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Ecological "Windows of opportunity" influence biofilm prokaryotic diversity differently in glacial and non-glacial Alpine streams

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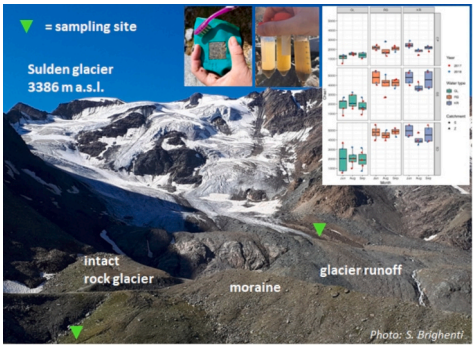
HIGHLIGHTS

- We meta-barcode prokaryotes of epilithic and sediment biofilm in different Alpine stream types.
- We confirm kryal and krenal streams as endmember of benthic prokaryotic α- and β-diversity.
- Windows of opportunity (WOs) appear able to increase microbial diversity besides biomass.
- Response of prokaryotic diversity to WOs is stronger in rock glacial and krenal than in kryal streams.
- Rock glacial runoffs are suggested as key climate refugia for cold stenotherm microorganisms.

GRAPHICAL ABSTRACT



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ABSTRACT

In glacier-fed streams, the Windows of Opportunity (WOs) are periods of mild environmental conditions supporting the seasonal development of benthic microorganisms. WOs have been defined based on changes in biofilm biomass, but the responses of microbial diversity to WOs in Alpine streams have been overlooked. A two year (2017–2018) metabarcoding of epilithic and epipsammic biofilm prokaryotes was conducted in Alpine streams fed by glaciers (kryal), rock glaciers (rock glacial), or groundwater/precipitation (krenal) in two catchments of the Central-Eastern European Alps (Italy), aiming at testing the hypothesis that: 1) environmental WOs enhance not only the biomass but also the α-diversity of the prokaryotic biofilm in all stream types, 2) diversity and phenology of prokaryotic biofilm are mainly influenced by the physical habitat in glacial streams, and by water chemistry in the other two stream types. The study confirmed kryal and krenal streams as endmembers of epilithic and sediment prokaryotic α- and β-diversity, with rock glacial streams sharing a large proportion of taxa with the two other stream types. Alpha-diversity appeared to respond to ecological WOs, but, contrary to expectations, seasonality was less pronounced in the turbid kryal than in the clear streams. This was attributed to the small size of the glaciers feeding the studied kryal streams, whose discharge dynamics were those typical of the late phase of deglaciation. Prokaryotic α-diversity of non-glacial streams tended to be higher

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in early summer than in early autumn. Our findings, while confirming that high altitude streams are heavily threatened by climate change, underscore the still neglected role of rock glacier runoffs as climate refugia for the most stenothermic benthic aquatic microorganism. This advocates the need to define and test strategies for protecting these ecosystems for preserving, restoring, and connecting cold Alpine aquatic biodiversity in the context of the progressing global warming.

1. Introduction

The structure and diversity of the benthic microbiome of cold aquatic ecosystems, including Alpine headwaters, has remained largely unexplored and little understood until recently (Ezzat et al., 2022). Biofilm microbial communities are key elements of local biodiversity and drivers of biogeochemistry and metabolism of cold freshwater (Battin et al., 2016). This role is accentuated by the short residence time and high variability of the rarefied microbial assemblages in cold stream water (Logue et al., 2004; Ezzat et al., 2022), and by the much smaller abundance and diversity of multicellular organisms in comparison to temperate aquatic ecosystems (Hotaling et al., 2017). Nonetheless, the understanding of biofilm microbial diversity, phenology, and response to climate warming still remains incomplete and patchy for Alpine headwater (Margesin and Collins, 2019; Bourquin et al., 2022).

The phenology of the biota in glacier-fed streams is strongly constrained by the variability of the physical properties of glacial runoff, especially water temperature and discharge, light intensity (photosynthetically active and UV wavelengths), and sediment transport, and by the nutrient availability (Elser et al., 2020). Yet, environmental conditions are usually milder in late spring/early summer (between the end of snowmelt and the beginning of glacier ablation) and in late summer/autumn (between the end of ablation and the first snow cover) than during the summer glacial ablation These milder periods are defined "Windows of Opportunity" (WOs, Uehlinger et al., 2010) since they allow short but rapid growth of biofilm. This is firstly composed of prokaryotic and eukaryotic primary producers (Battin et al., 2004; Rott et al., 2006), which enable the growth of heterotrophic prokaryotes and the development of complex biofilm architecture (Battin et al., 2016; Busi et al., 2022). The late summer WO is considered as the primary one, while the early summer WO more strongly depends on stochastic meteorological factors and is irregularly paralleled by microbial growth (Battin et al., 2004). The WOs have been conceptually modelled based on field surveys of primary producers in glacier-fed streams (kryal habitats; Ward, 1994) of the European Alps (Uehlinger et al., 2010), while scarce field evidence is available for non-glacial streams (e.g., Perić et al., 2015). Nonetheless, WOs are expected to be less pronounced there, and especially in groundwater-fed springs (krenal habitats; Ward, 1994) which are characterised by moderate physical and chemical seasonal changes (Cantonati et al., 2012; Freimann et al., 2013). In addition, it is not yet clear whether and to what extent the seasonal growth occurring during the WOs is paralleled by changes in the taxonomic composition and biodiversity of biofilm microorganisms, especially prokaryotes.

Closing the knowledge gaps on diversity and phenology of Alpine aquatic microorganisms is of paramount importance for predicting future ecological trajectories in relation to the ongoing global warming and deglaciation (e.g., Huss et al., 2017; Hock et al., 2019). Numerous glaciers have already passed their "peak-water", i.e., the maximum meltwater production that characterises the intermediate stages of glacier retreat (e.g., Gleick and Palaniappan, 2010; Huss and Hock, 2018). Therefore, the eco-hydrological role of glacier-fed streams is fading in numerous Alpine catchments (Jones et al., 2019), while the influence of other cryosphere components, including permafrost, is increasing (Luo et al., 2022). Since the thawing rate of sub-surface permafrost ice is 10–100 times slower than that of glaciers (Haeberli et al., 2017), a transition from glacial towards periglacial and paraglacial processes is occurring at global scale, with permafrost-related landforms increasingly shaping the ecohydrology of Alpine river systems (Haeberli et al., 2017; Milner et al., 2017). Among permafrost-related landforms, rock glaciers, i.e., creeping accumulations of rocky debris and sediment containing permafrost ice (Jones et al., 2018), are increasingly influencing the availability of water resources, representing important mountain aquifers under prolonged hydrological decline (Jones et al., 2019; Hayashi, 2020). These phenomena are increasingly accompanied by stochastic precipitation regimes that make freshwater physical and hydrological variability more unpredictable at both seasonal and inter-annual scale (Jones et al., 2018; Hock et al., 2019).

As summarised by Brighenti et al. (2019a), headwaters emerging from rock-glaciers, alpine slopes (krenal), or mainly fed by snowmelt and precipitations (rhitral), are characterised by peculiar physical and chemical conditions in comparison to glacier-fed headwaters (kryal). In particular, springs emerging from rock glaciers are cold (<2.0 °C) and clear (<5 NTU) (Carturan et al., 2016; Brighenti et al., 2019b) and can be enriched in solutes (electrical conductivity up to ~1000 μS cm^{-1}), including trace elements in catchments with crystalline bedrock (Colombo et al., 2018; Rotta et al., 2018; Brighenti et al., 2021a). Recent studies also outlined the biological peculiarity of rock glacial streams, where taxonomic composition and diversity of bacteria and diatoms in biofilm is intermediate between those of krenal and kryal habitats (Wilhelm et al., 2013; Fegel et al., 2016; Tolotti et al., 2020). The future physical and chemical changes expected for Alpine headwaters will trigger variations in abundance, diversity, and seasonal dynamics of aquatic microorganism, with great implications for the ecological integrity and functionality of Alpine aquatic ecosystems (Hotaling et al., 2017; Milner et al., 2017). Alpha diversity will temporarily increase in kryal headwaters as a response to the ameliorated habitat conditions (Brighenti et al., 2019a). However, the ecological niches of cold stenotherms will contract, threatening many species with extinction (Fell et al., 2017). Conversely, benthic β-diversity is expected to decrease due to the habitat homogenisation of deglaciated areas and the expansion of tolerant species (Fell et al., 2017; Brighenti et al., 2020). Biodiversity loss and habitat homogenisation may be partially compensated by rock glacial runoffs, which are increasingly considered as ecological refugia for cold adapted aquatic organisms (Brighenti et al., 2021a, 2021b). However, the scarcity of field and experimental observations still limit the understanding of the ecological role of rock glacial headwaters and the prediction capacity for the response of microbial communities to seasonal dynamics in the context of global warming (Busi et al., 2022).

In this study we investigated how the physical and chemical seasonality of Alpine streams influences the biofilm prokaryotic diversity in catchments over the peak water (sensu Huss and Hock, 2018) of the Central-Eastern Italian Alps. We focussed on the biofilm because attached prokaryotes dominate the microbial life in Alpine streams, while stream water communities are scarce and transient (e.g., Ezzat et al., 2022). We investigated two adjacent catchments where we surveyed the epilithic and sediment biofilm of kryal, rock glacial, and krenal streams from June to September 2017 and 2018. We tested the following hypotheses:

HP-1) Windows of Opportunity (WOs) enhance biofilm microbial α-diversity, in addition to the microbial growth, especially in glacial and rock glacial streams, which are characterised by harsh environmental conditions in comparison to krenal streams.

HP-2) Biofilm prokaryotic diversity and phenology are mainly influenced by the physical habitat in kryal streams, and by water chemistry in rock glacial and krenal streams.

2. Methods

2.1. Study sites

We selected two sub-catchments (Fig. 1), i.e., Upper Sulden (hereafter "S", area 20 km^2, maximum altitude 3386 m a.s.l.) and Zay (hereafter "Z", area 11 km^2, maximum altitude 3546 m a.s.l.), in the Sulden river catchment in the Ortler-Cevedale massif, Central-Eastern Italian Alps. The area hosts several glaciers and intact rock glaciers above ~2700 m a.s.l. (Autonomous Province of Bolzano/Bozen - APB, 2018a). The Sulden catchment is located on a crystalline basement (the Campo Nappe, mainly composed of orthogneiss and quartz phyllites) of the three Austroalpine domain tectonic units that merge in the Sulden Valley (Montrasio et al., 2015).

The area covered by glaciers is around 32 % for the Upper Sulden and 8 % for the Zay basin (Autonomous Province of Bolzano/Bozen - APB, 2018b). The N-oriented Suldenferner (1.05 km^2, debris free) and the Außerer Zay (0.3 km^2, partially debris covered) were chosen for the present study. Both glaciers retreated by several hundred meters since 1987 and have already passed the "peak-water" stage, so that their influence on the hydrology of their respective glacier-fed streams is moderate (Brighenti et al., 2019b). The main streams of both catchments receive the contribution of rock glacial runoff and slope springs (krenal) in their mid to lower sections (Fig. 1). Further details on the study area and the sampling stations are available in Brighenti et al. (2019b, 2020).

2.2. Sampling and laboratory analyses

In each sub-catchment (S and Z) we sampled headwaters of different origin (Table 1): the upper glacier runoff (kryal = GL-A at the glacier snout, and GL-B at 700 m (S) and 340 m (Z) downstream), the runoff of an intact rock glacier (rock glacial = RG, area of 0.072 km^2, front at 2586 m a.s.l. in Upper Sulden, and 0.09 km^2, front at 2719 m a.s.l. in Zay), and a spring without any presence of glaciers nor rock glaciers in the upstream draining area (krenal = KR). The downstream kryal section of each glacial stream was surveyed to cope with the expected scarce biofilm development at the glacier snout. The krenal streams were selected to maximise the physical and chemical differences of the surveyed stream types.

All sampling sites were surveyed during the three key hydrological stages of the high-Alpine summer of 2017 and 2018, i.e., during snow melt (second half of June), maximum glacier ablation (early August),

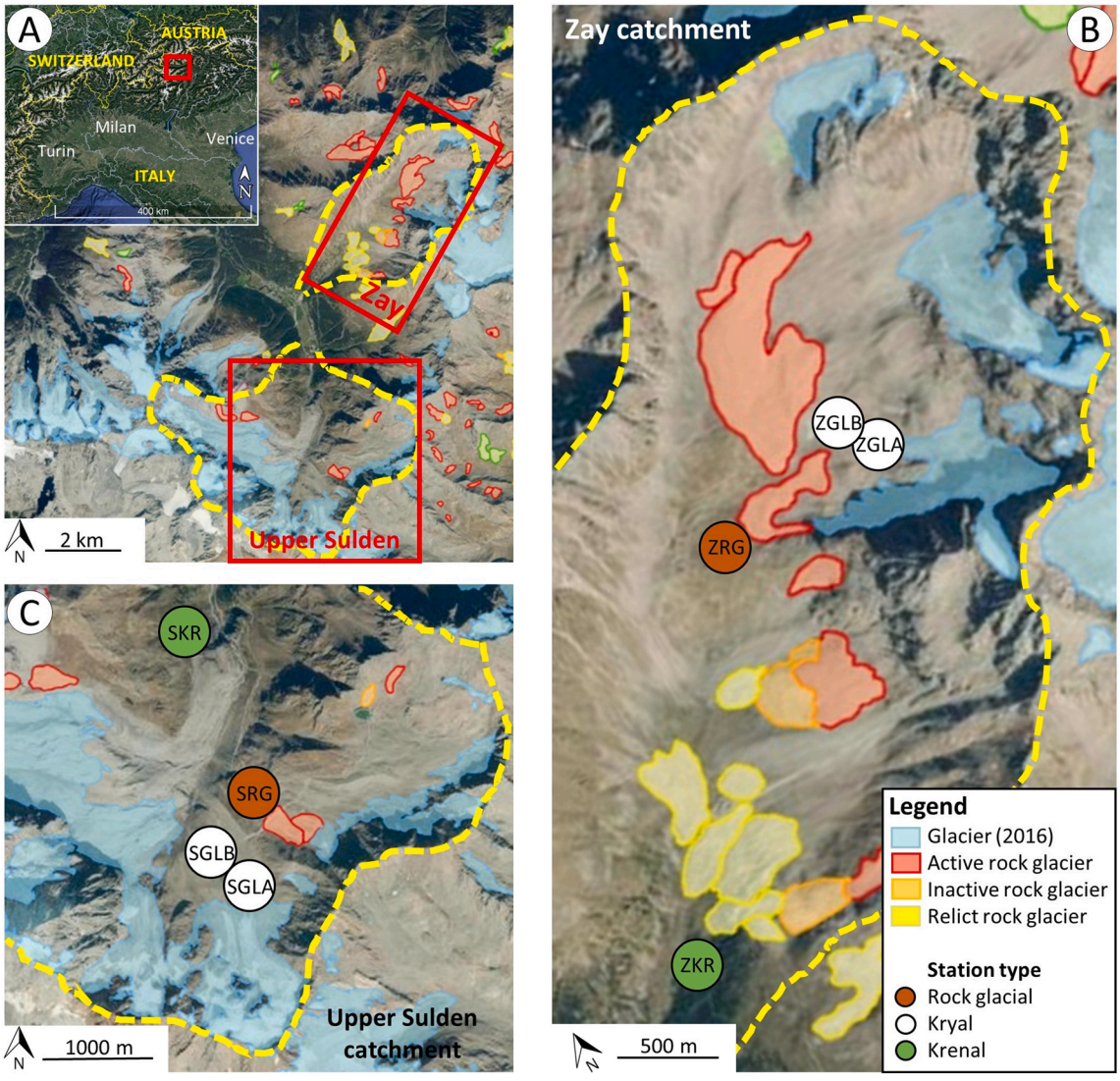

Fig. 1. Study area. (a) Geographic location of the two catchments studied within the Italian Central-Eastern Alps; (b-c) Detail of the areas included within each red rectangle in (a) showing the schematic geomorphological setting and location of the sampling stations in the Upper Sulden (S) and Zay (Z) catchments, respectively. GLA and GLB: upper kryal sampling sites; RG: rock glacial streams; KR: krenal streams; For further detail on sites and stations codes, see the Methods section. Active, inactive, and relict rock glaciers are defined based on their ice content and creeping movements, as in Kofler et al. (2020).

Table 1
Principal habitat characteristics of the surveyed headwaters in the Sulden (S) and Zay (Z) catchments. Lat/Lon: UTM32N, WGS84, coordinates. Lit: lithology of the underlain catchment as in Montrasio et al. (2015) according to the decreasing proportion of dominant rocks. Alti: elevation in m a.s.l.. Slop: % average slope by clivometry (Autonomous Province of Bolzano/Bozen - APB, 2018a). Veg: riparian vegetation, with 0 = absent, 1 = discontinuous alpine heath, 2 = continuous alpine heath, 3 = alpine heat with sparse shrubs and dwarf trees, 4 = mixed trees/shrubs canopy. Moos: percent of the channel covered by aquatic mosses, with 0 = 0–5 %, 1 = 5–10 %, 2 = 10–30 %, 3 = 30–50 %. Q: water discharge range as estimated from field measurements (Gordon et al., 1992) in June and September 2018. $Pfan_{bot}$: bottom component of the Pfankuch Index (Pfankuch, 1975) used as a proxy for the channel instability (Brighenti et al., 2019b).

Site	Type	Lat/Lon	Lit	Alti (m)	Slop (%)	Veg	Moos (%)	Q (L sec^{-1})	$Pfan_{bot}$
S-GLA	Upper kryal	622,586/5148841	Orthogneisses, Phyllites, Amphibolites	2710	15	0	0	1–136	58
S-GLB	Upper kryal	622,250/5149314	Orthogneisses, Phyllites, Quartzites	2560	10	0	0	96–458	59
S-RG	Rock glacial	622,737/5149517	Orthogneisses, Quartzphyllites, MIcaschists	2586	25	2	3	29–46	18
S-KR	Krenal	622,298/5151224	Orthogneisses, Quartz phyllites, Amphibolites	2105	65	3	2	29–32	37
Z-GLA	Upper kryal	625,249/5156172	Orthogneisses	2845	55	0	0	5–30	54
Z-GLB	Upper kryal	625,039/5156377	Orthogneisses	2780	18	1	0	4–38	50
Z-RG	Rock glacial	624,401/5156088	Orthogneisses	2718	2	2	1	28–173	30
Z-KR	Krenal	622,936/5154422	Orthogneisses, Flaser paragneisses	2160	22	4	3	8–10	18

and flow recession (early September). At each sampling session, water temperature, electrical conductivity (EC) and turbidity were measured with portable probes (WTW-Cond-3310 and WTW-Turb-430IR), and water velocity with a digital water velocity meter (Global Water Flow Probe, College Station, Texas, USA). Water discharge was estimated based on the salt-dilution method (Gordon et al., 1992) or with section/depth measurements (Brighenti et al., 2019b).

Water samples for chemical analyses (pH, alkalinity, EC, major ions, soluble reactive and total N, P, and SiO_2) were collected at each survey from each station and preserved at 4 °C until the analyses performed at the Hydrochemistry lab of the Edmund Mach Foundation following standard methods (American Public Health Association (APHA), 2017). Water samples for the determination of trace element concentrations were filtered through cellulose acetate filters (0.45 μm pore size) in pre-acidified (HNO_3 65 %) polyethylene bottles and fixed in the field with HNO_3 65 % at a final concentration of 2 %. The concentrations of 31 elements (complete list in Brighenti et al., 2019b) were determined using an ICP-MS ICAP-Q Thermo Fischer analyser at EcoResearch (I), following standard methods (American Public Health Association (APHA), 2017). Water samples for the determination of Dissolved Organic Carbon (DOC) were collected in acidified polyethylene bottles and preserved at 4 °C until the analysis conducted at the Dolomiti Energia Holding, Trento (I). The concentrations of water Total Suspended Solids (TSS) and Ash Free Dry Mass (AFDM) were calculated according to Hauer and Lamberti (2011) by filtering up to 4 L water in the field through pre-calibrated WHATMAN GF/C glass fibre filters (1.1 μm pore size) that were stored frozen (− 20 °C) and under dark conditions until the lab analyses.

At each sampling session, the biomass of the epilithic biofilm was estimated by determining the AFDM content (Battin et al., 2004; Logue et al., 2004) in the material obtained by scraping a known area - delimited using flexible plastic frames - of 3–5 cobbles randomly collected along a ~ 10 m long stream stretch. The biomass of biofilm eukaryotic and prokaryotic primary producers, mainly composed of microalgae and cyanobacteria (Battin et al., 2016), was estimated by determining the chlorophyll-*a* concentration of samples collected in the same way. The material was filtered in the field through pre weighted WHATMAN GF/C glass fibre filters and stored at −20 °C until analysed at the Biological Laboratory of the Edmund Mach Foundation (FEM). Biofilm AFDM was determined according to Hauer and Lamberti (2011) and expressed as g cm^{-2}, chlorophyll-*a* according to Lorenzen (1967) and expressed as mg cm^{-2}.

The collection and processing of the benthic biofilm for the metabarcoding of prokaryotes followed the same protocol used in Tolotti et al. (2020). The epilithic biofilm (EP) was collected by thoroughly brushing with a sterile toothbrush up to 20 pebbles collected from both the midchannel and banks of a 10 m long stream stretch. The epipsammic components of the stream biofilm were collected from the same 10 m stretch by syphoning with sterile plastic pipettes the superficial (SS: 0–2 cm) and deeper (SD: 5–10 cm) streambed sediment layers. Cross-contamination was carefully avoided during sampling. The epilithic and epipsammic samples were freeze-dried at the FEM labs and homogenised aliquots (150–250 mg) were extracted using the DNeasy Power Soil Kit (QIAGEN, USA) following the manufacturer's instruction. The extracts were checked and quantified with a NanoDrop spectrophotometer ND-8000 (Thermo-Fischer Scientific Inc. Ma., USA).

2.3. Library preparation and high throughput sequencing

The DNA extracted from the biofilm samples was purified using the 1.8× Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), following the manufacturer's instructions. The specific prokaryotic primer sets 515F (5' GTGYCAGCMGCCGCGGTAA 3′) and 806R (5' GGACTACNVGGGTWTCTAAT 3′) (Caporaso et al., 2011), with degenerate bases as suggested by Apprill et al. (2015) and the overhang Illumina adapters were used. The amplicon library preparation and sequencing were performed as described by Tolotti et al. (2020).

The nucleotide sequence data reported are available in the ENA/EMBL-EBI database under accession number PRJEB74693.

2.4. Data analysis

Differences in the physical, chemical, and biological properties of the surveyed stream types were assessed by the Kruskal-Wallis H test (ANOVA on ranks) after checking the dataset for normal distribution (Shapiro-Wilkinson test) and variance equality (Bennet test). The Wilcoxon rank-sum test was run for pairwise comparison of median values of water types (both overall regardless seasons and across seasons). A Bonferroni correction was applied to retain only the most significant differences.

Raw 16S rRNA gene sequences were processed using the open-source MICCA (v1.7.2) software (Albanese et al., 2015). Paired-end reads were first truncated at the 3′ end to a length of 250 bp and assembled using the procedure described in Edgar and Flyvbjerg (2015). Assembled reads with an overlap length smaller than 80 and with >24 mismatches were discarded. After forward and reverse primer trimming, merged reads shorter than 250 bp and with an expected error rate higher than 0.5 % were removed. Filtered sequences were clustered into Amplicon Sequence Variants (ASV) using the UNOISE (doi:https://doi.org/10.1101/081257) algorithm available in MICCA. Taxonomic assignment was carried out using the RDP classifier v. 2.12 (Wang et al., 2007). Multiple sequence alignments (MSA) were performed on the representative sequences using MUSCLE v.3.8.31 (Edgar, 2004) and the phylogenetic tree was inferred using FastTree v2.1.8 (Price et al., 2010). Non-bacterial ASVs (i.e. reads classified as chloroplast) were discarded from the 16S rRNA amplicon dataset in all downstream analyses. After checking the distribution of sample sequencing depth (1655–122,673, median = 61,962 reads), ASVs were rarefied without replacement to

20,000 sequences per sample.

Prokaryotic β-diversity was explored by t-distributed Stochastic Neighbour Embedding (t-SNE) analysis, a nonlinear dimensionality reduction technique (van der Maaten and Hinton, 2008), here based on the Bray-Curtis dissimilarity matrix. Alpha-diversity was quantified based on the richness of the observed number of ASVs by the Chao Index (Kim et al., 2017). Seasonal changes of α-diversity within stream types and within sample types were tested by applying pairwise Wilcoxson rank-sum test. The differential relative abundance analysis was performed at the genus level by ANCOM BC (Analysis of Compositions of Microbiomes with Bias Correction, Lin and Peddada, 2020) to assess for seasonal changes in the biofilm prokaryotic taxonomic composition in the different stream types.

Significant relationships (TICe Storey's q-value <0.01) between the relative abundance of the prokaryotic genera and the environmental variables was explored, separately for each sampled substratum (EP, SS, SD), using MICTools (Albanese et al., 2018). Spearman rank correlation analysis (ρ coefficients) was performed to explore the relationships between biofilm epilithic and sediment prokaryotic α-diversity and environmental settings of the different streams. No correction was applied to this test based on the small number of cases for each sample category. All statistical tests were considered significant if $p < 0.05$.

Data analyses were performed in the RStudio environment (R Core Team, 2017) using the *phyloseq* package (McMurdie and Holmes, 2013); the plots were drawn using the *ggplot2* package (Wickham, 2009).

3. Results

3.1. Water physical, chemical and biological characteristics

The three types of surveyed streams displayed different environmental characteristics (Figs. 2 and S1). RG runoffs were significantly colder than KR streams, with temperature steadily around 1 °C for the whole study period (Fig. 2). Due to their gentler terrain slope (Table 1), RG were also significantly ($p < 0.05$) slower than GL streams, with water velocity steadily <0.3 m sec^{-1} (Fig. 2). No significant seasonal changes emerged for velocity of each stream type. As expected, water turbidity of GL streams was significantly ($p < 0.05$) higher in June and August (up to 300 NTU) than in September (median 15 NTU, Fig. 2), while mean turbidity of RG and KR (1 and 20 NTU) was significantly ($p < 0.001$) lower than in GL in June and August, but not during the flow recession period.

EC showed a comparable range of variability in GL and RG streams, with increasing values from the snow melt period in June (minimum of 10 μS cm^{-1}) to the flow recession in September (Fig. 2). KR values oscillated around 170 μS cm^{-1} during the whole study period and did not significantly differ from GL and RG median values (Fig. 2). Median concentrations of Dissolved Inorganic Nitrogen (DIN) were significantly ($p < 0.05$) lower in GL and RG than in KR streams only in June and August, with RG streams showing intermediate values ranging from around 110 to 200 μg L^{-1} (Fig. 2). DIN showed minimum and highest median concentrations in August and September, respectively, in all stream types, but the difference between the two months was significant ($p < 0.01$) only in GL streams (Fig. 2). Median Total Phosphorus (TP) concentrations were significantly ($p < 0.001$) higher in GL than in RG and KR streams, where TP concentrations were one order of magnitude lower (< 15 μg L^{-1}) for the whole survey period. Concentrations of PO_4-P (not shown) steadily remained below 2 μg L^{-1} in all stream types, with many values below the detection limit of the instruments (0.5 μg L^{-1}). Median silica concentrations showed a significantly ($p < 0.01$) increasing trend from GL to KR streams, with RG and KR streams showing lower median values and higher variance in September (Fig. 2). The range of variability of the total concentrations of trace elements was comparable in GL and RG streams, both reaching very high concentrations in September 2017 (i.e., 1359 and 600 μg L^{-1}, respectively, Fig. 2), while trace element concentrations where significantly ($p < 0.05$) lower during the whole study period in the KR samples (~ 50 μg L^{-1}, Fig. 2). Seasonal patterns and median concentrations of trace elements with widespread technogenic dispersal (e.g., Cd, Cu, Ni, Zn, see Fig. S1) were comparable to those of total trace elements in KR and RG streams, while GL showed significantly ($p < 0.05$) higher median concentrations of elements of prevalent lithological origin (i.e., Sr, As, Rb, Fig. S1).

DOC concentrations ranged from 0.2 to ~4 mg L^{-1} (Fig. 2) in all stream types, except for a few values >5 mg L^{-1} recorded in June 2017, with no significant overall differences among stream types and among seasonal levels in each stream type. Conversely, median water AFDM concentrations were significantly ($p < 0.001$) higher in GL than in RG and KR streams due to peak values in August (Fig. S1). Both biofilm AFDM and chlorophyll-*a* concentrations were quite low in all stream types (0.1–2.5 mg cm^{-2} and ~ 0.0–2.6 g m^{-2}, respectively, Fig. 2), but especially in the GL ($p < 0.001$). Both GL and RG streams showed slightly lower, though not significantly, median AFDM values in August, while chlorophyll concentrations peaked either in June (RG) or in September (GL and KR).

3.2. Biofilm prokaryotic taxonomic composition, abundance, and diversity

The two-year survey of the eight sampling stations yielded a total of 141 biofilm samples (Fig. S2), since no biofilm sample could be collected from the Zay GL-A site in June 2017 due to snow cover persisting over the stream. Twenty-one glacial samples (9 from Upper Sulden and 15 from Zay, Fig. S2) were eliminated before processing the raw sequences, as they yielded <7000 reads each. Rarefaction resulted in removing four more GL-A samples (Fig. S2) as well as 35 ASVs which were no longer present in any sample after random subsampling. All remaining glacial samples were grouped together (GL) without further considering their distance from the glacial front, and prokaryotic taxonomic composition and diversity was analysed in a subset of 116 samples.

The metabarcoding of the V4 region of the prokaryotic 16S rRNA gene yielded 14,518 ASV, assigned to 412 coded genera and 28 phyla. Only eight phyla were recorded in all samples (Fig. 3a). *Pseudomonadota* had the highest median relative abundance (47.5 %): *Alpha-, Beta-, Gamma-, and Deltaproteobacteria* were widely widespread (100 % occupancy), but their median relative abundance dropped from around 20 % (*Alpha-* and *Betaproteobacteria*), to 1 % (*Deltaproteobacteria*, Fig. S3). *Oligoflexia* and *Epsilonproteobacteria* were less common and much less abundant (Fig. S3). *Bacteroidota* represented the second-most abundant phylum, although it reached a median share of only 16.4 %. All the remaining phyla showed median relative abundance <7 %, with 15 phyla not reaching the threshold of 1 % (Fig. 3a). For rare phyla (median relative abundance <0.1 %), *Candidatus* Latescibacteria was the only one present in all stream types and substrata, while all the remaining ones were absent in the epilithic samples of at least one stream type (Fig. S4). Most of the abundant phyla showed comparable median abundances in the epilithic and sediment biofilm of the three stream types, but especially in the glacial streams. Exceptions are represented by *Chloroflexota* and *Nistrospirota*, which were significantly ($p < 0.05$) more abundant in the sediment of KR streams, and *Deinococcota* which was significantly more abundant in the epilithon of RG streams. *Archaea*, although recorded in almost all the analysed samples, showed overall low abundances, especially in the epilithic biofilm of all stream types (Fig. S5).

The tSNE analysis (Fig. 3b) clustered the epilithic and sediment biofilm samples according to the stream type. The epilithic GL samples were clearly separated, independently from the catchment, from the KR and RG samples that formed a second mixed group. The sediment samples were more clearly clustered according to stream types, with RG and KR samples grouping also according to catchment. The median dissimilarity between epilithic prokaryotic assemblages in different stream types confirmed significantly larger differences between GL and RG and KR samples, respectively, than between KR and RG in 2017

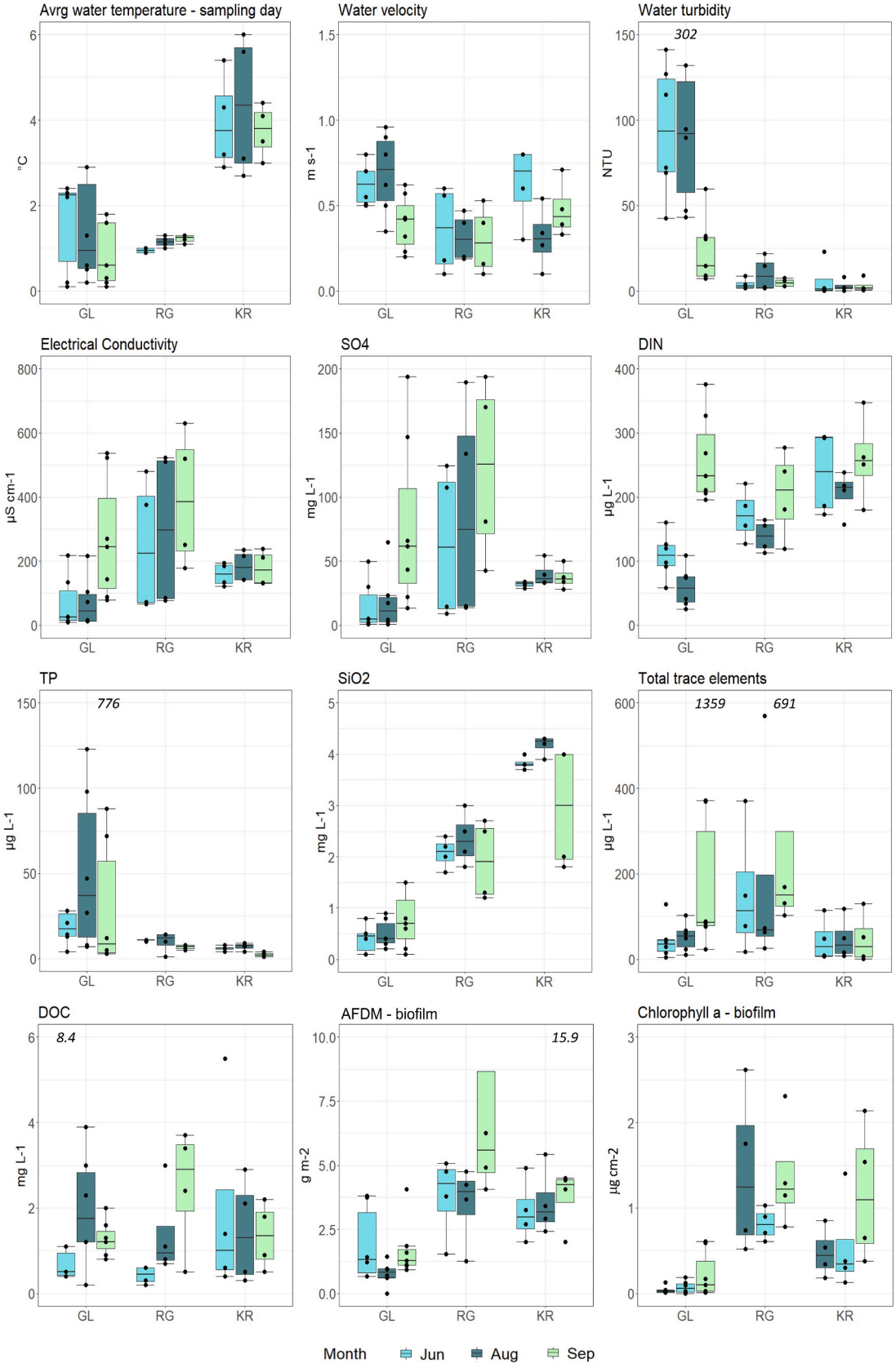

Fig. 2. Combined box plots representing median values and seasonal variability of key physical, chemical, and biological characteristics determined in the surveyed stream types during the summer of the two-year study period. GL: glacial; RG: rock-glacial; KR: krenal; Avrg: average; EC: electrical conductivity measured in the lab at 20 °C; DIN: dissolved inorganic nitrogen; TP: total phosphorus; Total trace metals: sum of the concentrations of all trace elements detected in each sample; DOC: stream water Dissolved Organic Carbon; AFDM biofilm: Ash Free Dry Mass measured in the epilithic biofilm.

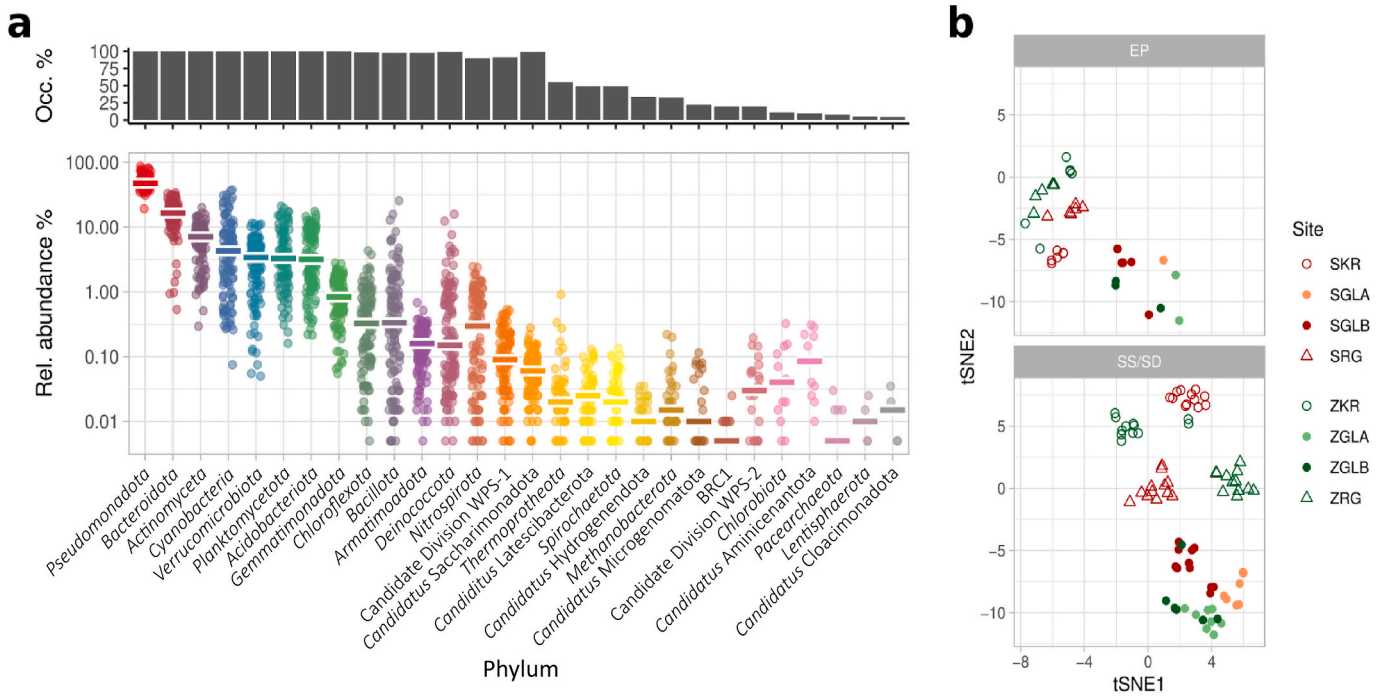

Fig. 3. a) Percent of occupancy (Occ. %) and relative abundances of the 28 phyla identified in the 116 analysed epilithic and sediment biofilm samples from the 2017–2018 survey of Alpine streams of different origin (a). Horizontal bars: median values. b) Estimation of the biofilm prokaryotic β-diversity of the surveyed stream types based on tSNE analysis for the epilithic (EP), and combined surface and deeper sediment (SS and SD, respectively) samples. Abbreviation of sampling sites and stations as in Fig. 1.

(Fig. S6). In the sediment, the dissimilarity was significantly higher between GL and KR samples (Fig. S6) than between the other stream combinations in both study years. The median Bray-Curtis dissimilarity between all temporal and spatial sample categories (Fig. S7) was low for samples differing by sampling year or month and increased for samples differing by catchment, substratum, and stream types, or differing by all the five categories.

The prokaryotic α-diversity in the epilithic biofilm displayed scarce variance and a moderate, though not significant, increase from GL to RG and KR streams (Fig. 4). In contrast, sediment prokaryotic assemblages showed higher variance and were significantly ($p < 0.05$) more diverse in KR and RG than in GL streams (Fig. 4). The seasonal patterns of prokaryotic α-diversity in the epilithic and sediment biofilm of KR streams were highly comparable, with highest diversity in June, a pronounced drop in August (significant only for the epilithon, $p < 0.05$) and a partial recovery in September. Prokaryotic α-diversity of RG streams similarly dropped from June to August in both the epilithon and the sediment, but without significant variation (Fig. 4). GL streams displayed no significant changes of prokaryotic α-diversity over time, although the epilithic glacial biofilm was nearly significantly ($p < 0.057$) more diverse in August than in June. The scatter plots of all biofilm α-diversity values in each stream type (Fig. S8) confirmed a higher diversity in June than in September in RG and especially KR streams, while GL streams showed no clear pattern. In August α-diversity was lower or equal than in September in the KR and, to a lesser extent, RG epilithon, while it tended to be higher than in September in the GL streams.

The ANCOM-BC statistics outlined small sets of rare (mean relative abundance ≤1 %) prokaryotic genera as responsible for the observed seasonal differences in α-diversity in the three stream types (Fig. 5). In the GL streams no significant difference was outlined between June and August, while only 11 genera showed significantly different relative abundance between June and September. A comparably small ($n = 10$) but taxonomically different set of genera was responsible for seasonal changes in the RG streams, either between June and August or between June and September (Fig. 5). In the KR streams seasonal changes were due to a larger set of genera ($N = 19$), the majority of which displayed significant differences in their relative abundance only between June and August.

3.3. Environmental drivers of biofilm prokaryotic α-diversity

The prokaryotic α-diversity of the stream epilithon was scarcely correlated with the environmental variables in all stream types, showing only a negatively significant correlation with Cl concentration for the RG samples, and with EC for the KR samples (Fig. 6). Correlations were overall stronger for the sediment α-diversity: in GL streams it was positively correlated with water temperature, and negatively with the concentrations of particulate and soluble nitrogen, and of trace elements more susceptible to atmospheric dispersal (Other-te). In RG streams, α-diversity was correlated negatively to water turbidity and positively to water velocity and some solute concentration (Ca^{2+}, Mg^{2+}, SO_4^{2-} and trace metals of prevalent geological origin). Finally, the sediment α-diversity of KR streams was negatively correlated only to the water solute concentration (i.e., EC, alkalinity, major ions). Weak correlations emerged between α-diversity and the variables related with the stream trophic status (especially DOC, P, and dissolved N) and the biofilm biomass. The sole exception is represented by the significant negative correlation between prokaryotic α-diversity and biofilm AFDM concentrations in the superficial sediment of the RG streams (Fig. 6).

The correlation analysis between the relative abundance of the prokaryotic genera and the stream environmental settings provided a similar picture of overall scarce relationships (Fig. S9). In the epilithic biofilm, only a few genera mainly belonging to the most widespread *Pseudomonadota*, as well as to *Acidobacteriota*, *Planctomycetota*, and *Cyanobacteria*, showed significant correlations with water turbidity, temperature, and with variables related to the development of the epilithic biofilms (i.e., SiO_2, chlorophyll-*a*, and AFDM concentrations). In both the superficial and deep sediment biofilm, a larger number of genera belonging to those same phyla and to several rare phyla, were

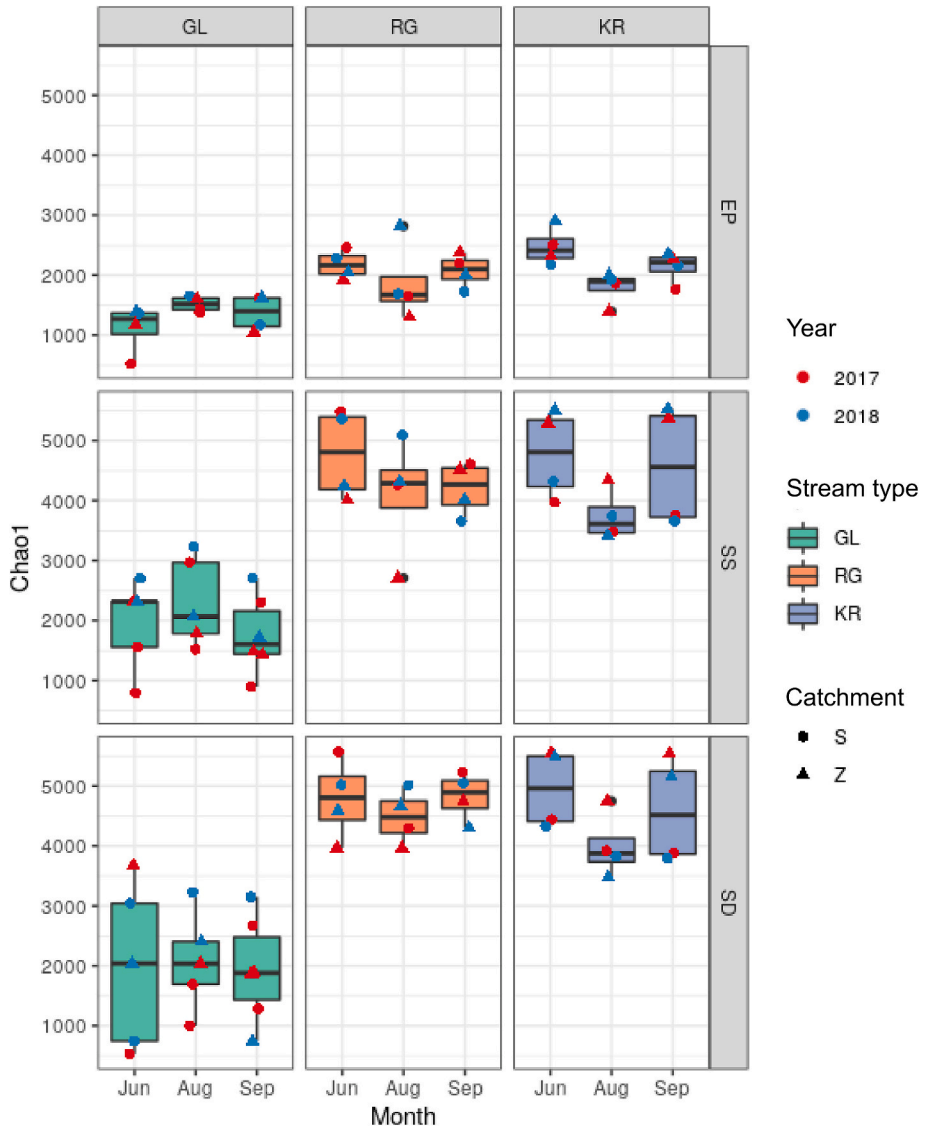

Fig. 4. Distribution of prokaryotic α-diversity (Chao Index) in the epilithic and sediment biofilm samples (EP, SS, SD) collected from each stream type (GL, RG, KR) in different moments (June, August, September) of the Alpine vegetative season of 2017 and 2018. Horizontal bars: median values. Each box plot shows α-diversity values of samples collected from each stream type in the two studied catchments and in the two survey years. Abbreviations for stream catchment, stream type, and sampled substratum as in Figs. 1, 2, 3.

correlated to the same environmental variables as well as to TP and several ions (Fig. S9).

4. Discussion

4.1. Habitat settings of different Alpine headwaters

Our results confirmed previous findings on the different habitat settings of kryal, rock glacial, and krenal streams (Rotta et al., 2018; Brighenti et al., 2019b, 2021a). Physical properties differed greatly between glacier-fed and rock glacial streams, with the latter being very cold, clear, and characterised by stable streambed and intermediate nutrient availability (i.e., DIN and SiO_2) in comparison to kryal and krenal streams. Concentrations of solutes, including trace elements, where highest in rock-glacial streams, in agreement with results of recent synchronic investigations on Alpine headwaters of different origin (e.g., Thies et al., 2013; Bearzot et al., 2023; Schreder et al., 2023). The very high solute concentrations in both rock glacial and glacier-fed runoffs are likely related to different, yet under investigated, weathering processes occurring in thawing rock glaciers as well as beneath the glaciers (Salerno et al., 2016; Brighenti et al., 2023). Our results emphasize that this topic deserves more attention by future research, given the potential implications of increasing trace element concentrations for aquatic organisms and human health in mountain areas (Huss et al., 2017; Brighenti et al., 2021b).

4.2. Taxonomic composition and diversity patterns of biofilm prokaryotes

The prokaryotic taxonomic composition at phylum level recorded in the biofilm of the surveyed streams was largely comparable to what reported in previous works on alpine streams of different origin (Fegel et al., 2016; Tolotti et al., 2020; Busi et al., 2022). Moreover, the streams share a core taxonomic structure with other cryosphere ecosystems at the global level (Bourquin et al., 2022). Indeed, both epilithic and sediment communities were dominated by *Pseudomonadota*, *Bacteroidota*, *Actinomycetota* and *Cyanobacteria*, accompanied by a set of widespread but less abundant phyla (such as *Verrucomicrobiota*, *Planctomycetota* and *Acidobacteriota*). As recorded by previous research (Wilhelm et al., 2014; Tolotti et al., 2020; Ezzat et al., 2022), the biofilm microbial assemblages of the three stream types differed by the relative

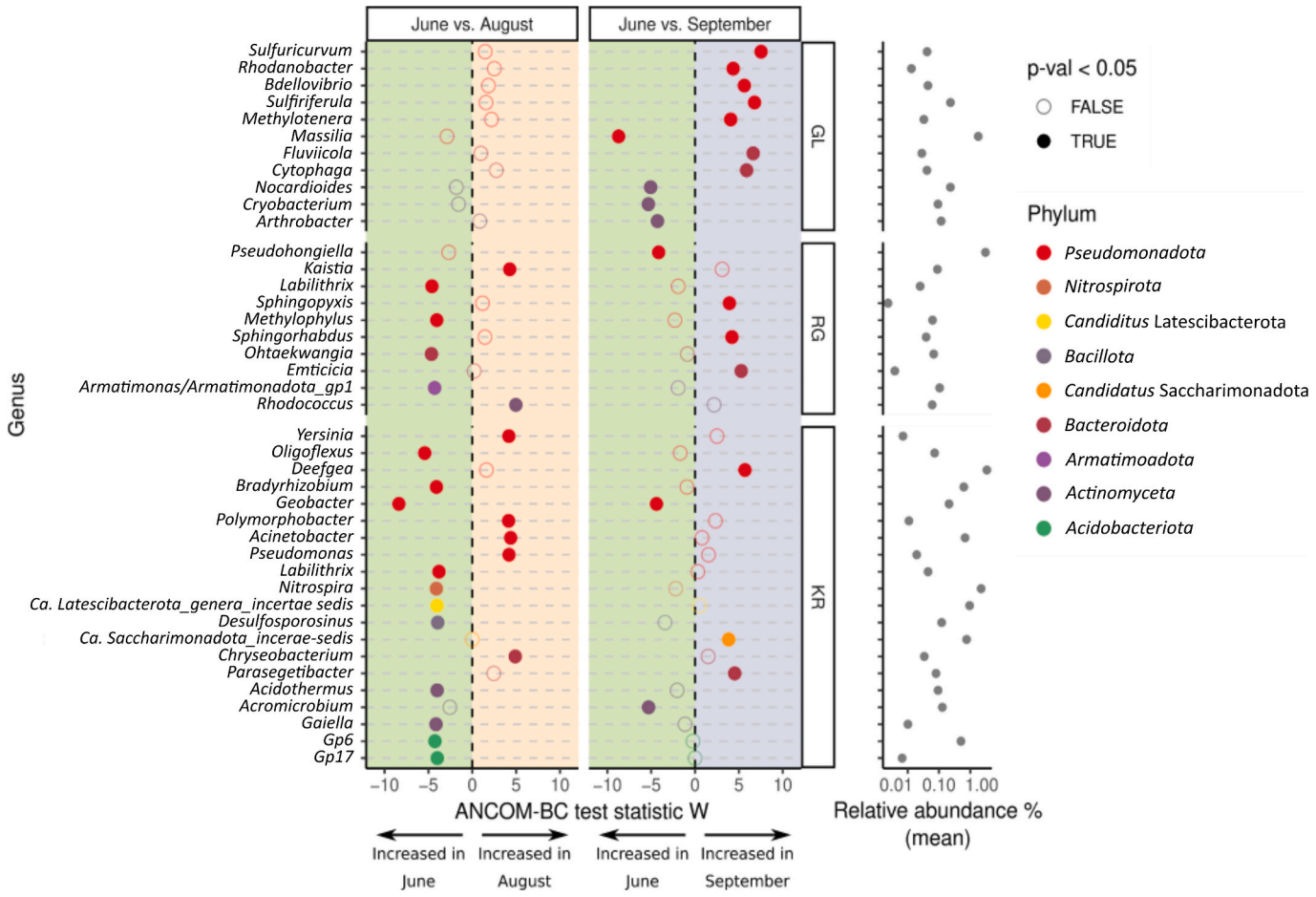

Fig. 5. Genera whose mean relative abundance significantly differed in in June vs. August and in June vs. September in the three stream types, with substrate types and sampling years considered together. Filled and empty bullets: significant ($p < 0.05$) and non-significant differences, respectively. The mean relative abundance of each genus in all samples analysed for each stream type is shown. Stream type abbreviations as in Fig. 2.

abundance of the most common phyla as well as by the presence of different pools of rare phyla, the latter including three archaeal phyla, which were very rare in all stream types and substrata, as typically observed in cryospheric aquatic habitats (Wilhelm et al., 2014; Margesin and Collins, 2019).

The biofilm prokaryotic β-diversity confirmed previous evidence that kryal and krenal streams represent the endmembers of prokaryotic taxonomic composition in both the epilithic and sediment biofilm, while rock glacial communities occupy an intermediate position and share a large portion of taxa with glacial and, mainly, krenal streams (Fegel et al., 2016; Hotaling et al., 2019; Tolotti et al., 2020). This pattern appears related to the contrasting physical condition of the investigated stream types, since high water velocity and mineral turbidity, as well as channel instability, are key determinants of the epilithic dynamics in glacier-fed streams (Cauvy-Fraunié et al., 2016; Hoyle et al., 2017).

The epilithic prokaryotic assemblages identified in the two kryal streams were taxonomically quite homogeneous on a spatial basis. Indeed, harsh conditions of glacier-fed headwaters select small pools of highly specialized or highly tolerant organisms (e.g. Milner et al., 2017) that are comparable at the global scale (Gesierich and Rott, 2012; Hotaling et al., 2019; Busi et al., 2022). In addition, the scarce taxonomic variability we found in kryal biofilm may be related to the fact that the surveyed streams are fed by small glaciers which are "over the peak water" (Brighenti et al., 2019b). During this late deglaciation stage, a June to September survey period may be not sufficient to capture the entire summer environmental and ecological variability of kryal streams evolving towards non-glacial headwaters (Engel et al., 2019). The very similar prokaryotic assemblages of krenal and rock glacial epilithic biofilms may be due to the comparable water clarity and channel stability of these streams, which also enhances the growth of aquatic mosses that represent an important source of autochthonous organic matter in Alpine headwaters (Rott et al., 2006; Milner et al., 2017; Brighenti et al., 2020).

The sediment prokaryotic assemblages outlined a similar picture, with taxonomically homogeneous kryal samples and samples collected from non-glacial streams differing more clearly between stream type and catchment. This pattern agrees with previous investigation in the region (Tolotti et al., 2020) suggesting that the environmental constraints selecting the epilithic microorganisms in glacial streams are also effective in homogenising the biofilm microbial assemblages of the superficial stream sediment.

The taxonomic dissimilarities recorded between different stream types and substrata suggests that biofilm prokaryotic variability of the surveyed Alpine headwaters is more related to spatial factors, especially substratum and stream type, than to interannual and seasonal variability. This agrees with field observations revealing low γ-diversity of Alpine aquatic microbiota at catchment/regional level, but high β-diversity related to the habitat settings of different streams (e.g., Hotaling et al., 2019; Tolotti et al., 2020; Bourquin et al., 2022). Such a dominant role of the spatial microbial variability underscores the pronounced individuality of communities in Alpine non-glacial headwaters (e.g., Freimann et al., 2014; Battin et al., 2016; Esposito et al., 2016), also found for benthic diatoms (Gesierich and Rott, 2012; Thies et al., 2013; Rotta et al., 2018) and invertebrates (Brighenti et al., 2020).

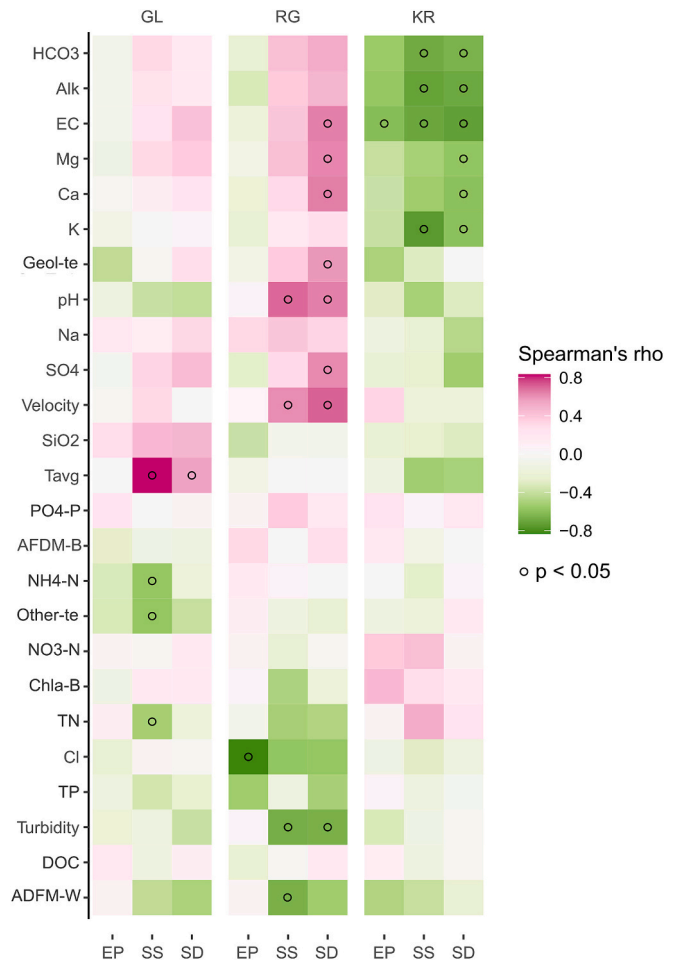

Fig. 6. Correlations (Spearman's ρ) between prokaryotic α-diversity and environmental variables measured in the three Alpine stream types surveyed in 2017–2018. Significant ($p < 0.05$) coefficients are marked by an empty circle. Geol-te: cumulative concentration of trace elements of prevalent geological origin, such as Sr, Rb, As (see Fig. S1); Other-te: cumulative concentration of trace elements with widespread technogenic dispersal (e.g., Ni, Cu, Mn, Zn, see Fig. S1). Abbreviations of stream type and sampled substrata as in Figs. 2 and 3.

In agreement with recent findings (Fegel et al., 2016: Tolotti et al., 2020; Busi et al., 2022), the present study outlined overall higher prokaryotic α-diversity in the biofilm of rock glacial and krenal than in kryal streams. The stream biofilm microbial diversity is considered as the result of an assembling process driven by the action of environmental conditions and biotic interactions over the stream water metacommunity, the size and diversity of which depend on the diversity and connectivity of the upstream aquatic and terrestrial habitats (Besemer et al., 2012). Therefore, the low prokaryotic α-diversity in the epilithic biofilm of glacier-fed streams can be explained by the combined effects of harsh habitat conditions and low upstream habitat diversity, which select small pools of highly specialized taxa (Wilhelm et al., 2013; Ezzat et al., 2022). Conversely, the milder habitat settings of krenal streams sustain larger pools of less specialized taxonomic groups with high metabolic redundancy (Fell et al., 2017), which are selected from larger upstream metacommunities. The colonization of different and abundant niches in krenal and rock glacial stream sediments can further explain the higher α-diversity of the epipsammic communities dwelling in these two habitats when compared with the epilithic communities (Anesio et al., 2017; Ezzat et al., 2022). Moreover, while sand and fine minerals are unstable, the surface of larger sediment grains may function as a "seed bank", i.e., as an inoculum reserve which can boost the taxonomic turnover and diversity typically observed in Alpine stream sediment (Wilhelm et al., 2014).

4.3. Windows of opportunity (WOs) and biofilm prokaryotic α-diversity

During the WOs, biofilm heterotrophic prokaryotes follow the development of epilithic primary producers (e.g., Boix Canadell et al., 2021; Brandani et al., 2022; Busi et al., 2022) which represent the major source of autochthonous organic matter and habitat in high altitude streams where allochthonous carbon inputs are very scarce (Logue et al., 2004; Battin et al., 2016). As expected, epilithic AFDM and chlorophyll-*a* concentrations (as indirect proxy of prokaryotic biomass) indicated a scarcer periphyton development in the turbid and unstable glacier-fed streams than in the clear rock glacial and krenal habitats. Moreover, in our study, early summer and early autumn appeared as more suitable than August for the periphyton development in all the three stream types, suggesting that these periods offer WOs for biofilm prokaryotic microbial growth in both glacial and non-glacial Alpine streams. Indeed, in the turbid glacial streams the low Photosynthetic Active Radiation (PAR) can limit the primary production during the summer ablation (Elser et al., 2020), whereas benthic microorganisms may be limited by the intense mid-summer UV radiation in the clear and shallow non-glacial waters (Kelly et al., 2003; Uehlinger et al., 2010). The low mid-summer nitrogen availability may also limit the growth of primary producers (Schimel et al., 2007).

The occurrence of both early summer and early autumn WOs agrees with previous findings from kryal (Battin et al., 2004; Uehlinger et al., 2010) and rock glacial (Perić et al., 2015) streams, but disagrees with studies from mountain streams and springs, that mainly reports increasing biofilm biomass towards the end of the vegetative season (e.g., Battin et al., 2004; Logue et al., 2004; Rott et al., 2006). In kryal habitats, the little seasonal changes in epilithic and sediment α-diversity may be due to the overwhelming role of physical constraints in depressing the development of the microbial biofilm throughout the Alpine summer, as mentioned in the previous paragraph. The absence of prokaryotic genera with significant differences in their relative abundance between June and August supports this hypothesis, while the small pool of prokaryotic genera differing between August and September indicates that only the autumn flow recession allows for some reorganization of the biofilm prokaryotic assemblages. Surprisingly, the pattern of seasonal changes in prokaryotic α-diversity expected for glacial streams was instead recorded in the epilithic and sediment assemblages of rock glacial and, especially, krenal streams, where the α-diversity was higher in June and September and dropped in August.

As typical for Alpine running waters (e.g., Sheik et al., 2015; Hotaling et al., 2019; Ezzat et al., 2022), the temporal variability of the biofilm prokaryotic diversity was not due to major taxonomic turnover, but to changes in the relative abundance of small pools of rare taxa. Nonetheless, a stronger taxonomical rearrangement occurred from June to August than from June to September in rock glacial and krenal streams. This suggests early summer as a diversity hotspot period in non-glacial streams, where the seasonal progression implies minor taxonomic rearrangement. The low mid-summer biofilm α-diversity in non-glacial habitats may be related to the mid-summer drop of epilithic primary production and the consequent scarcity in autochthonous carbon resources fuelling the prokaryotic development within the biofilm matrix.

In general, our first hypothesis (HP-1) that environmental WOs influence microbial α-diversity besides biomass in Alpine streams can be confirmed for non-glacial habitats but not for glacial ones. The unexpected scarce microbial response to WOs in glacial streams suggests that climate change and Alpine deglaciation tackle the predictability of WOs and of aquatic microbial diversity. The shift from snow to rain precipitation patterns (Berghuijs et al., 2014) and the increasing occurrence of snow drought and warm springtime in the European Alps (Colombo et al., 2023) are anticipating and accelerating the melt of winter snow.

This allows headwater streams to start flowing earlier and biofilm microorganisms to grow and express the maximum diversity already in early summer, as observed in the present study. The reduced snow melt also decreases groundwater recharge, negatively affecting the hydrological and chemical stability of Alpine springs (Hayashi, 2020). Conversely, the increasingly warm Alpine summers (e.g., Nigrelli and Chiarle, 2023) prolong the base flow conditions and the related enrichment of solutes in rock glacial streams, while making the smaller Alpine headwaters increasingly intermittent (Robinson et al., 2015; Siebers et al., 2020; Leathers et al., 2023). Low water discharge and dry conditions are key limiting factors of microbial life in cold aquatic environments (Bakermans, 2017) and, as such, desiccation usually leads to decreasing biofilm α-diversity through the selection of tolerant microorganisms with metabolic capacity to cope with dry conditions (Sabater et al., 2016).

Except under extreme conditions related to stream desiccation, climate change is expected to drive a short time net increase in microbial α-diversity in Alpine streams with decreasing glacial influence (Fell et al., 2017), as an effect of the substitution of specialized psychrophilic taxa by generalist and ubiquitous taxa typical of milder habitats (e.g., Wilhelm et al., 2013; Hotaling et al., 2019). At a longer time-scale, however, γ-diversity is expected to decrease due to the habitat homogenisation caused by the gradual transformation of kryal headwaters in clear streams increasingly influenced by precipitations and groundwater. Within this context, rock glacial streams are gaining importance as ecological refugia (Keppel et al., 2012) for stenothermic aquatic organisms threatened with extinction by the glacier retreat, that may survive in these very cold but clear streams (Brighenti et al., 2021b).

4.4. Environmental constraints of biofilm prokaryotic α-diversity

In agreement with the moderate physical and chemical seasonality outlined for the glacier-fed streams, their biofilm prokaryotic α-diversity, and especially that of epilithon, expressed scarce relationships with the environmental settings. Comparable weak relationships were found for the epilithic prokaryotic α-diversity of the non-glacial stream types, whereas the sediment diversity of krenal and especially rock glacial streams showed significant correlations with water velocity and turbidity, pH, and solute concentrations. Interestingly, biofilm prokaryotic α-diversity showed opposite relationship with solute concentrations in rock glacial and krenal streams. However, we still have no sufficient data to provide a sound ecological explanation for this phenomenon. The lacking relation between diversity and trophic variables (i.e., nutrients, DOC, AFDM, chlorophyll-*a*) in all the stream types may be related to the overall oligotrophy of alpine streams (Logue et al., 2004).

The differing strength of the correlations with the environmental seasonality outlined for the epilithic and sediment prokaryotic α-diversity may be related to the complexity of the biofilm architecture (Battin et al., 2016; Busi et al., 2022). Epilithic stream biofilm is a consortium made of autotrophs (algae and cyanobacteria) and heterotrophs (bacteria, fungi, and protozoa) embedded in a matrix of hydrated extracellular polymeric substances (EPS). This matrix favours cell interactions and the retention of the extracellular enzymes for the utilisation of ambient organic matter and nutrients (Flemming and Wingender, 2010). Moreover, it contributes to protect the cells from the negative effects of UV radiation, chemicals (Bourquin et al., 2022; Busi et al., 2022), and toxicants such as heavy metals (Sabater et al., 2016). Such a protective role of the EPS appears supported, in our study, by the small number and cumulative abundance of genera which showed significant correlation to physical or chemical variables in the epilithic in comparison to the sediment biofilm. The strong relationship outlined between sediment prokaryotic diversity and concentrations of solutes may be explained by a looser extracellular matrix related to the scarcity of photosynthetic microorganisms in the stream sediments. The algal scarcity in the sediment also reduces the possibility for the microbial assemblages to rely on cellular exudates as a major source of organic carbon, thus making them more dependent from allochthonous resources and more responsive to the environmental constraints (Wilhelm et al., 2014).

These observations only partially support our second hypothesis (HP-2) that biofilm prokaryotic diversity and phenology are influenced mainly by the physical habitat in glacial streams, and by water chemistry in non-glacial streams. While this hypothesis can be retained for non-glacial habitats, for kryal streams only the prokaryotic β-diversity can be explained in terms of physical constraints, whereas α-diversity appears weakly related to both physical and chemical variables. This underscores that the seasonal changes in biofilm prokaryotic α-diversity are not mainly driven by physical, chemical, or trophic shifts, but by the fine tuning of environmental settings, taxonomic composition, and microorganism interactions mediated by the biofilm architecture.

5. Conclusions

The present study provides novel insights into the seasonal patterns of biofilm prokaryotic α-diversity in different stream types during the Alpine summer and into the possible influence of environmental WOs on Alpine microbial diversity. We confirmed recent findings about the intermediate character of habitat conditions and biofilm prokaryotic diversity in rock glacial in comparison to kryal and krenal streams. The present survey also increases the knowledge on possible effects of climate change and Alpine deglaciation over taxonomic composition, diversity, and dynamics of cold aquatic microbial communities, and underscores the potential role of rock glacial runoffs as climate refugia for cryophile aquatic microorganisms. Due to the slow thawing rate of subsurface ice, and to the increasing water storage capacity of rock glacier under progressive ice loss, these landforms also represent a widespread buffer against water scarcity in mountain areas, thus playing a crucial role in preserving both psychrophilic aquatic biodiversity and local human activities, such as semi-wild breeding, Alpine pasture, and mountain tourism. However, awareness about the ecosystem services of rock glacial headwaters in a context of global warming is still scarce. This advocates further extensive studies of Alpine headwaters aimed at improving and generalising the knowledge of abiotic and biotic dynamics, the latter including metabolic functions of cold microbial diversity and responses to global warming. Such information is essential to define and test strategies for the protection of cryosphere-dependent Alpine headwaters and for exploiting and strengthening the role of rock-glacial streams as climate refugia for protecting, restoring, and connecting cold Alpine aquatic biodiversity. Indeed, the management and protection of cryosphere-dependent mountain headwaters are particularly challenging. In the European Alps, for instance, only a small fraction of glaciers, rock glaciers and their runoffs are protected (Wilkes et al., 2023), and no action has been conducted to mitigate the effects of climate change on Alpine aquatic biodiversity (Gobbi et al., 2021). The development of sound managing strategies also implies establishing networks of long-term monitoring of chemical and biological dynamics rock glacial runoffs, similarly to what has been done for the geo-physical and kinematic aspects of mountain permafrost.

CRediT authorship contribution statement

Monica Tolotti: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Stefano Brighenti:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Maria Cristina Bruno:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition. **Leonardo Cerasino:** Writing – review & editing, Formal analysis, Data curation. **Massimo Pindo:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation. **Werner Tirler:** Writing – review & editing, Methodology, Formal analysis. **Davide Albanese:** Writing – review &

editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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