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RESEARCH ARTICLE

Response of biofilm growth to experimental warming in a temperate stream

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Abstract

Biofilm is an important component of small streams, and it is highly sensitive to variations in water temperature. Therefore, it is expectable that the warming predicted for this century will be reflected in its communities. In this study, we investigated the effects of experimental warming on biofilm growth in a small forest stream in Central Portugal. The stream was longitudinally divided in two halves, both at ambient temperature during ambient period; the following months (warmed period), one stream half remained at ambient temperature (control half) and the other half was experimentally warmed by ~3 °C (experimental half), following a before-after control-impact design. Biofilm variables (biomass, chlorophyll-*a* and chlorophyll-*c* concentrations, autotrophic index, and diatom density) were determined from epilithic samples collected from both stream halves three times during the ambient period and five times during the warmed period. The experimental warming led to a significant increase in biomass, chlorophyll-*a*, chlorophyll-*c* concentration, and diatom density, especially in the winter months. Future warming, especially during the colder months, may thus stimulate biofilm growth, which may strengthen the autotrophic pathways of these systems traditionally based on detritus.

KEYWORDS

BACI design, biofilm biomass, chlorophyll, diatom density, temperature

1 | INTRODUCTION

Temperature is an environmental factor of key importance in determining the activities of organisms, and running water inhabitants are likely to be particularly vulnerable to changing thermal regimes (Giller et al., 2004; Friberg et al., 2009). The mean global air temperature is predicted to increase between 1.1 and 4.8 °C by 2100 (IPCC, 2014), which will be closely followed by an increase in stream water temperature (Langan et al., 2001; Morrill, Bales, & Conklin, 2005; Koycheva & Karney, 2009). Water warming can be expected to act as a stressor for freshwater communities because organisms have thermal optima over which physiological mechanisms, such as respiration and growth, might be affected (Romaní et al., 2016).

Stream biofilms growing on substrates are mainly composed of bacteria, algae, cyanobacteria, fungi, and protozoa embedded in a polysaccharide matrix (Lock, Walace, Costerton, Ventullon, & Charlton, 1984). They have a short generation time and are highly responsive to changes in environmental conditions (Romaní, 2010). The attached algae, or periphyton, within this biofilm has an important role in aquatic systems as primary producers in lotic environments (Weizel, 1979),

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and it serves as food source for invertebrates and some fish and can also play and active role in phosphorous interception in ecosystems (Dodds, 2003).

In fact, spatial and temporal variations in temperature strongly influence biological responses of periphyton (DeNicola, 1996), and increases in water temperature may stimulate benthic primary productivity (Barranguet, Kromkompp, & Peene, 1998; Acuña, Wolf, & Uehlinger, 2008; Díaz-Villanueva, Font, Schwartz, & Romaní, 2011). Water temperature is determined primarily by direct solar radiation and large spatial scale factors such as latitude, elevation, and morphometry (DeNicola, 1996). Weatherley and Omerod (1990) also indicated that the presence of canopy cover in deciduous forest tends to result in lower mean and maximum summer temperatures, less diurnal variation, and higher mean and maximum winter temperatures in streams.

The effects of water temperature on periphyton communities have been described in different studies in combination with other factors such as grazing (Cao, Li, & Jeppensen, 2014; Gregory, 1980; Hill, Ryon, & Schilling, 1995), nutrient enrichment (Gregory, 1980; Liess *et al*., 2009), fine sediments deposition (Piggott, Salis, Lear, Townsend, & Matthaei, 2015), and also multiple factors that can affect the seasonal variation in periphyton (Rosemond, 1994). Approaches addressing the effects of light irradiance on algal communities suggest that the response of periphyton differs substantially from the one typically reported for phytoplankton (Boston & Hill, 1991). This difference is probably related with matrix structure of the biofilm that influences particular environmental conditions on these communities that are different from planktonic communities. Other studies addressing the effects of light irradiance on algal communities, interacting with organic carbon and inorganic nutrients (Rier & Stevenson, 2002), detect positive relationships between light and growth of algae and bacteria.

In small forest streams, diatoms generally dominate the algal component of the biofilms when the climax communities are established (Biggs, 2000) and also when the water temperature ranges between 5 and 20 °C (Lamberti & Resh, 1985). Diatom communities are an important component of aquatic ecosystems (Cascallar et al., 2003; Romaní, 2010) because they are at the base of food webs and are the primary food resource for many common groups of freshwater invertebrates (McCormick & Stevenson, 1998). They may also be used as biotic indicators of ecological condition as they respond to human and natural disturbance (Stevenson, 2014).

Laboratory studies allow the isolation of the effect of temperature on biofilms but limit our ability to scale up the results due to the simplification of the systems tested. Field studies are more realistic and allow to overcome some of the limitations of laboratorial experiments; nevertheless, they are more difficult to control due to variation of other environmental variables (e.g., light availability and canopy cover), which can interfere in the results.

Our goal was to determine the effects of a ~3 °C increase in water temperature, as predicted under global warming (IPCC, 2014), in the growth of the biofilm of a small forest stream, under realistic field conditions, taking into account seasonality. Therefore, we performed an in situ experiment where the stream water temperature was raised ~3 °C above ambient temperature. We hypothesized that water temperature increase (a) would cause an increase in biofilm total biomass (includes biomass of algae, bacteria, fungi, microscopic fauna, and detritus) and (b) would cause higher increase in chlorophyll concentration and diatom density in the colder season.

2 | MATERIALS AND METHODS

2.1 | Study site characterization and experimental design

This study was carried out in Candal stream, Lousã Mountain, central Portugal (40°4′44″N, 8°12′10″W, 620 m.a.s.l.; Figure 1). This is a second-order stream, which drains an area of about 0.8 km^2 covered by mixed deciduous forest, where human activity is scarce. In this area, the bedrock is schist, and the stream substrate is composed mainly of cobbles and pebbles. The selected stream reach (22-m long, 1-m wide, and ~10-cm deep) was longitudinally divided in two halves using local schist tiles driven into the sediment and roughly cemented to the bedrock.

From November 2010 to March 2011, both stream halves were kept at ambient temperature (ambient period), whereas from April 2011 to February 2012 (warmed period), one half (experimental half) was warmed by ~3 °C above the temperature registered in the other

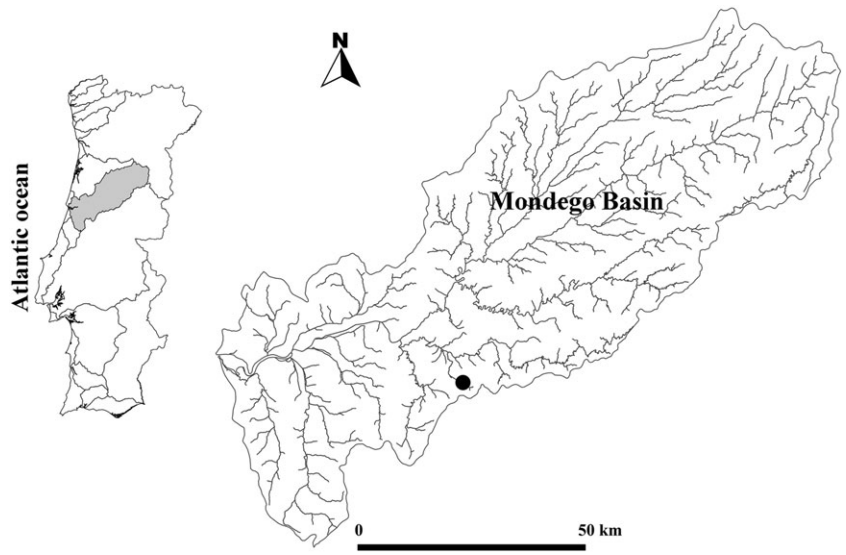

FIGURE 1 Location of the study site (black circle) in Candal stream, Lousã Mountain, Central Portugal

half (control half), following a before-after control-impact design. Warming of one half was possible with the help of a flowing-through tank with 30 electrical resistors supplied by 42-kW power; the control half received water from a bypass tank without electrical resistors. Both tanks were located upstream of the study reach and were equipped with inlet and outlet valves that guaranteed a similar flow in both stream halves (for more details on the experimental design, see Canhoto, de Lima, & de Almeida, 2013). The increase in water temperature by ~3 °C was based on predictions for air temperature in Portugal by the end of this century (Miranda, Coelho, Tomé, & Valente, 2002) and on the relationship between stream water and air temperature reported for similar streams (Morrill et al., 2005).

Eighteen schist slates (individual upper surface area: 12 × 9 cm = 108 cm^2) were introduced in each stream half and allowed to incubate between 30 to 60 days (incubation time) depending on the month. The stones were incubated in three occasions before warming (December 10, January 11, and March 11) and in five occasions during warming (April 11, May 11, June 11, December 11, and February 12). During the summer of 2011 (July–September), the stream dried out, so schist slates could only be incubated again in October after water resumed. These were sampled in December 2011. The incubation duration was selected on the basis of previous studies that suggest that biofilm can be considered "mature" after ~4 weeks of colonization (Ács, Kiss, Szabo, & Makk, 2000; CEN TC230 N68, 2003; Szabó et al., 2008). At each sampling occasion, three schist slates were selected randomly, and the upper surface was scraped with a soft toothbrush (total area per sample: 108 × 3 = 324 cm^2), rinsed with distilled water to remove the biofilm, and combined into a flask to make a single sample (~70 ml). This procedure was repeated with different schist slates to generate three samples (each with 324 cm^2), pseudo-replicates, for the determination of total biomass and chlorophyll concentration and other three samples for the determination of diatom density. The diatom samples were preserved with Lugol solution (10%). All samples were transported to the laboratory in a cooler.

2.2 | Stream characterization

During the field experiment, water temperature was recorded hourly in both stream halves with submersed data loggers (Hobo Pendant UA-001-08, Onset Computer Corp., Massachusetts, USA). Dissolved oxygen (Oxi 3210, WTW, Weihleim, Germany), pH (pH 3110, WTW, Weihleim, Germany), and electric conductivity (LF 330, WTW, Weihleim, Germany) were measured in situ with portable devices at weekly–biweekly intervals. Water samples (300 ml) were collected each week from each stream half, filtered through glass fibre filters (47-mm diam., pore size 0.7 μm; Millipore APFF04700, Millipore Corp., Massachusetts, USA), transported on ice to the laboratory, and frozen for posterior determination of nutrient concentrations. Nitrate, nitrite, and ammonium concentrations were determined by ionic chromatography (Dionex DX-120, Sunnyvale, California, USA). Alkalinity was determined by titration of 100 ml of stream water with 0.02 N sulphuric acid to an end point of pH 4.2 (APHA, 2005). Discharge was determined volumetrically at the output of the valves that fed each stream half (Gore, 1996).

Due to significant seasonal differences in the length of daylight in temperate areas, the number of daily light hours in the study site was taken into account (http://www.sunearthtools.com; accessed on May 10, 2013). For each sampling period, the total number of light hours was calculated approximately by averaging the daily number of light hours of the period of incubation of the schist slates in the warmed period because the samples were incubated in different months and seasons: April with 13 hr of daily light irradiance, May with 13.5 hr, June with 14 hr, December with 11 hr, and February with 10.5 hr.

2.3 | Biofilm characterization

Samples for total biomass and chlorophyll determinations (see Section 2.1 for details on the sampling procedure: n = 48) were promptly filtered through ignited, preweighed glass fibre filters (47-mm diam., 0.7-μm pore size; Millipore APFF04700, Millipore Corp., Massachusetts, USA), and the filters were enclosed individually in Petri dishes. The Petri dishes were wrapped with aluminium foil for protection against light and frozen at −18 °C until used. Filters were lyophilized overnight (LY3TTE, Snijders Scientific, Tilburg, the Netherlands), weighed (± 0.01 mg) for determination of total biofilm dry mass (DM), cut in half, and each half weighed for determination of partial DM. Half of each filter was ignited (550 °C, 4 hr) and reweighed for determination of partial ash mass. Total biomass, as ash-free dry mass (AFDM), was determined by the difference between partial DM and partial ash mass, corrected for the total DM, and the results were expressed as mg AFDM cm^{-2}. The other half of each filter was used for extraction of clorophyll. The method reported in APHA (2005) was used for chlorophyll-*a* (chl-*a*) and chlorophyll-*c* (chl-*c*) extraction. Half of each filter was introduced into a centrifuge tube and incubated with 10 ml of 90% acetone at 4 °C for 20 hr. Tubes were removed from the fridge, allowed to reach room temperature, supplied with 2 ml of 90% acetone and centrifuged (Sigma 316-P, Osterode am Harz, Germany) at 4000 rpm for 10 min. The absorbance of the supernatant was determined with a spectrophotometer (Jenway 6715B0, Staffordshire, UK) at 630, 646, 664, and 750 nm. The concentration of chlorophyll-*a* (chl-*a*) and chlorophyll-*c* (chl-*c*) (in μg chlorophyll cm^{-2}) was calculated following APHA (2005).

Total biomass gives the amount of organic matter in a sample and includes biomass of algae, bacteria, fungi, microscopic fauna, and detritus (APHA, 2005). The amount of chlorophyll in a sample should be proportional to algae biomass (chl-*a* ranges from 0.5% to 2% of total algal biomass, depending on the taxonomical groups present, light, and nutrients; APHA, 2005). The ratio biofilm biomass/chl-*a* (both measures in the same unit) gives the autotrophic index (AI), which is a good measure to forewarn impending shifts in dominance of functional groups (i.e., AI >200 indicates a high proportion of heterotrophic, non-chlorophyllous organisms, or organic detritus; APHA, 2005). As diatoms also contain chl-*c*, in addition to chl-*a*, there is a positive correlation between the former photosynthetic pigment and diatom abundance in streams (Szabó, 2011).

Samples for diatom density determination (n = 48) were centrifuged and concentrated to a final volume of 15 ml containing a total area of 324 cm^2. An aliquot (1–3 ml) of the pellet from each sample was treated with nitric acid (HNO_3) at 65% and potassium dichromate ($K_2Cr_2O_7$) at room temperature for 24 hr for the removal of organic content including other algae. Oxidation by-products were removed

by multiple centrifugations (1,500 rpm) followed by rinsing with distilled water. Permanent slides were prepared with a total volume of 25 µl from the treated sample, mounted with a high-resolution diatom resin (Naphrax®, refractive index of 1.74), and scanned under a light microscope (LM – Leitz Biomed 20 EB) equipped with an immersion objective of 100× (numerical aperture: 1.32). All unbroken diatom valves were identified (Krammer & Lange-Bertalot, 1986–1991; Prygiel & Coste, 2000) and counted, and diatom cell densities (no. cells cm^{-2}) were calculated.

2.4 | Data analysis

Differences in the physico-chemical variables between months (warmed period) and stream halves (control vs. experimental) were assessed by analysis of variance (ANOVA). Homogeneity of variances and normality of the water physico-chemical variables and of the biological variables were assessed with Levene's test and pp-plot test ($\alpha = 0.050$), respectively. When the variables did not comply with the assumptions, a Mann Whitney *U* nonparametric test was performed to compare the two groups (control and experimental). The biological data were log-transformed to achieve normality and homoscedasticity when necessary.

Differences in the biofilm variables between the two stream halves before and during experimental warming were assessed by ANOVAs (two-way ANOVAs, season and stream half as factors) to discern whether temperature effects differed between seasons (or were dependent on season) and after analysis of covariance (ANCOVA) that included continuous variables. This analysis allowed us to assess if differences in the biofilm variables between the two stream halves (control vs. experimental) were mainly due to differences in the water temperature or to other factors such as the incubation period, average of daily hours of light, or to the interaction between temperature and the other factors. The relationship between diatom density (log-transformed) and light hours was assessed by linear regression. All analyses were performed with the STATISTICA 7 software (StatSoft Inc., Tulsa, Oklahoma, USA).

3 | RESULTS

3.1 | Stream water characterization

The homogeneity of variances and normality for the physico-chemical variables in the ambient and warmed period were achieved in all cases except for the normality of the alkalinity data. So it was decided to use a nonparametric test, the Mann-Whitney *U*-test, with two groups (control and experimental). During the ambient period, no significant differences were found between the physico-chemical variables of the control and experimental halves; in the warmed period, there were only significant differences in the water temperature. During the ambient period, water temperature was similar in both stream halves (Mann-Whitney *U*, $Z = 0.027$, $p = .98$), whereas during the warmed period, water temperature was significantly higher (by 2.1–3.1 °C; Table 1) in the experimental than in the control half (Mann–Whitney *U*, $Z = -3.04$, $p = .0024$). Throughout the study, discharge was low (≤3.2 l s^{-1}), and stream water was well oxygenated (≥72.7% dissolved oxygen), circumneutral ($6.9 \leq pH \leq 7.6$), oligotrophic (68–594 µg NO_3^- l^{-1}), and had low conductivity (≤27.8 µS cm^{-1}) and alkalinity (≤8.3 mg $CaCO_3$ l^{-1}) (Table 1).

3.2 | Biofilm biomass

During the warmed period, total biomass was significantly different between stream halves taking into account season (spring vs. winter; ANOVA, $F = 10.94$, $p = .003$; Table 2). In the warmed period, biofilm biomass ranged between 0.017 and 0.089 mg AFDM cm^{-2} in the control half and between 0.039 and 0.142 mg AFDM cm^{-2} in the experimental half (Figure 2a). In the ambient period, mean values of biofilm biomass ranged between 0.02 and 0.12 mg AFDM cm^{-2} in the control half and between 0.060 and 0.170 mg AFDM cm^{-2} in the experimental half (Figure 2a) and did not significantly differ between stream halves (ANCOVA, $F = 0.149$, $p = .705$; Table 3). The incubation duration positively influenced the biofilm biomass during the ambient period (ANCOVA, $F = 5.163$, $p = .041$; Table 3). The values of biofilm biomass were significantly higher in the experimental than in the control half (ANCOVA, $F = 4.672$, $p = .041$; Table 3). The interactions "stream half × temperature" (both ambient and warmed period) and "stream half × light hours" (warmed period) were non-significant (Table 3).

3.3 | *Chlorophyll*-a

During the warmed period, chl-*a* concentration was significantly different between stream halves taking into account season (spring vs. winter; ANOVA, $F = 37.52$, $p < .001$; Table 2). During the ambient period, chl-*a* concentration was low (<0.05 µg cm^{-2}; Figure 2b) and didn't differ significantly between stream halves (ANCOVA, $F = 2.642$, $p = .128$; Table 3). Contrarily, during the warmed period, chl-*a* concentration was significantly higher in the experimental (0.014–0.259 µg cm^{-2}) than in the control half (0.010–0.074 µg cm^{-2}) (ANCOVA, $F = 17.182$, $p < .001$; Table 3) (Figure 2b). Significant differences between experimental and control halves (ANCOVA, $F = 17.182$, $p < .001$; Table 3) could not be explained only by the differences in water temperature between stream halves (ANCOVA, $F = 0.013$, $p = .909$; Table 3).

3.4 | *Chlorophyll*-c

During the warmed period, chl-*c* concentration was significantly different between stream halves taking into account season (spring vs. winter; ANOVA, $F = 6.56$, $p = .018$; Table 2). During the ambient period, chl-*c* concentration was low (<0.010 µg cm^{-2}; Figure 2c) and similar in both stream halves (ANCOVA, $F = 0.828$, $p = .379$; Table 3) but was significantly stimulated by the incubation duration (ANCOVA, $F = 8.238$, $p = .013$; Table 3). In the warmed period, chl-*c* concentration increased in the experimental over the control half during the winter months (December 11 and February 12; Figure 2c), but no significant overall differences were found between stream halves (ANCOVA, $F = 3.970$, $p = .061$; Table 3). Incubation duration (ANCOVA, $F = 17.736$, $p < .001$) and water temperature (ANCOVA, $F = 7.490$, $p = .013$) significantly affected chl-*c* concentration during the warmed period (Table 3).

TABLE 1 Water temperature and other physico-chemical characteristics (mean ± SE) of the control and experimental stream halves during the ambient and warmed periods

Date	Water temperature (°C)		Discharge ($l\ s^{-1}$)		Oxygen (%)		Conductivity ($\mu S\ cm^{-1}$)		Alkalinity (mg $CaCO_3\ l^{-1}$)		pH		NO_3^- ($\mu g\ l^{-1}$)	
	Control	Exp	Control	Exp	Control	Exp	Control	Exp	Control	Exp	Control	Exp	Control	Exp
Ambient period														
December 10	8.8 ± 0.2	9.2 ± 0.2	3.2 ± 0.2	3.2 ± 0.3	80.4 ± 5.9	78.3 ± 4.2	25.8 ± 0.2	26.2 ± 0.5	4.3 ± 1.7	4.0 ± 1.2	7.6 ± 0.2	7.6 ± 0.1	208 ± 102	220 ± 100
January 11	8.6 ± 0.3	9.3 ± 0.2	2.7 ± 1.2	2.6 ± 1.4	75.4 ± 5.9	72.7 ± 4.9	–	–	4.4 ± 0.4	6.4 ± 2.9	7.3 ± 0.2	6.9 ± 0.4	452 ± 92	566 ± 272
March 11	8.5 ± 0.1	8.8 ± 0.1	2.9 ± 0.9	2.9 ± 1.1	85.3 ± 14.8	84.7 ± 9.1	26.0 ± 0.4	26.3 ± 0.6	5.1 ± 0.4	4.9 ± 0.2	7.3 ± 0.3	7.3 ± 0.2	263 ± 240	255 ± 257
Warmed period														
April 11	11.0 ± 0.1	13.8 ± 0.3	1.3 ± 0.2	1.4 ± 0.4	91.3 ± 1.3	85.9 ± 1.0	26.7 ± 0.2	27.3 ± 0.5	4.9 ± 0.0	5.3 ± 0.0	7.3 ± 0.1	7.3 ± 0.1	80 ± 2	112 ± 6
May 11	12.2 ± 0.2	15.3 ± 0.3	2.1 ± 0.8	1.9 ± 0.7	98.6 ± 3.1	97.2 ± 3.9	27.1 ± 0.2	27.5 ± 0.2	4.7 ± 0.3	4.4 ± 0.2	7.2 ± 0.2	7.1 ± 0.3	70 ± 24	71 ± 38
June 11	12.8 ± 0.2	15.8 ± 0.3	2.5 ± 0.0	2.0 ± 0.5	104.6 ± 4.7	102.2 ± 2.9	27.4 ± 0.2	27.8 ± 0.5	4.7 ± 0.2	4.9 ± 0.5	7.1 ± 0.2	7.0 ± 0.1	72 ± 12	68 ± 15
December 11	9.3 ± 0.1	12.1 ± 0.3	2.3 ± 0.2	2.5 ± 0.3	93.2 ± 9.6	99.8 ± 15.6	25.3 ± 0.5	25.5 ± 0.4	6.0 ± 0.5	6.2 ± 0.6	7.2 ± 0.1	7.1 ± 0.1	350 ± 39	431 ± 113
February 12	5.5 ± 0.2	7.6 ± 0.4	2.3 ± 0.1	2.3 ± 0.1	108.2 ± 3.2	108.2 ± 2.8	26.4 ± 0.2	26.5 ± 0.2	8.0 ± 0.7	8.3 ± 0.4	7.4 ± 0.3	7.4 ± 0.3	344 ± 49	594 ± 283

Note. Nitrite and ammonium were always below detection limit (<100 and <50 $\mu g\ l^{-1}$, respectively).

3.5 | Autotrophic index (AI)

In the warmed period, AI significantly differed between stream halves when we compared seasons (spring vs. winter; ANOVA, $F = 8.28$, $p = .009$; Table 2). The AI values recorded in the ambient period did not differ significantly between stream halves (ANCOVA, $F = 1.286$, $p = .277$; Table 3). In the warmed period, AI ranged between 0.05 – 0.15 in the control half and between 0.02 – 0.18 in the experimental half and AI significantly differed between halves (ANCOVA, $F = 5.753$, $p = .025$; Table 3).

3.6 | Diatom density

During the warmed period, diatom density was significantly different between stream halves taking into account season (spring vs. winter; ANOVA, $F = 24.17$, $p < .001$; Table 2). In the ambient period, mean diatom densities ranged between 500 and 2,100 cells cm^{-2} in the control half and between 600 and 2,700 cells cm^{-2} in the experimental half (Figure 2d) being similar between the two halves (ANCOVA, $F = 0.272$, $p = .611$; Table 3). The control half of the warmed period (April 11, May 11, and June 11; spring) had diatom densities that were very similar to those found in the ambient period (Figure 2b). In the warmed period, mean diatom densities ranged between 500 and 3,100 cells cm^{-2} in the control half and between 2,190 and 15,760 cells cm^{-2} in the experimental half (Figure 2d). All individual factors, except incubation duration, significantly affected diatom density (Table 3). Among the interactions, only "stream half × light hours" had a significant effect on diatom density (ANCOVA, $F = 10.552$, $p = .004$; Table 3). Diatom density was significantly stimulated by the experimental increase in water temperature, but only in the winter months of the warmed period (Figure 2d). Experimental warming was more effective on winter diatom densities (Figure 3) when the number of daily hours of light was about 10.5–11 hr. The increase in the water temperature during the winter months clearly reversed the positive correlation between diatom density and daily hours of light found in the control half (Figure 3). In the control half, diatom density was highest in the spring months (April 11, May 11, and June 11) whereas in the experimental half, the highest densities were found in winter (December 11 and February 12).

4 | DISCUSSION

Our work addressed the effects of experimental warming on stream biofilms and in particular on diatom density, under field conditions and taking into account seasonal variation. We found that an increase in water temperature of a small temperate mountain stream by ~3 °C led to an increase in biofilm biomass, chl-*a* concentration and diatom density, especially in the winter months. An increase in water temperature can affect the steps of biofilm development, determining changes in the speed of accrual for the different organisms of the biofilm community (Romaní, 2010). In general, warming up to the thermal optimum can accelerate organismal growth, and the observed small increase in biofilm biomass would be expected. Previous studies showed that microbial colonization of the substratum occurred earlier when the temperature of flowing water was increased by 3 °C (Díaz-Villanueva et al., 2011) and that the density of prokaryotes and chl-*a*

TABLE 2 Results of the analysis of variance for testing the effects of season (spring vs. winter) and stream half (control vs. experimental), and the interactions stream half × season on log transformed data of biofilm biomass, chl-*a*, chl-*c*, AI, and diatom density for the warmed period

		Biomass			Chl-*a*			Chl-*c*			AI			Diatom density		
Source of variation	Df	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*
Season	1	0.044	1.870	.186	0.303	8.977	**.007**	1.217	9.040	**.007**	0.578	12.507	**.002**	0.016	0.214	.648
Stream half	1	0.240	10.199	**.004**	0.230	6.820	**.016**	0.273	2.030	.169	<0.001	0.002	.965	1.993	26.621	**<.001**
Stream half × season	1	0.257	10.938	**.003**	1.268	37.522	**<.001**	0.883	6.559	**.018**	0.383	8.284	**.009**	1.809	24.168	**<.001**
Error	21	0.024	–	–	0.034	–	–	0.135	–	–	0.046	–	–	0.075	–	–

Note. Significant differences ($p < .050$) are given in bold.

AI = autotrophic index; chl-*a* = chlorophyll-*a*; chl-*c* = chlorophyll-*c*.

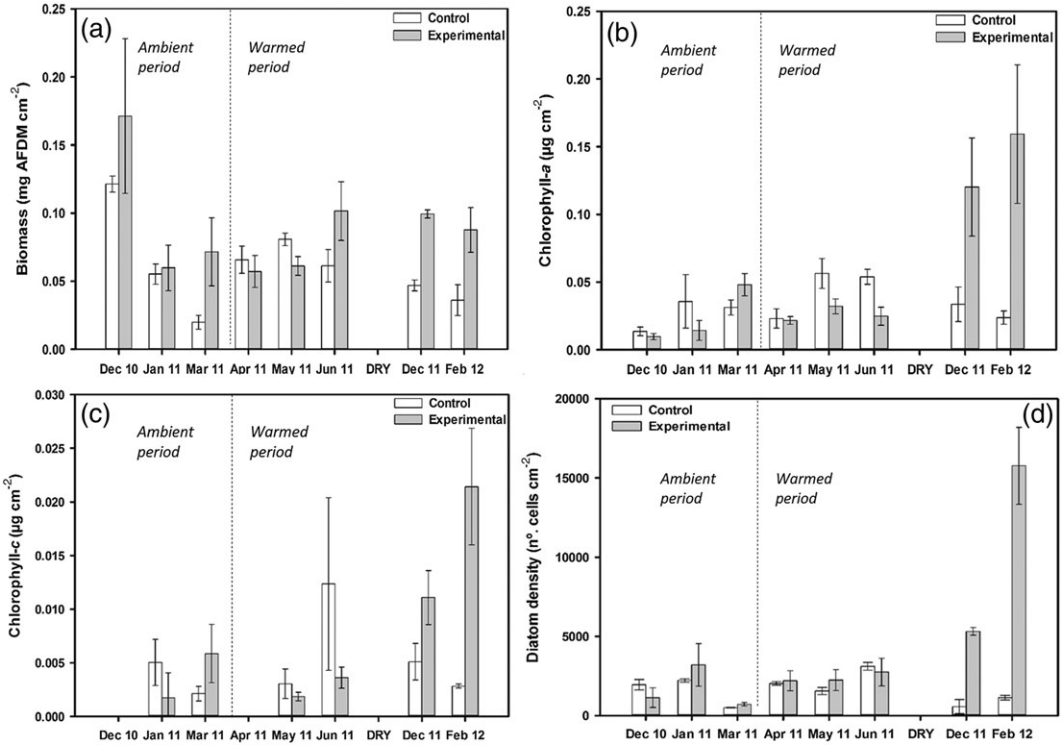

FIGURE 2 Comparison of the different variables in the control and the experimental stream halves during the ambient and warmed periods (mean ± SE). (a) Biomass, (b) diatom density, (c) chlorophyll-*a*, and (d) chlorophyll-*c*

concentration were highest in the biofilm when water was warmed by 3 °C above ambient temperature (Ylla, Canhoto, & Romaní, 2014). Piggott et al. (2015) hypothesize that the increase in temperature would have generally positive effects on green algae and cyanobacteria and negative effects on diatoms. Nonetheless, as recently stated, it is difficult to predict the response of biofilm to warming (Romaní et al., 2016). However, on the basis of our results, we verified that the experimental increase in water temperature during the winter months had a strong positive effect on diatom density.

The importance of light irradiance has been recognized in studies comparing forested and deforested stream reaches (Allan, 2004; Hill et al., 1995; Sweeney et al., 2004). In field experiments, it is difficult to separate interactions between temperature and other variables such as nutrients, light, and trophic interactions (DeNicola, 1996), and for this reason, we included light as a covariable in the analysis of our results concerning the warming effect. Uehlinger, Robinson, Hieber, and Zah (2010) refer that low temperature and low light intensities during winter apparently impose minor constrains on biofilm in alpine streams. Nevertheless, in this work, the lower water temperature and the lower irradiance in the control half in winter, during warming, have similar values in some of the measured variables in the biofilm (i.e., diatom density and chl-*a* concentration) in comparison to the control half in winter, before warming. On the contrary, there was a clear increase in chl-*a*, chl-*c* concentrations and diatom density in the experimental stream half compared to the control during the same winter months (December 11 and February 12) of the warmed period and also if compared with winter months of the period before warming. This increase could not be statistically explained by the water temperature increase, considering the whole period of experimental warming (from April 11 to February 12), but if we consider only the winter months of the warmed period, there are significant differences between control and experimental halves in all variables. The biomass accrual in the experimental half during December 11 and February 12 was due to the photoautotrophs' growth, particularly diatom communities (low AI values

TABLE 3 Results of the analysis of covariance for testing the effects of incubation duration, water temperature, light hours (duration of daily sunlight; only during warmed period), stream half (control vs. experimental), and the interactions stream half × temperature and stream half × light hours (only during warmed period) on log transformed data of biofilm biomass, chl-*a*, chl-*c*, and AI for ambient and warmed period

		Biomass			Chl-*a*			Chl-*c*			AI			Diatom density		
Source of variation	Df	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*
Ambient period																
Incubation duration	1	0.310	5.163	**.041**	0.744	7.373	**.018**	0.000	8.238	**.013**	2.015	14.576	**.002**	0.032	0.342	.569
Temperature	1	0.007	0.112	.743	0.005	0.049	.829	0.000	0.018	.895	0.000	0.001	.975	0.082	0.879	.366
Stream half	1	0.009	0.149	.705	0.267	2.642	.128	0.000	0.828	.379	0.178	1.286	.277	0.025	0.272	.611
Stream half × temp	1	0.008	0.141	.713	0.260	2.581	.132	0.000	0.804	.386	0.175	1.266	.281	0.023	0.241	.632
Error	12	0.060	–	–	0.101	–	–	0.000	–	–	0.138	–	–	0.094	–	–
Warmed period																
Incubation duration	1	0.010	0.416	.525	0.156	3.486	.075	1.394	17.736	**.000**	0.088	1.701	.205	0.177	3.788	.065
Temperature	1	0.045	1.869	.185	0.127	2.824	.106	0.589	7.490	**.013**	0.021	0.407	.530	0.240	5.133	**.034**
Light hours	1	0.000	0.000	.983	0.000	0.001	.976	0.323	4.109	.057	0.000	0.000	.989	0.535	11.456	**.003**
Stream half	1	0.112	4.672	**.041**	0.771	17.182	**<.001**	0.312	3.970	.061	0.296	5.753	**.025**	1.608	34.415	**<.001**
Stream half × light hours	1	0.029	1.235	.278	0.190	4.227	.051	0.031	0.400	.535	0.070	1.352	.257	0.493	10.552	**.004**
Stream half × temp	1	0.000	0.004	.953	0.001	0.013	.909	0.066	0.837	.372	0.001	0.022	.883	0.060	1.277	.270
Error	22	0.024	–	–	0.045	–	–	0.079	–	–	0.052	–	–	0.047	–	–

Note. Significant differences ($p < .050$) are given in bold.

AI = autotrophic index; chl-*a* = chlorophyll-*a*; chl-*c* = chlorophyll-*c*.

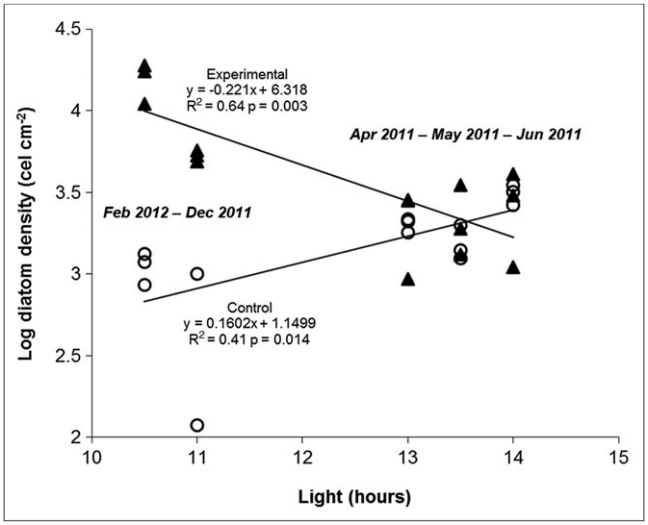

FIGURE 3 Relationship between diatom density and the mean daylight hours during the incubation period, in control (white circle) and experimental (black triangle) halves during the warmed period. The linear regression equation and coefficient of determination (R^2) are also shown

and high chl-*c* concentration). Similarly, Ferreira and Canhoto (2015) found an effect of warming on litter decomposition in winter but not in spring or autumn. As the present results were gathered in the same stream, we can say that this forest stream may be most affected by warming during cold seasons in which activities may be more limited by temperature.

The number of studies on the effects of temperature on the biofilm is small, which limits the discussion of our results, and thus, any extrapolation to other systems needs to be made cautiously. The increase in water temperature, in cold months, led to a decrease in the AI. That is, the same temperature increase appeared to have different influences in the photoautotrophic and heterotrophic components of the biofilm. The higher values of the biomass that were found in the experimental half compared to the control half during the winter months of the warmed period may be in accordance with the findings of Romaní (2010), as bacteria could have colonized the substratum in the stream faster and earlier at higher temperatures, but in the mature biofilm, no differences were found in total densities. The heterotrophic organisms in the experimental half may have colonized the substratum faster and may have allowed a colonization of the autotrophic organisms, which was translated by the observed higher chlorophyll and biomass values.

Although warming effects on biofilm are expected to be more relevant under eutrophic conditions (Díaz-Villanueva et al., 2011), our results demonstrate that an increase of water temperature in oligotrophic headwater streams can significantly stimulate biomass, chlorophyll winter production, and diatom density. The results of this study become especially relevant when considering that during the 20th century, most of Europe experienced increases in average annual surface water temperature, with stronger warming in winter than in summer for most regions (Alcamo et al., 2007). Routinely, studies in freshwater ecosystems do not measure diatom density due to the time needed for counting a known area or volume of a sample (in this case, a known volume and therefore all the diatom valves in the dried sample drop), but in this study, the response of this biofilm variable was a highly consistent and clear indicator of the effects of warming in lotic systems. Autotrophic (vs. heterotrophic) chains may acquire a higher relative importance in warming scenarios due to this bottom-up effect. Our results indicate that an increase in water temperature in small headwater streams can significantly stimulate algae growth in the cold months. Therefore, winter warming may compensate the natural reduction of the number of light hours and stimulate metabolic rates and a rapid division of unicellular algae.

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