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Temperature Effects on Growth Rates and Predation Loss of Bacterial Groups in Marine Ecosystems

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Abstract

This study investigated the effects of experimental warming on the major bacterial phylogenetic groups in the marine environment, focusing on their growth and grazing rates, phylogenetic community composition and grazing preferences. A series of growth and grazing experiments were performed in microcosms at a temperature increase of 3°C. The growth rates of all bacterial groups were enhanced by temperature increase. SAR11 and Roseobacter were particularly sensitive to temperature changes, while Gammaproteobacteria were the least affected. Protozoan grazing mainly affected fast-growing groups such as Roseobacter and Gammaproteobacteria, while slower-growing groups such as SAR11 were less affected. In most cases, the growth rates of the bacteria increased more with temperature than the grazing rates of predators. This indicates that temperature has a stronger influence on bacterial growth than on predator activity. At higher temperatures the grazing pressure of nanoflagellates (NF) on Roseobacter and Bacteroidetes increased while Gammaproteobacteria were almost exclusively grazed by ciliates (CIL). As a result, the dominant predator of certain groups and the composition of prey changed with temperature increase. The changes in bacterial community composition at higher temperatures were caused both by the direct effect of temperature on bacterial growth and by shifts in predator grazing preferences. Furthermore, our results suggest that bacterial growth and grazing behaviour of bacterial predators do not necessarily occur simultaneously at higher temperatures and highlight the importance of considering bacterial phylogenetic composition when predicting bacterial response to warming.

Keywords: experimental warming; CARD FISH; bacterial phylogenetic groups; growth rate; grazing rate; grazing preference.

Introduction

The rise in sea surface temperatures, which is one of the main effects of climate change, is expected to have a significant impact on the marine environment (Wernberg *et al*., 2013). Microorganisms in particular are responsible for the functioning of marine ecosystems driving biogeochemical cycles, fluxes and processes (Falkowski *et al*., 2008). Therefore, it is crucial to understand how they respond to environmental changes, especially in oligotrophic environments where the microbial food web is the dominant trophic pathway (Christaki *et al*., 2011a; Šolić *et al.,* 2018a; 2020; 2021).

Temperature is one of the most important regulators of microbial metabolic processes, including production, growth and respiration (Huete-Stauffer & Morán, 2012; Vázquez-Domínguez *et al*., 2012; Šolić *et al*., 2017). Temperature also influences various ecological and biological interactions within the complex microbial network, such as resource availability, predation by bacterivorous protists and viral lysis (Pernthaler, 2005; Kirchman *et al*., 2009). The sensitivity of these microbial metabolic processes to temperature can vary greatly, which ultimately determines how the microbial community will respond to warming (López -Urrutia *et al*., 2006; O'Connor *et al*., 2009).

Studies have shown that temperature can increase the growth rate, production and respiration of bacteria and alter the size and the composition of marine microbial communities (Morán *et al*., 2015; 2018; Arandia-Gorostidi *et al.,* 2017; Šolić *et al.,* 2017; Smith *et al*., 2019; Gu *et al*., 2020; 2024). Recent research suggests that the response of the major phylogenetic groups to high temperatures could also be influenced by phylogeny, although to our knowledge this information has only been recorded occasionally, especially in marine systems (Arandia-Gorostidi *et al*., 2017; 2020). Consequently, our knowledge about the effects of temperature on the growth rates of dom-

inant phylogenetic groups within bacterial communities remains limited.

Predation by nanoflagellates (NF) and ciliates (CIL) as well as viral infections are known to limit bacterial growth. Until now, feeding by protists was considered non-selective. However, recent research indicates that protists preferentially graze on larger, typically more active bacteria (Koton-Czarnecka & Chróst, 2003; Weinbauer *et al.,* 2019; Rychert, 2022) and that grazing can influence bacterial diversity. This influence appears to be stronger in freshwater environments (Pernthaler *et al*., 2001; Šimek *et al*., 2001) than in marine waters (Bonilla Findji *et al*., 2009; Baltar *et al*., 2016; Weinbauer *et al*., 2019). Nevertheless, the role of grazing in limiting the growth of phylogenetically distinct bacterial groups is still largely unexplored, and there are few studies on the effects of predation on dominant phylogenetic groups under different temperature conditions (Yokokawa & Nagata, 2005; Gu *et al*., 2020; Sánchez *et al*., 2020). Understanding the impact of increased temperature on grazing dynamics is crucial for clarifying the role of grazing in regulating microbial communities and nutrient cycling in a warming ocean.

Some marine regions have already been identified as climate change hotspots. The Mediterranean as a whole and the Adriatic Sea in particular are considered to be particularly vulnerable to projected climate change (Gualdi *et al*., 2013; Chust *et al*., 2014). Recent studies conducted in this area have already observed the effects of global change on the ecosystem. These impacts include an upward trend in sea surface temperature (Grbec *et al*., 2019), changes in thermal conditions (Matić *et al*., 2011), a weakening of thermohaline circulation (Vilibić *et al*., 2013) and a decrease in dissolved oxygen levels in deeper layers (Vilibić *et al*., 2012). Climate change in the Adriatic also affects the biological processes within the planktonic food web. This in turn affects the carbon cycle and energy flow within the ecosystem (Grbec *et al*., 2009; Ninčević Gladan *et al*., 2010; Šolić *et al*., 2017; 2018b; Kovač *et al*., 2018). In addition, future environmental changes in the Adriatic Sea could alter the structure and functions of the microbial web, as well as the carbon fluxes (Šolić *et al*., 2018b, 2019, 2020).

We assumed that different phylogenetic groups of bacteria would respond differently to factors that affect their growth such as temperature and predators. To investigate this, we conducted growth and grazing experiments in a temperature-controlled microcosm and simulated a 3°C temperature rise. This increase in sea surface temperature was predicted for the end of the 21st century (IPCC, 2019).

The experiments aimed to investigate how experimental warming affects (I) the specific growth and grazing rates of the most common phylogenetic bacterial groups (II), the phylogenetic composition of the bacterial community and (III) the phylogenetic-specific grazing preference of protozoa.

To our knowledge, this is the first study conducted to investigate how an experimental temperature increase affects both *in situ* growth and grazing rates of the major phylogenetic bacterial groups in temperate marine waters.

This study is part of a larger experimental study (Šolić *et al*., 2020) that addressed the temperature sensitivity of the picoplankton community. The study investigated changes in growth and grazing rates, growth efficiency and production, and carbon biomass flux through the microbial food web following an experimental increase in temperature. However, focusing only on whole community responses can overlook differences at the finer taxonomic level. Therefore, we extended our research to specifically investigate how different phylogenetic groups of bacteria respond to temperature increases.

Materials and Methods

Location and sampling

We conducted our research in April when the water temperature was below 16°C, as a previous study in this area has shown that temperature increases at temperatures below 16°C can lead to a notable increase in microbial growth (Šolić *et al*., 2017). Seawater for the experiments was collected from a depth of 1 meter in Kaštela Bay (middle Adriatic, 43.5167° N, 16.3667° E) in April 2019 using an acid-cleaned 20 L polycarbonate cylindrical plastic vessel (Fig. S1). A total of 100 litres of seawater was transported to the laboratory within 1 hour and immediately carefully filtered through a nylon sieve with a mesh size of 200 µm to remove large mesozooplanktonic grazers and particles. Temperature and salinity were measured *in situ* using CTD multiparameter probes (Idronaut and SeaBird) with an accuracy greater than 0.01°C and 0.02, respectively. Nutrient concentrations (nitrate ions/NO_3^-, nitrite ions/NO_2^-, ammonium ions/ NH_4^+, and phosphate ions/PO_4^{3-}) were determined using a Bran-Luebbe AutoAnalyser (II and III models) and standard colorimetric methods (Grasshoff, 1976). The concentrations of nutrients during the experiment are presented in Table S1. Salinity was 34.26.

Fractionation experiments

Figure S2 shows the scheme of the experimental design. The experimental technique involved size fractionation of natural seawater followed by incubation at two different temperatures. This allowed us to assess the relative effects of temperature and predators on different bacterial phylogenetic groups. For the growth/grazing size fractionation experiments (Wright & Coffin, 1984; Rassoulzadegan & Sheldon, 1986), the prefiltered water was successively filtered through membrane filters with pore sizes of 10 µm or 2 µm, resulting in two fractions with different bacterial predators. The seawater was therefore subjected to three experimental treatments: (i) seawater filtered through a 200 µm size filter containing NF and microzooplankton (mostly CIL, <200 µm fraction); (ii) seawater filtered through a 10 µm pore size filter con-

taining <10 µm grazers (mainly NF, <10 µm fraction); and (iii) seawater filtered through a 2 µm pore size filter with reduced NF abundance (<2 µm fraction). Filtration through <2 µm filters successfully eliminated more than 90% of NF, suggesting that the chosen pore size was suitable for assessing grazing pressure on free-living bacteria in the Adriatic Sea (Šolić *et al*., 2017). The abundance of free-living prokaryotic cells did not change noticeably after the size filtrations (Table 1). Each size fraction was transferred to pre-cleaned 5-liter flasks and incubated in triplicate in two temperature-controlled incubators set at the ambient temperature (T_{amb}, 14°C) and 3°C above the ambient temperature (T_{amb}+3). The temperature chambers were equipped with six daylight lamps (LH -TS LED 18 W, 6000 K) and programmed to follow the day-night cycle of the day of sampling (12:12). The flasks were incubated at a light intensity of 53 µmol photons m^{-2} s^{-1}.

Since temperature plays a fundamental role in nutrient-rich environments and has little or no effect on bacterial growth when resources are scarce (Calvo-Díaz *et al*., 2014; Morán *et al*., 2018), all flasks were initially supplied with 5 µM nitrate ($NaNO_3$) and 0.25 µM phosphate (KH_2PO_4). Preliminary experiments have shown that the addition of selected nutrients in the indicated ratio allows exponential cell growth from 0-72 hours, so that growth rates can be determined. Subsamples for all analyses were taken at the beginning (T0) and regular intervals during incubation (T24, T48, T72 and T96 h). Subsamples for CIL counts were taken at T0 and T96. During incubation, the bottles were gently shaken. The experiments were carried out until the abundance of bacteria reached a plateau and started to decrease (96 h). During incubation at elevated temperatures, the microorganisms were subjected to a gradual increase in temperature.

Enumeration of microorganisms

Subsamples for microorganism enumeration were preserved in 2% formaldehyde and stored at 4°C until

Table 1. The initial composition of microbial community in different fractions: microbial abundances (abundance± standard deviations) and percentages (%) of different bacterial groups detected by each probe (represented as percentages of DAPI- positive cells) at ambient (T_{amb}) and a 3°C increased temperature (T_{amb}+3). HB- heterotrophic bacteria; NF- nanoflagellates; DAPI -DAPI positive cells; Eub -number of cells hybridized with probe EUB338 targeting most Eubacteria; CF - number of cells hybridized with probe CF319a targeting Bacteroidetes; Gamma - number of cells hybridized with probe GAM42a targeting Gammaproteobacteria; Ros- number of cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 - number of cells hybridized with probe SAR11-441R targeting SAR11 clade.

Group (units):	**T_{am}b**			**T_{amb}+3**		
	< 2 µm fraction	**< 10 µm fraction**	**< 200 µm fraction**	**< 2 µm fraction**	**< 10 µm fraction**	**< 200 µm fraction**
HB (x10^6 cells mL^{-1})	0.45±0.01	0.47±0.01	0.48±0.01	0.47±0.01	0.48±0.01	0.48±0.01
NF (x10^3 cells mL^{-1})	0.09±0.01	0.99±0.02	1.34±0.03	0.12±0.05	1.02±0.07	1.44±0.11
DAPI (x10^6 cells mL^{-1})	0.30±0.02	0.32 ±0.01	0.30 ±0.03	0.31 ±0.02	0.34 ±0.03	0.34±0.01
Eub (x10^5 cells mL^{-1})	1.80±0.45	1.88±0.42	1.92±0.50	2.00±0.70	1.71±0.60	1.63±0.52
CF (x10^5 cells mL^{-1})	0.57±0.03	0.48±0.02	0.62±0.02	0.73±0.07	0.70±0.06	0.68±0.03
Gam (x10^5 cells mL^{-1})	0.13±0.02	0.16±0.03	0.15±0.05	0.13±0.07	0.10±0.04	0.14±0.01
Ros (x10^5 cells mL^{-1})	0.23±0.07	0.22±0.08	0.26±0.01	0.18±0.01	0.29±0.03	0.19±0.05
SAR11 (x10^5 cells mL^{-1})	0.92±0.09	0.95±0.06	0.98±0.04	0.70±0.09	0.91±0.03	1.10±0.05
Eub (%)	59.15	58.55	63.33	64.15	50.77	47.54
CF (%)	18.94	15.21	20.54	23.47	20.79	19.81
Gam (%)	4.45	5.07	5.18	4.23	3.17	4.26
Ros (%)	7.87	5.14	8.72	6.04	8.76	5.65
SAR11 (%)	30.51	29.77	32.44	22.61	27.12	31.96

analysis, which took place within 5-10 days. Heterotrophic bacteria (HB) and NF were enumerated using the Beckman Coulter CytoFLEX cytometer at a fast flow rate of 60 µL min^{-1}, following the protocols of Marie *et al*. (1997) for bacteria and Christaki *et al*. (2011b) for NF. Each sample was run in triplicate.

The samples were examined under a microscope to confirm that only free-living bacteria were counted. For CIL counts, the samples were treated with acidic Lugol's solution (2% final concentration) and stored in the dark at 4°C until counting, which occurred up to two weeks later. This process eliminated adult copepods, and a small number of nauplii remained in the samples at low concentrations. A 2-litre sample was allowed to settle in cylinders for 48 hours according to Utermöhl's method (1958), after which it was decanted to a volume of 200 mL. Prior to microscopic analysis the volume was further reduced to 20 mL. An inverted microscope (Olympus IMT-2) with phase contrast at magnifications of 200x and 400x was used to count the cells. The number of CIL and tintinnids was reported as number of cells per litre.

Catalysed reporter deposition-fluorescence in situ hybridization (CARD-FISH)

The abundance of specific phylogenetic groups of bacterioplankton was quantified using the CARD-FISH method, following the protocol of Pernthaler *et al*. (2002). The specific probes and hybridization conditions used are presented in Table S2. The preliminary experiments showed that the mean detection rate using probes EUB I-III was 10-20% lower, on average, compared to probe EUB338 (data not shown).

Samples were fixed with a 2% formaldehyde solution (final concentration), filtered through white polycarbonate filters with a pore size of 0.2 µm, and stored at -20°C until analysis. For CARD-FISH, the filters were embedded in 0.2% (w/w) low melting point agarose and permeabilized with lysozyme (10 mg mL^{-1}) for 60 minutes at 37°C. After inactivation of endogenous peroxidase (incubation in 0.01 M HCl for 10 min), filter sections were hybridized with horseradish peroxidase-labelled probes (50 ng mL^{-1} final concentration) for 2 h at 35°C. Fluorescein isothiocyanate-labelled tyramides (FITC) were used to amplify the CARD-FISH signal by incubating the filter sections for 30 min at 37°C in the dark. After washing, filter sections were counterstained with a 4,6-diamino-2-phenylindole (DAPI)-Vectashield-Citifluor mixture containing 1 mg mL^{-1} DAPI. The number of DAPI- and FITC-positive cells was determined manually using an epifluorescence microscope (Olympus BX51, 1000x magnification). At least 700 DAPI-positive cells per sample were counted. The number of FITC-positive cells varied depending on the relative proportion of each subgroup and ranged from 70 to 500 cells per sample. The relative abundances of each group were defined as the ratio of the number of cells hybridized with specific probe to the number of DAPI-positive cells (% DAPI). A nonsense probe (NON338) was also used to assess non-specific binding, and this signal was less than 1%. To estimate the variability of the hybridization efficiency of the EUB 338 probe, we performed three parallel hybridizations on the filters taken at T0 from each treatment. The coefficient of variation in the number of cells hybridized with the probe was 4.1-5.2%. Due to lack of resources and time, we were unable to perform replicates of the CARD-FISH samples, which would likely yield an even more accurate and statistically significant estimate of the results.

Calculation of growth and grazing rates

Growth rates (μ, day^{-1}) of NF, DAPI- and probe-positive cells were calculated for each treatment. To estimate growth rates, we followed the method of Landry & Hassett (1982) and calculated the slope of the linear regressions of the natural log-transformed abundances against the exponential growth period:

$$\mu = \frac{(\ln Nt - \ln N_0)}{t}$$

where N_0 and N_t are the cell abundance at the beginning and end of the exponential growth period, respectively, and t is the duration of the exponential growth period in days. For the bacteria, the exponential growth period extended over the first 0 to 48 hours. For NFs, the exponential growth occurred between 24 and 72 hours.

We refer to the 'net growth rates' of the bacteria as the changes in abundance in the presence of grazers (in the < 10 µm and < 200 µm fractions) and to the 'maximal growth rates' as those observed in the < 2 µm fraction.

Grazing mortality rates (g, day^{-1}) were calculated from the difference between growth rates in the predator-reduced fraction and growth rates in the presence of the predator:

$$g = \mu\,(\text{ungrazed}) - \mu\,(\text{grazed})$$

Grazing rates of NF on bacteria (g_{NF}) were estimated by calculating the difference between the growth rates of bacteria in the <2 µm and <10 µm fraction. The total grazing rates (g_{TOT}) of bacteria were estimated by calculating the difference between the growth rates of bacteria in the <2 µm and <200 µm fractions. The grazing rates of CIL on bacteria (g_{CIL}) were estimated as the difference between g_{TOT} and g_{NF}. Bacterial grazing by NF (G_{NF}) or CIL (G_{CIL}) was calculated as:

$$G = g \times P$$

where g is the grazing rate of predators (NF or CIL) and P the geometric mean of predP istor abundance. Total grazing (G_{TOT}) is the sum of (G_{NF}) and (G_{CIL}).

Calculation of the grazing selectivity index

The Manly-Chesson selectivity index (alpha index, α) was calculated for each bacterial group to determine the extent of predator preference (both NF and CIL) for a

particular prey type (Manly, 1974; Chesson,+--/71983):

$$\alpha_i = \frac{d_i/e_i}{\sum_{i=1}^{m} d_i/e_i}$$

where d_i is the relative abundance of prey in the predators' diet, e_i is the relative abundance of prey in the environment, and m is the number of prey types in the environment. The index assumes a neutral preference for a particular prey type when $\alpha = 1/m$ (grazing is directly proportional to relative abundance in the environment). Values of $\alpha = > 1/m$ indicate a grazing preference and values of $\alpha = < 1/m$ indicate avoidance of a particular prey group.

Assessing differences in community composition between treatments

To examine the taxonomic composition similarities between the initial communities and the communities formed at the end of the experiment (at T96), we performed a hierarchical cluster analysis using the Bray-Curtis distance matrix for square root transformed abundance data in PRIMER v.7 (Clarke & Warwick, 2001). To determine the significance of the dendrogram branches resulting from the cluster analysis, we conducted the Similarity Profile Permutation Test (SIMPROF). To assess the statistical significance of temperature, time, and grazing effects on bacterial community composition, we conducted a permutational multivariate analysis of variance (PERMANOVA) using the Bray-Curtis distance on a square root transformed dataset in PRIMER7 (Clarke & Gorley, 2015). The results are presented in Table 2. The PERMANOVA design incorporated fixed factors of temperature, grazing and time, with 9999 permutations, sums of squares Type III (partial), and employed the permutation method of Permutation of residuals under a reduced model (Anderson, 2017).

Results

Initial bacterial community

The initial bacterial community was similar in all three treatments, indicating that filtration through filters with different pore sizes did not result in noticeable losses of bacterial cells (Table 1). Filtration through 2 µm filters successfully eliminated more than 95% of NF. The percentage of cells detected with the general eubacterial probe EUB338 was lower at the beginning of the experiment (from 47.54 to 64.15%), but increased steadily to 88.9% over the course of the experiment. The abundance of each phylogenetic group was comparable across treatments, indicating a similar community composition at a broad phylogenetic group level. The highest number of cells was hybridised with probe SAR11-441R (between 0.70 x 10^5 and 1.10 x 10^5 cells mL^{-1}), followed by probe CF319a (0.48-0.73 x 10^5 cells mL^{-1}). The probes GAM42a and ROS537 hybridised only 3.17-5.18% (0.10-0.15 x 10^5 cells mL^{-1}) and 5.14-8.76% (0.22-0.29 x 10^5 cells mL^{-1}) of DAPI-positive cells, respectively.

Influence of temperature on bacterial abundance

The changes in bacterial and NF abundances over time are shown in Figure 1. The changes in microzooplankton abundances are presented in Figure 2. CIL and tintinnids were only found in the <200 µm fraction.

At T_{amb}, abundances of all bacterial groups increased rapidly in the first 48 hours of incubation and then gradually decreased. The NF peak occurred in all fractions 2 days after the bacterial peak. Filtration led to rapid growth of all groups except SAR11 and Bacteroidetes within the first 48 hours. When the grazing pressure exerted by NF was reduced (in the <2 µm fraction), there was a noticeable increase in Roseobacter abundance. Removal of CIL led to a rapid increase in Gammaproteobacteria after only

Table 2. Results of permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance of square root transformed dataset. Fixed factors were temperature (TE), grazing (GR) and time (TI) [9999 permutations, sums of squares Type III (partial) and permutation method "Permutation of residuals under a reduced model"].

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
TE	1	250.03	250.03	13.2	**0.033 ***	9393
GR	2	72.96	36.48	1.9259	0.281	6973
TI	1	3166.7	3166.7	167.18	**0.003 ****	8936
TE x GR	2	75.879	37.939	2.003	0.266	9945
TE x TI	1	202.4	202.4	10.686	**0.047 ***	2067
GR x TI	2	66.086	33.043	1.7445	0.311	9964
Res	2	37.883	18.941			
Total	11	3871.9				

*P value < 0.05
**P value < 0.005

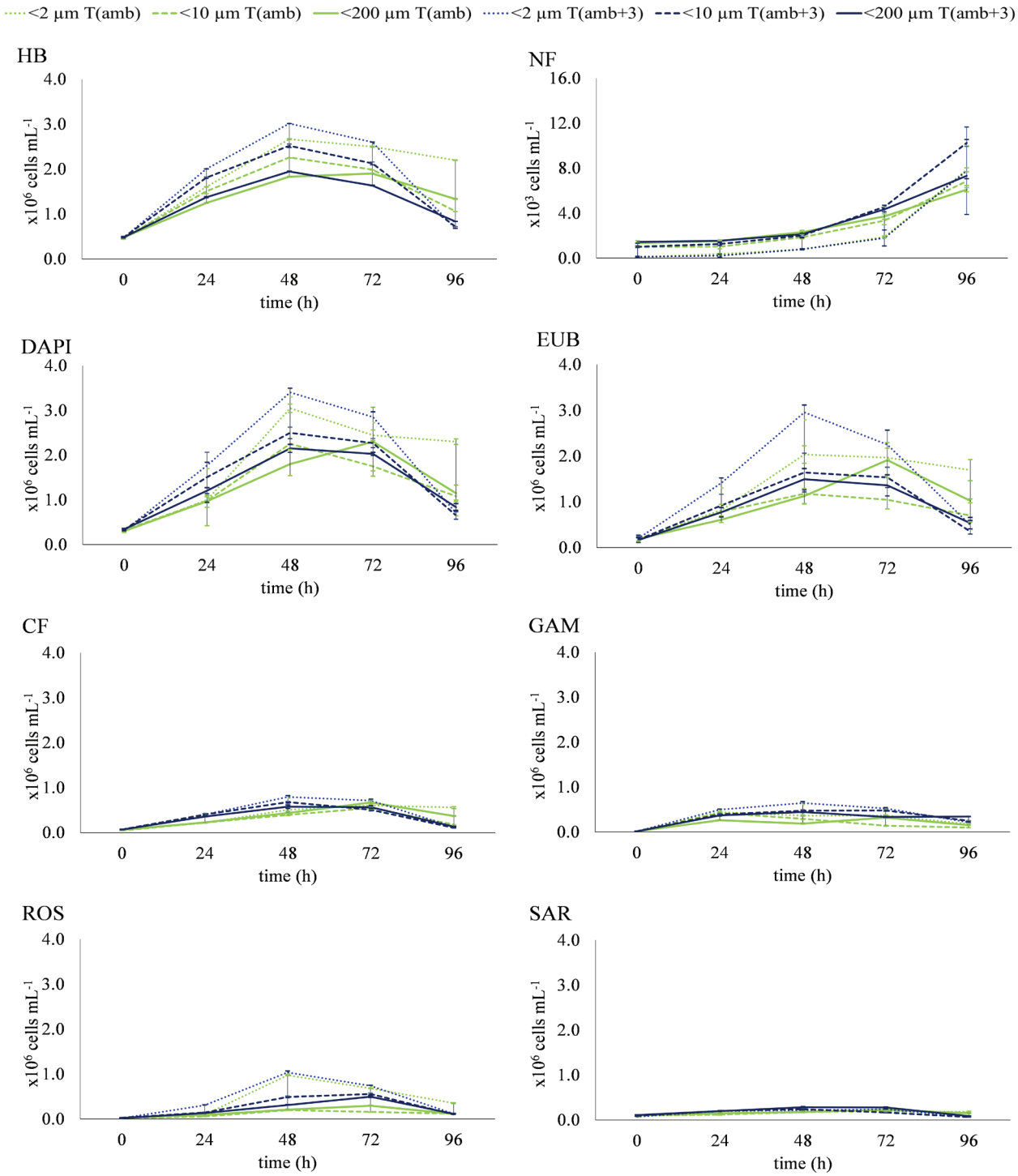

Fig. 1: Abundances of heterotrophic bacteria (HB), nanoflagellates (NF) and cells hybridized with different probes during the experiment in different treatments at ambient temperature (T_{amb}) and a 3°C increase in temperature (T_{amb}+3). DAPI - DAPI positive cells; EUB - number of cells hybridized with probe EUB338 targeting most Eubacteria; CF - number of cells hybridized with probe CF319a targeting Bacteroidetes; GAM - number of cells hybridized with probe GAM42a targeting Gammaproteobacteria; ROS - number of cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 - number of cells hybridized with probe SAR11-441R targeting SAR11 clade.

24 hours (in the both <2 µm and <10 µm fractions).

Incubation at higher temperatures increased the abundance of all groups in all treatments. Both experimental manipulations (temperature and predator reduction) further increased abundance of all groups in the first 48h (the period of exponential growth), with the exception of SAR11. The abundance of Roseobacter and Gammaproteobacteria increased more with temperature increase than with the removal of the predators.

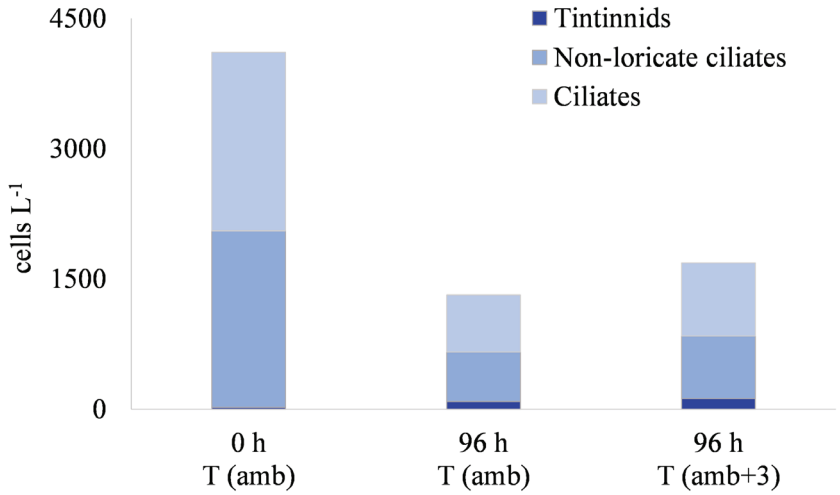

Fig. 2: Abundances of ciliates, non-loricate ciliates and tintinnids during the experiment in <200 µm fraction at ambient temperature (T_{amb}) and a 3°C increase in temperature (T_{amb}+3).

Influence of temperature on group-specific growth and grazing rates

In the treatment without manipulation, the growth rates of DAPI-positive cells (1.152 day^{-1}) were similar to those of Eubacteria (1.209 day^{-1}, Table 3). Gammaproteobacteria, Bacteroidetes and Roseobacter grew much faster than DAPI-positive cells. In contrast, SAR11 grew the slowest, at rates well below those of the community as a whole. In the absence of predators, all bacterial groups grew much faster and their net growth rates were lower in the <10 µm and <200 µm fractions compared to the <2 µm fraction.

Temperature increased the growth rates of all groups (Fig. 3, Table 3), with SAR11 and Roseobacter showing the highest percentage increases (60% and 34%, respectively). Bacteroidetes and Gammaproteobacteria showed the smallest increase in growth rate across treatments (9-14%). Despite the relatively small increase in growth rates with temperature, the Gammaproteobacteria remained the fastest growing group at (T_{amb}+3). Although the growth rates of SAR11 increased most with temperature, this group remained the slowest growing.

The impact of temperature increase on growth rates was comparatively smaller than predator reduction for all groups except SAR11 (Fig. 3). When bacteria were exposed to both experimental manipulations the highest growth rates were observed for all groups. Roseobacter and SAR11 showed the highest increase in growth rates (95% and 90%, respectively), while the Bacteroidetes and Gammaproteobacteria groups showed a relatively lower increase (23% and 28%, respectively).

Table 3. Growth (μ) and grazing (g) rates at ambient temperature (T_{amb}) and a 3°C increase in temperature (T_{amb}+3) of bacterial groups hybridized with different probes (DAPI - DAPI positive cells; Eub - cells hybridized with probe EUB338 targeting most Eubacteria; CF - cells hybridized with probe CF319a targeting Bacteroidetes; Gam - cells hybridized with probe GAM42a targeting Gammaproteobacteria; Ros - cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 - cells hybridized with probe SAR11-441R targeting SAR11 clade).

Bacterial group	Maximum growth rate μ (day^{-1})		Net growth rate μ (day^{-1})		Net growth rate μ (day^{-1})		g_{HNF} (day^{-1})		g_{CIL} (day^{-1})	
			<10 µm		<200 µm					
	T_{amb}	T_{amb}+3	T_{amb}	T_{amb}+3	T_{amb}	T_{amb}+3	T_{amb}	T_{amb}+3	T_{amb}	T_{amb}+3
DAPI	1.152	1.195	0.974	1.002	0.891	0.917	0.117	0.103	0.109	0.085
Eub	1.209	1.346	0.917	1.131	0.885	1.107	0.291	0.215	0.032	0.024
CF	1.090	1.194	1.045	1.137	0.974	1.069	0.045	0.057	0.072	0.067
Gam	3.489	3.632	3.253	3.598	2.817	3.224	0.236	0.033	0.437	0.694
Ros	1.854	2.005	1.077	1.405	1.031	1.386	0.777	0.600	0.046	0.020
SAR11	0.371	0.564	0.300	0.484	0.297	0.473	0.071	0.080	0.003	0.001

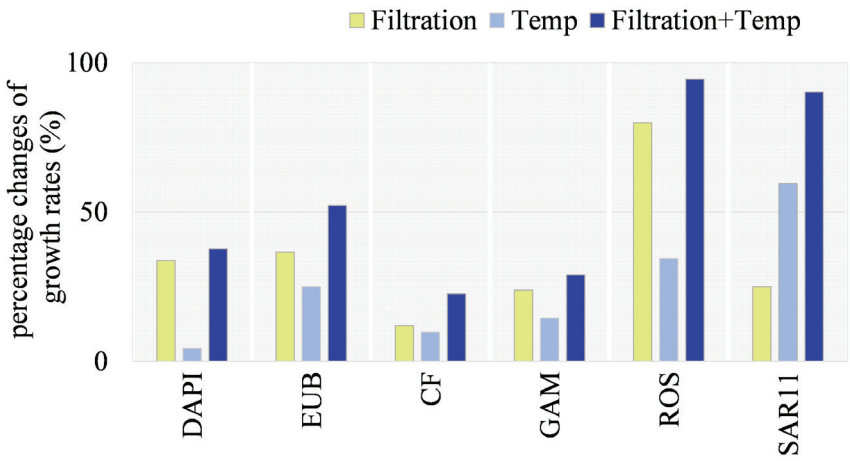

Fig. 3: Percentage change in growth rates of cells hybridized with different probes after experimental manipulations. HB - DAPI positive cells; CF - cells hybridized with probe CF319a targeting Bacteroidetes; GAM - cells hybridized with probe GAM42a targeting Gammaproteobacteria; ROS - cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 - cells hybridized with probe SAR11-441R targeting SAR11 clade.

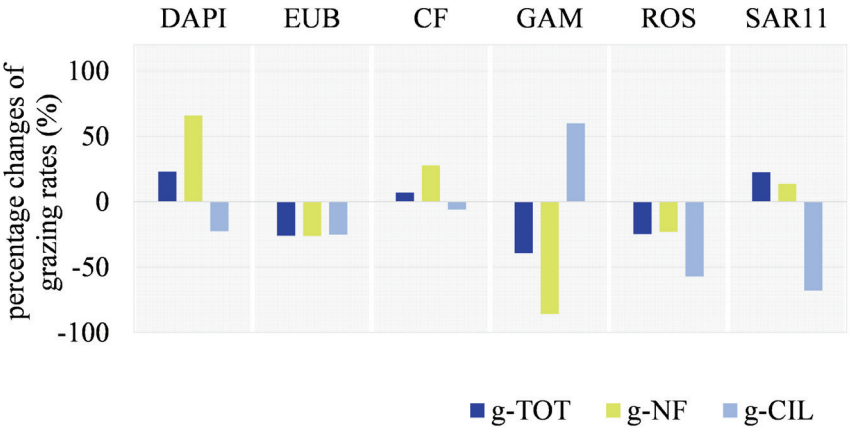

Fig. 4: Percentage change of grazing rates of cells hybridized with different probes after experimental manipulations. DAPI -DAPI positive cells; EUB -number of cells hybridized with probe EUB338 targeting most Eubacteria; CF -number of cells hybridized with probe CF319a targeting Bacteroidetes; GAM -number of cells hybridized with probe GAM42a targeting Gammaproteobacteria; ROS -number of cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 -number of cells hybridized with probe SAR11-441R targeting SAR11 clade; g-TOT - total grazing; g-NF -grazing of nanoflagellates; g -CIL-grazing of ciliates.

The grazing rates of NF and the total grazing rates were both lower than the maximum growth rates of the bacteria, resulting in positive net growth of bacteria (Table 3). The grazing rates of NF were higher than those of CIL for DAPI-positive cells, Eubacteria, Roseobacter and SAR11. NF grazed different groups of bacteria at rates between 0.07 day^{-1} (SAR11) and 0.78 day^{-1} (Roseobacter). CIL grazing rates were highest for Gammaproteobacteria (0.44 day^{-1}). Experimental warming resulted in different changes in grazing rates among different bacterial groups, indicating considerable heterogeneity even at higher taxonomic levels (Fig. 4). Temperature decreased both the total and individual predator grazing rates on Eubacteria and Roseobacter. However, it increased the grazing rate of NF on DAPI-positive cells by 66% and the grazing rate of CIL on Gammaproteobacteria by 86%. Grazing rates of CIL on SAR11 and Roseobacter decreased with temperature by 68% and 58%, respectively. Overall, the total grazing rates changed to a lesser extent than the grazing rates of the individual predators.

Influence of temperature on phylogenetic composition

Figure 5 shows the percentage of relative abundance of each phylogenetic group of bacteria during the experiment. At T_{amb} during the period of exponential growth, there was a sudden shift in all treatments, mainly due to the decrease of SAR11 and the development of Gammaproteobacteria. The proportion of SAR11, which accounted for the largest proportion of the bacteria in the original populations (30-32% of the DAPI-positive cells), decreased after 48 hours and accounted for only 6-9% of DAPI-positive cells in all treatments. Gammaproteobac-

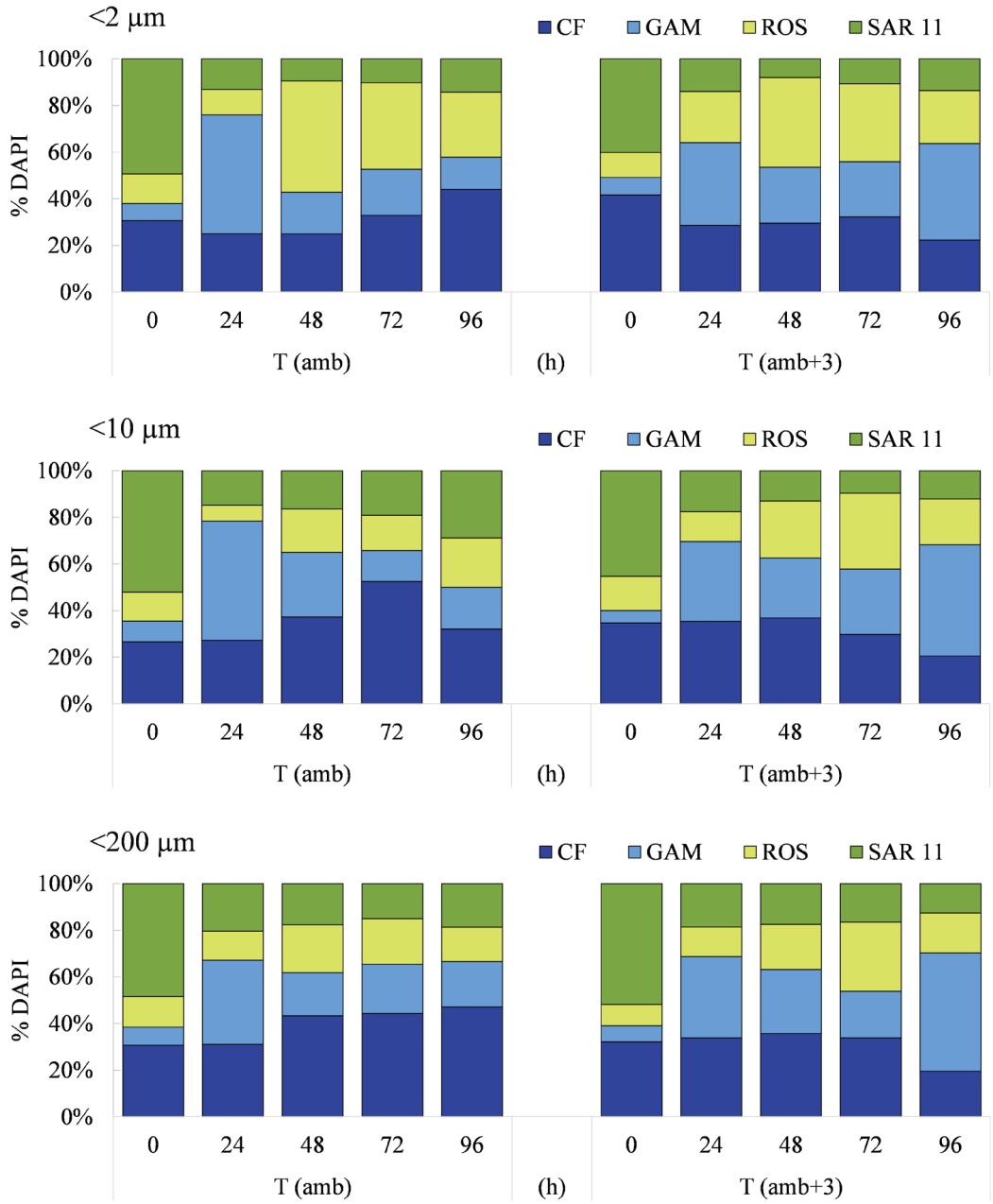

Fig. 5: Relative abundance of cells hybridized with different probes during the experiment in different treatments at ambient temperature (T_{amb}) and a 3°C increase in temperature (T_{amb}+3). CF - number of cells hybridized with probe CF319a targeting Bacteroidetes; GAM - number of cells hybridized with probe GAM42a targeting Gammaproteobacteria; ROS - number of cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 -number of cells hybridized with probe SAR11-441R targeting SAR11 clade.

teria, which made up 4-5% of the original communities, showed an apparent increase in their relative proportion after only 24 hours in all treatments. This increase was greatest when no CIL were present (<2 µm and <10 µm fractions) and accounted for 43-44% of all bacteria, whereas it was only 27% when CIL were present. Roseobacter accounted for 6-9% of all bacteria in the initial communities and reached 32% of all bacteria in the <2 µm fraction after 48 hours. However, in the presence of NF (<10 µm and <200 µm fraction), the initial proportion of Roseobacter was maintained throughout the experiment. In the presence of both predators, the proportion of Bacteroidetes merely increased after 48 hours (from 20% to 24%).

Cluster analysis revealed that the initial communities differed from those at the end of the experiment ($\pi = 4.9$ p = 0.001, CLUSTER analysis, Fig. 6 and Fig. 5). This was mainly due to the continued decline of SAR11 and the development of Roseobacter and Gammaproteobacteria. Moreover, communities incubated at T_{amb}+3 that formed at the end of the experiment clustered together and were more similar to each other than to the final communities incubated at T_{amb}. However, no statistically significant differences were found between the different size fractions.

The PERMANOVA analysis showed that temperature and time and their interaction significantly influenced the phylogenetic composition of the bacterial communities at the end of the experiment (Table 2). Grazing and its

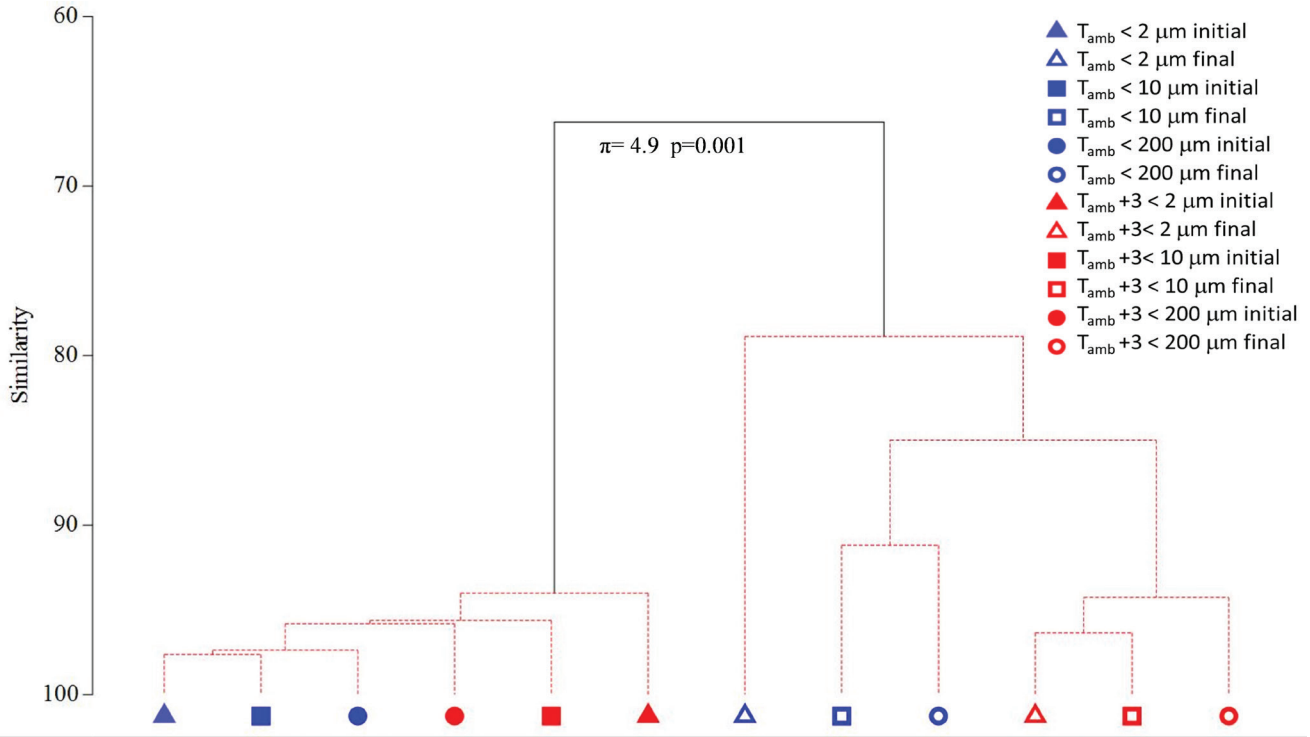

Fig. 6: Dendrogram representing the similarity of the bacterial communities' structure using as proxy the abundances of bacterial groups hybridized with different probes determined by CARD-FISH at ambient temperature (T_{amb}) and a 3°C increase in temperature (T_{amb}+3) at T0 ('initial') and T96 ('final') of the experiment in different fractions. Groups that are significantly different are denoted by thick black lines whereas red dashed lines indicate similar groups (using SIMPROF test).

interactions with two other factors were not statistically significant.

Influence of temperature on dominant predator, prey selection and diet composition

At T_{amb} the NF were the main grazers of SAR11 and Roseobacter. CIL contributed slightly more than NF to the total grazing of Bacteroidetes and Gammaproteobacteria, with the proportion of grazed cells ranging from 64% to 66% (Fig. 7). Temperature increased the grazing pressure of NF on Roseobacter and Bacteroidetes and the grazing pressure of CIL on Gammaproteobacteria and SAR11. Incubation at higher temperatures also led to a shift in the dominant predator for Bacteroidetes and Gammaproteobacteria. After the temperature increase, Gammaproteobacteria were almost exclusively grazed by CIL (96%).

To determine whether a particular group of bacteria was preferentially grazed by NF and CIL, we calculated the grazing selectivity index (Manly-Chesson selectivity index). According to the results of the selectivity index, NF showed a different feeding strategy towards Roseobacter and SAR11, avoiding SAR11 as preferred prey while actively grazing Roseobacter (Fig. 8). Consequently, the diet of NF consisted mainly of Roseobacter (58%), while the contribution of SAR11 was negligible (10%) (Fig. 9). Neither NF nor CIL showed a grazing preference for Bacteroidetes, and the contribution of this group to the total number of cells ingested by NF and CIL was low (9% and 14%, respectively). NF randomly selected Gammaproteobacteria as prey, but CIL showed a strong preference for this group. Consequently, this group made up a large proportion of the CIL diet (69%). When the temperature increased, the proportion of Roseobacter in the NF diet also increased. In contrast, Gammaproteobacteria completely disappeared from the NF diet, limited to 2% (see Fig. 9). This suggests that NF actively avoided Gammaproteobacteria at higher temperatures, as indicated by the Manly-Chesson index (Fig. 8). On the other hand, the proportion of Gammaproteobacteria in the CIL diet increased with temperature. This increase occurred mainly at the expense of Roseobacter and SAR11, and accounted for 80% of the ingested bacterial cells.

Discussion

This study expands upon previous research by investigating the impact of temperature and grazers on bacterial communities in the Adriatic (e.g., Šolić *et al*., 2020). It specifically aims to analyse the response of bacterial communities at a more specific taxonomic level than previously studied.

Experimental approach and methodological constraints

Microcosms in the laboratory introduce bias in the interpretation of results due to sample manipulation and limited incubation time. The size fractionation experimental technique, which uses filters with different pore sizes, may alter the relative composition of prey and grazer communities compared to those occurring natu-

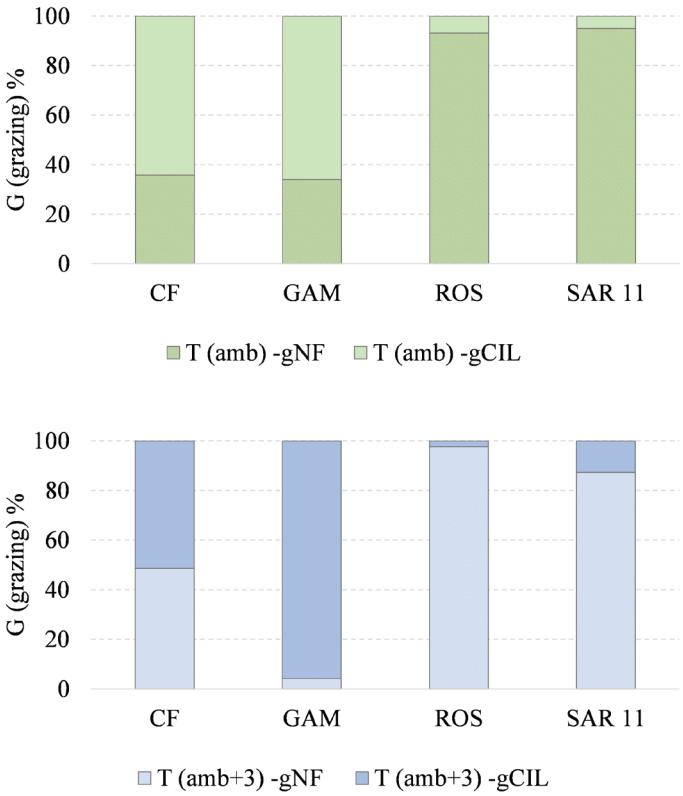

Fig. 7: The contribution of nanoflagellate (gNF) and ciliate (gCIL) grazing to total grazing (G) of cells hybridized with different probes at ambient temperature (T_{amb}) and a 3°C increase in temperature (T_{amb}+3). CF - cells hybridized with probe CF319a targeting Bacteroidetes; GAM - cells hybridized with probe GAM42a targeting Gammaproteobacteria; ROS - cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 - cells hybridized with probe SAR11-441R targeting SAR11 clade.

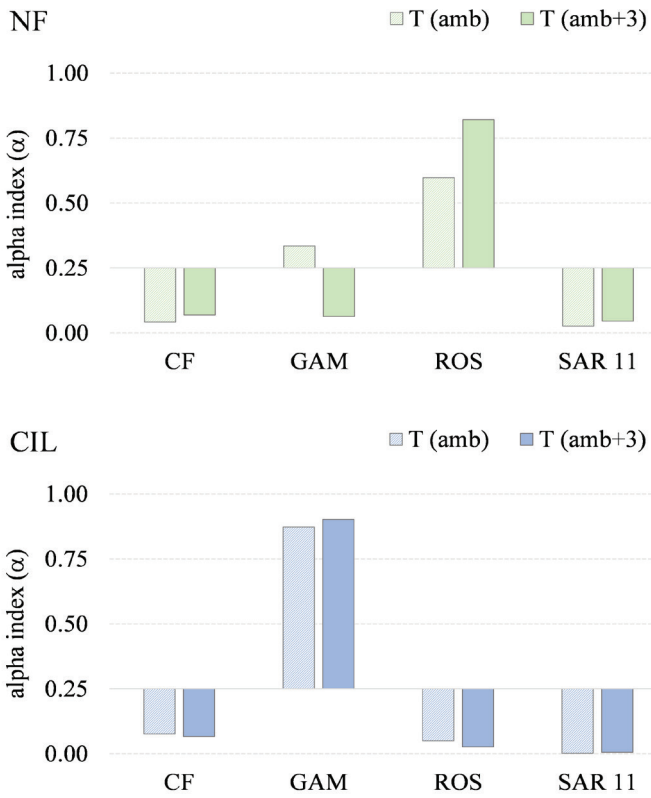

Fig. 8: Manly-Chesson selectivity index (α index) for nanoflagellates (NF) and ciliates (CIL) calculated for ambient temperature (T_{amb}) and a 3°C increase in temperature (T_{amb}+3). The horizontal line is the 0.25 value, which is the line of 'neutral grazing'. Values of $\alpha > 0.25$ indicate grazing preference and values of $\alpha < 0.25$ indicate avoidance of a particular prey group. CF - cells hybridized with probe CF319a targeting Bacteroidetes; GAM - cells hybridized with probe GAM42a targeting Gammaproteobacteria; ROS - cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 - cells hybridized with probe SAR11-441R targeting SAR11 clade.

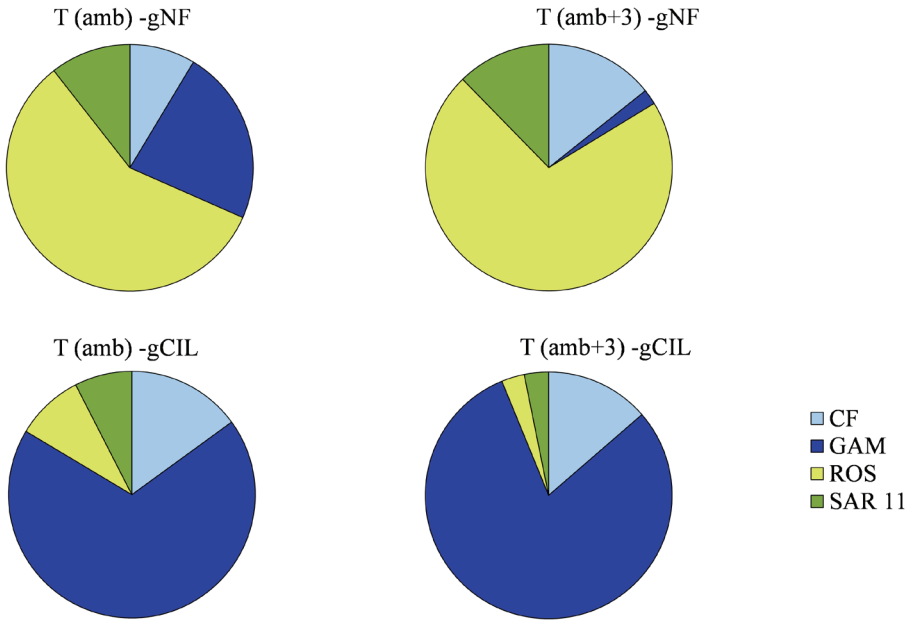

Fig. 9: Relative contribution of cells hybridized with different probes to the diet of nanoflagellates (*g*NF) and ciliates (*g*CIL) at ambient temperature (T_{amb}) and a 3°C increase in temperature (T_{amb}+3). CF - cells hybridized with probe CF319a targeting Bacteroidetes; GAM - cells hybridized with probe GAM42a targeting Gammaproteobacteria; ROS - cells hybridized with probe ROS537 targeting Roseobacter clade; SAR11 - cells hybridized with probe SAR11-441R targeting SAR11 clade.

rally. This could be a result of removing cells attached to particles or increasing dissolved organic matter concentrations due to damage of phytoplankton cells (Ferguson *et al.,* 1984). Additionally, complete separation of grazers from prey is not possible with this technique, as organisms overlap in size. However, filtering samples through different pore sizes did not notably decrease bacterial cell counts in our experiments (Table 1). Furthermore, filtration through < 2 µm filters successfully removed over 90% of NF. This clearly demonstrates that the selected pore size was appropriate for measuring grazing pressure on free-living bacteria in the Adriatic Sea (Šolić *et al*., 2017). The initial abundances of each group were comparable in all treatments, suggesting a similar community composition at the broad phylogenetic group level. As the number of EUB338-hybridised cells steadily increased over the course of the experiment, we assume that initially a large part of the prokaryotic community was inactive, dormant or very low in number of ribosomes per cell that could be detected by the CARD-FISH method.

These methodological limitations could also explain the observed differences in the growth and grazing rates of these groups and their responses to elevated temperatures. We also acknowledge that the measured growth and grazing rates may be underestimated due to incomplete removal of predators by filtration and the effects of viral lysis. Additionally, the phytoplankton cells in the <200 µm fraction may have provided additional labile dissolved carbon to the bacteria, which could influence their growth. This could have led to an underestimation of grazing rates, given that they were calculated as differences in growth rates between different size fractions. In addition, the outcomes of short-term experiments involving sudden shifts in environmental conditions restrict our capacity to predict long-term temperature changes. However, despite these methodological limitations, short-term experiments remain valuable for identifying potential trends in change.

Growth rates of bacterial phylogenetic groups

The growth rates of specific phylogenetic groups of bacteria are consistent with a limited number of studies conducted in the natural environment (Teira *et al*., 2009; Ferrera *et al*., 2011; Sánchez *et al*., 2020). Our findings indicate that Gammaproteobacteria and Roseobacter have much higher growth rates compared to others, and exceed the growth rates of DAPI-positive cells. Moreover, other studies have shown that the growth rates of Gammaproteobacteria are well above the community average (Ferrera *et al*., 2011; Sánchez *et al*., 2017, 2020). This group is considered to contain r-strategist members, responding rapidly to nutrient pulses (Eilers *et al*., 2000; Pinhassi & Berman, 2003; Fecskeová *et al*., 2021), which gives them a competitive advantage when resources are abundant. The accelerated growth of Gammaproteobacteria within < 20 h after incubation has also been observed in similar microcosm studies (Eilers *et al*., 2000; Batani *et al*., 2016), suggesting that they are able to efficiently utilize available nutrients compared to other bacterial groups. However, in oligotrophic environments such as the original community, r-strategists such as Gammaproteobacteria are typically present in low abundance and are outgrown by more numerous but slower-growing groups that are better adapted to low nutrient concentrations, such as the SAR11 group. Initially, the SAR11 group was the most abundant group in the community, but only after 24 hours of incubation it was outcompeted by all other groups. This is due to the fact that the SAR11

group continued to grow at very low rates (0.29-0.37 day^{-1}). Previous studies have also shown that the SAR11 group generally has the lowest growth rates compared to other groups (Sánchez *et al*., 2020; Campbell *et al*., 2011; Salter *et al*., 2015). In contrast to SAR11, the Roseobacter class, which was initially present in low numbers, grew at much higher rates (1.03-1.85 day^{-1}), a phenomenon observed at the same site (Fecskeová *et al*., 2021) and in other environments (Teira *et al*., 2009; Ferrera *et al*., 2011; Giebel *et al*., 2011). Although both subgroups belong to the same large phylogenetic group, the Alphaproteobacteria, they have different functional growth characteristics that reflect their different life strategies and allow them to thrive in different ecological niches (Alonso & Pernthaler, 2006; Fuhrman & Hagström, 2008; Giebel *et al*., 2011; Gifford *et al*., 2013). These results also show that groups with small genomes consistently dominate in terms of abundance, while large cells capable of rapid growth are typically the least abundant groups (Thingstad *et al*., 2022).

The wide range in growth rates among phylogenetic groups observed in this and in other studies (Yokokawa *et al*., 2004) may indicate substantial differences in activity between groups (Cottrell & Kirchman, 2004; Sintes & Herndl, 2006; Kirchman, 2016), or the presence of different percentages of active cells within each taxonomic group. It may also be due to differences in the uptake of different forms of dissolved organic matter (Cottrell & Kirchman, 2000; Alonso-Sáez & Gasol, 2007). In a study conducted at the same site, Fecskeová *et al*. (2021) found that individual phylotypes within each phylogenetic group respond differently to nutrient addition, possibly contributing to the observed differences in growth rates. Besides competition for nutrients, predation or viral lysis also play an important role in determining the outcome of competition between different bacterial taxa. Therefore, bacteria with high specific growth rates do not necessarily always dominate the environment (Cottrell & Kirchman, 2004; Yokokawa & Nagata, 2005; Alonso-Sáez & Gasol, 2007).

Grazing rates of bacterial phylogenetic groups and prey selectivity

The interaction between protists and bacteria is influenced by various factors, including bacterial cell size, abundance, activity, phylogenetic composition, morphological plasticity and the taxonomic composition of protist communities (Fenchel, 1986; Posch *et al*., 2001; Pernthaler, 2005). Protists preferentially graze on more active cells, while smaller and less active cells are relatively less susceptible to grazing (Hahn & Höfle, 2001; Pernthaler, 2005). A number of studies that estimated *in situ* grazing rates of bacterial groups in marine environments (Suzuki, 1999; Yokokawa & Nagata, 2005; Sánchez *et al*., 2020) have shown that grazing rates vary little between groups, which was also demonstrated by our study. However, the increase in growth rates and abundance of certain groups when predators were reduced suggests that protozoa preferentially graze on specific phylogenetic groups and ignore or avoid others. Fast-growing groups (Roseobacter and Gammaproteobacteria), which were rare in the original community, were heavily grazed upon by NF or CIL, while more abundant groups such as Bacteroidetes and the slower-growing SAR11 group were only minimally affected by predation. Consequently, these fast-growing groups accounted for a large proportion of the grazed cells. Additionally, the grazing selectivity index (α index) showed that protozoans consumed a much higher proportion of Gammaproteobacteria and Roseobacter than their proportions in the environment, while SAR11 and certain taxa within the Bacteroidetes were avoided, despite their relatively high concentrations in the environment. These observations suggest that the relative proportion of Roseobacter and Gammaproteobacteria ingested was primarily determined by their high growth rates (i.e., relative changes in their production) rather than their abundance in the environment. This supports the hypothesis that grazers selectively consume more active bacterial cells which tend to be larger (Sherr *et al*., 1992; Gonzalez *et al*., 1990; Suzuki, 1999). Smaller and slower growing cells, such as SAR11 and certain Bacteroidetes (Jurgens *et al*., 1999), are relatively well-protected from grazing, which probably explains their higher abundances in the original community. Moreover, some intrinsic properties of SAR11, such as its less hydrophobic surface compared to that of other similarly sized planktonic bacteria makes it more resistant to grazing (Dadon-Pilosof *et al*., 2017). These results are consistent with the findings of a previous study conducted at the same site (Fecskeová *et al*., 2021), which distinguished between phylotypes that are highly sensitive to grazing by NF (Roseobacter and Gammaproteobacteria) and those that show a weak response to predator removal (SAR11).

Although the differences in growth and grazing rates between bacterial groups suggest that predators preferentially graze certain bacterial groups in manipulation experiments, these results may not reflect the relationships between bacteria and their grazers in the natural environment. Viral-induced mortality which can lead to different mortality rates in different bacterial groups cannot be ruled out.

The effect of temperature on growth and grazing rates

Temperature directly affects the metabolic activity of aquatic bacteria. A better assessment of this can be made by conducting short-term, temperature-controlled incubation experiments where bacterial growth is less influenced by environmental parameters; however, it still responds to other factors such as interactions with phytoplankton and predators (Marrasé *et al*., 1992; Sarmento *et al*., 2010). In our short-term experiment, warming increased the growth rates of all groups and SAR11 and Roseobacter were more sensitive to temperature than others as they displayed the highest growth rates. Different sensitivity of SAR11 and Roseobacter to temperature has also been observed in other studies (Teira *et al.*, 2009;

Arandia-Gorostidi *et al*., 2017; Gu *et al.*, 2020). However, despite a 60% increase in growth rates, SAR11 still grew at much slower rates compared to other groups. Those values were consistent with literature values obtained at other Mediterranean sites during warmer seasons (Teira *et al*., 2009; Sánchez *et al*., 2020). In the same study, Šolić *et al*. (2020) found that the growth rates of LNA (low nucleic acid content) bacteria, which include the SAR11 clade (Mary *et al*., 2006; Vila-Costa *et al*., 2012), also increased notably with temperature, while HNA (high nucleic acid content) cells did not respond to the temperature increase. Other authors have also observed a higher temperature sensitivity in LNA cells compared to HNA cells (Morán *et al*., 2011; Huete-Stauffer & Morán, 2012; Courboulès *et al*., 2022). We assume that experimental warming caused a transition from cold-water to warm-water ecotypes within the SAR11, which have the potential for higher growth rates under warmer conditions (Lindh *et al*., 2015; Yeh & Fuhrman, 2022). Higher temperature sensitivity of Roseobacter compared to other groups, such as Gammaproteobacteria and Bacteroidetes, was also observed by Arandia-Gorostidi *et al*. (2017), suggesting higher growth potential of Roseobacter possibly due to versatile mechanisms employed for energy and carbon acquisition under changing conditions (Buchan *et al*., 2005; Newton *et al*., 2010; Tada *et al*., 2010).

If bacterial growth rates are higher than the mortality rates caused by viral lysis and grazing, we would expect to observe greater bacterial abundances at higher temperatures. Our results may support this hypothesis, as the growth rates of all bacterial groups increased more with temperature compared to grazing-induced losses. The small increase in predator feeding rates as a function of temperature suggests that temperature has a faster effect on bacterial growth than on protist activity (Sarmento *et al*., 2010; von Scheibner *et al*., 2014). Several factors contribute to this and are related to the metabolic and ecological differences between the organisms studied. Bacteria respond more efficiently and faster to temperature increases due to their simpler metabolic processes and higher metabolic rates. In contrast, grazers are limited by their more complex physiology and slower responses to environmental changes (Rose & Caron, 2007). A greater influence of temperature on bacterial growth compared to protist activity has already been shown in previous studies conducted in the same region (Šolić *et al*., 2018b, 2019). However, caution is required when extrapolating results obtained using the size fractionation technique to the natural environment. In natural ecosystems, the Lotka-Volterra predator-prey model predicts cyclical fluctuations in predator and prey populations and our result may merely indicate a temporal decoupling of bacteria and their predators. This decoupling could also be an indirect effect of bacterial utilisation of dissolved organic matter excreted by sloppy feeding or by increased phytoplankton production as a result of grazing. This means that grazing rates may have been underestimated. However, in our study, it is impossible to distinguish between these different feedback mechanisms that led to increased bacterial growth in the presence of grazers at higher temperature. Although our results suggest a decoupling due to higher temperatures, additional observations over seasons or longer-term experiments might be required to fully understand population dynamics and the eventual return to a natural equilibrium.

Changes in phylogenetic composition and predator diet

The PERMANOVA analysis showed that the bacteria incubated at different temperatures had a different community composition that did not depend on grazing pressure. This indicates that even small temperature changes can lead to different responses of individual bacterial phylogenetic groups. These responses were mainly due to the fact that the growth rates of all bacterial groups increased more with temperature than the losses caused by grazing. Therefore, grazing pressure had a less significant effect on bacterial community composition. However, trophic interactions between bacteria and their predators were affected by temperature. In response, the diet of the predators changed.

Different responses of individual bacterial groups to predators highlight the different survival strategies of bacteria. Predation is a driver of the genetic and functional structure of bacterial communities (Weinbauer *et al*., 2019; Bell *et al*., 2010, de la Cruz Barron *et al*., 2023). However, the relationship between functional traits of bacteria that allow them to evade predators and bacterial phylogeny is not yet fully understood (Tamames *et al*., 2016). Furthermore, the predators themselves responded to the temperature increase, as evidenced by changes in their grazing activities and prey selectivity. Thus, temperature and predation acted together to alter bacterial community composition, with these factors affecting individual bacterial groups in different ways.

However, it is difficult to generalise about how temperature-induced phylogenetic changes in bacterial communities affect their functioning in the environment due to the high functional diversity that characterises marine bacteria. The structural and functional changes in response to temperature fluctuations are influenced by factors such as the thermal preferences of the different bacterial taxa, their adaptability to temperature fluctuations, and the ecological dynamics of the marine environment. If the community consists of resistant, resilient, or functionally similar taxa, changes in phylogenetic composition may have minimal impact on ecosystem process rates (Allison & Martiny, 2008). Although the mechanisms and factors controlling bacterial response to temperature fluctuations are unclear, recent studies suggest that climate-induced temperature fluctuations may influence biogeochemical cycles mediated by bacterial communities (Ward *et al*., 2017).

Despite advances in understanding the response of bacterial composition to environmental change, a predictive framework is needed to interpret the functional implications of these changes. This requires interdisciplinary research to establish links between the phylogenetic responses of bacterial communities and their function

within ecosystems (Amend *et al.,* 2016; Morrissey *et al.,* 2016).

In summary, our results support previous research indicating that experimental warming can affect bacterial communities in marine environments. The observed changes in the growth and grazing rates of specific bacterial groups, bacterial community composition, and shifts in grazing preferences of different bacterial predators in response to temperature, emphasise the important role of temperature in regulating trophic interactions between predators and their prey. Furthermore, our results suggest that bacterial growth and the grazing behaviour of bacterial predators do not necessarily occur simultaneously at higher temperatures. This highlights the importance of considering bacterial phylogenetic composition when predicting bacterial response to long-term warming.

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Supplementary Data

The following supplementary information is available online for the article:

Fig. S1: Map of the investigated area.

Fig. S2: Scheme of the experimental design.

Table S1. Concentrations of nutrients (average ± standard deviation) during the experiment at ambient (T_{amb}) and a 3°C increased temperature (T_{amb}+3).

Table S2. Oligonucleotide probes and hybridization conditions used for CARD-FISH. FA (%): final concentration of formamide used in the hybridization buffer.