs. About 31.5% of them (79 OTUs) showed long persistence, while the remaining showed short persistence. Long-persistence OTUs featured high relative abundances and belonged to groups that also displayed the same behaviour. Furthermore, among the 89 rare OTUs that appeared in at least 10 samples, we detected nine that were moderately seasonal and displayed short persistence.

3.4 | Community seasonality

Besides the recurrence already found for different taxa, we aimed at identifying seasonality in whole community turnover. For that we calculated mean Bray–Curtis dissimilarities between samples separated by different time lags. Communities separated 12 months and their multiples (24, 36 and so on) showed the lowest dissimilarity (BC ~0.7), while those separated by 6, 18, 30 months and so on showed the highest dissimilarity in both pico- and nanoeukaryotes (BC ~0.9; Figure 3). Thus, both size fractions displayed annual seasonality. Furthermore, community differentiation did not increase with time, as Bray–Curtis distances between samples separated by

1 year were very similar to those from samples separated by several years. Interestingly, despite the observed cycling, community differentiation remained rather high during the 10 years, with averaged Bray–Curtis dissimilarities ranging from 0.7 to 0.9.

Further analysis of whole community turnover unveiled recurrent configurations throughout the 10 years for picoeukaryotic and nanoeukaryotic assemblages, which overall presented different compositions (Figure S4). Within each size fraction, winter and summer assemblages formed well-marked groups and differed clearly among them (ANOSIM test: R_{pico} = 0.72; R_{nano} = 0.71, $p < 0.001$; Table S3; Figure 4), while spring and autumn assemblages represented transient and less delineated states between winter and summer clusters (Figure 4). Interestingly, winter communities were more similar among themselves when compared to other intraseasonal variability (Figure S5). To investigate the existence of season-specific OTUs supporting these different assemblages, additional IndVal analyses for picoeukaryotes were run. We detected 56 season-specific OTUs, most of them associated to winter and summer communities (29 and 14 OTUs, respectively; Table S4).

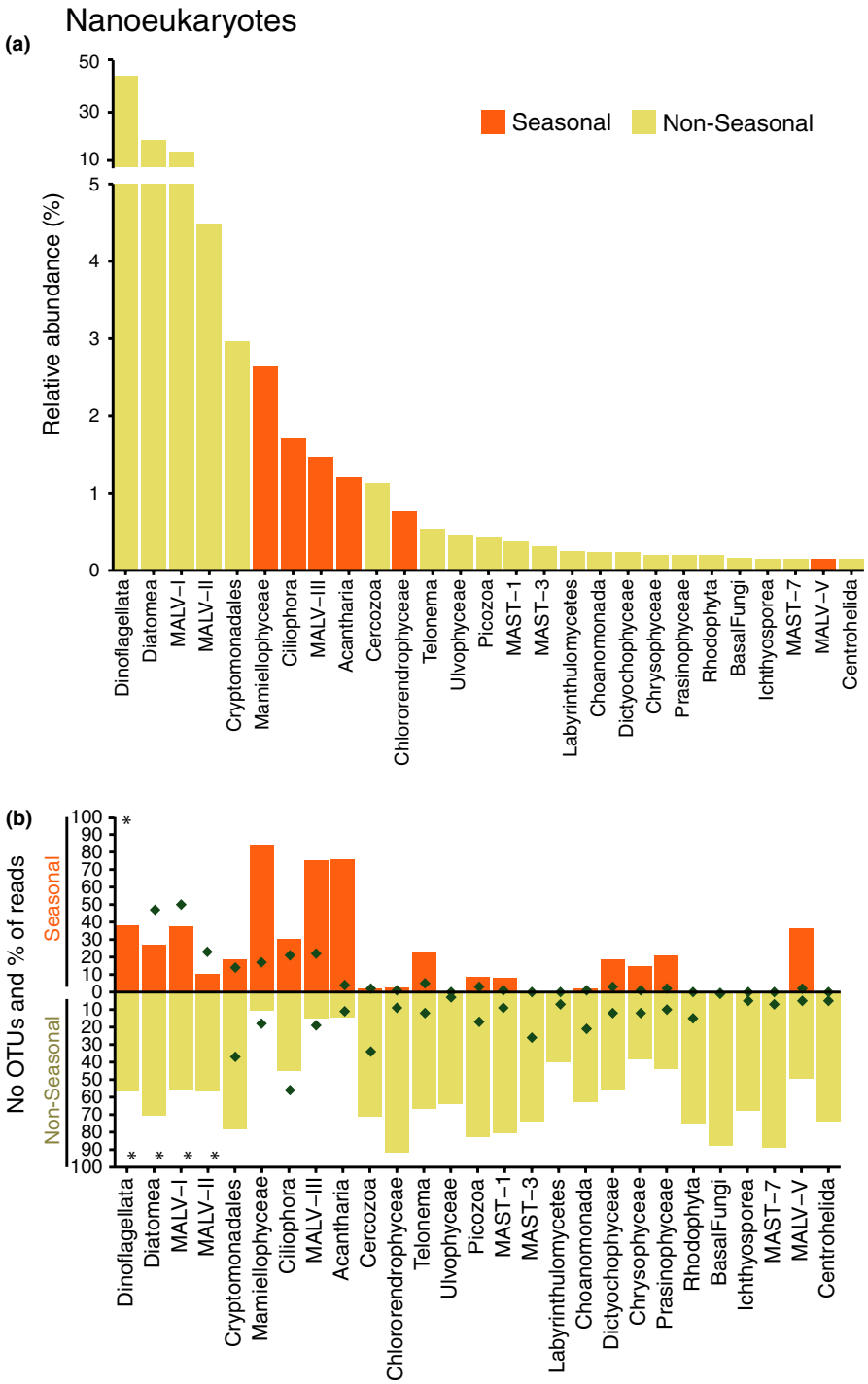

FIGURE 2 Taxonomic groups constituting the nanoeukaryotic community in the BBMO and indications of their seasonality. (a) Mean relative abundances of groups accounting for >0.2% of the reads are shown. Orange bars indicate groups that exhibit seasonality. (b) Seasonal (above central line) and non-seasonal (below central line) signal for the main groups, showing the number of OTUs (dots) and their percentage of reads within the taxonomic group (bars). Only OTUs present in >7 samples were considered (* indicate more than 100 OTUs)

Seasonal patterns at the whole community level were driven by the most abundant OTUs, which have a stronger weight than rare OTUs when using Bray–Curtis dissimilarities. Therefore, it was relevant to investigate whether the rare biosphere exhibited any seasonality. Within picoeukaryotes, 3,095 OTUs were considered permanently rare. Similar to what we found for the entire community, we observed two main rare sub-community states associated with winter and summer (Figure S6a), with spring and autumn communities representing transient states. We also found that the averaged Bray–Curtis values were most similar between rare communities separated by 1 year (and their multiples), and most different when separated by half a year (Figure S6b). Bray–Curtis values between rare assemblages were higher than the ones for the entire community (from 0.9 to almost 1), indicating that despite the evidence pointing to seasonality, the rare sub-communities were very different from year to year.

3.5 | Environmental selection and community turnover

The annual cyclic fluctuations of environmental conditions at the BBMO site are expected to exert cyclic abiotic selection on its microbiota. The days were longer in early summer; water temperature was maximal two months later, and inorganic nutrients, particularly nitrate, nitrite and silicate, peaked in winter (Figure S7). In order to determine which environmental variables exerted the strongest selection, they were fitted to NMDS separately for picoeukaryotes and nanoeukaryotes (Figure 4). In both cases, day length and temperature were the variables better correlated with community turnover (day length: $r^2 = 0.62/0.45$, temperature: $r^2 = 0.56/0.52$, $p < 0.001$ for pico- and nanoplankton; Table S5). Given that both day length and temperature covary, we carried out partial Mantel tests to determine the effect of each variable when removing the effect of the other. In

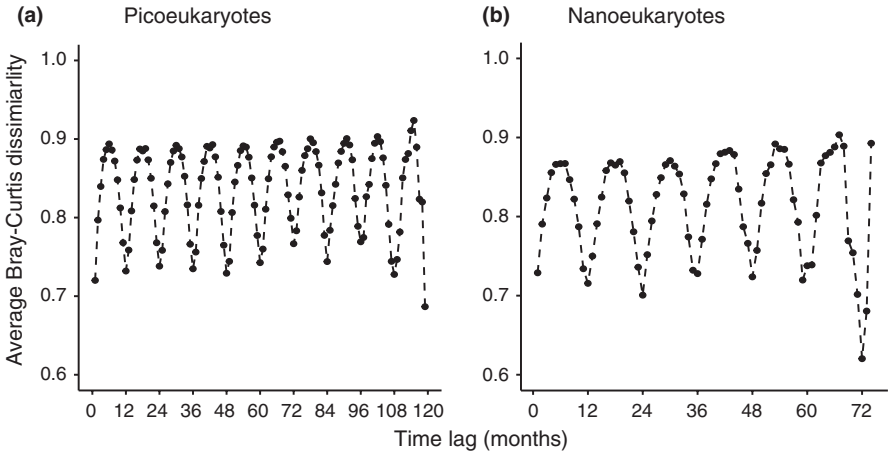

FIGURE 3 Interannual recurrence of communities of picoeukaryotes (a) and nanoeukaryotes (b), shown by the average Bray–Curtis dissimilarities of all pairs of communities separated by a given number of months, from 1 to 119 in (a) and from 1 to 74 in (b)

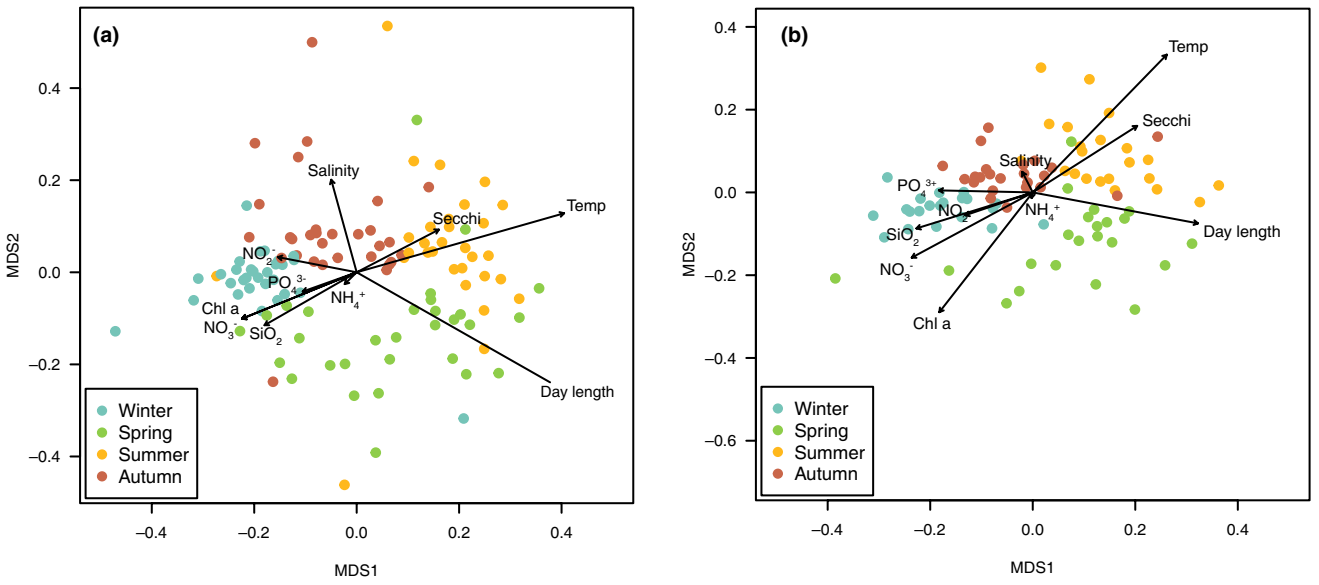

FIGURE 4 Similarity of protist communities (NMDS analysis) in monthly samples taken during 10 years at the BBMO for picoeukaryotes (a) and nanoeukaryotes (b). Arrows indicate the environmental parameters that are predominantly associated with community variance. Note that the community presents two main configurations across the 10 years that correspond to winter and summer months, while autumn and spring months represent transitional states (Temp: temperature; chla: chlorophyll *a*)

partial Mantel tests, both variables still displayed a moderate significant correlation with community composition (r = 0.44 temperature | day length, r = 0.40 day length | temperature, p = 0.001). The remaining environmental variables displayed weaker or non-significant correlations with community composition (Table S5). Additional analyses indicated that a large part of the community variance (~77% in PERMANOVA) was not explained by any of the measured environmental variables (e.g., inorganic nutrients, salinity nor chlorophyll a; Table S6). Using PERMANOVA tests, day length and temperature explained together only 16% of community variance ($p < 0.001$), a value that increased to 26% when performing the analyses only with recurrent OTUs. Even though environmental variables explained a minor part of community turnover, results from the RC_{bray} analyses indicated that 93% of the measured β-diversity differed from what it would be expected under random community assembly, suggesting the action of environmental selection. Specifically, 86% of the β-diversity comparisons presented $RC_{bray} > +0.95$ (i.e., more different than chance), and 7% $RC_{bray} < -0.95$ (i.e., less different than chance). Furthermore, by using the individual correlations obtained with the eLSA analyses, we detected 2,375 OTUs, which tended to be abundant, that were positively or negatively correlated with the analysed environmental variables (Table S7). Specifically, 4% of the OTUs, representing ~47% of the total sequence abundance, were positively or negatively correlated with temperature or day length, indicating they are at least partially structured by abiotic selection.

3.6 | Diversity patterns

Based on rarefaction curves, most individual samples (~80%) were close to richness saturation (Figure S8). We also found richness saturation when analysing the entire data set of pico- and nanoeukaryotes (Figure S9a), indicating that we recovered most of the diversity present in the BBMO throughout the 10 years. In accumulation curves, richness increased rapidly until approximately the 60th sample (i.e., month of sampling), with subsequent samples contributing with few new additional OTUs (Figure S9b). Finally, alpha diversity presented clear temporal trends. For both size fractions, averaged richness and Shannon indices were highest during autumn and winter months and significantly lower during spring (Figure 5, $p < 0.05$ Wilcoxon test). No statistical differences in alpha diversity indices were found between pico- and nanoeukaryotes, neither when comparing all samples together nor when comparing each of the seasons separately (Wilcoxon tests, $p > 0.05$).

4 | DISCUSSION

Long-term recurrent patterns in microbial community dynamics have been reported previously (Bunse & Pinhassi, 2017; Fuhrman et al., 2015). Yet, earlier studies have provided limited information on the proportions of the community that display recurrent dynamics (Winder & Cloern, 2010). In addition, few previous studies, mostly focused on bacteria, have tried to understand microbial seasonality in terms of the dynamics of the composing taxonomic groups and species (Cram et al., 2015; Gilbert et al., 2012). Here, using data from one of the longest protistan time series analysed to date, we determined the proportions of the community (OTUs and taxonomic groups) that present recurrent versus non-recurrent dynamics. Furthermore, using a newly developed RI, we explore how the recurrent dynamics of OTUs and taxonomic groups translate into repeatable community patterns.

4.1 | Quantifying community seasonality and recurrence

We introduce the new RI to quantify the recurrence of a given taxon within a temporal series. In our 10-years study, we found recurrence in only 13% and 19% of the pico- and nanoeukaryotic OTUs, respectively (yet accounting for 39% and 37% of the abundance). We also identified recurrence at the taxonomic group level, as in the case of Mamiellophyceae and MALV-III that featured strong seasonality. As expected, most OTUs within these groups were seasonal, indicating that seasonality was a conserved class-level trait. The strong seasonality of Mamiellophyceae has already been reported, with species of this group showing a preference for low temperatures in coastal areas (Foulon et al., 2008). In contrast, the strong seasonality of MALV-III is intriguing, given that nothing is known on the ecology of this group (Guillou et al., 2008). Interestingly, recurrent OTUs within non-recurrent taxonomic groups were also found, and this was explained by different OTU dynamics within the groups. For instance, we detected some OTUs of MALV-II with a preference for low temperatures and others for high temperatures.

In most cases, recurrent groups and OTUs displayed one peak of abundance per year despite our RI can detect other patterns of abundance dynamics (e.g., two peaks per year, one peak every 2 years and so on). In a study using chlorophyll *a* as a proxy of phytoplankton biomass in 125 time series studies, Winder and Cloern (2010) found that having one peak per year was the most common pattern among phytoplankton (detected in ~48% of the time series analysed). Thus, OTUs having a single peak of abundance per year could be the norm across microbial assemblages in surface ocean temperate waters. This does not have to be the case for the whole water column, as it has been observed that seasonality is attenuated at deeper waters (Hernández-Ruiz et al., 2018; Kim et al., 2014).

Our results demonstrate that pico- and nanoeukaryotic communities displayed annual seasonality throughout the 10 years. Furthermore, as shown by the Raup–Crick analyses, we found that β-diversity was significantly different from chance, indicating that community dynamics were not random (Chase et al., 2011; Stegen et al., 2013). Interestingly, there was little interannual variability in the amount of community differentiation over the 10 years. This suggests seasonality for at least some abundant taxa, absence of large immigration and similar selection throughout the 10 years. A different result was found by Chow et al. (2013) and Cram et al. (2015) in surface marine prokaryotic communities, as dissimilarity increased slightly through the 10 years sampled. Both in the previous studies

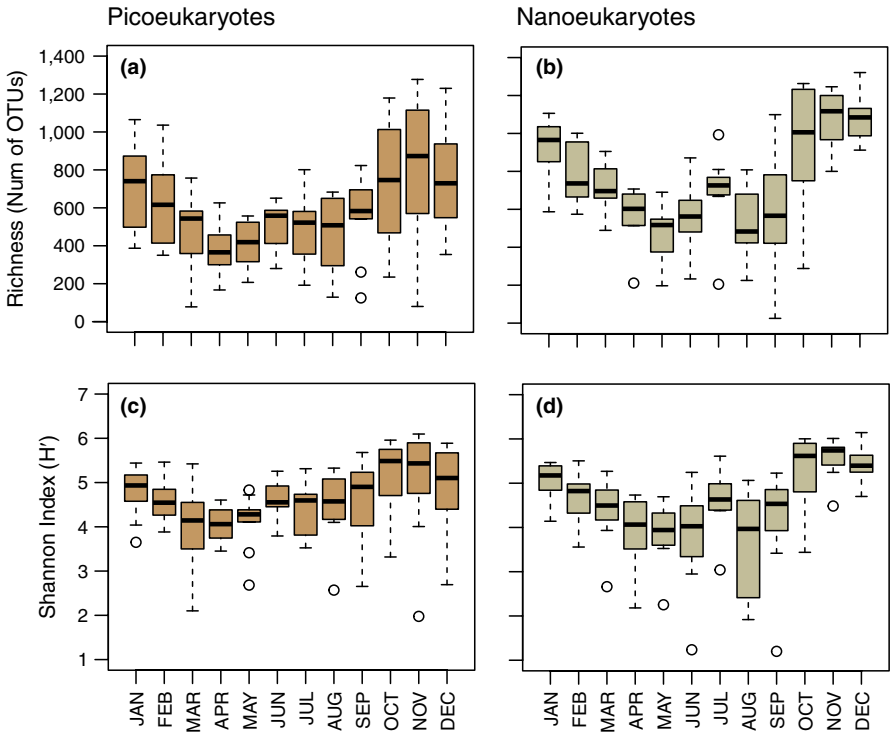

FIGURE 5 Monthly variation along 10 years of alpha diversity in BBMO protist communities. Boxplots display the temporal variability of the richness (a, b) and Shannon indices (c, d) for the picoeukaryotes and nanoeukaryotes. Note that the studied communities present a systematic annual oscillation between low and high richness

and in our own data, Bray–Curtis dissimilarities between any pair of samples taken during the same month but in different years were relatively high (ranging between 0.6 and 0.8), indicating that seasonal communities are far from identical during their annual dynamics. Similar Bray–Curtis values (0.7–0.9) were found in shorter temporal studies in freshwater systems (Simon et al., 2015), suggesting that there could be a regular dissimilarity value for eukaryotic assemblages. Our findings indicate that there is not a unique community state to return, allowing for multiple community configurations that likely reflect historical processes or ecological drift (Chase, 2003). But, despite this variability, we observe recurrent pattern every 12 months.

Seasonal taxa presented different persistence times in the system, which could point to different ecological strategies. Short-persistence taxa may reflect a fast growth under the presence of specific resources and a fast decrease in their abundance due to a high predation, competitive pressure or viral mortality. On the other hand, long-persistence taxa could reflect slow growth accompanied with relatively low predation or competition pressures, thus maintaining taxa in the system for relatively longer periods. Long-persistence taxa may also have their growth rates tightly associated with specific environmental variables (e.g., temperature), with their abundances thus reflecting environmental selection. We have also found that 1.6% of the OTUs were conditionally rare taxa, a magnitude coinciding with that observed by Shade and Gilbert (2015) for prokaryotes. These are considered opportunistic OTUs that can sporadically increase in their abundance triggered by environmental cues.

About 24% of the OTUs were permanently rare (i.e., they were never abundant throughout the 10 years), and nine of them showed seasonality. Similar patterns were observed in the rare biosphere of coastal marine bacterioplankton (Alonso-Sáez et al., 2015). The permanently rare community mirrored the seasonality of the whole community, suggesting that similar processes drive community turnover in abundant and rare species. Yet, the overall dissimilarity among samples from the rare sub-community was higher than in the whole community, suggesting a larger stochasticity in rare community dynamics. Our observations support the idea that microbial communities include rare species that are metabolically active, as suggested by their seasonality, but never become abundant as they may be adapted to a low abundance life (Logares et al., 2015) or subjected to high mortality rates.

4.2 | Community and OTU response to environmental variation

Similar to bacterioplankton (Bunse & Pinhassi, 2017; Fuhrman et al., 2015), we hypothesized that environmental selection would be a major force driving protist community seasonality. Indeed, the grouping of protist assemblages into winter and summer states points to environmental selection as a driving force. However, the measured environmental factors explained a minor fraction of community variability along the 10 years, which agrees with previous studies showing that abiotic fluctuations explained only 22%–30% of protist community seasonality (Genitsaris et al., 2015; Simon et al., 2015). These values are lower to those found in prokaryotic communities (i.e., 40% in El-Swais, Dunn, Bielawski, Li, & Walsh, 2015). A possible explanation is that environmental selection has a different importance in microbial eukaryotic and prokaryotic plankton (Logares et al., 2018). But also, biotic factors not considered here, such as prey abundance or viral and

predatory mortality, could be explaining changes in community composition. Interestingly, we found that a small fraction of picoeukaryotic OTUs (4%), yet representing 47% of the total abundance, correlated positively or negatively with temperature and day length. It has been observed that temperature is an important factor structuring bacterioplankton communities across space (Sunagawa et al., 2015; Zinser et al., 2007) and time (Chow et al., 2013; Fuhrman et al., 2006). It seems that environmental selection associated with variables fluctuating between summer and winter could be acting on a subset of taxa in the community. Thus, differential responses of individual OTUs to environmental variation may partially explain the low correlation between whole community and environmental parameters, as different OTU dynamics may cancel out or generate noise in whole community analyses. It is also possible that there is a time lag between environmental variation and community response, which would not be captured by our analyses.

Furthermore, the distinct summer and winter community states suggest that the intensity of environmental selection may change throughout the year, being stronger in summer and winter and weaker in spring and autumn. This is consistent with the larger number of OTUs exclusively associated with summer and winter months as compared to those associated with autumn and spring. In addition, the fact that winter communities were the most similar along the 10 years indicate a stronger environmental selection during this period compared to summer. Autumn and spring may be intrinsically more variable seasons, with episodic rains and less constant temperatures/irradiances, and this is why they appeared as transitional states. Communities featuring two main states and two transitional states have been reported for Atlantic Ocean bacterioplankton (Ward et al., 2017), while the analysis of protist dynamics during 2.5 years in the English Channel revealed three seasonal states corresponding to summer, autumn–winter and spring (Genitsaris et al., 2015). Overall, the existence of recurrent states associated with different seasons suggests that environmental selection drives, to certain extent, community dynamics. Ecological interactions (biotic selection) can also have a role in community turnover, yet it has been suggested that they affect dynamics in periods ranging from days to weeks (Bunse & Pinhassi, 2017).

Despite the patterns found, when using 18S rRNA genes to assess community structure we need to be aware of the possible limitations of the approach. There might be PCR biases (Wintzingerode, Göbel, & Stackebrandt, 1997), by which some phylotypes can be amplified preferentially, whereas others may remain undetected. For instance, the primers we used (Stoeck et al., 2010) underestimate the phytoplankton group Prymnesiophyceae, known to abundant in marine waters in general and in the BBMO in particular (Unrein, Gasol, Not, Forn, & Massana, 2014). A slight modification of the reverse primer has been proposed to solve this problem (Balzano, Abs, & Leterme, 2015). When investigating microbial eukaryotes, another potential bias may be caused by the large variation of the rDNA operon copy number among taxa, ranging from few copies per cell in small algae to thousands copies in some dinoflagellates (Zhu, Massana, Not, Marie, & Vaulot, 2005). In our study, MALV-I and MALV-II represent a large fraction of the total sequence data probably due to a very high rDNA copy number per cell (Massana et al., 2015). Despite this, as MALV-I and MALV-II were relatively constant through time in our study, they likely had a small influence in the changes of the relative abundance of the other taxa, so the seasonality detected here would not be affected. Additional biases may be introduced during size fractionation, as DNA from large organisms may leak to smaller fractions. This probably explains the presence of dinoflagellates, ciliates and diatoms in the picoeukaryotic fraction. Besides all the potential biases, the robust seasonality found in our data, which is consistent with previous studies, contributes to understand the seasonality of microbial OTUs, taxonomic groups and communities.

In sum, our work contributes to start linking the temporal dynamics observed in whole microbial communities with that of their composing taxa. We found that microbial plankton communities include taxonomic groups and species with recurrent as well as non-recurrent dynamics. In particular, our quantifications estimate that 13% of the picoeukaryotic and 19% of the nanoeukaryotic OTUs, representing ~40% of the sequence abundance in both cases, were seasonal or recurrent. Thus, we did not find recurrence for most taxa in our system. To our knowledge, this is the first time that recurrent and non-recurrent patterns are quantified at the OTU and taxonomic group level in planktonic microbial communities. Future studies need to determine whether these patterns characterize microbial plankton communities across temperate zones.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

C.R.G., R.L. and R.M. designed the study. V.B., I.F. and A.R. collected the data and extracted the DNA. J.M.G. and R.M. sustained the long-term sampling at Blanes Bay. J.M.G. and E.G. provided the environmental and ecological context. A.K.K. did the eLSA analysis. C.R.G., R.L. and R.M. analysed the data, interpreted the results and discussed and wrote the manuscript. All authors contributed substantially to manuscript revisions.

DATA ACCESSIBILITY

The sequence data are publicly available at the European Nucleotide Archive with Accession No. PRJEB23788.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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