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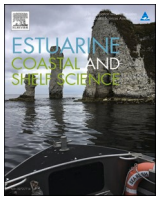

# Contribution of different bacterial groups in the carbon flow through the microbial food web

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ABSTRACT

Bacteria greatly contributes to the transfer of dissolved organic carbon to the upper trophic levels. Nevertheless, little is known about the contribution of specific bacterial groups. Here, we conducted three seasonal experiments (both, microcosm and mesocosm) in the coastal area of the central Adriatic with the aim of determining the contribution of selected bacterial groups to carbon flow through the microbial food web. We assessed the growth rates of four bacterial groups (Bacteroidota, SAR11, Rhodobacteraceae, and Gammaproteobacteria), as well as aerobic anoxygenic phototrophs, and their contributions to biomass production and carbon transfer to upper trophic levels. For the first time, we reported a significant contribution of aerobic anoxygenic phototrophs to the carbon flow, especially during the summer.

Under initial winter conditions, SAR11 contributed the most to bacterial biomass production, while Gammaproteobacteria were the primary contributors to grazing loss and served as the preferred prey. As temperature increased, the contribution of Gammaproteobacteria and Bacteroidota to bacterial biomass production also increased. Regarding grazing loss, SAR11 and Bacteroidota were the dominant contributors in spring, whereas Gammaproteobacteria and Rhodobacteraceae played the most significant role during summer. Under nutrient-enriched conditions, SAR11 contributed the most to both bacterial biomass production and grazing loss during winter. However, with rising temperatures, Gammaproteobacteria, Rhodobacteraceae and Bacteroidetes became the primary contributors to bacterial biomass production and Gammaproteobacteria and Bacteroidetes in grazing loss. Our estimates suggest that Gammaproteobacteria and the SAR11 clade were more significantly impacted by grazing, whereas Bacteroidota and Rhodobacteraceae were equally influenced by both nutrient availability and grazing pressure.

Therefore, the results obtained in this study are useful for evaluating the potential contributions of diverse bacterial groups to carbon cycling in marine ecosystems under changing environmental conditions, particularly during shifts in seawater temperature. We further analyzed the dynamics of bacterial community composition in relation to varying environmental factors.

## 1. Introduction

Marine waters are inhabited by a highly diverse array of microorganisms. Primary production is mainly conducted in the euphotic zone by eukaryotic algae and prokaryotic cyanobacteria. Produced organic carbon is then recycled by heterotrophic organisms (Ducklow et al., 1986; Cole et al., 1988). The study of the carbon cycle in marine microbiology is crucial for understanding how microbial processes

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regulate carbon fluxes in ocean ecosystems, impacting global climate and biogeochemical cycles. Microbial community contains a large and diverse assembly of bacteria with different carbon requirements and distinct contribution to the aquatic carbon cycle such as autotrophy, heterotrophy, and photoheterotrophy. Heterotrophic bacteria are responsible for organic carbon utilization and nutrient remineralization (Pomeroy et al., 2007). Moreover, they largely contribute in biomass and energy production and transfer in oligotrophic areas such as the central Adriatic Sea (Legendre and Rassoulzadegan, 1995; Magazzù and Decembrini, 1995; Li, 1998; Zubkov et al., 2000; Li and Harrison, 2001; Grob et al., 2007; Vilibić et al., 2012; Šantić et al., 2013; Šolić et al., 2015.). Also, different bacterial groups are, due to their different physiology, controlled by different physicochemical and biological factors (Fuhrman and Hagström, 2008; Gasol et al., 2008). Therefore, determining the growth rate of individual groups is crucial for better understanding their differential environmental roles and contribution to the biochemical carbon cycle.

Marine bacteria belong to several major ubiquitous groups: Pseudomonadota, Bacteroidota, Planctomycetota, and Actinobacteriota (Giovannoni and Stingl, 2005, 2007; Luna, 2015). The actual contribution of these groups varies depending on the location, distance from land and other physicochemical and biological factors (Giovannoni and Stingl, 2005; Luna, 2015). In the studied coastal area of the central Adriatic, where Skejić et al. (2024) identified oligotrophication, with a pronounced decline in phytoplankton abundance, consistent pattern of bacterial groups was observed using the CARD-FISH method, with SAR11 dominating, followed by other groups (Šantić et al., 2023; Šestanović et al., 2025). The results of 16S rRNA gene metabarcoding and a qualitative description of the microbial community, further confirmed the dominance of Pseudomonadota (with Alphaproteobacteria and Gammaproteobacteria as the main classes), followed by Cyanobacteriota and Bacteroidota (Šantić et al., 2023).

Additionally, aerobic anoxygenic phototrophic (AAP) bacteria, a polyphyletic metabolic group, play a crucial role in the marine carbon cycle (Koblížek, 2015). This functional group is able to harvest light energy using bacteriochlorophyll-containing photosynthetic complexes to supply their primarily heterotrophic metabolism (Harashima et al., 1978; Shiba et al., 1979; Piwosz et al., 2018). AAP bacteria are widely distributed in habitats with different trophic status and represent 1–30% of total bacteria in the euphotic zone (Koblížek, 2015; Vrdoljak Tomaš et al., 2023). They are on average larger (Stegman et al., 2014) and grow faster (Ferrera et al., 2011, 2017; Fecskeová et al., 2021) than average heterotrophic bacteria (Stegman et al., 2014; Vrdoljak Tomaš et al., 2019). Regardless of the recent discoveries of the bacterial community composition and dynamics in the Adriatic Sea and the great contribution to the aquatic carbon cycle (Korlević et al., 2015; Babić et al., 2018; Fecskeová et al., 2021; Šantić et al., 2021, 2023; Matek et al., 2023), little is known about the specific contribution of the relevant taxonomic and functional groups (Šestanović et al., 2025). We hypothesize that the previously described dominant groups, will have a large contribution to the carbon transfer towards the upper trophic levels in the coastal area of the central Adriatic Sea. Additionally, we tested whether environmental factors have an impact on the production and grazing of specific groups. To address that, we deciphered the seasonal growth rates of different bacterial groups, including the AAP community and monitored the changes in bacterial community composition under the influence of different environmental factors.

## 2. Material and methods

### 2.1. Experimental settings

Three manipulation mesocosm experiments were conducted on February 2nd (winter), May 5th (spring) and July 7th (summer) of 2023. Seawater was taken from the central part of Kaštela Bay (N 43°31′6″; E 16°22′54″) at 8 a.m. from 0.5 m depth and filtered through a 150 μm mesh to remove larger plankton, and detritus. The collected water samples were divided in six polyethylene translucent bags (Hedwin Cubitainer LDPE 20 L), each filled with 15 L of water representing: (1) control sample, (2) control sample with added inorganic nutrients (0.25 μM $Na_2HPO_4$, 3 μM $NH_4Cl$), (3) control sample with added inorganic nutrients and glucose (0.25 μM $Na_2HPO_4$, 3 μM $NH_4Cl$, 10 μM glucose). Treatments (4), (5) and (6) repeated (1), (2) and (3) respectively, with an additional filtration through 1.2 μm pore size filters (mixed cellulose ester, 47 mm, Whatman) to remove bacterial predators. The bags were placed in closed tanks with natural light and sea flow (Table 1). Samples were collected immediately (0 h) and after 6 h, 24 h, 30 h and 48 h.

In parallel, we performed three seasonal microcosm experiments with a volume of 1.5 L in triplicate in incubators in three treatments: 1) filtration through 150 μm to remove larger organisms, phytoplankton, zooplankton, and detritus, 2) filtration through 10 μm to remove mostly ciliates, and 3) filtration through 1.2 μm to remove mostly heterotrophic nanoflagellates (Šolić et al., 2017, 2020). Subsamples were taken at 0 h, 2 h, 4 h, 6 h, 10 h, 22 h, 24 h, 26 h and 28 h. The incubator was illuminated with 6 LED lamps (LH-TS LED 18 W, spectral temp. 6000 K) programmed to simulate day-night cycle. During incubation, the bottles were gently mixed. All incubation bottles were acid-washed with 10 % HCl and rinsed with Milli-Q water.

### 2.2. Determination of growth and grazing parameters

The biomass of total bacteria ($B_{CTC}$) were calculated using the conversion factor 20 fg C $cell^{-1}$ (Lee and Fuhrman, 1987; Kirchman et al.,

**Table 1**
Environmental and biological parameters of the initial samples in the different experiments.

| Variable (median) | Winter | Spring | Summer |
|---|---|---|---|
| Temperature (°C) | 12.7 | 18.1 | 24.9 |
| Salinity | 36.2 | 32 | 34.9 |
| PAR (μmol *photon* $m^{-2}s^{-1}$) | 11.252–33.57 | 87.3946–145.7121 | 4.1895–97.4541 |
| SRP (μM) | 0.07 | 0.05 | 0.02 |
| $NH_4^+$(μM) | 0.20 | 0.36 | 0.23 |
| $NO_3^-$(μM) | 0.32 | 0.32 | 0.091 |
| $NO_2^-$(μM) | 0.31 | 0.03 | 0.085 |
| Chlorophyll *a* (μg $L^{-1}$) | – | 0.68 | 0.17 |
| Bacteriochlorophyll *a* (pM) | 0.634 | 4.125 | 4.646 |
| $P_T$ (μg $CL^{-1}$ $day^{-1}$) | 0.285 | 0.46 | 0.64 |
| $P_L$ (μg $CL^{-1}$ $day^{-1}$) | 0.517 | 2.95 | 2.15 |
| Prokaryote abundance (x $10^6$ cell $mL^{-1}$) (μ $day^{-1}$) | 0.25 (0.80 $day^{-1}$) | 0.57 (0.48 $day^{-1}$) | 0.60 (0.56 $day^{-1}$) |
| HNA prokaryotic cells (%) | 31.70 | 82.10 | 55.61 |
| AAP abundance ($10^3$ cell $mL^{-1}$) (μ $day^{-1}$) | 15.4 (0.80 $day^{-1}$) | 66.4 (0.60 $day^{-1}$) | 83.1 (0.75 $day^{-1}$) |
| Eubacteria (%) | 43.46 | 65.63 | 47.11 |
| Bacteroidota (%) (μ $day^{-1}$) | 10.27 (2.54 $day^{-1}$) | 24.83 (0.30 $day^{-1}$) | 18.51 (0.97 $day^{-1}$) |
| SAR11 (%) (μ $day^{-1}$) | 23.34 (1.60 $day^{-1}$) | 8.66 (0.30 $day^{-1}$) | 5.85 (1.20 $day^{-1}$) |
| Rhodobacteraceae (%) (μ $day^{-1}$) | 16.83 (1.20 $day^{-1}$) | 8.70 (0.55 $day^{-1}$) | 10.61 (0.12 $day^{-1}$) |
| Gammaproteobacteria (%) (μ $day^{-1}$) | 9.57 (1.35 $day^{-1}$) | 22.37 (0.75 $day^{-1}$) | 21.52 (0.68 $day^{-1}$) |
| *Synechococcus* abundance ($10^3$ cell $mL^{-1}$) | 5.92 | 66.77 | 207.67 |
| *Prochlorococcus* abundance ($10^3$ cell $mL^{-1}$) | 4.72 | 3.99 | 20.55 |
| Picoeukaryotes ($10^3$ cell $mL^{-1}$) | 3.47 | 1.36 | 0.65 |
| HNF abundances ($10^3$ cell $mL^{-1}$) | 0.42 | 0.40 | 0.52 |

1993) while the biomass of aerobic anoxygenic phototrophs was determined using a biomass factor calculated from biovolume (Simon and Azam, 1989; Vrdoljak Tomaš et al., 2019). The growth rates (μ, $day^{-1}$) of the total heterotrophic bacteria and the individual phylogenetic groups were calculated for each treatment during the period of their exponential growth rate for each incubation treatment. Following the method of Landry and Hassett (1982), growth rates were estimated as the slope of the linear regressions of the natural log-transformed abundances against the exponential growth period: $\mu=(\ln N_t - \ln N_0)/t$ where $N_0$ and $N_t$ are the cell abundance at the beginning and end of the exponential growth period, and t is the duration of exponential growth in days.

Total bacteria grazing rates (gTOT) were estimated as the difference between the growth rates of bacteria in the <1.2 μm and <150 μm fractions. Grazing rates of heterotrophic nanoflagellates (HNF) on bacteria (gHNF) were estimated by calculating the difference between the growth rates of bacteria in the <1.2 μm and <10 μm fraction. The grazing rates of larger predators (≥10 μM) on bacteria (g LP) were estimated as the difference between gTOT and gHNF (Šolić et al., 2020; Šestanović et al., 2025). Production rates ($P_{CTC}$, μg C $L^{-1}day^{-1}$) and losses due to grazing (G, μg C $L^{-1}day^{-1}$) were estimated using the following equations according to Landry et al. (1995): $P = \mu \times B_{CTC}$ and $G = g \times B_{CTC}$ were $B_{CTC}$ is the cell biomass (μg C $L^{-1}$) at sampling time.

### 2.3. Calculation of the grazing selectivity index

The Chesson-Manly selectivity index (alpha index, α) was calculated for each bacterial group to determine the extent of predator preference for a particular prey type (Manly, 1974; Chesson,1983).

The index assumes a neutral preference for a particular prey type when $\alpha = 1/n$ (grazing is directly proportional to relative abundance in the environment). Values of $\alpha = > 1/n$ indicate a grazing preference and values of $\alpha = < 1/n$ indicate avoidance of a particular prey group.

### 2.4. Picoplankton community structure

All members of picoplankton community (*Synechococcus*, *Prochlorococcus*, picoeukaryotes, heterotrophic bacteria, high and low nucleic acid content (HNA, LNA) bacteria), including HNF and AAPs were determined using flow cytometry (Gasol and Morán, 2016) and infra-red microscopy (Mašín et al., 2006). For details, please see supplement information.

### 2.5. Bacteriochlorophyll a concentration

Between 300 and 2000 mL of water sample were collected onto polyethersulfone filters (25 mm diameter, 0.45 μm pore size, Merck Millipore) to retain marine AAPs (Lami et al., 2009). Filters were fast-frozen and stored in cryogenic vials in liquid nitrogen until pigment extraction could be performed using 7:2 acetone:methanol (v/v) and analyzed using high performance liquid chromatography (HPLC) as described in Gazulla et al. (2023).

### 2.6. Bacterial production

Besides production rates calculated from bacterial cell number mentioned above ($P_{CTC} = \mu$ x $B_{CTC}$), bacterial cell production was estimated by measuring the incorporation of $^3$H-thymidine ($P_T$) into bacterial DNA (Fuhrman & Azam,1982). Bacterial protein synthesis was estimated by measuring the incorporation of $^3$H-labeled leucine ($P_L$) into bacterial cells (Smith, 1992). For details, please see supplement information.

### 2.7. Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH)

Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) was performed according to the standard protocol (Pernthaler et al., 2002) with slight modifications (Supplement information).

### 2.8. Statistical analyses of the impact of environmental factors on growth rates

Principal component analysis (PCA) to link biotic and environmental factors with different growth rates of specific bacterial group was performed using CANOCO software (http://www.canoco5.com/), v5 (ter Braak &Š;milauer, 2012). The data was transformed and standardized, and model results were reproduced in ordination biplots, summarizing the main trends in the data. PRIMER v7 + PERMANOVA (Clarke and Gorley, 2015) was used to test whether the season, inorganic nutrients, glucose or predators significantly affected the bacterial growth rates.

### 2.9. Sampling, DNA extraction and sequencing

Seawater was prefiltered through a 20 μm plankton net and from 200 $mL^{-1}$ to 1 L were immediately filtered through 0.22 μm polyethersulfone membrane filters (PES, 47 mm in diameter, FiltraTECH, France, Ref. MF047PE022). DNA was sampled at 0, 6, 24, and 48 h and extraction was performed as described in our previous work (Šantić et al., 2023).

16S rRNA regions V4 and V5 were amplified using specific primers 515F-Y (5′-GTGYCAGCMGCCGCGGTAA-3′) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3′) with added barcode. The V4-V5 hypervariable regions were chosen because they differentiate bacterial and archaeal taxa (Parada et al., 2016) PCR reactions were performed using Phusion® High - Fidelity PCR Master Mix (New England Biolabs) with temperature conditions of 98 °C for 1 min, 30 cycles of 10 s denaturation at 98 °C, 30 s annealing at 50 °C, 30 s elongation at 72 °C, and final elongation at 72 °C for 5 min. The amplicons were purified using magnetic beads and mixed equimolarly. Sequencing library was generated and quality was checked using Qubit, RT-PCR and bioanalyzer and sequenced on Illumina platform NovaSeq 6000 using paired-end 250bp kit at Novogene Europe (Cambridge, UK). Raw reads ranged from 81 962 to 174 948 (mean 130 653) per sample and can be found in PRJNA1220662 accession number in NCBI SRA repository.

### 2.10. Bioinformatics

Barcode and primer sequences were removed and paired-end reads were merged using FLASH (V1.2.1.1) software at Novogene. Amplicon sequence variants (ASVs) were created using DADA2 and chimera removal (v. 2023.2.0) with default parameters (Callahan et al., 2016). Supplement Table 1 contains information about percentage of filtration of chimera and denoised reads. ASV classification into taxa was performed using naive Bayes classifier built on a RESCRIPt formatted SILVA (v.138) database with extracted amplicon region of the 16S gene (Bokulich et al., 2018; Robeson et al., 2021; Quast et al., 2012). Phylogeny was analyzed using MAFFT multiple sequence alignment and fasttree phylogenetic tree building (Katoh and Standley, 2013; Price et al., 2010). The above steps (ASV creation, classification and phylogenetic analysis) were performed using QIIME2 (version 2024.2) (Bolyen et al., 2019).

Further data analysis was performed in R using RStudio (Team, 2013). Packages phyloseq (v. 1.44.0), vegan (v. 2.6–4), microbiome (v. 1.22.0) and qiime2R (v. 0.99.6) were used for microbiome data manipulation and analysis (McMurdie and Holmes, 2013; Oksanen, 2010; Lahti et al., 2017; Bisanz, 2018). Chloroplast and mitochondria reads were filtered out. The final ASV table contained from 71 322 to

172 304 read counts per sample. ASV table was rarefied to 71 322 counts for all samples for alpha diversity measures calculation. The final number of ASVs was 18 429, while final number of reads in all samples was 9 106 483. PCA from the ASV community composition was generated on centred log-ratio transformed reads. The 10 ASVs driving the major dissimilarities were included in the plot with their taxonomic affiliation. Packages ggplot (v. 3.5.0) and microViz (v. 0.12.1) were used for graphical representation of results (Parada et al., 2016; Wickham and Wickham, 2016).

### 2.11. Environmental parameters

Temperature and salinity were measured with a SeaBird 25 CTD profiler with an accuracy of $> \pm$ 0.01 °C and ±0.02, respectively. Concentrations of inorganic nutrients were determined using the modified autoanalyzer method (Koroleff and Grasshoff, 1976). The detection limits for the method were 0.001 μM for nitrites ($NO_2^-$), 0.01 μM for nitrates ($NO_3^-$), 0.01 μM for ammonium ion ($NH_4^+$), and 0.02 μM for soluble reactive phosphorus (SRP). Chlorophyll *a* (Chl *a*) was determined from 500-mL subsamples filtered through Whatman GF/F glass fiber filters and stored at −20 °C. The filters were homogenized, extracted in 90% acetone, and then analyzed fluorometrically using a Turner TD -700 laboratory fluorometer calibrated with pure Chl *a* (Sigma) (Strickland and Parsons, 1972).

## 3. Results

### 3.1. Environmental parameters and bacterial community structure

Our manipulation experiments were conducted in February, May and July 2023 representing winter, spring and summer season (Table 1). The total prokaryotes abundance was elevated during the warmer period, along with increased values of thymidine incorporation, abundances of *Synechococcus*, *Prochlorococcus*, and AAP bacteria. Picoeukaryotes reached their highest abundance during winter. Leucine incorporation values were highest in spring when HNA bacteria also reached 82% of the prokaryotic population. Growth rates for total bacteria and AAP bacteria were highest in the winter experiment, with both groups reaching 0.80 $day^{-1}$. The bacteriochlorophyll *a* (BChl *a*) concentration in the sea water, which is an indicator of the photoheterotrophy occurring in the environment, was lower in winter (0.634 pM) than in the other seasons (4.125 pM and 4.646 pM in spring and summer, respectively) (Supplementary Fig. 1). Although spring and summer presented similar initial concentration, the photoheterotrophy presented a different response with a seasonal component. Nevertheless, higher concentration of BChl *a* occurred in the presence of inorganic and organic nutrients as well as with the reduction of the grazing pressure.

The proportions of specific groups determined by CARD-FISH method in initial samples showed the following patterns. Within the prokaryotic population, clade SAR11 predominated during winter, Bacteroidota predominated in spring, and Gammaproteobacteria predominated in summer. Eubacteria were recorded within a median range of 43.46%–65.63%, consistent with our previous studies (Šantić et al., 2023).

In the conducted experiments, the highest values of growth rates for all groups were obtained in the winter experiment (Table 1), ranging from 1.20 $day^{-1}$ (Rhodobacteraceae) to 2.54 $day^{-1}$ (Bacteroidota).

In additional fractionation experiments, the temperature and salinity were as follows: winter 13.5 °C and 36.5, spring 16.4 °C and 36, summer 23.9 °C and 32.9.

Abundances of total prokaryotes, Rhodobacteraceae-ROS537, SAR11-SAR11, Bacteriodetes-CF319a, Gammaproteobacteria-Gam42a and AAPs along different combination of temperature during the incubation time (A-February, B-May, C- July) in control samples (C), added inorganic nutrients (IN), added inorganic nutrients with glucose (ING) and without predator (PF) are shown in Supplementary Fig. 2.

### 3.2. Assessment of the impact of environmental factors on carbon flow through bacterial and AAP community

In the mesocosm experiments, we investigated the influence of environmental factors on carbon flow through a prokaryotic community, with the main focus on contribution of AAP bacteria. Bacterial biomass production was assessed using thymidine and leucine incorporation measurements, as well as by calculating changes in prokaryote abundance using the cell-to-carbon conversion factor. Bacterial production, measured through leucine incorporation rates and thymidine incorporation rates, exhibited diverse responses to different conditions (Fig. 1). The highest $P_L$ values and the highest proportions of HNA were recorded in the winter-spring period under conditions without nutrient limitations. The highest $P_T$ value was recorded during winter, under conditions of unlimited inorganic nutrients and glucose, regardless of predation. The summer experiment showed that both bacterial productions evaluated responded faster to the addition of inorganic nutrients than to the addition of inorganic nutrients with glucose.

Across the temperature range studied (12.7 °C–24.9 °C), $P_{CTC}$ ranged from 3.82 μg C $L^{-1}day^{-1}$ to 21.43 μg C $L^{-1}$ $day^{-1}$ with AAP bacteria contributing from 9% to 33.52% to this production (Fig. 2A). Grazing-induced loss of carbon from the entire bacterial community (G) reached up to 18 μg C $L^{-1}$ $day^{-1}$, with AAP representing from 9% to 70% of this loss. Increases in $P_{CTC}$, AAP contribution, and G were observed with rising environmental temperatures. However, the highest AAP contribution to G were recorded during the winter experiment (Fig. 2B).

The effects of inorganic nutrient addition increased $P_{CTC}$, especially in the presence of glucose, and during warmer periods without predator presence. AAP exhibited a more pronounced increase in its contribution to $P_{CTC}$ during warmer periods and under predator-free conditions compared to inorganic nutrient and glucose supplementation (Fig. 2A). G values were higher during warmer periods compared to colder ones, particularly in spring under nutrient-unlimited conditions (Fig. 2B) with more biomass removed by grazing from HNF compared to grazing by larger predators.

In microcosm grazing experiments with different predator fractions, we employed three treatments: a control sample (C), a sample with HNF, and a sample of seawater without predators (PF). Results indicate an increase in $P_{CTC}$ during warmer seasons compared to winter, particularly in treatments with predator presence. The highest and similar contribution of AAP bacteria in $P_{CTC}$ were observed during spring in the control treatment and in the treatment without predators (Fig. 3A). Looking at the contribution of AAP in grazing-induced loss, a slight elevation in grazing by all present predators (most likely with ciliates) compared to only flagellates is evident. Additionally, AAP were responsible for a 40 % of bacterial biomass loss during the summer period in the control experiment (Fig. 3B).

### 3.3. Assessment of the impact of environmental factors on carbon flow through bacterial community with emphasis on bacterial groups

In the control treatments during winter, SAR11 dominated prokaryotic biomass production rates, while Gammaproteobacteria contributed the most to grazing losses and were the preferred prey. As temperature increased, the contribution of Gammaproteobacteria and Bacteroidota to biomass production rates also increased. Regarding grazing losses, SAR11 and Bacteroidetes were the main contributor in spring, whereas Gammaproteobacteria and Rhodobacteraceae played the most significant role during summer (Fig. 4A; Table 2).

In inorganic nutrients enrichment, SAR11 contributed the most to biomass production rates and grazing losses as the preferred prey during winter. With increasing temperature, Gammaproteobacteria, Bacteroidetes and Rhodobacteraceae became the dominant contributors to biomass production rates. Regarding grazing losses, Gammaproteobacteria contributed the most in spring and were the preferred prey, while during summer, Gammaproteobacteria and Bacteroidetes groups

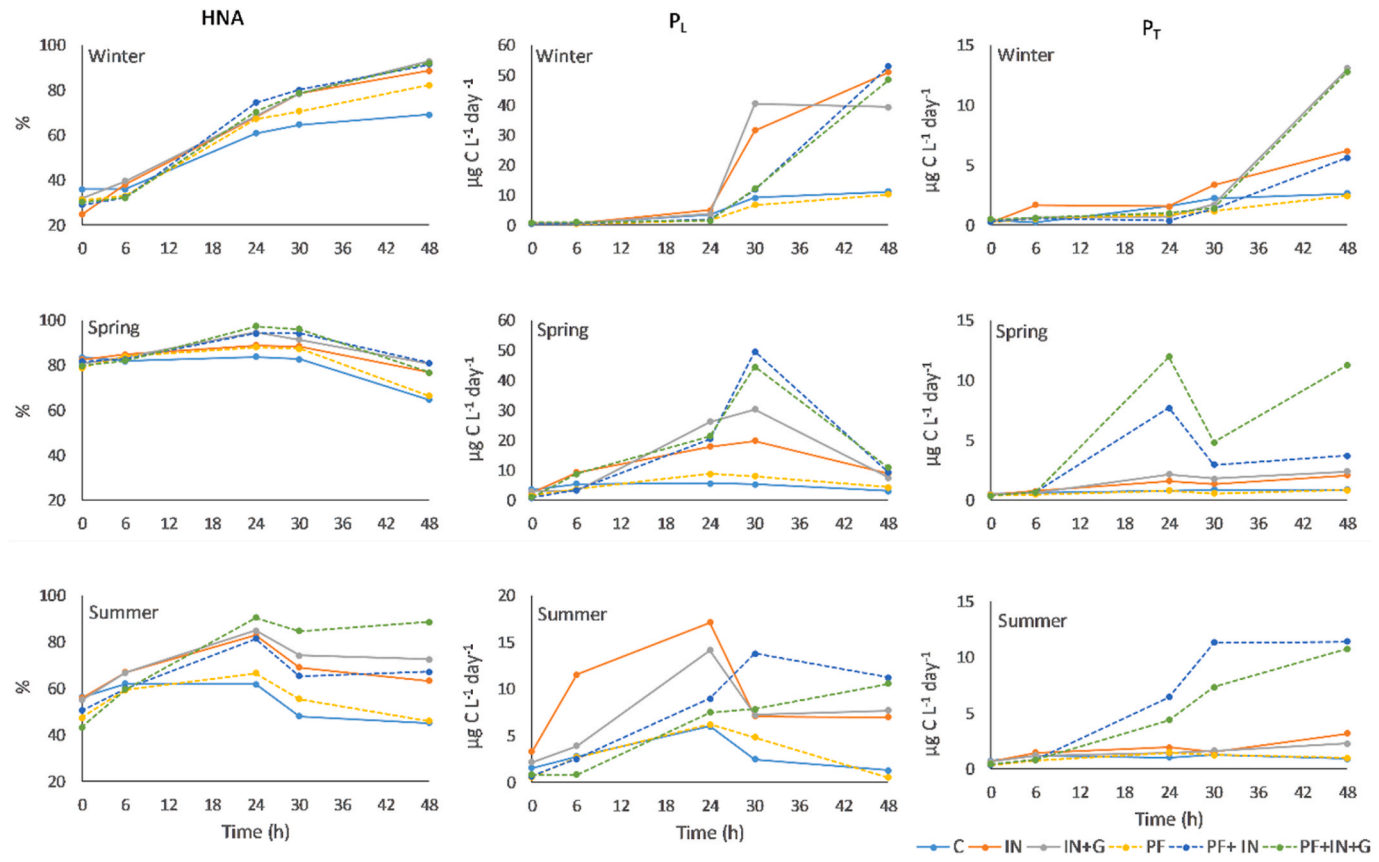


**Fig. 1.** Left panel: Percentage of high nucleic acid content (HNA) cells for each treatment and experiment. Middle panel: Bacterial heterotrophic production measured as leucine incorporation rates (PL) during the incubation for each treatment and experiment. Right panel: Bacterial heterotrophic production measured as thymidine incorporation rates (PT) during the incubation for each treatment and experiment. C, control; IN, inorganic nutrients added; IN + G, inorganic nutrients and glucose added; PF, predator free; PF + IN, predator free and inorganic nutrients treatment; PF + IN + G, predator free and inorganic nutrients and glucose added.

contributed equally to carbon flux through grazing (Fig. 4B; Table 2).

In conditions enriched with inorganic nutrients and glucose, SAR11 predominated notably in both prokaryotic biomass production and biomass loss due to grazing during winter, indicating it as the preferred prey (Table 2). With increasing temperature, an increased contribution of Gammaproteobacteria, Bacteroidota, and Rhodobacteraceae to production rates was observed, along with a greater contribution of the Bacteroidota phylum and Gammaproteobacteria to grazing losses (Fig. 4C; Table 2). Growth and grazing rates of all observed bacterial groups are shown in Supplementary Table 2.

### 3.4. Assessment of the impact of environmental factors on growth rates

Permanova results (Supplementary Table 3) show that the most significant factor influencing the growth rates of the bacterial groups is inorganic nutrient limitation (P = 0.013, Pseudo-F: 31.711), followed by the influence of predators (P: 0.049; Pseudo-F: 13.486).

The bacterial community was subjected to variable environmental conditions throughout the experiments. To link biotic and environmental factors with different growth rates of specific bacterial groups, we conducted PCA. PCA1 accounted for 48.23% of the variation and demonstrated an increase in growth rates in the samples with added nutrients, in those ones lacking control by grazing, and in the samples from the warmer seasons (Fig. 5). SAR11 and the Rhodobacteraceae group exhibited similar patterns across environmental conditions, distinct from other groups.

### 3.5. Assessment of the impact of environmental factors on prokaryotic diversity

Observing bacterial diversity (Supplement Fig. 3) in the initial sample, the highest alpha diversity values were recorded during winter (median Shannon: 4.756; median Pielou's: 0.779; median ASV: 450), while the lowest diversity was observed during the summer experiment (median Shannon: 4.135; median Pielou's: 0.708; median ASV: 337). In all three experiments, a decrease in bacterial diversity (as indicated by all three metrics) was observed with an increase in bacterial numbers (0–24 h) and the addition of inorganic nutrients, especially inorganic nutrients with glucose.

The diversity in the initial sample and in the sample filtered through 1.2 µm pores during winter was similar, while diversity in the filtered samples was lower compared to the initial sample during warmer periods.

In all initial conditions, at class level Alphaproteobacteria predominates in community and in all experiments and treatments, there was an increase in temperature leads to the predominance of Gammaproteobacteria and Bacteroidia in the community composition. At class level, Gammaproteobacteria and Alphaproteobacteria prevail, particilarly the first at the end of the experiment and in the predator free treatment with nutrients and glucose. During winter there is Thermoplasmata which dissapears early in the experiment. Bacteroidia fluctuate during summer in the <150 µm treatments (Fig. 6A). At order level, Enterobacterales prevail among season and treatments. SAR11 clade increased during warmer periods and in the predator free treatments. Flavobacteriales are more abundant in winter and spring. Pseudomonadales remain abundant throughout the experiment, preffering winter

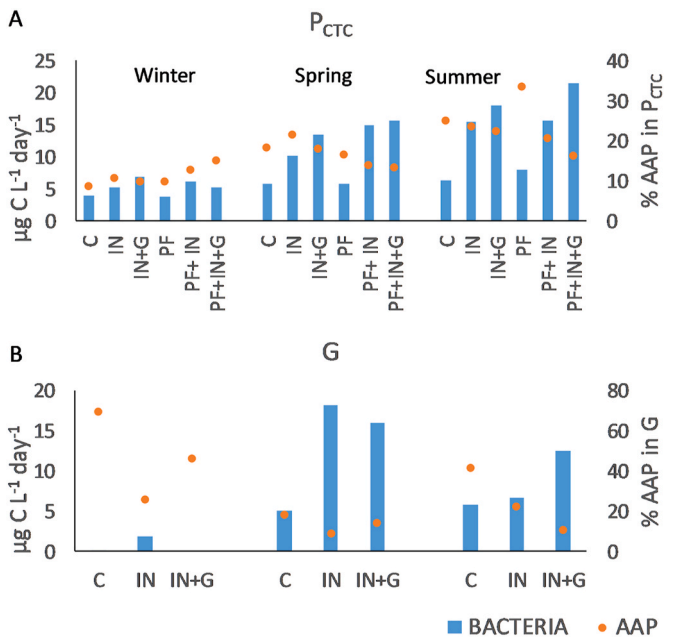


**Fig. 2.** A) Bacterial production ($P_{CTC}$) and the AAP contribution within it during the incubation for each treatment and experiment. Treatments were categorized as follows: C, control; IN, inorganic nutrients added; IN + G, inorganic nutrients and glucose added; PF, predator-free; PF + IN, predator-free with inorganic nutrients treatment; PF + IN + G, predator-free with inorganic nutrients and glucose added.
B) Losses due to grazing (G) and the AAP contribution within it during the incubation for each treatment and experiment. Treatments were categorized as follows: C, control; IN, inorganic nutrients added; IN + G, inorganic nutrients and glucose added.

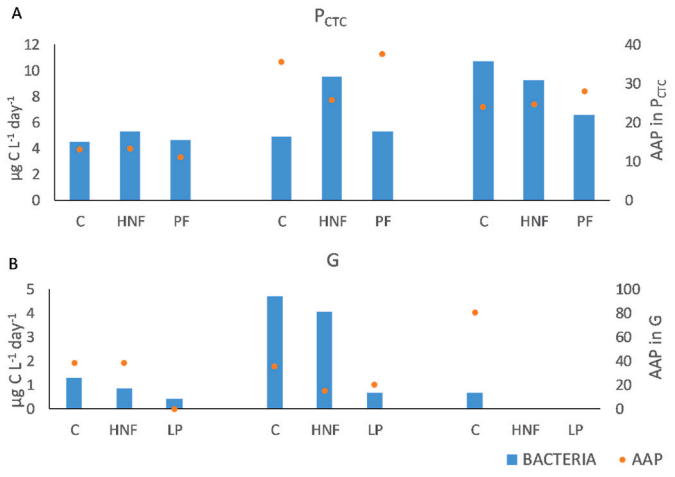


**Fig. 3.** A) Bacterial production ($P_{CTC}$, bars) and the contribution of AAP to the bacterial production (circles) for each treatment and experiment. Treatments were categorized as follows: C, control; HNF, with heterotrophic nanoflagellates; PF, predator-free. B) Losses due to grazing (G) and the AAP contribution within it during the incubation for each treatment and experiment. Treatments were categorized as follows: C, control, grazing by all predators; HNF, grazing by heterotrophic nanoflagellates; LP, grazing by predators larger than ≥10 μm.

and predator free treatment. Marine group II is visible only in winter (Fig. 6B).

At genus level, in spring there is increae in Glaciecola with added nutrients and nutrients and glucose together. *Synechococcus* and Vibrio are abundant in summer, particularly in control treatments, with the latter exhibited the highest proportio with added nutrients and glucose. Clade Ia is abundant at the beginning of the winter experiment, with Glaciecola prevailing towards the end (Fig. 6C). To investigate the possible influence of environmental factors (seasons, availability of inorganic nutrients, inorganic nutrients and glucose) on the community composition at the top 10 ASVs, we conducted principal component analysis (PCA). PC1 explains 37.7 % of the variance while PC2 explains 14%. PC1 shows a clearer separation of the most abundant ASVs based on season rather than nutrient availability (Fig. 7). Additionaly, Jaccard and Bray-Curtis distance show clear separation of samples according to seasons (Supplementary Fig. 4).

## 4. Discussion

Our study aimed to assess the contribution of the bacterial community and its individual members to carbon production in marine ecosystems. Furthermore, it investigates the transfer of carbon through predation to higher trophic levels, identifying the key bacterial groups and environmental conditions that contribute most to this transfer. Despite pronounced differences in growth rates and biomass production among different functional groups, the overall bacterial biomass in marine ecosystems remains relatively stable, likely due to balanced biomass production and grazing (Jürgens and Massana, 2008; Ferrera et al., 2017; Sánchez et al., 2017). By conducting experiments, our intention was to determine the influence of nutrients, predation, and temperature as a trigger for all biochemical reactions (White et al., 1991; Pomeroy and Wiebe, 2001). Aware of all methodological limitations (e.g., without biological replicates, filtration through 1.2 μm does not remove all predators since smaller ones may pass through, as observed in previous experiments, and filtration removes up to 30% of the bacterial population in the coastal Adriatic (data not shown); limitations of the CARD-FISH method), we conducted three seasonal experiments to assess the afore mentioned points in the coastal area of the central Adriatic Sea.

### 4.1. Assessment of the dynamics of community structure

In the initial samples, higher prokaryotic abundance was observed during warmer periods, while picoeukaryote blooms occurred in winter, consistent with previous research (Šantić et al., 2014). Elevated picoeukaryote abundances were also linked to higher phosphate and nitrite concentrations (Matek et al., 2023). Regardless of the season, class Alphaproteobacteria dominated the picoplankton community, consistent with findings from the Mediterranean (Alonso-Sáez et al., 2007). Furthermore, seasonal distribution of different bacterial groups showed SAR11 and Rhodobacteraceae predominating in colder periods, while Gammaproteobacteria and Bacteroidota dominated warmer periods. This pattern aligns with previous studies (Babić et al., 2018; Šantić et al., 2023; Šestanović et al., 2025), which showed the dominance of SAR11, a typical oligotrophic clade, in the Adriatic during winter. The highest growth rates in our study occurred during winter, coinciding with the lowest abundances of all investigated CARD-FISH groups. The Bacteroidota group had the highest growth rate, followed by the SAR11 clade. Similar patterns were observed in the NW Mediterranean (Sánchez et al., 2017, 2020). Growth rates reflect survival strategies: fast-growing, metabolically versatile bacteria (r-strategists) and slower-growing, smaller bacteria (K-strategists). Our findings contradict previous Mediterranean studies, which suggested that the SAR11 clade had the slowest growth rate compared to other bacterioplankton groups (Ferrera et al., 2011; Sánchez et al., 2020). Seasonal dynamics were observed, with the highest bacterial diversity during colder periods. Diversity decreased with increasing nutrient availability and prokaryotic abundance, aligning with previous research (Šantić et al., 2023). This trend is supported by Pommier et al. (2010), who reported a negative correlation between bacterial diversity, biomass, and heterotrophic production. In winter, diversity was similar between initial and

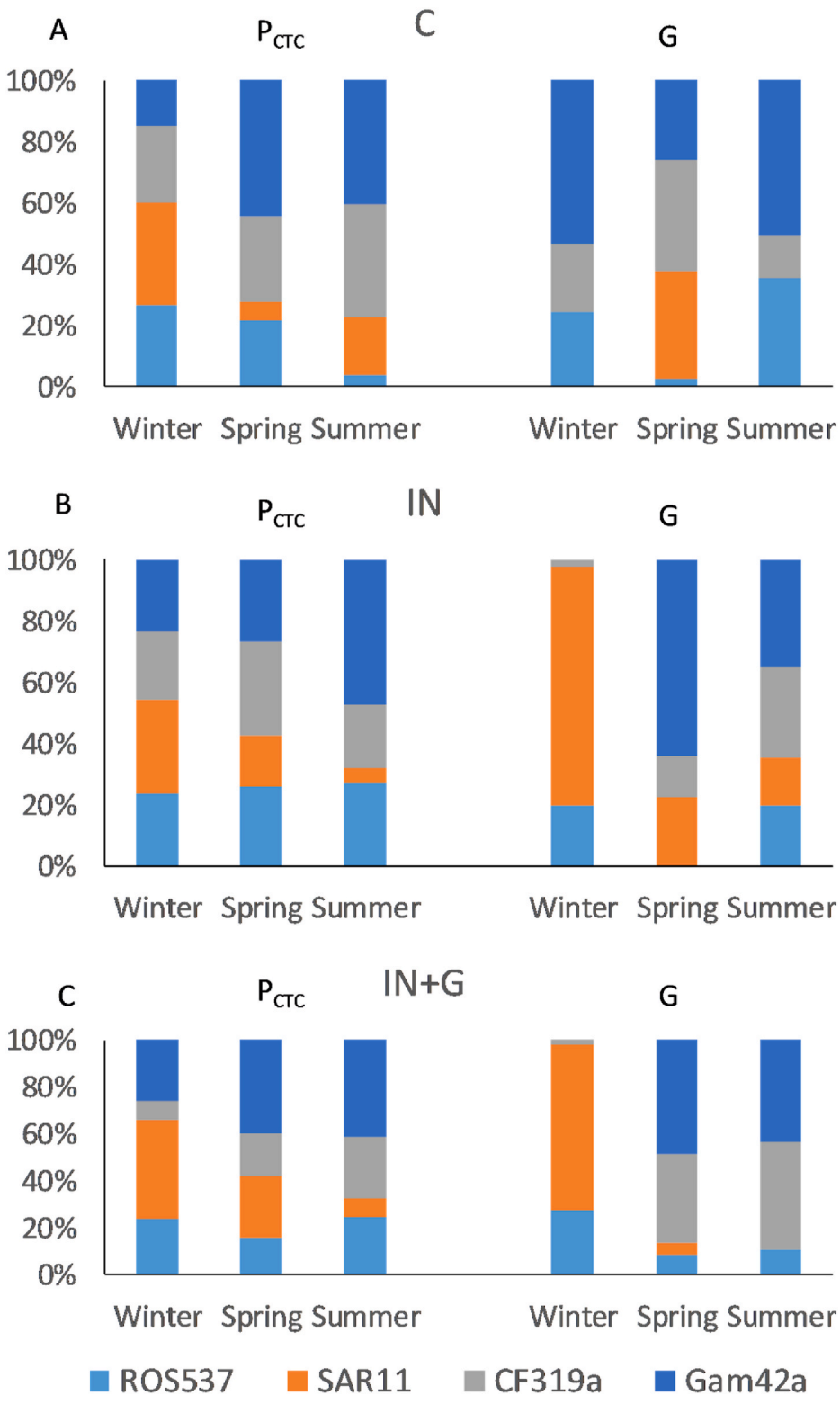


**Fig. 4.** Proportion of specific CARD-FISH identified groups in bacterial production ($P_{CTC}$) and grazing losses (G) during winter, spring and summer across three treatments: A) control conditions (C); B) IN, inorganic nutrients added; C) IN + G, inorganic nutrients and glucose added.

filtered samples, while in warmer periods, it was higher in the initial samples. Increased temperatures promote differentiation between particle-attached and free-living bacteria due to enhanced primary production and an increase in particulate organic matter availability. This suggests that bacterial cells grew and attached to particles in spring to exploit phytoplankton resources, influencing diversity in filtered samples (Arandia-Gorostidi et al., 2017; Wang et al., 2024). In general, dominance of Gammaproteobacteria and Alphaproteobacteria, as well as order Enterobacterales is evident, with seasonal variations in SAR11, Flavobacteriales, and Marine Group II. Archeal Marine group II is known to degrade organic carbon (Zhang et al., 2015). Recently archeal genera were described in the Adriatic Sea (Šantić et al., 2013).

**Table 2**
Alpha index: Values above 0.25 indicate prey preference (bold); values below 0.25 indicate prey avoidance. Treatments were categorized as follows: C, control; IN, inorganic nutrients added; IN + G, inorganic nutrients and glucose added.

| | C | | | IN | | | IN + G | | |
|---|---|---|---|---|---|---|---|---|---|
| | Winter | Spring | Summer | Winter | Spring | Summer | Winter | Spring | Summer |
| ROS537 | 0.14 | 0.02 | **0.50** | 0.23 | 0.00 | 0.23 | **0.37** | 0.20 | 0.18 |
| SAR11 | 0.00 | **0.66** | 0.00 | **0.42** | **0.40** | 0.25 | **0.50** | 0.09 | 0.00 |
| CF319a | **0.27** | 0.14 | 0.15 | 0.16 | 0.09 | **0.26** | 0.13 | **0.32** | **0.34** |
| Gam42a | **0.59** | 0.17 | **0.35** | 0.20 | **0.51** | **0.26** | 0.00 | **0.39** | **0.48** |

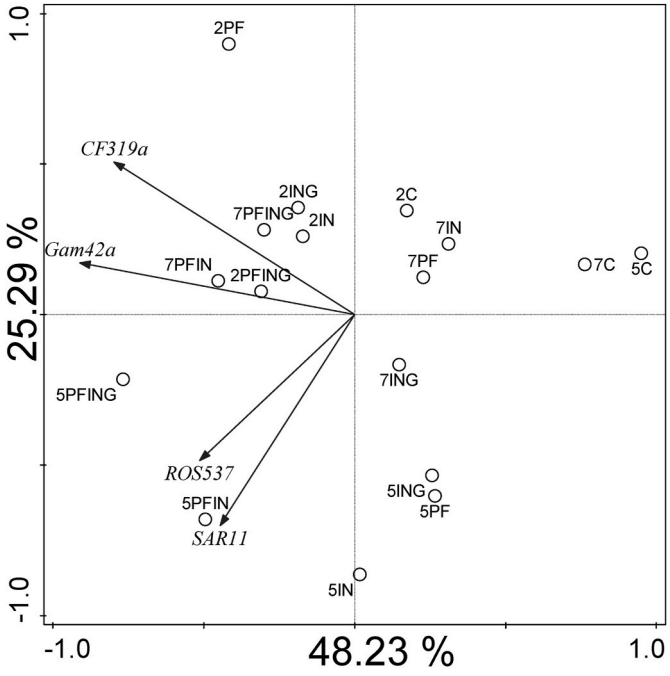


**Fig. 5.** Principal component analysis of specific bacterial growth rates in this study (Bacteriodetes-CF319a, SAR11-SAR11, Rhodobacteraceae-ROS537 and Gammaproteobacteria-Gam42a) along different combination of temperature (month:2-February; 5-May; 7-July), in control samples (C), added inorganic nutrients (IN), added inorganic nutrients with glucose (ING) and without predator (PF).

Thermoplasmatota is very common and important archaeal phylum in the marine nitrogen cycle, as well as Nitrososphaera.Chemo-organotrophic Flavobacteriales and Enterobacterales are noticed, suggesting potential associations with organic matter degradation or pollution.

### 4.2. Assessment of the dynamics of bacterial production

The results of our experiments indicate the influence of temperature, nutrients, and predation on the dynamics of the entire bacterial population. In most aquatic environments, bacteria are primarily controlled by nutrients in eutrophic areas, while predation predominates in oligotrophic regions (Billen et al., 1990; Gasol, 1994; Gasol et al., 2002; Šolić et al., 2009). This study is the first to measure both types of bacterial production (leucine and thymidine incorporation) in the investigated area. The highest leucine incorporation occurred when HNA bacteria dominated the community, particularly in conditions with sufficient inorganic nutrients and no predation. Thymidine incorporation was highest in initial summer samples, with the highest experimental values observed during winter under nutrient-unlimited conditions and in summer without predators and nutrient limitations. The association of HNA bacteria with high leucine and thymidine incorporation, along with previous findings linking LNA bacteria to high thymidine values (Šantić et al., 2012, 2013), highlights the significance of both groups in marine ecosystems. The view that LNA bacteria represent dead or inactive cells has been abandoned (Mary et al., 2006; Zubkov et al., 2006; Hill et al., 2010). Both HNA and LNA bacteria contribute to bacterial activity (Bouvier et al., 2007), with HNA cells generally dominating leucine incorporation due to their larger size (Talarmin et al., 2011). The factors influencing the predominance of these groups remain unclear, as both are affected by temperature and predation (Morán and Calvo-Diaz, 2009; Morán et al., 2010; Šolić et al., 2020).

### 4.3. Assessment of carbon flow through the bacterial community with emphasis on different bacterial groups

Under initial winter conditions, as well as under nutrient-enriched conditions, SAR11 contributed most to prokaryotic biomass production. Gammaproteobacteria and Bacteroidota increased their contribution with rising temperatures, and Rhodobacteraceae in a nutrient-enriched environment.

Rhodobacterales metabolize a wide variety of organic compounds (Newton et al., 2010) and are sensitive to grazing (Fecskeová et al., 2021). It is well-known that some Gammaproteobacteria and Bacteroidota thrive in warmer, nutrient-rich environments (Teira et al., 2008; Fecskeová et al., 2021; Šantić et al., 2023). Gammaproteobacteria showed an increase in their contribution to bacterial biomass production across all three treatments with rising temperature. This is consistent with their known opportunistic behaviour (Allers et al., 2007; Teira et al., 2008) and rapid response to environmental changes (Beardsley et al., 2003), allowing them to dominate under specific conditions (Ferrera et al., 2011). Changes in environmental factors did not significantly affect the diversity of contributions to bacterial biomass production. In this study, in warm, nutrient-rich conditions without predator, bacterial growth rates showed the highest values (Bacteroidetes, SAR11, Rhodobacteraceae, and Gammaproteobacteria), while in similar experiments conducted in the Mediterranean, predation was found to be a more important ecological factor compared to nutrients (Sánchez et al., 2017).

Regarding grazing losses under initial winter conditions, Gammaproteobacteria were the primary contributors and served as the preferred prey. SAR11 and Bacteroidota were the dominant contributors in spring, while Gammaproteobacteria and Rhodobacteraceae played the most significant role during summer. Under nutrient-enriched conditions, SAR11 contributed the most to grazing loss during winter. However, with rising temperatures, Gammaproteobacteria and Bacteroidota became the primary contributors to grazing loss. Our study shows that bacterial group composition in grazing loss varies with the trophic status of the environment. Gammaproteobacteria has proven to be the preferred prey regardless of season or nutrient addition, consistent with previous studies (Šestanović et al., 2025). Previous research in the Mediterranean has shown that representatives of the Bacteroidota are controlled by both available nutrients and predation, and, as in our study, they represent a good prey (Ferrera et al., 2011). Regardless of the environment (control, with added inorganic nutrients, inorganic nutrients and glucose) where the bacterial community was situated, in this study, during winter and spring representatives of the SAR11 clade were

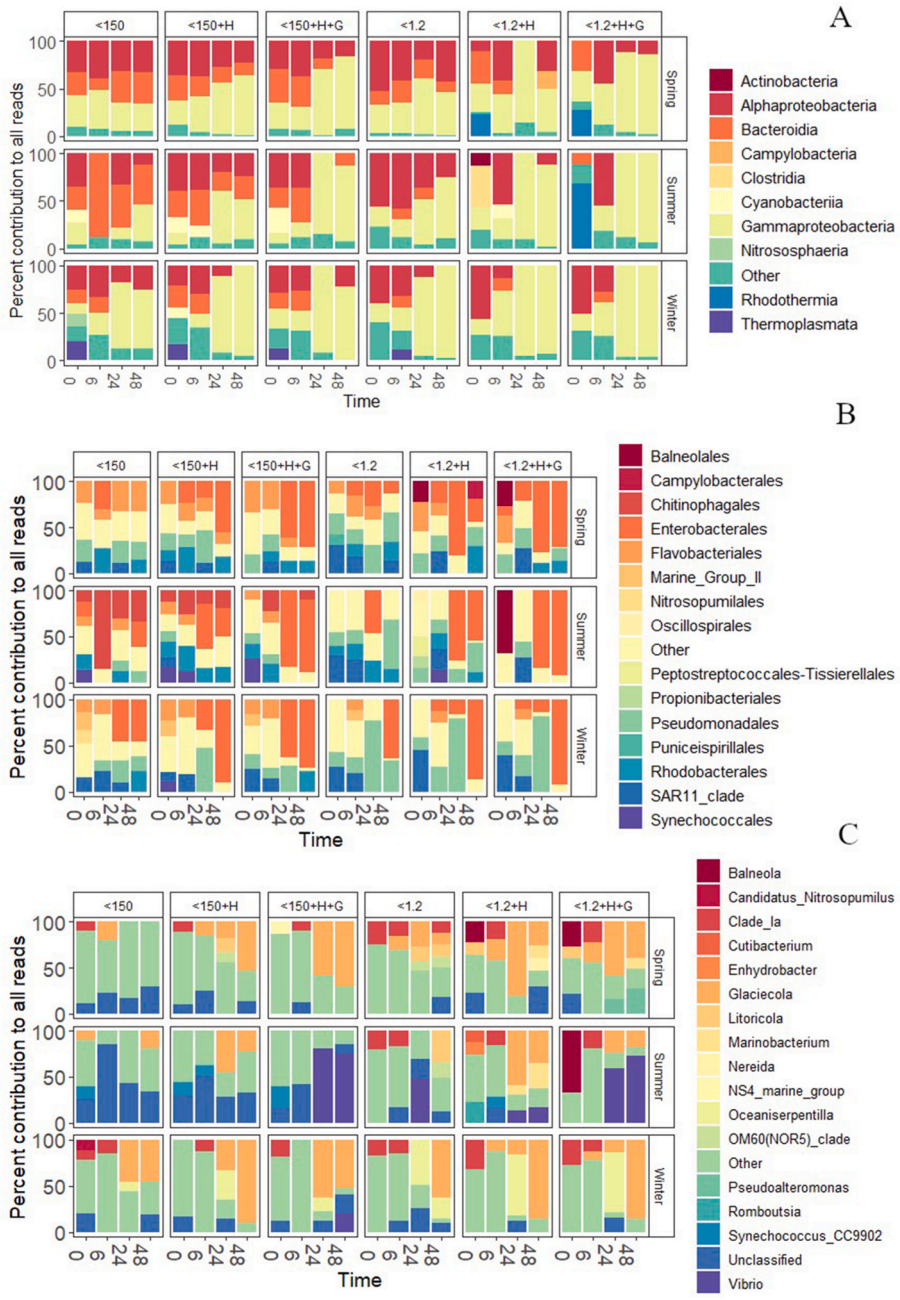


**Fig. 6.** Prokaryotic community composition at the: A) class level shown on top 10 class level; B) order level; C) genus level under different conditions (control conditions (C < 150 μm); inorganic nutrients added (C + IN); IN + G, inorganic nutrients and glucose added (C + IN + G); predator-free condition (PF); inorganic nutrients added (PF + IN); inorganic nutrients and glucose added (PF + IN + G)).

identified as the preferred prey. This aligns with previous research in the Mediterranean, where SAR11 was shown to be a good prey, indicating that predation is an important factor in its control (Ferrera et al., 2011; Sánchez et al., 2020). Furthermore, our study found that the Rhodobacteraceae and SAR11 clades had the same response across the conditions they were exposed to, whereas in previous similar studies, these groups exhibited opposite responses to environmental changes (Teira et al., 2009; Ferrera et al., 2011; Sánchez et al., 2017). These differences are most likely due to the genetic diversity within those groups, since members from the same genus might have differential behaviour (Villena-Alemany et al., 2024), as well as the seasonality of the community composition (Stojan et al., 2024).

### *4.4. Assessment of carbon flow through the bacterial community with emphasis on AAP bacteria*

The results of this study, for the first time, reveal the role of AAP bacteria in carbon flow within the microbial food web in the Adriatic Sea. We observed an increase in $P_{CTC}$ values, the contribution of AAP bacteria to bacterial production, and grazing losses with rising temperatures. The highest bacterial biomass production occurred in summer, coinciding with AAP bacteria contributing up to 33.52% of production. Grazing losses peaked in spring, while in summer, AAP bacteria accounted for up to 40% of bacterial losses. These findings align with research in the Catalan Sea, where AAP bacteria contributed 27–69% of bacterial production (Hojerová et al., 2011). Notably, during winter, AAP bacteria represented up to 70% of bacterial losses due to

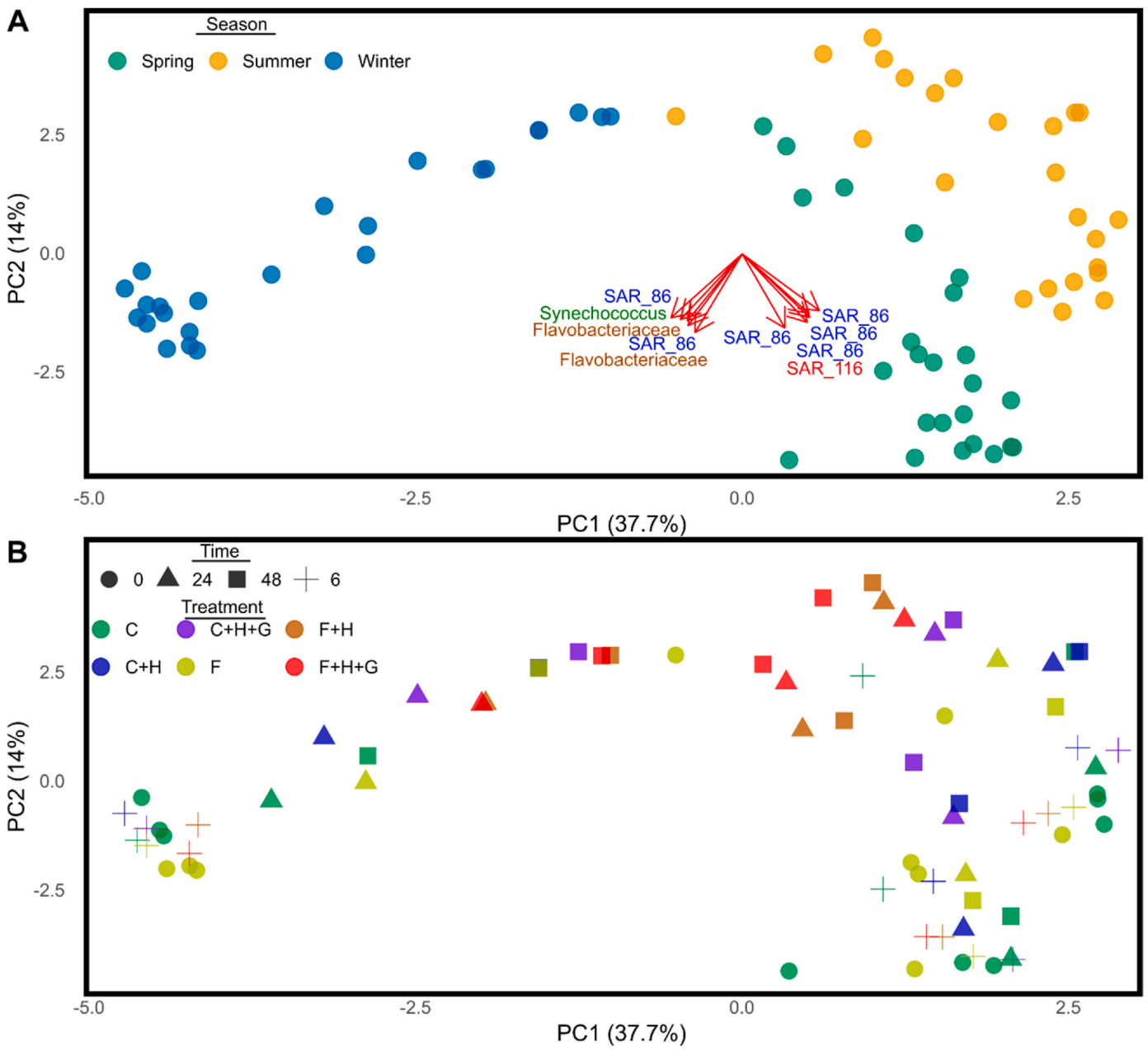


**Fig. 7.** Principal component analysis of the top 10 ASVs A) seasons; B) treatments under different conditions (control conditions (C < 150 μm); inorganic nutrients added (C + IN); IN + G, inorganic nutrients and glucose added (C + IN + G); predator-free condition (PF); inorganic nutrients added (PF + IN); inorganic nutrients and glucose added (PF + IN + G)).

grazing. Despite AAP abundances being lower than total bacteria, their growth rates were highest in winter in control experiments. In addition to heterotrophic flagellates, predators larger than 10 μm also removed AAP biomass through grazing. These results confirm that grazing is a key factor in controlling AAP populations, with larger, fast-growing cells being favoured prey (Brüwer et al., 2023; Stoecker and Capuzzo, 1990; Ferrera et al., 2011). Photoheterotrophy plays an important role in the carbon cycle (Piwosz et al., 2022), with warmer temperatures and nutrient inputs enhancing its contribution to ecosystem functioning. Given global warming and coastal eutrophication, photoheterotrophy's role in ecosystems is expected to increase. Consistent with previous studies (Koblížek et al., 2007, 2024), grazing significantly reduces AAP biomass and BChl *a* concentration. Seasonality strongly influences photoheterotrophy, and further studies on AAP community composition are recommended. Our results highlight that AAP bacteria contribute significantly to organic matter recycling in marine environments, beyond their abundance alone (Koblížek et al., 2007; Ferrera et al., 2011).

## 5. Conclusions

Our results demonstrate that nutrient availability, predation, and temperature play crucial roles in controlling bacterial population growth throughout the seasons. AAP bacteria exhibited the most pronounced increase in their contribution to biomass production with rising environmental temperatures, particularly in nutrient-enriched environments. However, they were identified as significant prey during winter and summer under control conditions, suggesting that they were more strongly influenced by grazing during the warmer part of the experiment. AAP bacteria were notably preyed upon by HNF and predators larger than 10 μm.

The composition of bacterial groups associated with grazing loss

varies depending on the trophic status of the environment. Representatives of the SAR11 clade were identified as preferred prey during winter and spring across all three treatments, indicating that they were under greater predation pressure than nutrient limitation. The simultaneous increase in the proportion of Gammaproteobacteria in biomass production across all three treatments, along with their greater contribution to grazing losses during spring and summer, suggests that this bacterial group was more strongly affected by predation pressure than by nutrient availability.

Bacteroidota showed increases in both biomass production and grazing losses with rising temperatures across all treatments, suggesting that these bacteria are regulated equally by nutrient availability and predation.

Rhodobacteraceae exhibited an increase in grazing losses with rising environmental temperature under initial conditions, whereas under nutrient-enriched conditions, they showed a slight increase in biomass production with increasing temperature.

In summary, this study enhances our understanding of the contributions of bacterial groups to the marine carbon cycle under changing environmental conditions, providing insight into their roles in the context of ongoing climate change.

## CRediT authorship contribution statement

**Danijela Šantić:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Iva Stojan:** Software, Formal analysis, Data curation. **Ana Vrdoljak Tomaš:** Writing – review & editing, Formal analysis. **Blanka Milić Roje:** Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Izabela Mujakić:** Writing – review & editing, Formal analysis. **Cristian Villena-Alemany:** Writing – review & editing, Visualization, Formal analysis, Software. **Michal Koblížek:** Writing – review & editing, Resources, Funding acquisition. **Slaven Jozić:** Formal analysis. **Mladen Šolić:** Writing – review & editing. **Stefanija Šestanović:** Formal analysis.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Bioinformatic work was performed using the services of SRCE (University of Zagreb University Computing Centre) (https://www.srce.unizg.hr/en/advanced-computing).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2025.109228.

## Data availability

Data will be made available on request.

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