PRIMARY RESEARCH PAPER

Temporal patterns of stream biofilm in a mountain catchment: one-year monthly samplings across streams of the Orobic Alps (Northern Italy)

Luca Bonacina · **Riccardo Fornaroli** ·
Valeria Mezzanotte · **Francesca Marazzi**

Abstract Periphyton is the dominant primary producer in mountain streams and sustains the higher trophic levels. While certain periphyton groups, particularly diatoms, have received extensive study, the comprehensive characterization of the entire community has been largely neglected. This study aims to investigate the temporal pattern of biofilm in mountain streams characterized by different water thermal regimes. A one-year quantitative campaign, involving monthly samplings, was conducted in five subalpine streams in Northern Italy's Orobic Alps to collect epilithic biofilm from a wide surface area. The total biomass was quantified and the periphyton was analyzed both for composition (diatoms, green algae, cyanobacteria, and red algae) and for pigments. Disturbance, water temperature, physico-chemical conditions, nutrients, substrate diversity, and light availability were assessed concurrently with biofilm samplings. Results show sharp biofilm variations over months. In all sites, the disturbance was the primary factor reducing biomass and pigment content. Annually, all sites experienced similar turnover in periphyton composition mainly associated with light and water temperature. Overall, the study indicates that frequent quantitative investigations of biofilm help understand intra-annual variations and identify key drivers. Such information is useful to understand the ecosystem processes and the food web dynamics.

Keywords Microalgae · Dam · Water temperature · Light · PAM fluorimetry · Bioassessment

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L. Bonacina (✉) · R. Fornaroli · V. Mezzanotte · F. Marazzi
Department of Earth and Environmental Sciences (DISAT), University of Milano-Bicocca, Piazza della Scienza 1, 20126 Milan, Italy
e-mail: l.bonacina9@campus.unimib.it; luca@bonacina.eu

Introduction

Biofilm is the assemblage of algae, bacteria, fungi, and meiofauna enclosed within a mucilaginous, polysaccharide matrix attached to wet surfaces (Hauer & Lamberti, 2017). The autotrophic component, called periphyton, encompasses a diverse range of organisms primarily from Bacillariophyta (diatoms), Chlorophyta (green algae), Cyanophyta (cyanobacteria), Rhodophyta (red algae), and Chrysophyta (gold algae) groups. Although primary producers are a minority in mountain lotic systems due to their constant instability, they play a vital role in supporting upper trophic levels and providing habitat for invertebrates, protists, and bacteria (Rott et al., 2006). In headwaters, periphyton can contribute up to 80% of autochthonous primary production (Hansson, 1992), serving as

a crucial energy for the trophic chain (Hauer & Lamberti, 2017). In mountain streams, flow is the primary factor controlling biofilm growth. The current exerts shear stress on biofilm causing cell sloughing (Power & Stewart, 1987a; Biggs, 1988). Extreme flows can have a strong negative impact on benthic biota that, due to high velocity, overturning of stones and tumbling abrasion, flows away (Power & Stewart, 1987b; Robinson & Rushforth, 1987). Such effects primarily result in decreased taxonomic richness and abundances as highlighted in several studies (Biggs & Smith, 2002; Wellnitz & Rader, 2003; Luttenton & Baisden, 2006). However, the size and type of substrate (rough or smooth) as well as the riverbed typology (armored or not) can play a pivotal role (McAuliffe, 1984; Biggs & Smith, 2002). The pattern of recolonization is taxon specific, depends on hydrology and substrate type, and is generally faster especially just after a flood and in streams where disturbances are frequent (Biggs & Smith, 2002). On the other hand, drought events also strongly influence biofilm, particularly affecting diatoms productivity and altering the composition of the assemblages (Falasco et al., 2020). Besides hydrology and substrate, light and nutrients also control periphyton growth. In shaded streams, periphyton growth is limited by insufficient light, while in unshaded, high-altitude streams, it is constrained by both low nutrient concentration and UV exposure (Rosemond, 1993; Hill et al., 1995; Mosisch et al., 1999; Sudlow et al., 2023). Nutrient availability is controlled by inputs from riparian and terrestrial environments (Hedin et al., 1998; Bernal et al., 2015), with phosphorus (PO_4^{3-}), nitrogen (NH_4^+, NO_3^-), and silica (SiO_2) being the primary factors. However, in many streams, silica concentration often exceed the minimum request (Robinson et al., 2000). Overall, mountain running waters not subjected to pasture are very poor of phosphate, while nitrogen, available generally in the form of nitrate (NO_3^-), comes from the atmospheric deposition. Finally, the water temperature affects the biomass of periphyton (Morin et al., 1999), controlling the rate of primary production through its effects on photosynthetic physiology (Medlyn et al., 2002). Moreover, the periphyton composition depends on the thermal preference of each taxon, and temperature variations promote changes in the dominance of the main groups (green algae, cyanobacteria, diatoms, and red algae) throughout the seasons (Allan & Castillo, 2007) and along the watercourses (Li et al., 2022).

Multiple factors influence the growth and composition of biofilm. However, research focusing on the fine temporal patterns of stream biofilm and providing an overall characterization of the community is relatively scarce. Most of this research has been conducted in New Zealand (Quinn & Hickey, 1990; Biggs, 1995) and South America (Necchi et al., 1995; Branco & Necchi, 1996, 1998; Pizarro & Alemanni, 2005). In contrast, investigations conducted in the Alps have predominantly centered around diatoms and cyanobacteria in Trentino springs (Italy) (Cantonati et al., 2005, 2012b; Gerecke et al., 2011), algae assemblages of Swiss glacier streams (Uehlinger, 1991, 2006; Hieber et al., 2005), and of Austrian watercourses (Rott et al., 2006; Rott & Wehr, 2016).

Given the importance of studying biofilm to gain insights into ecosystem processes and food web dynamics, this study seeks to explore intra-annual changes in epilithic biofilm and the associated environmental variables. The research study was conducted in five stream sites of the Serio catchment (Orobic Alps, Northern Italy), characterized by distinct thermal regimes. At each stream site, biofilm was surveyed monthly, over a year and different approaches were combined to investigate changes in periphyton through a fast assessment (without a taxonomic identification). Specifically, the research aims to describe within-site monthly variations in biofilm biomass, pigments content, and the relative abundance of periphyton groups, to compare annual differences among sites, and to assess the relationship between environmental factors and biofilm structure. We supposed temporal variations in total biomass and pigments' content would primarily result from floods, while monthly shifts in the relative abundance of periphyton groups would be influenced by variations in water temperature (i). As a result, we expected that sites experiencing high annual water temperature variability would display more pronounced temporal dissimilarity in periphyton composition, leading to spatial distinctions (ii). Furthermore, we hypothesize that differences in substrate, water quality, and light availability would explain variations in the biofilm structure among the sites (iii).
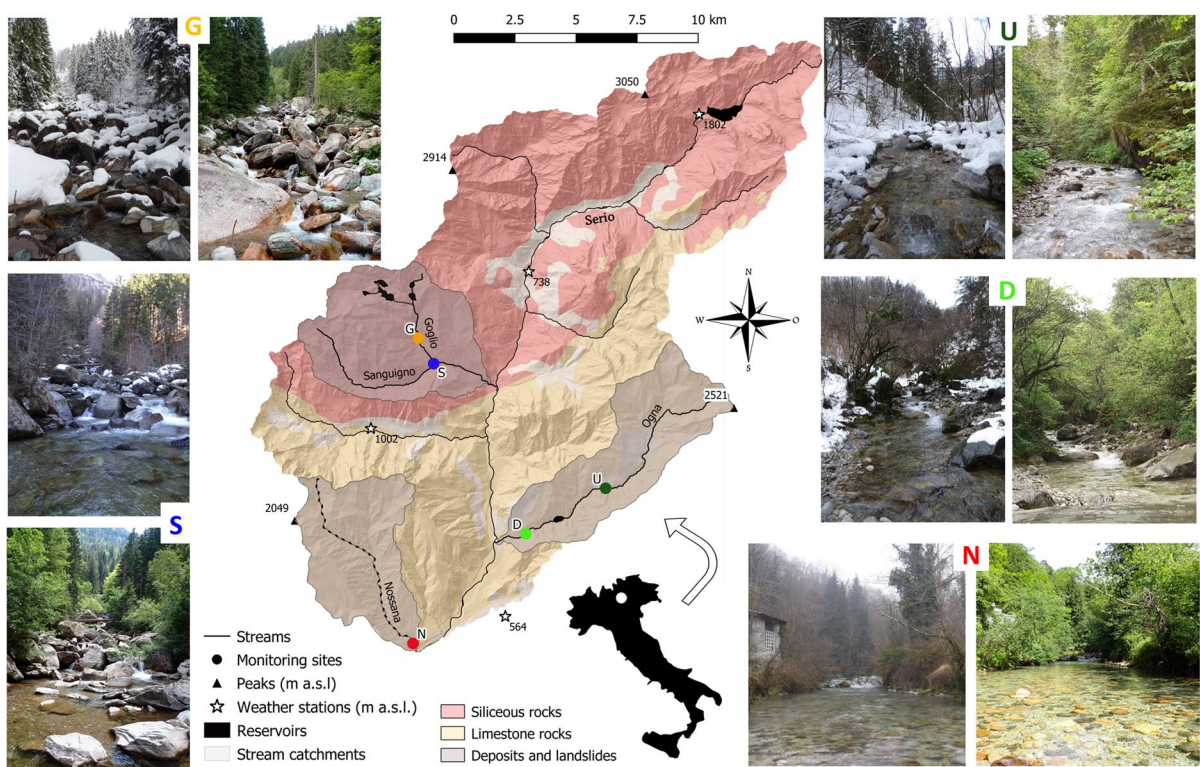
Methods

Monitoring plan and sites

One-year campaign (June 2020–May 2021) with monthly samplings was carried out in five stream sites in the upper Serio catchment (Orobic Alps, Northern Italy). Streams are characterized by different water sources (snowmelt/stormwater and groundwater-fed streams) and anthropogenic pressures (presence of reservoirs) leading to different annual water temperature variabilities (annual daily maximum-annual daily minimum) ranging from ~1 to 16 °C. Sites S and G were situated in the Goglio catchment: S at 975 m a.s.l., on a tributary stream not subjected to flow regulation, and G at 1128 m a.s.l., on the Goglio stream, approximately 2-km downstream of reservoirs (volume of 5.4 Mm³ and dam height exceeding 20 m). Both streams are fed by snowmelt/storm waters. Due to flow regulation, G displays a lower water temperature variability (~16 vs ~9 °C). Sites U and D were situated upstream (970 m a.s.l.) and downstream (583 m a.s.l.) of a small reservoir (volume of 0.15 Mm³ and dam height not exceeding 12 m) in the Ogna catchment, along a stream fed by snowmelt/storm and groundwaters. In this case, the downstream site exhibits slightly lower variability compared to the upstream one (~7 °C vs ~8 °C). The N site was located in the Nossana catchment at 468 m a.s.l. on a groundwater-fed stream characterized by a constant temperature (~8 °C), situated about 200 m from the spring. All the monitored streams have similar morphology and substrates, with sizes ranging from gravel (0.2–2 cm) to large pebbles (>40 cm). The study area, situated at altitudes ranging from 500 to 1200 m a.s.l, is covered by beeches and firs forests, while the riverbanks feature a variety of vegetation, including willows, alders, birches, hazels, maples, and shrubs (Fig. 1).

Biofilm sampling and quantification

Epilithic biofilm, i.e., that part of biofilm present on the stone surface in the river bed, was sampled

Fig. 1 Study area with pictures of the stream sites in winter and summer

following European and Italian methodology guidelines (APAT, 2007; CEN EN 14407, 2014). A dozen stones (~ 10–13), representing different microhabitats (akal/microlithal/mesolithal/macrolithal/megalithal) (Hering et al., 2004) were randomly selected from the riverbed to cover an area of about 0.5 m². All stones from a specific site were then scrubbed, and the biomass was collected in 500 ml of stream water, resulting in a single composite biofilm sample for each sampling event (site × date). A 100-ml suspension of biomass was analyzed for dry weight (dried for 24 h at 105 °C) and ashes (burned for 4 h at 550 °C). The total biofilm mass was calculated as the ash-free dry mass (AFDM) normalized by the sampled surface (g/m²) following Hauer & Lamberti (2017). To calculate the sampling surface, we took a picture of the collected stones arranged on a squared sheet and measured the total area in QGIS. On the same day of the sampling, 10 ml of biofilm solution were frozen (− 20 °C) for the pigment extraction, while the rest was chilled to 4 °C and brought to the laboratory to be processed within 24 h to determine the composition of the algae community as described in the next paragraph.

Periphyton composition

The composition of the periphyton component of biofilm was determined analyzing the biofilm suspension by the Phyto-PAM II instrument (Heinz Walz GmbH, Effeltrich, Germany) that distinguishes the fluorescence signals emitted by four different groups of photosynthetic organisms: cyanobacteria, green algae, diatoms (and dinoflagellates), and red algae (organisms with phycoerythrin). The operating principle of PAM instrument is the same as BenthoTorch, commonly used for rapid assessment of phototrophic communities on river bottom substrates (Kahlert & McKie, 2014), but Phyto-PAM II can distinguish also red algae. The percentage of each algal group was calculated, and the composition diversity was estimated using the Shannon index. To assess the overall annual temporal community composition dissimilarity (Legendre, 2019) within a site, we calculated the multi-date β-diversity (i.e., also known as multi-site β-diversity (Baselga et al., 2022)) using the Sorensen dissimilarity (BSor) and its components (turnover and nestedness).

Pigment determination

Pigment content was determined as follows: 10 ml of biofilm solution was centrifuged at $10,000\times g$ for 5 min; the supernatant was discarded and 10 ml of 99.9% methanol was added to the concentrated pellet, well mixed, and incubated at 45 °C for 24 h in dark. Pigment concentration was calculated according to the following equations as the mean of three replicates per sample (Lichtenthaler, 1987):

$$\text{Chlorophyll a (mg/l)} = 16.72 \, \text{Abs665} - 9.16 \, \text{Abs652}$$

$$\text{Chlorophyll b (mg/l)} = 34.09 \, \text{Abs652} - 15.28 \, \text{Abs665}$$

$$\begin{aligned} \text{Carotenoids (mg/l)} = (1000 \times \text{Abs470} - 1.63 \times (\text{Chl a}) \\ - 104.9 \times (\text{Chl b}))/221 \end{aligned}$$

where Abs665, Abs652, and Abs470 are the absorbances measured by a spectrophotometer (DR600TM UV–VIS Spectrophotometer Hach Lange) at 665-, 652-, and 470-nm wavelength, respectively. The concentration of each pigment was then reported to the sampled surface and expressed as mg/m².

Finally, the chlorophyll a concentration was used to calculate the autotrophic index (Weber, 1973) that estimated the ratio between heterotrophic organisms/organic detritus and autotrophic organisms.

$$\text{Autotrophic index} = \frac{\text{AFDM biomass} \left(\frac{mg}{m^2}\right)}{\text{Chlorophyll a} \left(\frac{mg}{m^2}\right)}$$

Environmental variables

Stream microhabitat availability (HABITAT) was assessed using the standardized multi-habitat methodology used for macroinvertebrates sampling (Hering et al., 2004). When sampling biofilm, the relative proportion of each microhabitat covering more than 10% of the stream sampling site was measured and the Simpson index was calculated for each habitat. The light exposure (EXPOSURE) was estimated as a percentage of the open sky, based on photographic pictures of the surrounding trees (0% = full canopy, 100% = no canopy) (see Cantonati and Pipp, 2000). Each water sample was characterized

for electric conductivity (CE), dissolved oxygen (DO), and oxygen saturation (O2) using a probe HACH-HQ40d (Loveland, USA). In addition, 0.5 l of water were collected and brought to the laboratory where pH was measured using a HANNA pH meter 211 (Woonsocket, USA), and nitrate nitrogen NO_3–N (NNO3) and Chemical Oxygen Demand (COD) were determined using spectrophotometric test kits (Hach Lange, Düsseldorf, Germany, LCK 340, and LCK1414, respectively). Total phosphorus (Ptot) and ammoniacal nitrogen NH_4–N (NNH4) concentrations were determined in the laboratory according to standard methods (APHA/AWWA/WEF, 2012). The water temperature was measured continuously (every 10 min) from Autumn 2019 to Autumn 2021 using iButton devices (DS1925L sensors: range − 40–80 °C, accuracy: ± 0.5 °C, resolution: ± 0.0625 °C) fixed in the riverbed of the five stream sites. The mean water temperature (TMean) of each sampling day was calculated as the mean of the 30 days before each sampling. Substrate movement associated with flood disturbance was measured using painted pebbles placed in the streambed according to Townsend et al. (1997). 15 locally sourced painted pebbles belonging to three size classes (50th, 75th, and 95th percentiles of the substrate size distribution) were placed in riffles, in random order in triplets (small, medium, and large), across the main flow of the stream at marked points on the stream bank. On each sampling day, the distance traveled by each stone was recorded and stones were placed back at their initial position (Death & Zimmermann, 2005). Flood disturbance (DISTURBANCE) was quantified for each sampling combining the displacement of the substrate with the moved mass by the following equation:

$$\text{Disturbance} = \frac{\sum_{i=1}^{n} m_i \times d_i}{\sum_{i=1}^{n} m_i}\%$$

where m is the pebble mass and d is the displacement (1 = moved or 0 = not moved). Pebbles that could not be recovered were not considered since they could have been washed away or buried by sediments. In most cases (75%), all the pebbles were found. As several floods could occur within one month, the metric DISTURBANCE can be used as a proxy of the overall sediment movement between two consecutive sampling dates. Light availability (HLIGHT) in

each site was calculated by the GRASS software. The "r.sun" function was used to obtain the hours of light at each site as a function of the day of the year and the topography (location and mountain shadowing). Each site was represented by an area of 250 m^2 described by a digital terrain model with a resolution of 5 m. The light availability at each sampling day was measured as the mean of the daily light in the 30 days before. This indicator, coupled with the exposure, was considered more representative than the photosynthetically active radiation (PAR) that could have been measured monthly at each site: indeed, punctual PAR measures are very sensitive to weather conditions and to the specific riverbed position (Melbourne & Daniel, 2003) and, thus, are not representative of monthly changes.

Data analysis

To describe the temporal patterns of environmental variables monthly values were represented for each site apart for water temperature for which daily measures were used. Non-parametric Kruskal–Wallis and Dunn's multiple comparison tests were performed to identify significant annual differences in the environmental variables among sites. The Bonferroni method was used to adjust p values for multiple comparisons. Analyses were performed using the "dunn.test" R package (Dinno, 2022). Similarly, the temporal variations of biofilm were represented by considering biomass/pigments and composition separately. First, the time series of biofilm biomass, periphyton pigments, and autotrophic index were plotted for each site. Then, the temporal change in community composition was represented by cumulative bar plots with the relative abundance of each algal group (cyanobacteria, green algae, diatoms, and red algae) for each site. To identify differences among temporal patterns of biomass/pigments in each site, Spearman correlations were used. The p-values were corrected for multiple inferences using Holm's method using the *rcorr.adjust* function from the "RcmdrMisc" package (Fox et al., 2022). To visualize spatial variations, boxplots of biofilm metrics (biomass, pigments, periphyton groups, Shannon, and autotrophic indices) were reported for each site and Kruskal–Wallis and Dunn's multiple comparison tests were performed to identify significant annual differences among sites.

To assess how environmental factors were associated with the biofilm structure, generalized linear mixed-effect models (GLMM) between biofilm metrics and environmental variables were developed and the relative contribution was determined. The analysis was performed using the "glm2" R package (Marschner & Donoghoe, 2022). Biomass, chlorophyll a, chlorophyll b, carotenoids, Shannon, and autotrophic indices were log+1 transformed, while the relative abundance of each group was logit transformed (*logit* function from the "car" package (Fox et al., 2022) to normalize residuals and equalize variances. As predictors, the more explicative variables according to the PCA (Fig. 1A) were used; DO and pH were excluded as they were highly correlated with TMean (*r* Pearson > 0.7) and exhibited always optimal values as well as O_2. HABITAT, COD, and NNH4 were also excluded as they did not show any temporal pattern and poorly contributed to the variance explained by the PCA. Thus, DISTURBANCE, CE, NNO_3, TMean, and the interaction term between EXPOSURE and HLIGHT were included as fixed effects, while sites were included as random effects on the intercept. To account for temporal and spatial variations, the SEASON, identified as winter (December–January–February), spring (March–April–May), summer (June–July–August), and autumn (September–October–November), and the SITE (S, G, U, D, N), were included as fixed effects. The dredge function within the "MuMIn" package (Barton, 2022) was used to derive the optimal set of fixed effects tested within each GLM. This function fits different models comprising all the combinations of fixed effects and ranks them by the Akaike Information Criterion corrected for small sample size (AICc). The most parsimonious model within 2 AICc units of the best (the model exhibiting the lowest AICc value) was selected as the "optimal" model. The explanatory power of the statistical models was derived from marginal pseudo r-squared values ($R^2_{marginal}$, see Nakagawa et al., 2017), which quantify the variance explained by the fixed effects, obtained using the *r.squaredGLMM* function in "MuMIn." The significance of each optimal model was obtained via likelihood ratio tests (see White et al., 2018); if SEASON or SITE was included in the optimal model, it was also included in the "null" model to test the joint effect of the other variables. Finally, to establish if differences in the water thermal regime could partially explain differences

in periphyton composition among sites the annual water thermal variability was correlated with the annual temporal dissimilarity (BSor). All the analyses were performed in R-project software (R Core Team, 2020). Please note that the analyses are constrained by a small sample size ($n = 60$) and the temporal autocorrelation among within-site samples, which restrict the statistical power. Consequently, our emphasis is on the observed patterns and associations rather than statistical significance.

Results

Patterns of environmental variables

The daily mean temperature ranged from ~0 °C to ~16 °C in *S*, between ~3 and 12 °C in *G*; between ~3 and 11 °C in *U*, it was almost constant (~8 °C in *N*); nevertheless, according to the Dunn's test, even with distinct thermal variability, all sites were characterized by a similar annual thermal average ($P = 0.03$, Fig. 2A). The water thermal variability was smaller in the sites with groundwater inputs (*U* and *D*) than in snowmelt/stormwater sites (*S* and *G*), while the stream fed exclusively by groundwater (NN) had a stable thermal profile. DO exhibited a temporal pattern opposite to the thermal one ($P = 0.05$, Fig. 2B), while pH followed the thermal behavior with slightly differences among *S*, *N*, and the other sites ($P = 0.08$, Fig. 2C). DISTURBANCE displayed strong temporal fluctuations, synchronous in all sites, with abrupt peaks in June, September, October, and May and maximum values (70–100%) in autumn without annual differences among sites ($P = 0.95$, Fig. 2D). HLIGHT strongly varied through the year reaching 8–12 h in summer and 0–3 h in winter ($P = 0.6$, Fig. 2E); of course, EXPOSURE had higher values in winter, in the absence of leaves on the trees, especially in Ogna sites (*U* and *D*) that were different from the Goglio sites (*S* and *G*) and the *N* site ($P = 0$, Fig. 2F). The other environmental variables did not show any seasonal pattern but displayed some annual differences among sites according to the Dunn's test. First, CE was below 85 µS/cm in sites dominated by snowmelt/stormwater (*S* and *G*), while it was above 200 µS/cm in sites influenced by groundwater inflows (*U*, *D*, *N*) and especially in the *D* site where it reached 480 µS/cm as annual average.
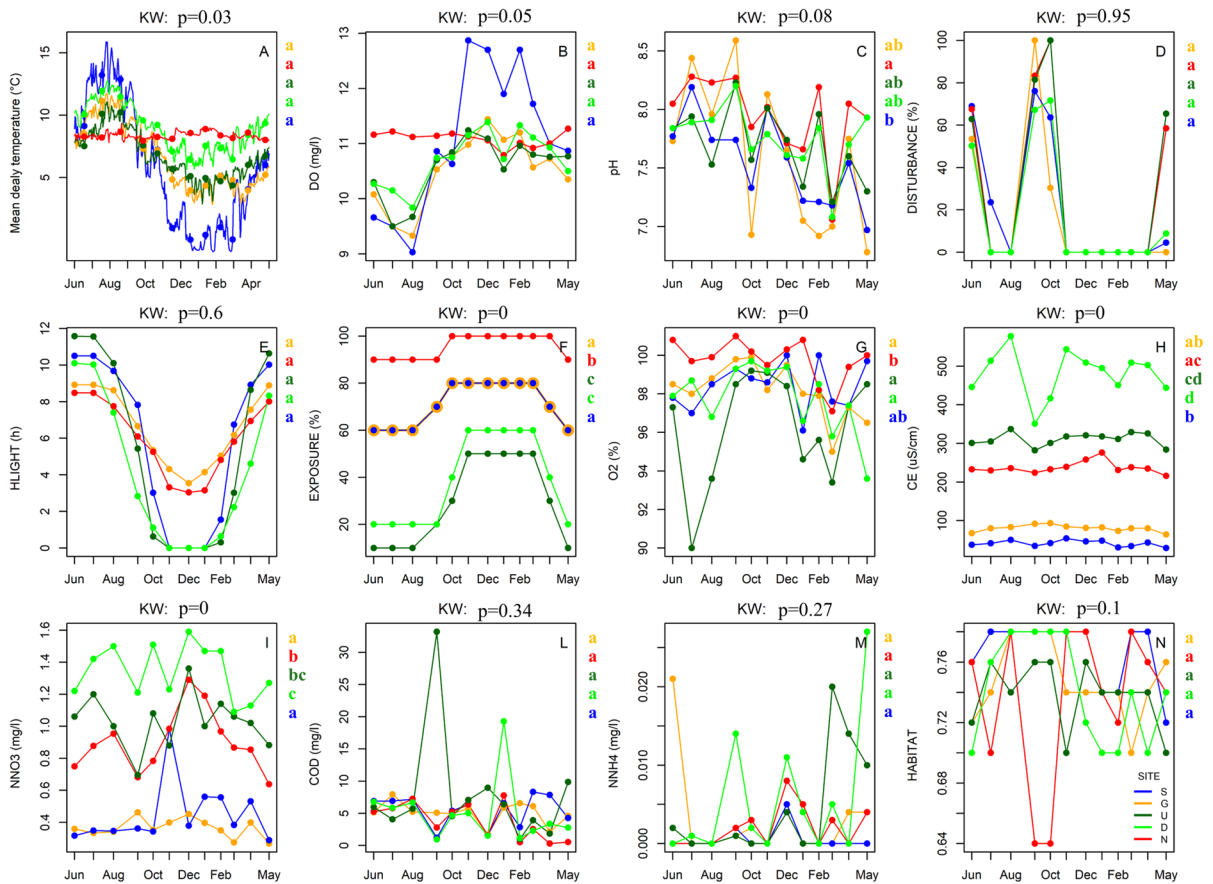
Fig. 2 Temporal pattern of environmental variables in each stream site. The *p* value of Kruskal–Wallis test is reported above each plot and colored lowercase letters indicate which comparisons are significant (Dunn's multiple comparison test, alpha = 0.05)

It was also slightly higher in regulated sites (*D* and *G*) than in the unregulated ones (*U* and *S*) (*P* = 0, Fig. 2H). Second, NNO$_3$ was lower (< 0.6 mg/l) in Goglio sites (*S* and *G*) than in the others and in *D* was higher (~ 1.3 mg/l annual mean) (*P* = 0, Fig. 2I). COD was below 10 mg/l in all sites, indicating a low concentration of oxidable compounds (*P* = 0.34, Fig. 2L), including ammoniacal nitrogen (NNH4 < 0.005 mg/l) (*P* = 0.27, Fig. 2M) and the concentration of total phosphorus (Ptot) was always below the methodological sensitivity (0.001 mg/l). The streambed HABITAT availability was consistent both among and within the sites (*P* = 0.01, Fig. 2N) (Table 1).

Biofilm temporal patterns

Photosynthetic pigments and total biomass showed similar temporal patterns in each site, as indicated by

their strong positive Spearman correlation (*p* always greater than 0.5, Table 1A). The highest values were observed in winter, while lower values occurred in summer and after the floods (Fig. 3A). By contrast, the autotrophic index was negatively correlated with biomass and photosynthetic pigments in all sites (*p* always lower than − 0.32, Table 1A) and had the highest values in summer and autumn (Fig. 3A). Winter promoted green algae, while summer cyanobacteria and red algae; diatoms were the dominant group in all sites (Fig. 3B). In *N*, an exceptional algal bloom occurred in November when the total biomass reached 90 g/m^2, while Chlorophyll *a* and *b* were 81 mg/m^2, and 73 mg/m^2, respectively. Annually, the sites exhibited similar changes in periphyton composition as the temporal dissimilarity was 0.63, 0.61, 0.57, 0.49, and 0.58 in *S*, *G*, *U*, *D*, and *N*, respectively. The total *β*-diversity (BSor) corresponded to
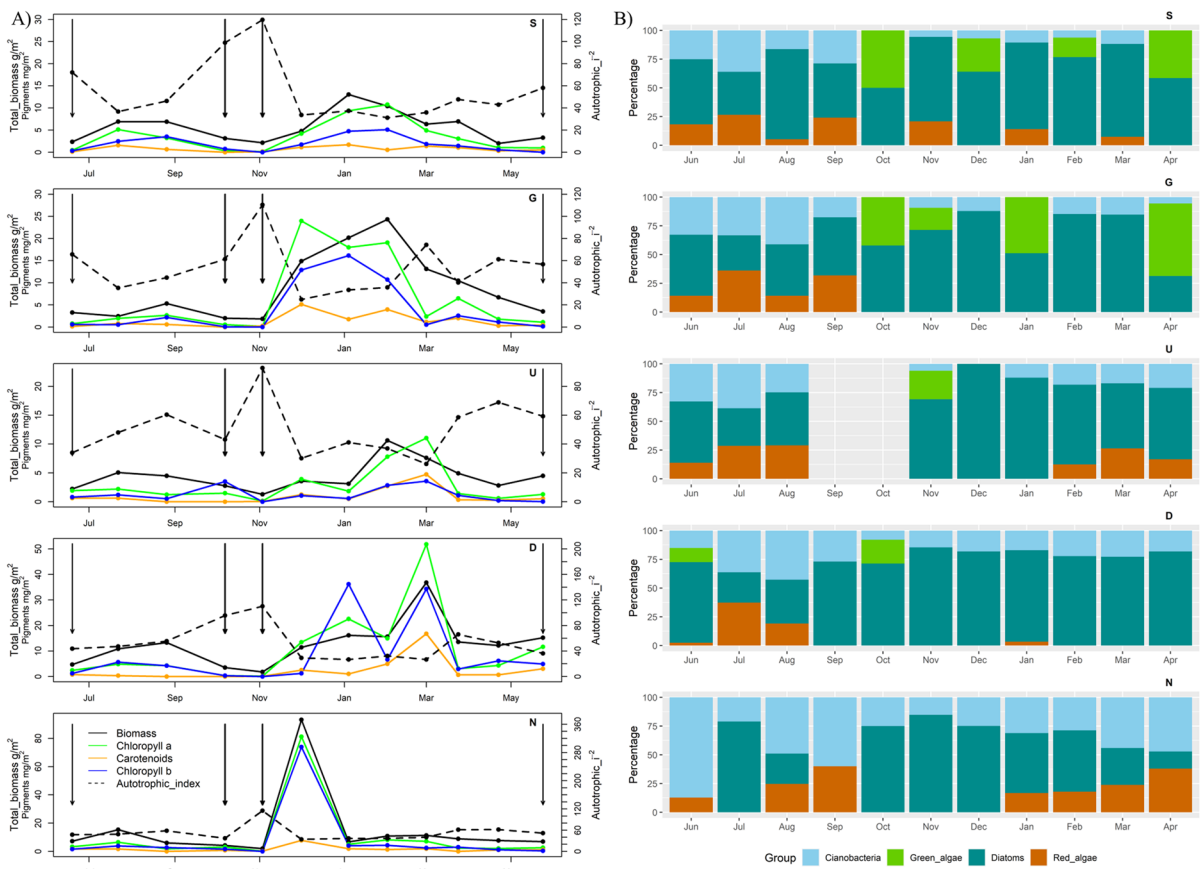
Fig. 3 Temporal pattern of **A** biofilm biomass, periphyton pigments, and autotrophic index and **B** periphyton community composition. Black arrows represent the sampling dates fol-

lowing a flood (with the highest occurring in September and October). Missing data in community composition were due to Phyto-PAM malfunction or loss of samples

the turnover component (the ratio between turnover and Sorensen indices was 0.97) outlining that the temporal changes in community composition were driven almost only by the replacement and not by the loss/gain of groups.

Biofilm spatial patterns

The overall epilithic biofilm biomass was 9.4 ± 12.7 g/m^2 on average (Table 2). According to the Dunn's test sites, D and U sites showed marked differences from the others, with lower and higher annual averages, respectively (the annual mean is 4.4 ± 2.5 g/m^2 and 12.9 ± 8.94 g/m^2, respectively, $P = 0.01$, Fig. 4A). Sites below dams (G and D) had higher annual biomass compared to their unregulated counterparts (S and U). The sites exhibited similar annual pigment

content ($P = 0.63$, 0.24, and 0.11 for carotenoids, chlorophyll a, and b, respectively, Fig. 4B). However, site U displayed the lowest content of chlorophyll a, carotenoids, and chlorophyll b (2.9 ± 3.2, 1.0 ± 1.4, and 1.3 ± 1.3 mg/m^2, respectively), while site D had the highest (11.1 ± 14.5, 2.6 ± 4.7, and 8.7 ± 12.6 mg/m^2, respectively). Diatoms were the most represented group (58.6%), followed by cyanobacteria (18.6%), red algae (17.1%), and green algae (14.1%), with high variability among samplings (Table 2). For example, green algae were slightly more abundant in G and S (15.7% and 11.4%, respectively) and absent in N. Red algae represented 22.7% of the community in N and U. Annually, sites displayed similar abundances of diatoms, green algae, and red algae ($P = 0.19$, $P = 0.12$, and $P = 021$, respectively, Fig. 4C) but not of cyanobacteria, which dominated in N and were scarce

Table 1 Environmental variables of the five stream sites expressed as annual average ± standard deviation and minimum and maximum values based on the monthly values

Environmental variables	Acronym		S	G	U	D	N
Mean monthly temperatures (°C)	Tmean	Mean±sd	5.5±4.9	6.5±2.6	6.6±1.8	8.9±1.8	8.4±0.2
		Min–max	−0.5 – 13.9	3.7 – 11.0	4.4 – 10.2	6.4 – 12.2	8.2 – 8.8
Electrical conductivity (µS/cm)	CE	Mean±sd	40.7±7.9	80.1±8.6	311.1±18.0	480.1±61.2	239.4±14.8
		Min–max	29.1 – 53.7	64.1 – 93.4	282.0 – 337.0	351.0 – 578.0	224.0 – 276.0
Oxygen saturation (%)	O2	Mean±sd	98.4±1.3	98.1±1.4	96.4±3.0	97.7±1.8	99.7±1.2
		Min–max	96.1 – 100.0	95.0 – 99.9	90.0 – 99.2	93.6 – 99.7	97.1 – 101.0
Dissolved oxygen (mg/l)	DO	Mean±sd	11.12±1.30	10.55±0.65	10.56±0.54	10.74±0.48	11.07±0.13
		Min–max	9.03 – 12.87	9.33 – 11.44	9.50 – 11.24	9.84 – 11.39	10.79 – 11.22
pH	pH	Mean±sd	7.54±0.37	7.58±0.63	7.66±0.32	7.75±0.27	7.94±0.36
		Min–max	6.97 – 8.19	6.78 – 8.59	7.21 - 8.23	7.08 – 8.20	7.06 – 8.28
Nitric nitrogen concentration (NO_3–N) (mg/l)	NNO3	Mean±sd	0.450±0.190	0.365±0.060	1.022±0.173	1.343±0.168	0.927±0.182
		Min–max	0.290 – 0.980	0.267 – 0.462	0.695 – 1.360	1.090 – 1.290	0.681 – 1.290
Ammonium nitrogen concentration (NH_4-N) (mg/l)	NNH4	Mean±sd	0.001±0.002	0.003±0.006	0.004±0.007	0.005±0.008	0.002 ±0.003
		Min–max	0.000 – 0.005	0.000 – 0.021	0.000 – 0.020	0.000 – 0.027	0.000 – 0.008
Total phosphorous (mg/l)	Ptot	Mean±sd	<0.001	<0.001	<0.001	<0.001	<0.001
		Min–max					
Chemical oxygen demand (mg/l)	COD	Mean±sd	5.46±2.43	5.09±1.74	8.33±8.56	5.03±4.95	4.13 ±2.68
		Min–max	1.21 – 8.34	1.68 – 7.96	1.86 – 33.20	0.95 – 19.30	0.29 – 7.78
Microhabitat equitability	HABITAT	Mean±sd	0.77±0.02	0.75±0.02	0.74±0.02	0.74±0.04	0.73±0.05
		Min–max	0.72 – 0.78	0.70 – 0.78	0.70 – 0.76	0.70 – 0.78	0.64 – 0.78
Disturbance (%)	DISTURBANCE	Mean±sd	19.7±30.9	15.3±31.6	28.2±40.2	16.5±29.0	22.8±39.7
		Min–max	0.0 – 76.1	0.0 – 100.0	0.0 – 100.0	0.0 – 71.7	0.0 – 100.0
Percentage of the sky not covered by tree canopy (%)	EXPOSURE	Mean±sd	71.7±9.4	71.7±9.4	30.8±18.3	40.0±19.1	95.8±5.2
		Min–max	60.0 – 80.0	60.0 – 80.0	10.0 – 50.0	20.0 – 60.0	90.0 – 100.0
Mean monthly hours of light (h)	HLIGHT	Mean±sd	5.7±4.5	6.5±2.0	5.2±5.0	3.9±4.0	5.9±2.1
		Min–max	0.0 – 10.5	3.5 – 8.9	0.0 – 11.6	0.0 – 10.1	3.0 – 8.5
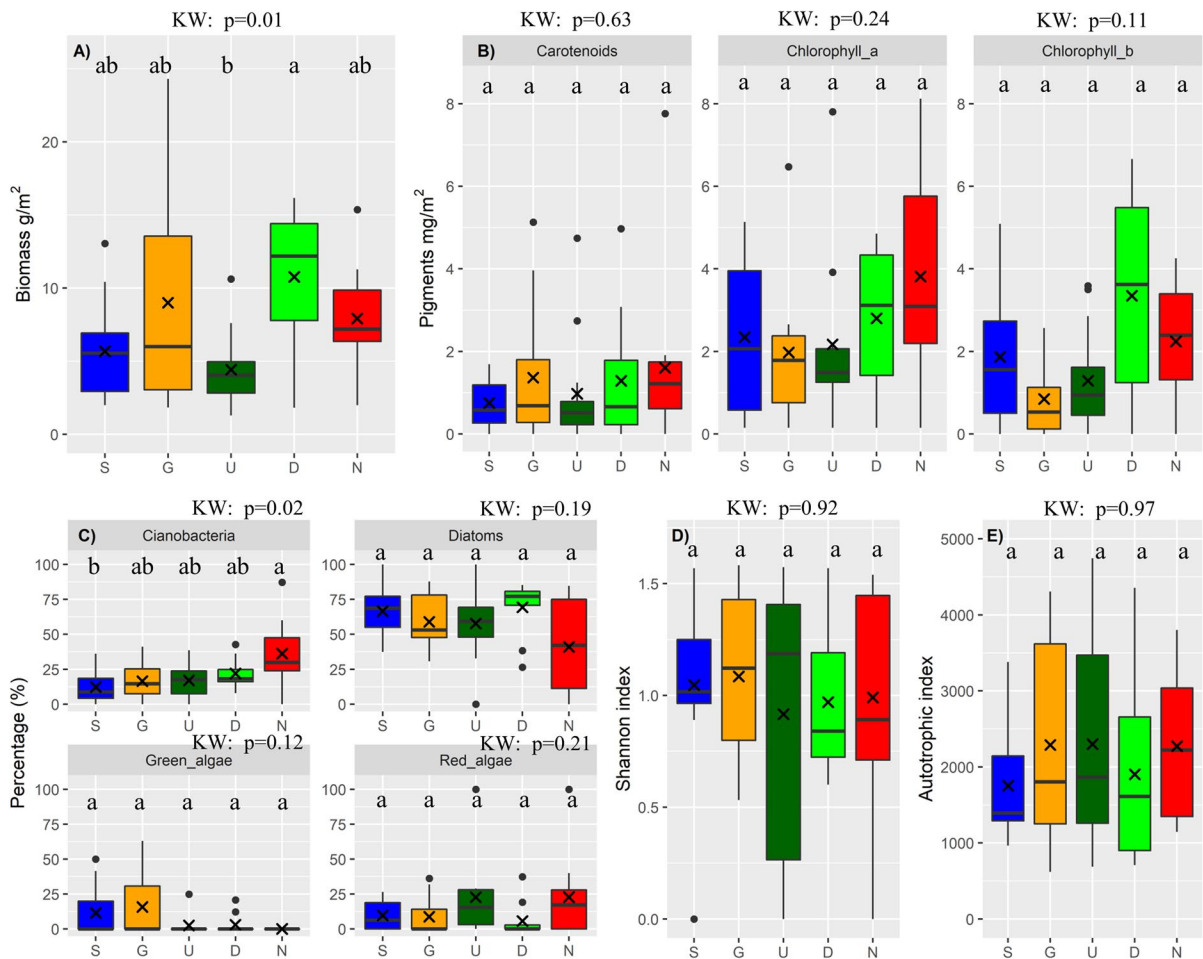
Fig. 4 Boxplots comparing the annual variability of **A** biofilm biomass, **B** pigment's component, **C** the percentage of the main groups, **D** Shannon index, and **E** Autotrophic index in the five stream sites. The *p* value of Kruskal–Wallis test is reported above each plot and lowercase letters indicate which comparisons are significant (Dunn's multiple comparison test, alpha = 0.05). Outliers were excluded for a better visualization

in S ($P = 0.02$, Fig. 4C). The Shannon and autotrophic indices were similar in all sites (~ 2800–3700) ($P = 0.92$ and 0.97, Fig. 4D and E, respectively).

Environmental factors and biofilm

In general, the selected environmental variables explained 0.11–0.51 of the biofilm metrics' variance except for the Shannon diversity index, which remained unexplained (Table 3). Biomass, Chlorophyll a, Chlorophyll b, Diatoms, and Carotenoids displayed strong negative association with DISTURBANCE ($P < 0.01$) in contrast to the Autotrophic index and the relative abundance of Red

algae that were positively associated ($P < 0.001$ and $0.05 \leq P < 0.1$, respectively). Light availability, expressed as the interaction between EXPOSURE and HLIGHT, was weakly negatively correlated with biomass and the relative abundance of diatoms ($0.01 \leq P < 0.05$), while it was strongly positively related to the relative abundance of cyanobacteria ($P < 0.001$). NNO_3 exhibited a weak positive association with chlorophyll b ($0.05 \leq P < 0.1$) and was strongly correlated with cyanobacteria abundance ($P < 0.001$). TMean poorly explained the relative abundance of green algae and red algae ($0.01 \leq P < 0.05$ and $0.05 \leq P < 0.1$, respectively). The time (SEASON) was pivotal to explain the amount of

Table 2 Biofilm metrics of the five stream sites expressed as the annual average ± standard deviation and minimum and maximum values based on the monthly samples

Biofilm metrics		S	G	U	D	N
Biomass (g/m^2)	Mean ± sd	5.68 ± 3.47	9.00 ± 7.62	4.42 ± 2.55	12.93 ± 8.94	15.03 ± 24.86
	Min–max	1.99 – 13.05	1.83 – 24.30	1.29 – 10.63	1.82 – 36.78	1.99 – 93.20
Chlorophyll a (mg/m^2)	Mean ± sd	3.63 ± 3.51	6.56 ± 8.57	2.91 ± 3.25	11.17 ± 14.50	10.27 ± 22.47
	Min–max	0.15 – 10.78	0.15 – 23.96	0.15 – 11.05	0.15 – 51.82	0.15 – 81.22
Carotenoids (mg/m^2)	Mean ± sd	0.75 ± 0.59	1.37 ± 1.63	0.98 ± 1.40	2.58 ± 4.72	1.61 ± 2.06
	Min–max	0.00 – 1.69	0.00 – 5.13	0.00 – 4.74	0.00 – 16.76	0.00 – 7.76
Chlorophyll b (mg/m^2)	Mean ± sd	1.87 ± 1.77	3.95 ± 5.78	1.29 ± 1.29	8.66 ± 12.62	8.21 ± 20.73
	Min–max	0.00 – 5.09	0.00 – 16.14	0.00 – 3.59	0.00 – 36.16	0.00 – 73.88
% Cyanobacteria	Mean ± sd	12.28 ± 11.98	16.50 ± 13.74	17.01 ± 12.99	22.00 ± 10.04	36.15 ± 23.04
	Min–max	0.00 – 36.06	0.00 – 41.07	0.00 – 38.65	7.93 – 42.68	0.00 – 87.31
% Green algae	Mean ± sd	11.45 ± 18.53	15.75 ± 24.03	2.48 ± 7.86	3.00 ± 6.93	0.00 ± 0.00
	Min–max	0.00 – 50.00	0.00 – 63.12	0.00 – 24.84	0.00 – 20.68	0.00 – 0.00
% Diatoms	Mean ± sd	66.57 ± 17.46	59.00 ± 20.71	57.77 ± 28.09	69.36 ± 19.07	41.06 ± 32.98
	Min–max	37.47 – 100.00	30.61 – 87.90	0.00 – 100.00	26.40 – 85.35	0.00 – 84.79
% Red algae	Mean ± sd	9.70 ± 10.44	8.76 ± 13.72	22.74 ± 29.48	5.65 ± 11.93	22.79 ± 28.17
	Min–max	0.00 – 26.47	0.00 – 36.15	0.00 – 100.00	0.00 – 37.34	0.00 – 100.00
Shannon index	Mean ± sd	1.05 ± 0.40	1.08 ± 0.38	0.92 ± 0.65	0.97 ± 0.34	0.99 ± 0.49
	Min–max	0.00 – 1.57	0.53 – 1.58	0.00 – 1.57	0.60 – 1.57	0.00 – 1.54
Autotrophic index	Mean ± sd	3755 ± 4161	3384 ± 3165	2825 ± 2218	3359 ± 3632	3188 ± 3315
	Min–max	966 – 14,309	621 – 12,194	688 – 8595	710 – 12,138	1148 – 13,250

Table 3 Results of the most parsimonious generalized linear mixed-effect models relating the different biofilm metrics to the main environmental variables

Response	Fixed terms	$R^2_{marginal}$
Biomass	**DISTURBANCE*** + EXPOSURE: HLIGHT* +** SITE	0.51
Chlorophyll a	**DISTURBANCE*** +** SEASON	0.44
Chlorophyll b	**DISTURBANCE** +** NNO3°	0.24
Carotenoids	**DISTURBANCE** +** SEASON	0.34
Shannon index		0.00
Autotrophic index	DISTURBANCE***	0.24
% Cyanobacteria	NNO3*** + EXPOSURE: HLIGHT***	0.25
% Diatoms	**DISTURBANCE** + EXPOSURE: HLIGHT***	0.25
% Green algae	**TMean***	0.11
% Red algae	TMean° + DISTURBANCE°	0.14

The bold fixed terms indicate negative regressions, while SEASON and SITE variables are underlined. Coefficients are reported in Online Table 2A

Asterisks represent the *P*-value: (°): $0.05 \leq P < 0.1$; (*): $0.01 \leq P < 0.05$; (**): $0.001 \leq P < 0.01$; (***): $P < 0.001$

Chlorophyll a and carotenoids, which exhibited different behaviors during winter compared to the other months. Space (SITE) was important in explaining differences in biofilm biomass (Online Table 2A). Annual water thermal variability and annual temporal diversity (BSor) were poorly correlated (R2 = 0.18, p = 0.48).

Discussion

Temporal patterns

One-year biofilm samplings revealed a pronounced seasonal pattern in total biomass and pigment concentration common to all sites. Autumn floods drastically reduced biofilm, altering the balance between autotrophic and heterotrophic organisms/organic detritus. Subsequently, in November, the autotrophic index peaked, followed by a biofilm recovery during winter, with increased pigment content persisting into spring. The results indicate that the biofilm biomass is primarily influenced by the disturbance, consistent with other studies conducted in mountain streams. For instance, Power and Stewart (1987a, b) observed a 55% decrease in algal coverage following severe floods in a Oklahoma stream (New Zealand). Similarly, Biggs (1995) reported a negative correlation between chlorophyll a and flood disturbance

frequency in various New Zealand streams. According to our field observations, the abrasion was the primary mechanism of biofilm removal because of the substrate overturning and tumbling. Although the pebble displacement method had already proven to be valid for the assessment of the ecological effect of floods (Power & Stewart, 1987a; Biggs, 1995), flow data, unfortunately unavailable here, would have contributed to explain biomass variations during free flood periods and similarly, the concentration of suspended solids. Indeed, biofilm biomass responds also to changes in current velocity and sediment scouring (Horner & Welch, 1981; Horner et al., 1990; Biggs, 1996; Francoeur & Biggs, 2006; Niedrist et al., 2018).

In winter, a sparce canopy can enhance biofilm growth due to the limited light in small streams under dense tree cover (DeNicola, 1996; McConnell & Singler, 1999; Larned & Santos, 2000). Consequently, periphyton tends to peak just before canopy development, declining through the summer, as observed in our surveyed streams, especially those with lower exposure (*S*, *D*, *U* and *D*) (Hill & Dimick, 2002). However, in this case, the time (SEASON) better explained the monthly changes in chlorophyll a and carotenoids than light availability (EXPOSURE: LIGHT), indicating similar temporal variations across all sites. Light availability impacts photosynthesis, which in turn affects carbon fixation and elemental

ratios (Sterner et al., 2007). Consequently, the sparse winter canopy may boost primary production and elevate autochthonous carbon content to the detriment of inorganic nutrients, with effects on the diet of consumers (Martyniuk et al., 2016). The opposite pattern observed between the autotrophic index and the pigment concentrations suggests changes in the benthic metabolism. Thus, floods shifted stream metabolism toward heterotrophy as primary production is more affected than ecosystem respiration (Uehlinger and Naegeli, 1998). Moreover, with this shift, heterotrophic bacteria and fungi associated with periphyton may emerge as significant resource for consumers, diminishing the relative importance of algal inputs (Hillebrand et al., 2002).

Following the autumn floods, the N site experienced an exceptional algal bloom, likely attributable to *Hydrurus foetidus* (Villars) Trevisan, which covered the entire riverbed. This fast colonization has been observed in glacier streams and karstic springs too (Uehlinger et al., 1998; Hieber et al., 2001; Cantonati et al., 2006; Rott et al., 2006). Reduced summer light availability was associated with cyanobacteria abundance, whereas higher temperatures, as indicated by Hieber et al. (2001) and Allan and Castillo (2007), were related to red algae dominance in spring and summer. In contrast, diatoms remained the dominant group for most months, with higher abundances in winter, when light availability and disturbance were low (Table 3). Green algae were notably present in winter, contrary to the expectation that they thrive in higher temperatures (Allan & Castillo, 2007). However, Hill et al., (1995) suggested that green algae also require high light intensity, often lacking in summer due to the canopy. Therefore, limited light availability may outweigh temperature as the key factor in these stream sites. However, given the documented variations in periphyton composition assessment using fluorimetric methods (Kahlert & McKie, 2014), we advise caution when interpretating community composition responses.

Spatial pattern

The annual biofilm biomass varied between 4.4 and 15.0 g/m^2, aligning with findings from studies in diverse environments, such as glacial streams (Joos, 2003; Robinson et al., 2016; Peszek et al., 2022), meadows streams (Elsaholi, 2011), and forested

streams (Biggs, 1988; Fernandes & Esteves, 2003). Annually, sites regulated by reservoirs (G and D) had higher biomass (9.0 and 12.9 g/m^2) compared to unregulated sites (S and U, with 5.7 and 4.4 g/m^2, respectively). This confirmed the positive impact of upstream regulation on the biofilm, attributed to enhanced flow stability, consistent with findings in the Soca River (Slovenia) by Smolar-Žvanut & Mikoš (2014). However, due to the absence of gauging sites, the assessment of small flow variations was not possible, and disturbance, estimated by pebble movement, revealed similar annual magnitude across all sites (Fig. 3D). Biofilm growth was not associated with nutrient concentration which is always very low (nitrate and phosphate concentrations were less than 1.4 mg/l and < 0.001 mg/l, respectively) as low-light availability and disturbance probably overrode nutrient limitations (Larned & Santos, 2000; Bernhardt & Likens, 2004).

Apart from biomass, the other biofilm metrics annually were similar among sites. For example, chlorophyll a content averaged 2.9 to 10.2 mg/m^2, in line with forest stream studies (Rier & Stevenson, 2002; Bernhardt & Likens, 2004; Pizarro & Alemanni, 2005; Francoeur & Biggs, 2006) but considerably lower than the 20–80 mg/m^2 observed in several unshaded streams (Biggs, 1988; Quinn et al., 1996; Cattaneo et al., 1997; Robinson et al., 2016). This discrepancy was attributed to the combined influence of disturbance and canopy cover, typically found in mountain-shaded streams. However, light utilization efficiency increases under low-light conditions leading to elevated cellular nutrition content which potentially benefit consumers through enhanced trophic energy transfer (Martyniuk et al., 2019). Additionally, the autotrophic index annually averaged around 3000, exceeding values typically found in mountain streams, despite known high seasonal variability (this study, Biggs, 1988; Joos, 2003). These high values suggest that primary producers have a diminished functional role within the biofilm matrix of the surveyed streams.

Contrary to our assumptions, periphyton groups were not associated with site-specific environmental conditions but primarily with factors, like light availability, water temperature, disturbance, and nitrate concentration, which exhibited marked temporal variations (Table 3). However, the N site showed a high percentage of cyanobacteria in summer (> 50%) and

red algae in late winter–spring (20%), consistent with observations in other groundwater streams (Hieber et al., 2001; Uehlinger, 2006). On the other hand, the absence of green algae may be attributed to the short distance from the source (~200 m), which limits the colonization by green algae from terrestrial environments. In contrast, the high abundance of green algae in *G* (15%) could be promoted by the colonization of Chlorophyceae and Conjugatophyceae from high-altitude reservoirs. These lakes host several species of green algae, including genera such as *Staurastrum, Staurodesmus, Crucigeniella, Planctosphaeria, and Sphaerocystis* (Gentili et al., 2001). Cyanobacteria were particularly abundant in *N* (36%), positively associated with light availability and nitrate concentration. This contrasts with prior studies where nitrate enrichment did not promote Cyanobacteria density, likely due to their nitrogen-fixing capacity (Allan & Castillo, 2007). Unlike our assumptions, all the investigated sites displayed similar annual turnover in periphyton composition, poorly related to the annual water thermal variability. However, we believe that a detailed taxonomic investigation is necessary to assess how periphyton taxa's occurrence and abundance respond to changes in water temperature. A species-based investigation may also be useful to detect differences in community composition related to lithology (limestone vs silica), hydrology, and shading as reported in several studies (Gesierich & Kofler, 2010; Larned, 2010; Cantonati et al., 2012a; Kamberovic et al., 2019). Overall, the biofilm variations observed within and among the surveyed streams suggest that, at small catchment scale, the seasonal variations outweigh the spatial ones. However, the limited number of sites (*n*=5) hindered a rigorous evaluation of biofilm patterns and the factors influencing them; therefore, further research including several sites is needed. Similarly, conducting investigations at the mesohabitat scale may provide a better understanding of the role of within stream reach variability in biofilm patterns at larger scale (across stream sites and samplings).

Conclusions

In a year-long survey with monthly samplings, marked temporal variability in epilithic biofilm was observed across all sites, mainly driven by disturbance and seasonality. Floods dropped the biofilm biomass, leading to a shift in the benthic metabolism toward higher heterotrophy. While various environmental factors affected periphyton community, water temperature, and light availability were the most influential in shaping its composition. A succession of the main groups was encountered through the seasons with green algae dominating in autumn, followed by cyanobacteria and red algae in spring–summer and summer–autumn, respectively. Spatially, higher biofilm biomass values were measured in regulated sites, and no green algae were found in the groundwater stream. Despite varying annual temperature variability at the sites, they displayed similar annual turnover in composition. Overall, the study highlights the importance of conducting a quantitative and frequent (monthly) investigation of biofilm to understand temporal changes. It also indicates that Phyto-PAM deconvolution is an effective tool to detect major changes in periphyton composition. Nevertheless, a deeper investigation involving a larger number of sites would be valuable for a comprehensive community characterization and a more thorough assessment of the role of each environmental driver.

Declarations

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