BRIEF REPORT

Annual recurrence of prokaryotic climax communities in shallow waters of the North Mediterranean

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Abstract

In temperate coastal environments, wide fluctuations of biotic and abiotic factors drive microbiome dynamics. To link recurrent ecological patterns with planktonic microbial communities, we analysed a monthly-sampled 3-year time series of 16S rRNA amplicon sequencing data, alongside environmental variables, collected at two stations in the northern Adriatic Sea. Time series multivariate analyses allowed us to identify three stable, mature communities (climaxes), whose recurrence was mainly driven by changes in photoperiod and temperature. Mixotrophs (e.g., Ca. *Nitrosopumilus*, SUP05 clade, and Marine Group II) thrived under oligotrophic, low-light conditions, whereas copiotrophs (e.g., NS4 and NS5 clades) bloomed at higher temperatures and substrate availability. The early spring climax was characterised by a more diverse set of amplicon sequence variants, including copiotrophs associated with phytoplankton-derived organic matter degradation, and photo-auto/heterotrophic organisms (e.g., *Synechococcus* sp., Roseobacter clade), whose rhythmicity was linked to photoperiod lengthening. Through the identification of recurrent climax assemblages, we begin to delineate a typology of ecosystem based on microbiome composition and functionality, allowing for the intercomparison of microbial assemblages among different biomes, a still underachieved goal in the omics era.

INTRODUCTION

Marine planktonic prokaryotes are pivotal players in the global cycling of elements due to their capability to produce, degrade, and mineralise organic matter (Gasol & Kirchman, 2018). In the sunlit portion of the ocean, the compositional dynamics of these microbes undergo seasonal variations with repeatable annual patterns (Bunse & Pinhassi, 2017) driven by the physical, chemical, and biological features of the environment (Yeh & Fuhrman, 2022). In addition to these patterns, short-term events such as phytoplankton blooms (Needham

et al., 2018), jellyfish blooms (Tinta et al., 2021), or changes in hydrological dynamics (Hernando-Morales et al., 2018) drive alternations in the community structure by favouring the growth of some taxa at the expense of others. On the other hand, long-term phenomena such as prolonged drought, warming, or ocean acidification can also affect the ecological success of given taxa, thus modifying the overall temporal successions of Bacteria and Archaea (Manna et al., 2021; Tsiola et al., 2023).

The concept of 'climax' dates back to the beginning of the XX century and refers to a biological community that has reached a mature, self-maintaining steady state (Clements, 1916 and references therein). On the

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contrary, 'successions' are the processes of change within a community induced by environmental variations. Therefore, a succession is supposed to continue until the species combination best suited to a given environment is established, that is, until a climax is reached (Drury & Nisbet, 1973). However, the definitions of these two processes or conditions have been revised and elaborated many times. While attempting to review different facets of the climax concept, Whittaker (1974) put the plankton in a corner of aclimaxes, where climax and succession are not distinguishable, because the generation times of dominant taxa are short relative to environmental changes. Similarly, Margalef (1963) suggested that plankton communities are kept under immature conditions by unpredictable abiotic factors (dispersal, turbulence) and that random components, rather than deterministic ones, are pivotal in their dynamic composition. It must be noted, however, that marine microbial ecology in those years was in its infancy and DNA-based approaches for studying biodiversity nowadays allow us to depict previously unnoticed ecological patterns in the dynamics of marine microbiomes.

Taking advantage of such novel approaches, Bunse and Pinhassi (2017) reviewed the typical annual succession of prokaryotic taxa in temperate marine systems. The spring phytoplankton bloom determines the onset of a community dominated by copiotrophic Flavobacteria and by members of the Roseobacter group (Rhodobacteraceae). The summer stratified period is often characterised by microbial assemblages dominated by oligotrophs and small-sized phototrophs, such as SAR86, SAR11 clades, and Cyanobacteria, although pronounced divergence from this scheme can characterise different sites and oceanic regions. The fall transition from summer to winter has been reported to be dominated by specific populations of Flavobacteria, SAR11, and Planctomycetes, thus pointing to microbial assemblages characterised by multiple trophic modes. Finally, a widespread feature of wintertime prokaryotic assemblages is the dominance of Archaea, namely members belonging to *Candidatus Nitrosopumilus* and the Marine Group II, able to use energy sources other than pure heterotrophy (Bunse & Pinhassi, 2017). However, it has recently been shown that even (taxonomically) closely related prokaryotes may show highly diverse environmental preferences, indicating that the use of broad taxonomic groups to depict spatio-temporal biodiversity gradients can be misleading (Auladell et al., 2022).

Given their highly dynamic, yet recurrent, nature in terms of physico-chemical as well as biological variability, coastal systems offer the unique opportunity to study the effects of environmental rhythmicity on prokaryotic community structure by searching for recurrence, successions, and climaxes. Exploiting these natural laboratories is therefore of pivotal importance to

set a baseline of knowledge to understand future modifications of these delicate environments. To investigate key patterns in the temporal dynamics of prokaryotes in temperate Mediterranean waters, we analysed a monthly-sampled 3-year time series of 16S rRNA gene amplicon sequencing data at two coastal stations. We specifically aimed at answering the following questions (i) Can we identify recurring climax assemblages? (ii) What are the drivers structuring these assemblages? and (iii) What is their role in the functioning of coastal ecosystems?

EXPERIMENTAL PROCEDURES

Study area and sampling design

The Gulf of Trieste (GoT) is a shallow (<25 m), river-influenced embayment in the northernmost sector of the Adriatic Sea. The main freshwater inputs come from the Isonzo and the Timavo rivers in the northern area (mean flows between 1998 and 2008 = 82 and 27 $m^3 s^{-1}$, respectively) (Figure 1; Cozzi et al., 2012). Atmospheric processes, discharge of continental waters, and the oceanographic circulation cause large variations in salinity (29–38.5), temperature (4–29.2°C), and dissolved organic carbon loads (49.8–162.3 μM); furthermore, the water column is stratified in summer and fully mixed in winter (Malačič et al., 2006; Manna et al., 2021). Throughout the year the system oscillates between oligo- to mesotrophic conditions; phytoplankton dynamics are characterised by the prevalence of *Synechococcus* during the nutrient-depleted summer season (Manna et al., 2021) and the onset of eukaryotic blooms dominated by haptophytes and diatoms during spring and autumn, respectively (Flander-Putrle et al., 2022). Likewise, early studies based on DNA fingerprinting and cloning techniques showed seasonal variations of bacterial communities at several locations and depths (Celussi & Cataletto, 2007; Tinta et al., 2015).

For this study seawater samples were collected at two sites included in the International Long-Term Ecological Research Network (LTER): station C1 (45°42′2.99″N; 13°42′36.00″E) is located along the eastern coast of the basin, is distant 0.2 km from the shore and is 17 m deep; station BF (45°32′55.50″N; 13°33′02.52″E) is 2.3 km distant from the southern coast and is 23 m deep (Figure 1). Both stations were sampled monthly from October 2018 to December 2021 (Table S1) using 5-L Niskin bottles at the surface (ca. 1 m) and 2 m above the seafloor (15 m at C1, 21 m at BF). Temperature and salinity values along the water column were recorded by CTD casts with an SBE 19plus (Seabird) and an MSS90 (Sea and Sun Technology GmbH) profiler at C1 and BF, respectively. Samples for inorganic macronutrients (ammonium, nitrate, nitrite, phosphate), chlorophyll *a* (Chl *a*), and dissolved organic nitrogen (DON) were collected and
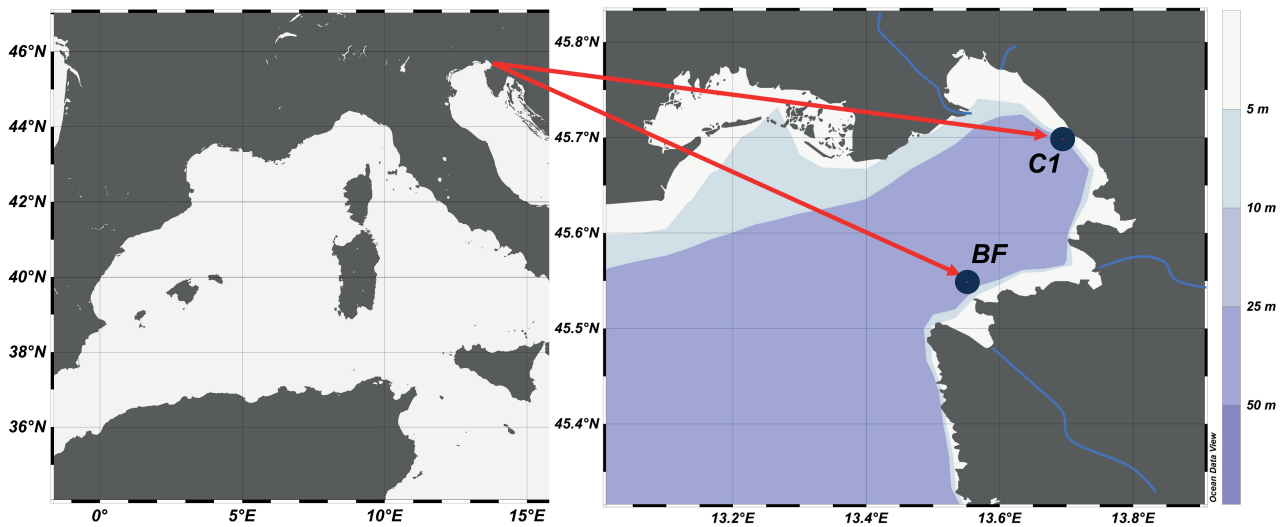
FIGURE 1 Map of the sampling locations in the Gulf of Trieste (Adriatic Sea; northern Mediterranean Sea). The main rivers are drawn in blue. Maps were drawn using the Ocean Data View (ODV) software (Schlitzer, 2022). Station coordinates are given in the main text; sampling metadata is provided in Table S1.

processed according to standard procedures, as described by Manna et al. (2021), Flander-Putrle et al. (2022) and Orel et al. (2022). Duration of daylight was computed for both stations through the application available at https://aa.usno.navy.mil/data/Dur_OneYear (accessed on March 10, 2023). Daylength increase was calculated as the difference in daylight duration between two consecutive sampling events. Seawater aliquots for microbial community analyses were collected in HCl-washed polycarbonate containers, transported to the laboratories into cooler boxes, and processed within 2 h after collection.

DNA extraction, 16S library preparation and sequencing

Sampled seawater was filtered until clogging on 0.2 μm pore-sized polyethersulfone filters (Supor-200, PALL) with final filtration volumes ranging between 1 and 3 L. DNA was extracted from the filters using the DNeasy Power-Water kit (Qiagen), yielding between 0.7–67.4 and 0.3–19.36 ng μL^{-1} for C1 and BF samples, respectively. The supplier's instructions were followed with slight modifications aimed at increasing the DNA yield and quality (detailed in Celussi et al., 2018). For the DNA metabarcoding analysis, the V4–V5 region of the 16S rRNA gene was amplified using 515-Y (5′-GTGYCAGCMGCCGCGGTAA-3′) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3′) primers (Parada et al., 2016). Libraries were prepared following the 16S Metagenomic Sequencing Library Preparation protocol. C1 samples were sequenced in multiple runs of 2 × 250 bp paired end reads at the genetic and epigenetic ARGO Open Lab Platform, Area Science Park, Trieste Italy, using either Illumina MiSeq or NovaSeq Systems. BF samples were sequenced in a single

batch of 2 × 300 bp paired end reads at the LGC Genomics GmbH, Berlin Germany, using Illumina MiSeq. Because the library size obtained with NovaSeq was up to 10-fold larger than that obtained with MiSeq, we randomly subsampled the reads obtained with NovaSeq to a library size of 250,000 using the 'Short-Read' package (Morgan et al., 2009). The lack of a batch effect was verified with PERMANOVA using the sequencing platform as a discriminating factor ($p > 0.05$, Table S2; Figure S1).

Bioinformatic pipeline

Reads processing, including primer removal, was performed in the R environment v 4.2.2 using the packages DADA2 v 1.26.0 and phyloseq v1.42.0 (Callahan et al., 2016; McMurdie & Holmes, 2013; R Core Team, 2022). After quality checking, reads were filtered using the default package parameters and truncated at positions 215 and 180, respectively. Error rate learning and amplicon sequence variants (ASVs) inference were performed separately for each sequencing run (Callahan et al., 2017). In the case of samples sequenced using the Novaseq system (which generates binned quality scores), error learning was performed by modifying the loess function (i.e., log-transformed totals as weights, degree = 1, span = 0.95) and forcing monotonicity of the estimated error rates. ASVs were derived by pooling samples to achieve the highest possible resolution (Bardenhorst et al., 2022). Sequencing runs were then consolidated into a single data set that was subjected to chimera removal (in consensus mode). Singletons and sequences in the non-target length range were filtered out. Taxonomy was assigned against the

reference database SILVA SSU v 138.1 (Quast et al., 2013; Yilmaz et al., 2014). A subset of sequences affiliated with poorly known genera was manually blasted against the GenBank (Altschul et al., 1990) and/or the Cyanorak (Garczarek et al., 2021) databases. ASVs with unassigned domains and those assigned as chloroplasts, mitochondria, or eukaryotes were removed. We defined abundant ASVs as those with a relative abundance ≥1% in at least one sample, using this subset for subsequent analyses. Read processing and filtering steps resulted in a dataset of 17,031,091 reads and 13,755 ASVs, with an average of 131,541 ± 62,817 reads and 1691 ± 892 ASVs per sample.

Community data analyses

To explore the relationship among samples we used a principal coordinates analysis (PCoA), computed from a Bray–Curtis distance matrix through the *capscale* function of the phyloseq package. On the PCoA plot, samples were distributed in a triangular pattern (see Section 3.1); therefore, the next steps were aimed at defining the 'climax' communities represented by the three vertices. First, the 3 most external samples (vertices) were identified according to their PCoA coordinates (the lowest Axis 2 value, the highest sum of Axis 1 + Axis 2, and finally the highest value of Axis 2 − Axis1). Subsequently, samples belonging to the 1st decile of the Bray–Curtis dissimilarity index calculated between one given vertex sample and all the other samples were considered as the 'core' of these 3 climax communities. Sample membership to a given climax is detailed in Table S1.

Communities' response to environmental forcings was investigated through a distance-based redundancy analysis (dbRDA) computed on the same distance matrix used for the PCoA. After checking for autocorrelation within environmental variables, daylight duration, day length increase, temperature, salinity, Chl *a*, inorganic nutrients, and DON concentrations were selected among the measured parameters and centred and scaled (i.e., zero mean and unit variance) prior to analysis. Samples with missing data were excluded from the analysis; for samples with data below the detection limit, the middle-bound approach was followed (DL/2). The function *anova.cca* from the vegan package (v2.6-4; Oksanen et al., 2022) was used to test for the significance of the dbRDA model ($p < 0.001$).

Identification of climax ASVs, seasonality, and environmental preferences

The indicator species analysis (*multipatt* function, indicspecies v1.7.12; De Cáceres et al., 2010) was used to identify the sets of ASVs significantly ($q < 0.05$ after false discovery rate correction) defining the three climax communities, keeping only those ASVs with a fidelity score greater than 0.8 (i.e., present in at least 80% of samples of a given climax). Aside from the abovementioned R packages, tidyverse v2.0.0 (Wickham et al., 2019) was used for data handling and visualisation.

Using the Lomb–Scargle periodogram, computed as implemented in the *lomb* package (v 2.1.0; Ruf, 1999), we tested whether indicator species displayed recurrent changes over time (i.e., seasonality). For each ASV the strength of recurrency (the peak normalised power, PN) was calculated and only those with PN higher than 10 and $q < 0.05$ (after false discovery rate correction) were deemed as seasonal.

With a differential abundance approach, computed using the package corncob (v. 0.3.1, Martin et al., 2020), we evaluated the response of ASV abundance to selected environmental forcing. For this approach, we selected day length, day length increase, temperature, salinity, chlorophyll *a*, dissolved inorganic macronutrients, and DON concentrations. Only significant models highlighting a differential abundance over the considered environmental forcing were selected for further discussion. Visualisation of both seasonal trends and environmental preferences was finalised through a GAM approach (mgcv package, v. 1.8-41; Pedersen et al., 2019).

RESULTS

Community dynamics

A general overview of the composition of prokaryotic assemblages in the study timeframe at both stations is reported as supplementary information in Figure S4.

PCoA ordination explained 48.2% of the total variance, partitioning microbial communities in a strongly recurrent seasonal pattern. Samples clustered during winter (i.e., December–January), summer (i.e., July–August), and spring (i.e., April–May), generating a triangular distribution in the multivariate space (Figure 2A), with 'climax' vertices interspersed by communities sampled during transitional months (e.g., March, June, and October; Figure 2A). A seventh cluster recruited summer samples retrieved below the thermocline, represented by very variable communities characterised by features that can be considered intermediate among the three climaxes (Figure 2A). This pattern was characterised equally by both stations as shown in Figure 3 and as evidenced by the correlation analysis reported in Figure S3.

The dbRDA ordination captured 41.1% of the total variance, showing the same triangular pattern evident for the PCoA (Figure 2A), strengthening the idea of recurrent climax communities. These were characterised
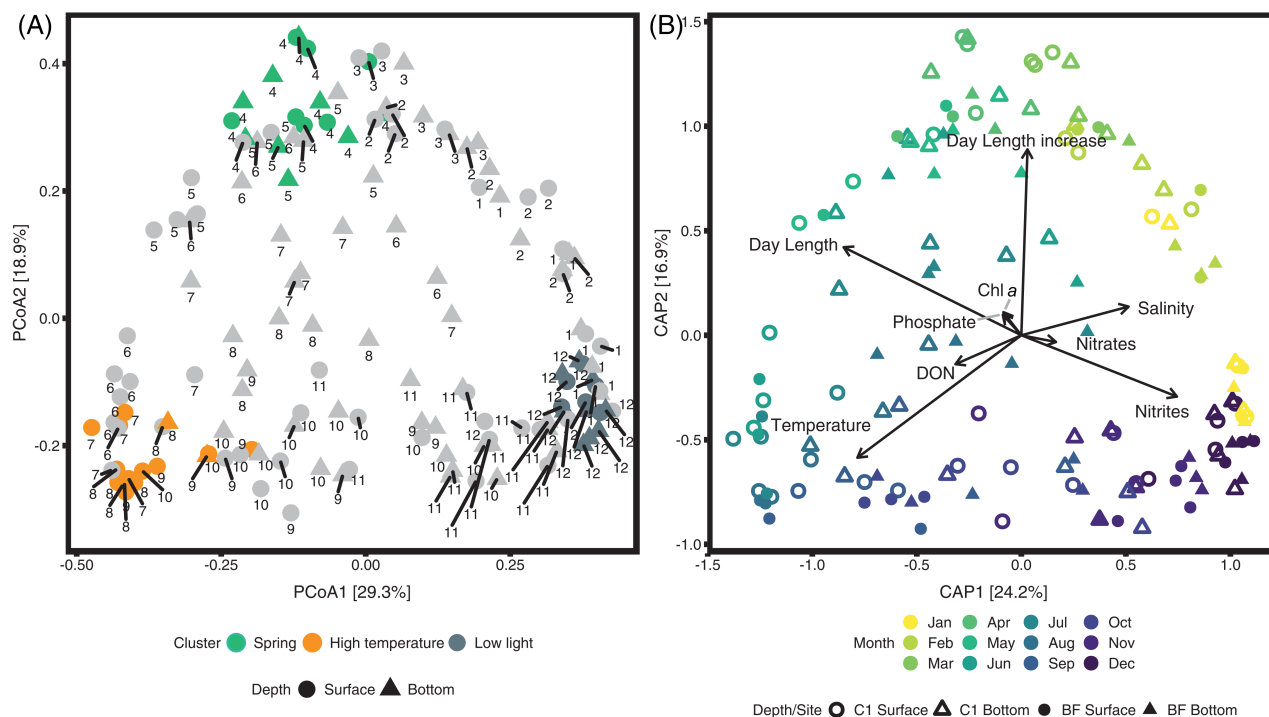
FIGURE 2 Principal coordinate analysis (PCoA, A) and distance-based redundancy analysis (db-RDA, B) of Bray–Curtis dissimilarities, based on ASV abundances (>1% in at least one sample, see Section 2.3), of prokaryotic communities sampled in the Gulf of Trieste (the Adriatic Sea, northern Mediterranean Sea). Each axis reports the percentage of explained variance; in (B), the variance is constrained by the set of environmental variables depicted on the plot and samples are colour and shape-coded according to sampling month, depth, and site. In (A), sample membership to each cluster is colour-coded to identify the three climax communities (see Section 2.5).

(1) by the shortest day length (i.e., 'Low light' community; Mann–Whitney test, $Z = 5.6$, $p < 0.001$, $n = 13$ vs. 137), (2) by the highest day length increase (i.e., 'Spring' community; Mann–Whitney test, $Z = -4.8$, $p < 0.001$, $n = 14$ vs. 136) and (3) by the highest seawater temperature (i.e., 'High temperature' community; $Z = -5.4$, $p < 0.001$, $n = 15$ vs. 133), respectively. Background information about the environmental variables is reported in Figure S4 and Table S1.

Indicator species of climax communities

The indicator species analysis yielded a total of 71 ASVs significantly associated with one single climax community. Samples belonging to the high-temperature cluster were composed of a set of 33 ASVs, largely dominated by two *Synechococcus* ASVs followed by members of the NS4 and NS5 marine group (Figures 3, S6) and *Cyanobium*. A downstream analysis on this latter genus, performed by blasting the *Cyanobium* sequences against the Cyanorak database, revealed a 100% similarity with two *Synechococcus* isolates. Archaeal ASVs belonging to the genus *Nitrosopumilus* and Marine Group II dominated the pool of 15 ASVs characteristic of low-light communities, accounting for an average relative

abundance of ~13% (Figures 3, S6). Spring climax communities were best identified by 23 ASVs, mostly represented by two ASVs (altogether representing a relative abundance = 13.4% ± 5.1%, Figures 3, S6) affiliated with the *Ascidiaceihabitans* genus. However, when blasting the sequences of the two ASVs against the GenBank database an identity of 100% and 98.9% with *Sulfitobacter* (a well-known genus, widespread in coastal environments) was found. Other ASVs within the Spring climax belonged to *Synechococcus*, NS4, and NS5 marine groups, which together made up ~17.5% of sequence relative abundance (4.63% ± 3.26%, 6.94% ± 6.14% and 6.00% ± 4.02% on average, respectively, Figures 3, S6).

Seasonality

Out of the 13,755 ASVs present in our dataset, ~16% were identified as seasonal (i.e., ASVs displaying recurrent changes throughout the years; PN > 10 and $q < 0.05$) representing, on average, 83.1% ± 10.4% of the reads' relative abundance. The vast majority of the 71 ASVs significantly defining the three climax communities were found to be seasonal, except for ASV6825, of the *Croceicoccus* genus, belonging to the high-temperature climax. By clustering seasonal trends of
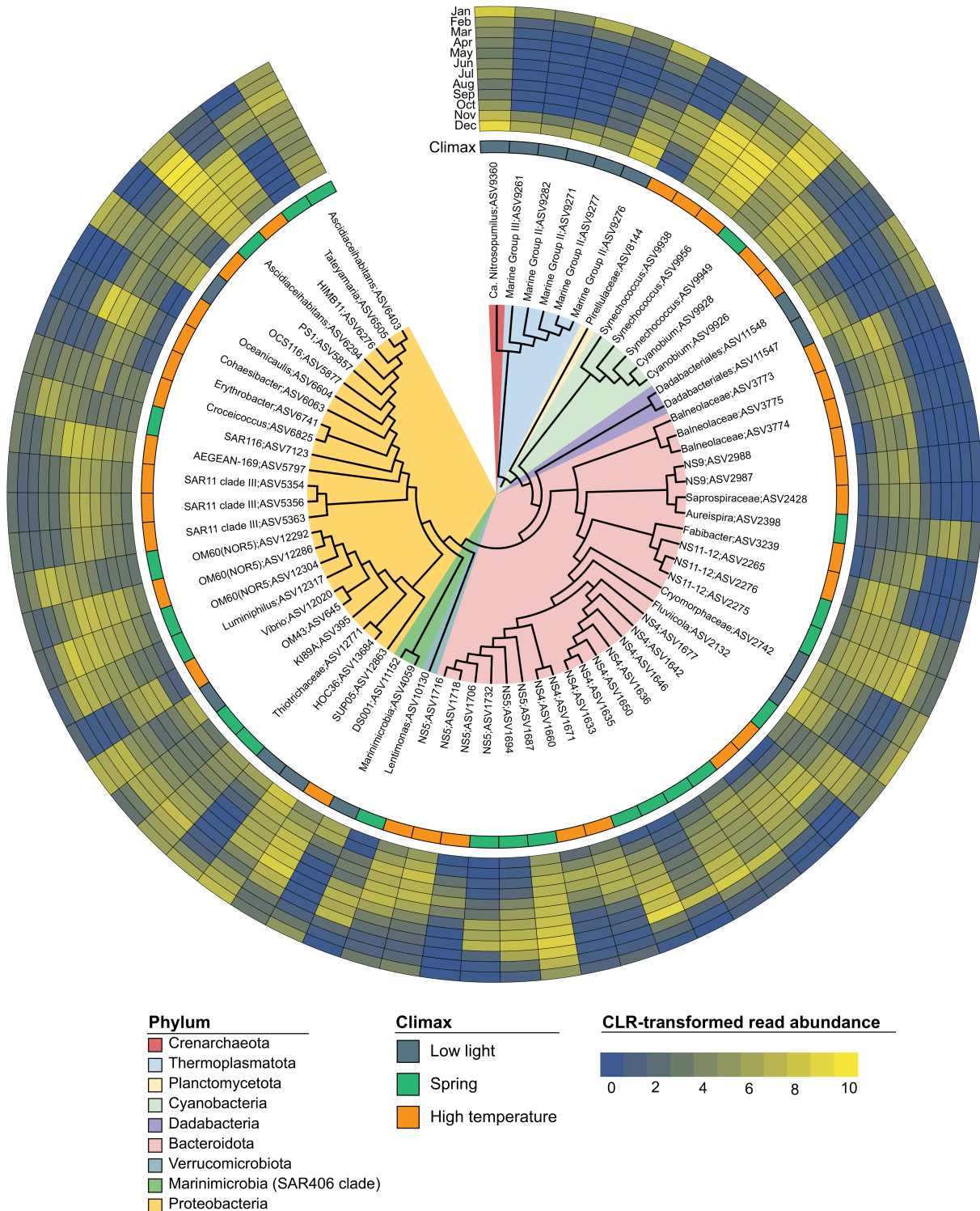
FIGURE 3 Phylogenetic tree of climax ASVs. Clade colours indicate taxonomic assignment at the phylum level. ASV climax membership is shown in the inner ring. The heatmap in the outer ring depicts the site-averaged monthly abundance patterns based on CLR-transformed raw reads. The tree was constructed using the maximum likelihood algorithm with MEGAX (Kumar et al., 2018) with default parameters and edited with Interactive Tree Of Life (iTOL) v5 (Letunic & Bork, 2021).

climax ASVs we identified four main groups (Figures 3, S7). Three of them matched with the three climax communities (i.e., High temperature, Low light, and Spring), with ASVs peaking in the respective seasonal window.

A fourth cluster emerged, grouping ASVs that were always present in our dataset (e.g., ASVs belonging to *Synechococcus* and *Ascidiaceihabitans* genera, Figures 3, S2, and S7) but only able to 'bloom' in
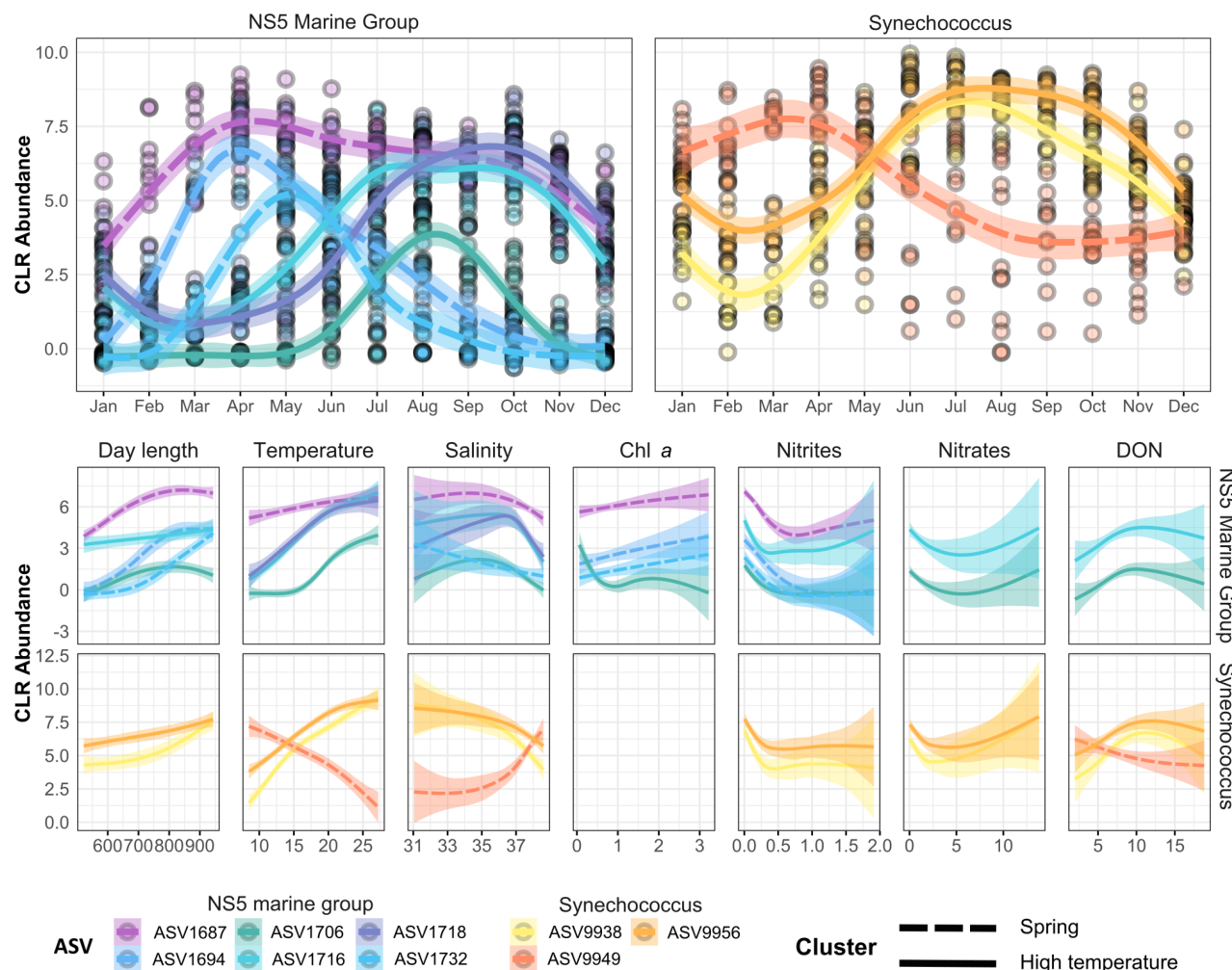
FIGURE 4 The generalised additive model smoothed seasonal trends (upper panel) and response curves for significant differential abundance models (lower panel) for ASVs within NS5 and *Synechococcus* genera. *X*-axis in the upper panels depict months; in the lower panels, the environmental parameters range is presented. *Y*-axis present the centred-logarithm ratio abundance. Shaded areas indicate 95% confidence intervals. Chl *a*: Chlorophyll *a*; DON: dissolved organic nitrogen. Since differential abundance models for phosphate and ammonium concentrations were not significant, response curves for these two variables are not visualised here.

specific time frames (i.e., summer and spring, respectively).

Environmental preferences

Day length, temperature, and salinity gradients resulted in significantly different climax ASV abundance. Within genera represented by multiple ASVs, the response curve showed non-cohesive behaviour (Figure S8). The spring *Synechococcus* ASV was negatively related to temperature, in contrast to what emerged for the summer ones (Figure 4). ASVs belonging to the NS4 marine group followed a similar pattern, with niche preferences matching with their assignment to Spring or high-temperature climaxes (Figure S8). Chl *a* response models showed significant positive relationships between photosynthetic biomass and the

abundance of ASVs characterising the Spring climax communities (i.e., NS4 and NS5 marine groups; Figures 4 and S8).

Finally, taxa specialised in organic matter degradation showed a positive response to high DON concentrations (i.e., NS4 and NS5 marine groups; Figures 4 and S8), whereas negative relationships were found for those ASVs defining Low light assemblages (i.e., Ca. *Nitrosopumilus* and SUP05; Figure S8).

DISCUSSION

In our time series analyses of prokaryotic diversity at two coastal sites in the north Mediterranean Sea we identified three annually recurrent sets of specific ASVs. Following the definition of 'cycloclimax' by Whittaker (1974) in his 'Climax Pattern Hypothesis', we

chose to name these communities as climax. Each of these three climaxes followed temporal patterns forced by different environmental settings. The Low light climax was characterised by the shortest day length, whereas the highest day length increase characterised the Spring climax. Finally, the highest seawater temperature underlined the development of the high-temperature climax. Nonetheless, despite this sharp temporal partitioning, closely related prokaryotes emerged as indicators of multiple climax assemblages, hinting at within-genus functional differentiation.

Mixotrophs dominate the Low-light climax

The Low light climax was characterised by a high relative abundance of mixotrophs, microbes that can complement their energy and C requirements obtained by the oxidation of organic matter with extra sources of energy and, in some cases, by fixing dissolved inorganic C (Erb, 2011). One example of mixotrophy is likely carried out by the archaeon *Nitrosopumilus* (mean RA: 9.0% ± 5.8%; Figure S6) which can oxidise ammonium, releasing nitrite into the environment, to obtain energy and utilise dissolved inorganic C for biomass build-up (Bayer et al., 2019; strains isolated in the study area). High nitrite concentrations were indeed strongly associated with Low light climax assemblages (Figure 3B) and in particular with Ca. *Nitrosopumilus* (Spearman's $\rho = 0.82$, $p < 0.001$, $n = 150$), as also shown by environmental niche modelling (Figure S8). Therefore, even if further dedicated studies are needed to corroborate this, building on top of other similar-minded studies (Kim et al., 2019), we speculate that nitrification processes carried out by these archaea may likely be responsible for the wintertime nitrite accumulation regularly observed in the study area (Figure 2B; Flander-Putrle et al., 2022). Members of this genus showed the narrowest seasonal distribution, almost disappearing during other periods of the year (Figures 3, S7), in accordance with previous findings (Pereira et al., 2021); indeed, the high relative abundance of this taxon between December and January appears to be a common feature of temperate coastal microbial communities (e.g., Bunse & Pinhassi, 2017; Kim et al., 2019; Korlević et al., 2022; Lambert et al., 2019). Culture-based experiments showed photoinhibition of ammonia-oxidising Archaea, even at low light intensities (Merbt et al., 2012), suggesting that the wintertime appearance of this archaeon is strongly driven by abiotic drivers.

Alongside *Nitrosopumilus*, members of the archaeal Marine Group II (MGII; Figure 3; mean RA 4.5% ± 3.1%; Figure S6) are common in surface temperate waters during winter (e.g., Bunse & Pinhassi, 2017; Korlević et al., 2022). The current lack of cultured representatives of this taxon limits the knowledge of their ecological role; however, most genome reconstructions

of euphotic-dwelling MG II suggest a heterotrophic lifestyle (Rinke et al., 2019 and references therein). Interestingly, MGII abundance was unrelated to Chl a or DON, suggesting that climax MGII ASVs of the northern Adriatic Sea may thrive using proteorhodopsin-mediated energy production (Rinke et al., 2019) or even exploit organic matter freshly produced by mixotrophs (Celussi et al., 2017).

Members of the clade SUP05, also indicators of the Low light climax, (Thioglobaceae, Gammaproteobacteria; Figure 3; mean RA: 3.0% ± 1.4%; Figure S6) can live autotrophically by oxidising hydrogen and reduced sulphur species (sulphide, thiosulfate, elemental sulphur) and fixing inorganic carbon (Morris & Spietz, 2022). The 16S-based approach of our study is not per se sufficient to highlight the trophic mode of the single, highly abundant ASV of SUP05 (Figure S6). Nonetheless, the oxygen concentration in the winter water column in winter is unlikely to promote the formation of H_2S; these organisms should thus rely on other reduced compounds, and organic matter, or thrive thanks to the exploitation of anoxic/hypoxic microniches within particulate matter (Bianchi et al., 1994). Surprisingly, SUP05 members in the sediments of the GoT are not abundant (<0.01%, unpublished data), therefore their resuspension from the seabed in such high numbers is unlikely.

Therefore, the Low light climax is characterised by microbes that can access energy sources other than organic carbon oxidation, thriving at low environmental concentrations of DOC in this period of the year (De Vittor et al., 2008). Thus, they would contribute to the maintenance of the low DOC baseline values through a balance of utilisation and production of de novo synthesised polymers (Celussi et al., 2017) in the absence of important phytoplankton production processes while contributing to increasing environmental nitrite levels.

The Spring climax is led by phytoplankton-associated bacteria

Changes in day length are linked to photoautotrophic microbe dynamics since they are dependent on light to perform photosynthesis (Marra & Heinemann, 1982). Furthermore, in the last few years, day length has emerged as a previously overlooked structuring factor for the whole prokaryotic community spatiotemporal dynamics, especially at the seasonal scale (e.g., Fuhrman et al., 2015; Lambert et al., 2019). The springtime day length increase triggers successional patterns in phototrophic communities which are intertwined with prokaryote dynamics, as well documented in several marine systems (e.g., Fandino et al., 2001; Teeling et al., 2012). ASVs belonging to the genus *Ascidiaceihabitans/Sulfitobacter* (Rhodobacteraceae) dominated the microbial community in the study area (Figure S2) and were highly representative of the Spring

climax (Figure 3; average 13.4% ± 5.1%; Figure S6). Members of the *Sulfitobacter* genus (Rhodobacteraceae) are known to live in tight association with phytoplankton, being particularly active in exploiting microalgae-derived organosulfur molecules such as dimethylsulfoniopropionate and taurine (Amin et al., 2015), eventually playing a pivotal role in blooms demise (Barak-Gavish et al., 2018). Even though our sampling frequency did not allow a clear identification of blooms during spring months (Figure S4), a previously published time series analysis has reported phytoplankton highest cell abundance during April and May in the area, mainly represented by diatoms and flagellates (Cerino et al., 2019). Likewise, ASVs belonging to the Flavobacteriales NS4 and NS5 Marine Groups were abundant in these months (Figure S2) making up most of the reads relative abundance in the Spring climax (6.9% ± 6.1% and 6.0% ± 4.0%, respectively; Figure S6). These bacteria are equipped with gene repertoires that allow them to exploit specific phytoplankton-derived substrates and for this reason are not uncommon during spring phytoplankton blooms (Díez-Vives et al., 2019; Korlević et al., 2022; Teeling et al., 2016).

Interestingly, one ASV affiliated to *Synechococcus* was also abundant in this cluster (4.6% ± 3.3%; Figures 3, S6), showing different environmental preferences compared to the other members of this genus (see Section 4.4; Figure 4). When blasting the *Synechococcus* ASV 9949 through Cyanorack (Garczarek et al., 2021) we obtained a 100% identity against the sequence of strain PROS-9-1, belonging to the cold-adapted Clade Ib (Farrant et al., 2016), as also highlighted by its preference for lower temperatures and higher salinity (Figure 4). Auladell et al. (2022) also found a match with this strain in their NW Mediterranean 16S rRNA metabarcoding dataset, suggesting that early spring *Synechococcus* bloom, due to cold-adapted ASVs, may be a common feature of temperate coastal areas, challenging the paradigm of the spring bloom led by larger phytoplankton (e.g., diatoms) as the cornerstone seasonal feature in these areas.

The prokaryotic Spring climax is thus strongly connected to phytoplankton (including *Synechococcus*) successions driven by the constant daily increase in light availability. It is characterised by microbes that are highly specialised in the processing of autochthonous organic matter, possibly determining the annual peak in polysaccharide degradation typical of this period of the year (Celussi et al., 2015; Celussi & Del Negro, 2012; Manna et al., 2022).

Photoautotrophs and the Flavobacteriales largely determine the High-temperature climax

Temperature is often one of the most prominent environmental factors driving spatiotemporal patterns of microbial communities (Fuhrman et al., 2015; Sunagawa et al., 2015). Indeed, summer prokaryotic assemblages in the northern Adriatic emerged as a climax (Figure 2A). In the study area, summer months are characterised by oligotrophic conditions in which small photoautotrophs (e.g., *Synechococcus*) are known to thrive, reaching high abundances (Figure S2) and sustaining most of the phototrophic biomass and primary production (Cibic et al., 2018; Fonda Umani et al., 2012). Consistently, two *Synechococcus* ASVs made up most of the high-temperature climax (16.4% ± 3.6%; Figure S6), in line with previous findings of multiple ribotypes of this cyanobacterium in the area (Figure S6; Paoli et al., 2008). Noteworthy, ASVs identified as *Cyanobium/Synechococcus* were also abundant (3.2% ± 1.6%; Figure S6), corroborating the pivotal role of these primary producers in the oligotrophic summer, as reported for other coastal Mediterranean sites (e.g., Auladell et al., 2022; Korlević et al., 2022). The high relative abundance of these primary producers (Figures S2 and S6) is putatively connected to the typical summer DOC accumulation (Manna et al., 2021), likely sustaining the development of heterotrophic or photoheterotrophic bacterial populations. In fact, besides cyanobacteria, the high-temperature climax was characterised by high abundances of ASVs belonging to the Rhodobacteraceae (former Roseobacter group, genus HIMB11, 2.0% ± 0.7%; Figure S2; Figure S6) as well as by members of the NS4 and NS5 Marine Group of the Flavobacteriales (2.4 ± 1.3 and 3.1% ± 1.4%, on average, respectively; Figures S2 and S6). The latter are taxa well known for their versatility in DOC processing, with specialised loci for specific substrate processing (Priest et al., 2022), suggesting that the community transitioning from the High temperature to the Low light climax may be driven by DOC dynamics.

Noteworthy, summer bottom samples (i.e., below the thermocline) were not included in the high-temperature climax, except for August and September 2021 at station C1, characterised by water column mixing events (Figure S5). This pattern suggests that the onset of summer stratification decouples surface and bottom community dynamics, as highlighted by multivariate ordinations (Figure 2). Consequently, prokaryotic assemblages below the thermocline may follow independent successional trajectories, which are dependent on the 'seed' communities segregated in the lower layer of the water column that experience different biogeochemical settings (e.g., fluxes of nutrients from sediments, episodic anoxic conditions, etc.; Tinta et al., 2015; Kralj et al., 2019). A metagenome-based investigation of depth-related functional patterns during thermal stratification in the Mediterranean Sea highlighted substrate processing and acquisition strategies partitioning along the water column (Haro-Moreno et al., 2018). Surface-segregated assemblages exploit organic carbon freshly produced by small photoautotrophs, whereas communities thriving below the

thermocline present less diverse, but more generalist sets of polysaccharide-degrading enzymes (Haro-Moreno et al., 2018). However, multiple depths are seldom considered in prokaryotic diversity time series (e.g., Auladell et al., 2022; Korlević et al., 2022; Lambert et al., 2019) and thus the effect of the summer stratification onset and development on microbial successional and functional dynamics are still poorly known.

The high-temperature climax that characterises mainly surface waters in late summer is therefore shaped by the interplay between primary producers (cyanobacteria), which are responsible for the DOC summertime accumulation, and by specialised degraders (e.g., Flavobacteriales) that anticipate the organic matter pool drawdown occurring in Autumn (Manna et al., 2021).

Rhythmicity and niche preferences of climax ASVs

Using the statistical approach of Lambert et al. (2019) and Auladell et al. (2022), ~16% of the ASVs composing our dataset were identified as annually recurring. While this number is considerably smaller than those previously reported (34% and 47% in Lambert et al., 2019 and Auladell et al., 2022, respectively), the rhythmic ASVs were highly representative of the sampled prokaryotic communities (83.1% ± 10.4% of the reads relative abundance). Interestingly, almost all the ASVs identified as indicators of the climax communities displayed a strong rhythmicity (Figure 3), evidencing how in the GoT few, yet very abundant, ASVs shape the ecosystem functioning all year round and with a pronounced interannual repeatable pattern. It has been shown that while co-occurrence patterns within the microbial food web rearrange themselves following environmental perturbations, key taxa re-occur from year to year exploiting the functional redundancy of co-occurring organisms to thrive (Lambert et al., 2021). Given the high dynamism of our study area, its wide interannual and seasonal biogeochemical variability may promote a lower degree of functional redundancy, favouring instead fewer ASVs, largely dominating the prokaryotic communities, and exploiting wider ecological niches.

Our decision to analyse the time series at the ASV level corroborates the recent findings by Auladell et al. (2022). With such an approach the authors evidenced sharp niche differentiation, as well as contrasting seasonal patterns, even among closely related taxa. In our dataset both NS5 Marine Group and *Synechococcus* characterised two climax assemblages (Spring and High temperature climaxes, Figure 3), showing intragenus variability in terms of environmental preferences (Figure 4).

The within-genus NS5 microdiversity emerging from our dataset (Figure 4) has been also highlighted by

genome-resolved successional-like dynamics within the NS5 marine group in the Arctic Ocean (Priest et al., 2022). The springtime abundance of NS5 ASVs 1694 and 1732 was positively related to Chl *a* concentration, indicating a role in the processing of phytoplankton bloom-derived organic matter. Furthermore, the tight timing of their succession (Figures 4 and S7) suggests a species-specific relationship with photoautotrophs, in line with previous metagenomic-based surveys during spring phytoplankton blooms (Teeling et al., 2016). ASVs 1706, 1718, and 1716 peaked over the summer months possibly in response to the summer organic matter accumulation, as partially suggested by the positive relationship with DON concentrations (Figure 4). Analysis of the functional potential of NS5 members has shown that some of them lack genes involved in flagellar assemblage or gliding motility and are thus considered free-living dwellers (Priest et al., 2022 and references therein). Thus, it is very likely that summer NS5 ASVs occupy the niche of free-living organic matter degraders, with successional dynamics linked to substrate availability, while Spring climax ASVs behave as early responders to freshly produced organic matter, as supported by the positive relationship found with Chl *a* for these ASVs (Figure 4) and by their wide repertoire of glycosyl-hydrolases (Priest et al., 2022; Teeling et al., 2016).

Different *Synechococcus* ASVs characterised Spring and High temperature climax communities (Figure 4), with clearly distinct environmental preferences (Figure 4). Our findings are in line with recent results highlighting how this cyanobacterium harbours intragenus functional microdiversity (Auladell et al., 2022; Auladell et al., 2023). As discussed in Section 4.2, the presence of a cold-adapted *Synechococcus* ASV thriving in early spring may be a common feature in Mediterranean coastal waters (Auladell et al., 2022). At the very least, this feature should be considered when discussing trophic dynamics of coastal temperate systems, as *Synechococcus*-derived organic matter may fuel heterotrophic microbial communities following environmental perturbations (i.e., Manna et al., 2021). Summer *Synechococcus* ASVs showed a marked environmental preference for higher temperatures, as well as higher abundances at low dissolved inorganic nitrogen and high DON concentrations (Figure 4), in line with the capability of this organism to grow under oligotrophic conditions possibly due to its ability to supplement its energetic requirements through the oxidation of small organic compounds (Paoli et al., 2008).

The presence of bacteria from the same genus in highly different environmental conditions again points to the recognition of specific niche preferences even in closely related microbes (Figure 4; Auladell et al., 2022).
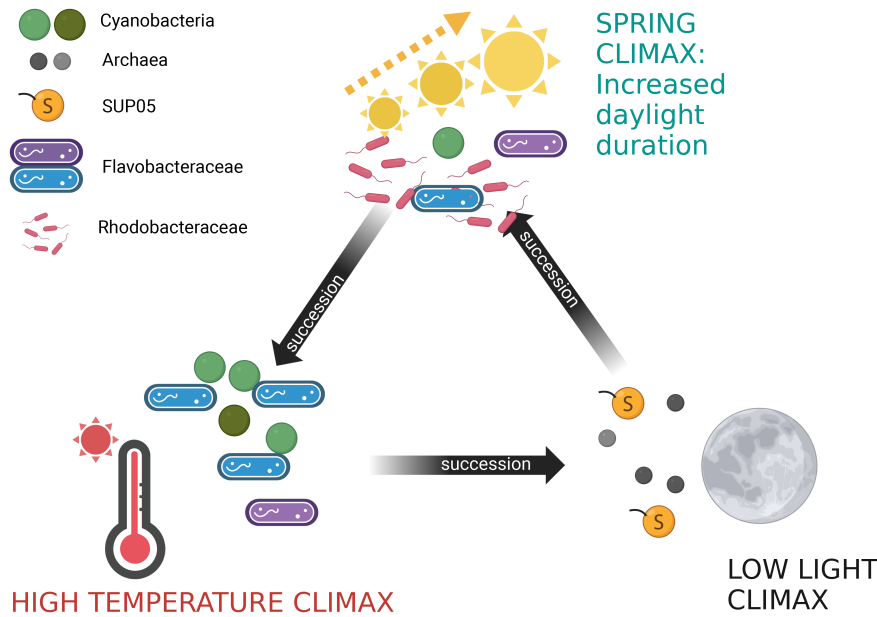
FIGURE 5   Recurrent environmental patterns structure temperate Mediterranean coastal microbial communities in climax assemblages. Springtime climax communities recruit copiotrophic taxa associated with phytoplankton-derived organic matter degradation (e.g., Flavobacteraceae—NS4 and NS5 Marine Groups, and Rhodobacteraceae), as well as photoautotrophic prokaryotes (i.e., *Synechococcus*) whose rhythmicity is linked to the increase in photoperiod length. Climax communities associated with summertime High-temperature climax exploit the dissolved organic matter reservoir with strong intragenus specialisation (e.g., NS5 marine group; Figure 4); furthermore, oligotrophic conditions (in terms of inorganic nutrients) favour *Synechococcus* domination among photoautotrophs. Assemblages climaxing in vernal months (Low light climax) thrive on mixotrophy, either directly fixing CO_2 (oxidising ammonium or sulphur species, e.g., Ca. *Nitrosopumilus* and SUP05) or supplementing organic matter oxidation with proteorhodopsin-mediated energy production (e.g., Marine Group II). Rhythmic climax assemblages are interspersed by successional dynamics widely varying on the interannual scale. Image created with BioRender.com.

Conceptualising rhythms and climaxes in coastal microbial communities

With a time-series approach, we identified recurrent assemblages in the northernmost area of the Mediterranean Sea, characterised by specific environmental drivers, whose dynamics are conceptualised in Figure 5. Daylight duration and temperature structured the sampled microbiome in three climax communities, each of them defined by a specific set of ASVs as well as by a dominant trophic mode. Members of the Low light assemblages (e.g., Ca. *Nitrosopumilus*, SUP05 and MGII, typical of January and December) exploited mixotrophy to thrive under low DOC conditions, whereas spring (March, April, and May) recurrent communities showed a high prevalence of copiotrophic taxa (e.g., Flavobacteraceae and Rhodobacteraceae), related to freshly produced organic matter following photoautotrophic biomass increase. ASVs found in the High-temperature climax (e.g., Flavobacteraceae, NS4, and NS5 Marine Group, abundant from July to October) were associated with dissolved organic matter degradation, showing tight successional dynamics within the same genus putatively related to different albeit intertwined degradation capabilities.

Using amplicon sequence variants as a fundamental taxonomic unit we were able to disentangle fine-grained successional dynamics within globally abundant genera, identifying their potential role in ecosystems and their interannual recurrence patterns. Despite the intercomparison of microbial assemblages across different biomes is still an underachieved goal due to the different methodologies used (e.g., size fractionation of seawater samples and primer set used), our findings begin to delineate a common framework for microbiome-based ecological viewpoints in temperate marine systems.

AUTHOR CONTRIBUTIONS

Mauro Celussi: Conceptualization; investigation; funding acquisition; writing – original draft; validation; writing – review and editing; visualization; supervision. **Vincenzo Manna:** Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; data curation. **Elisa Banchi:** Writing – review and editing; visualization; formal analysis; data curation; methodology. **Viviana Fonti:** Writing – review and editing; methodology; data curation; formal analysis. **Matteo Bazzaro:** Formal analysis; data curation. **Vesna Flander-Putrle:** Writing – review and editing; formal analysis; methodology; data curation. **Katja Klun:** Methodology; writing – review and editing; formal analysis; data curation. **Martina Kralj:** Writing – review and editing; data curation; formal analysis. **Neža Orel:**

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The 16S amplicon sequences generated for this study can be found in the Sequence Reads Archive (SRA) at NCBI under the BioProject accession numbers PRJNA818117, PRJNA767222, PRJNA818144, PRJEB60871. The environmental data are reported in Table S1.

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SUPPORTING INFORMATION

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