# Seasonal variations overwhelm temperature effects on microbial processes in headwater streams: insights from a temperate thermal spring

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**Abstract**
Carbon cycling in headwater streams is mostly driven by the decomposition of allochthonous organic matter, and to a lesser extent by primary production. Quantifying the influence of temperature on these processes is therefore essential to better anticipate the consequences of global warming for stream ecological functioning. In this study, we measured alder litter microbial decomposition and associated fungal biomass and diversity, using leaf discs enclosed in fine-mesh bags along a natural geothermal temperature gradient, in both spring and winter. We monitored the chlorophyll-*a* accrual in biofilms growing on ceramic tiles. The temperature gradient, from upstream to downstream, ranged from 15.3 to 14.2 °C in spring and 18.2 to 13.2 °C in winter. Autotrophs and heterotrophs exhibited contrasting responses to temperature. The expected positive effect of temperature was actually observed for chlorophyll-*a* accrual only, while an apparent temperature-independence of litter decomposition rate was found. Moreover, temperature effects on heterotrophic and autotrophic organisms depended on the season, with higher litter decomposition rates, sporulation rates, fungal biomass and chlorophyll-*a* in spring, despite a lower mean water temperature than in winter. Together, these results suggest that the influence of temperature remained largely overrode by seasonal effects. This result is likely due to annual variations in light availability, and may involve indirect positive interactions between microbial primary producers and decomposers.

**Keywords** Decomposition · Headwater streams · Microbial · Seasons · Temperature

## Introduction

Earth has been undergoing a greater rate of warming over the last century than at any other time during the last 1000 years (IPCC 2001). Since the nineteenth century, global mean air temperature has increased by ca. 0.75 °C, and is projected to keep increasing by a further 1.1–4.8 °C by 2100 (IPCC 2014). Since air and water temperatures are closely associated, stream water temperature will mirror this increase (Langan et al. 2001; Pilgrim et al. 1998; Stefan and Sinokrot 1993). Due to its fundamental role in biological processes (Friberg et al. 2009; Woodward et al. 2010), higher temperatures trigger various ecological responses. For instance, ecological responses can occur through changes in community dynamics, population density, species distribution and organismal phenology (Durance and Ormerod 2007; Frainer et al. 2017; Walther et al. 2002) and may lead to substantial modifications to ecosystem function.

In forested headwater streams, aquatic food webs are mostly maintained through allochthonous organic matter provided by riparian vegetation, with dead leaves representing the main source of carbon and energy (Benfield 2006). At the bottom of these food webs, aquatic hyphomycetes are the main drivers of microbial leaf-litter decomposition (Bärlocher 1985). Moreover, surface-attached biofilms, composed of diverse heterotrophic and autotrophic microorganisms (e.g. bacteria, archaea, protozoa, fungi and algae),

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s00027-019-0627-2) contains supplementary material, which is available to authorized users.

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can also play an important role in stream primary production (Weitzel 1979; Schnurr and Allen 2015). Both microbial compartments take part in aquatic food webs and contribute substantially to biochemical processes and ecosystem functioning (Besemer 2015; Romaní 2010). As they contribute in opposite ways to the carbon cycle (carbon fixation vs. carbon mineralization) and may have different sensitivities to temperature and seasonal variability, climate change could have critical effects on carbon cycle balance in streams (Song et al. 2018; Yvon-Durocher et al. 2010).

Due to the key ecological position of micro-organisms, many studies focused on the effect of rising temperature on microbially driven decomposition and aquatic hyphomycete communities. Results, though, remain equivocal. For example, Gonçalves et al. (2013) reported no effect of increased temperature on alder leaf microbial decomposition, but a faster microbial decomposition of oak leaves in microcosms. In contrast, in a global field experiment, Boyero et al. (2011) found an increase of alder microbial decomposition along a latitudinal gradient of temperature. Finally, Ferreira and Canhoto (2015) observed a stimulation of the microbial decomposition of oak in an experimentally warmed stream, but only in winter.

Inconsistencies are likely due, in part, to the use of different strategies to manipulate temperature across studies. To date, most of them were conducted either under controlled laboratory conditions (Dang et al. 2009; Ferreira and Chauvet 2011a, b; Fernandes et al. 2012, 2014; Geraldes et al. 2012; Gonçalves et al. 2013; Martínez et al. 2014; Mas-Martí et al. 2015), *in situ* using large-scale gradients (i.e., latitudinal or altitudinal; Boyero et al. 2011, 2016; Casas et al. 2013; Fenoy et al. 2016; Fleituch 2001; Martínez et al. 2014; Unterseher et al. 2016) or through experimental warming of a stream (Ferreira and Canhoto 2015).

Each of these interesting approaches has its own limitations: from the over-simplification of the systems (e.g. microcosms) to the inability to disentangle the effects of temperature from other abiotic and/or biotic drivers over biogeographic scales. Midway between those opposite strategies, natural geothermal gradients offer a promising approach to study and understand the effects of global warming on stream functioning by isolating temperature from other drivers and by including the complexity of natural systems (O'Gorman et al. 2014).

The main objective of this study was to investigate the effects of water temperature on alder leaf litter decomposition and on primary producers. To this end, we relied on a natural temperature gradient provided by a temperate geothermal spring exiting the ground at 21 °C all year round and progressively cooling downstream through contact with air, and to a lesser extent through inputs of colder runoff water. We replicated the experiment across two seasonal contexts (spring and winter), in order to enlarge the temperature gradient of ca. 1 °C in spring to ca. 5 °C in winter. We hypothesized that even small gradients in water temperature would lead (1) to an increase in leaf microbial decomposition (2) through changes in leaf-associated aquatic hyphomycete communities, and (3) to an increase of in situ algal production. Finally, we expected the effect of temperature to be more apparent in winter for decomposition (Ferreira and Canhoto 2015), because of the wider temperature gradient at this season, and in spring for primary production, due to higher solar radiation (Delgado et al. 2017; Olapade and Leff 2005).

## Materials and methods

### Study site and stream characterization

The study was carried out in spring (April–May 2016) and winter (January–February 2017) in Chaudefontaine, a first-order stream fed by a temperate geothermal source, located in Vecoux (Vosges Mountains, north-eastern France; 47°58′01.8″ N, 6°39′56.5″ E, 540 m a.s.l.). The spring outlet exhibits a constant temperature of 21 °C and provides a constant discharge throughout the year. The water of this small forested stream (length = 106 m) cools down from upstream to downstream through contact with air until it reaches a cold second-order stream. The watercourse is surrounded by a mixed coniferous forest mainly composed of silver fir (*Abies alba*), Norway spruce (*Picea abies*), black alder (*Alnus glutinosa*), beech (*Fagus sylvatica*) and underlain by granite. The riverbed of the stream mainly consists of sand and gravel with some cobbles.

The experiments were conducted at four sites, each separated by ca. 20.4 m, chosen along the stream section to capture the widest temperature range and maximize between-sites temperature differences. At each site, water temperature was recorded every 30 min with submerged data loggers (Hobo Pendant UA-001-08, Onset Computer Corp., Massachusetts, USA). The mean difference in water temperature (± SD) between sites 1 and 4 was 1.10 ± 0.33 °C in spring and 4.94 ± 1.27 °C in winter (Fig. 1).

Water samples (500 mL) from each site were collected on four occasions during the experiments. Stream pH was measured in the laboratory using a microprocessor pH meter (pH 3000, WTW) and acid-neutralizing capacity (ANC) was determined by Gran's titration. Conductivity was measured with a Metrohm Herisau Conductometer E518 (Herisau, Switzerland) at 25 °C. Concentrations in $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ and $K^{+}$ were determined by atomic absorption spectrophotometry (Aanalyst 100; Perkin Elmer and Varian SpetrAA-300) and concentrations in $Cl^{-}$, $NO_3^{-}$, $PO_4^{2-}$ and

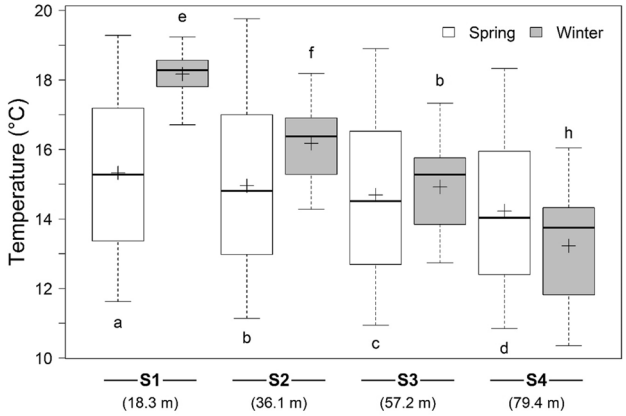


**Fig. 1** Water temperature at the four sites (ordered by increasing distance along the stream reach) across the two study periods. Each boxplot represent 1344 temperature observations collected during the experiments (central line is median; plus sign is mean; box top and bottom are first and third quartiles; whiskers are confidence limits). Sites with the same letter do not differ significantly (Kruskal–Wallis test, $p > 0.05$)

$SO_4^{2-}$ by ion chromatography (Dionex 1500i; Sunnyvale, USA; Clivot et al. 2013; Cornut et al. 2012) (Table 1).

## Leaf conditioning

Leaves of alder (*Alnus glutinosa* (L.) Gaertn.), a common riparian tree species in the area and in Europe, were collected just after abscission in October 2015 and 2016. For each season, 480 discs (diameter16 mm) were cut from the leaves with a cork-borer, avoiding the central vein, and air-dried in the dark at ambient temperature until needed. Discs were individually weighed (±0.1 mg) to determine initial dry mass (DM) and enclosed in fine-mesh bags composed of eight rows of five discs (40 discs; bag size: 13 × 22.8 cm, 0.25 mm mesh). Three bags (i.e. replicates) were submerged at each site. At each sampling date, two rows of five discs per replicate bag were sampled (i.e., one row for mass loss estimation and the other one for fungal biomass and diversity measurements), placed in individual ziplock bags containing stream water, and transported to the laboratory in a cooler. The samples were retrieved after 7, 14, 21 and 28 days.

## Leaf mass loss

At each sampling date, five discs from each bag were gently washed with distilled water, oven-dried at 60 °C for 72 h to constant mass, and weighed (±0.1 mg) to determine final DM. The discs were then ignited in a muffle furnace at 550 °C for 4 h to determine the ash-free dry mass (AFDM). Three unexposed sets of five discs were used to determine initial leaf AFDM.

**Table 1** Water parameters at the four different sites and averaged across the two seasons during the litter decomposition experiments

| Site | pH | Conductivity ($\mu S\ cm^{-1}$) | ANC ($\mu eq\ L^{-1}$) | $Cl^-$ ($mg\ L^{-1}$) | $NO_3^-$ ($mg\ L^{-1}$) | $SO_4^{2-}$ ($mg\ L^{-1}$) | $PO_4^{2-}$ ($mg\ L^{-1}$) | $Ca^{2+}$ ($mg\ L^{-1}$) | $Mg^{2+}$ ($mg\ L^{-1}$) | $Na^+$ ($mg\ L^{-1}$) | $K^+$ ($mg\ L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | 7.98 ± 0.03 | 135.7 ± 13.4 | 1186 ± 167 | 1.30 ± 0.50 | 2.68 ± 0.55 | 2.14 ± 0.18 | 0.04 ± 0.04 | 15.24 ± 1.86 | 3.61 ± 0.43 | 5.17 ± 0.21 | 1.98 ± 0.10 |
| S2 | 8.01 ± 0.05 | 135.3 ± 12.9 | 1195 ± 160 | 1.32 ± 0.51 | 2.69 ± 0.55 | 2.16 ± 0.20 | 0.03 ± 0.02 | 15.05 ± 1.88 | 3.56 ± 0.45 | 5.15 ± 0.21 | 1.98 ± 0.10 |
| S3 | 8.04 ± 0.07 | 133.3 ± 14.6 | 1175 ± 162 | 1.32 ± 0.50 | 2.65 ± 0.60 | 2.16 ± 0.20 | 0.03 ± 0.02 | 14.91 ± 1.98 | 3.55 ± 0.48 | 5.13 ± 0.24 | 1.94 ± 0.13 |
| S4 | 8.00 ± 0.08 | 135.0 ± 12.5 | 1190 ± 146 | 1.40 ± 0.57 | 2.65 ± 0.60 | 2.28 ± 0.38 | 0.05 ± 0.03 | 14.97 ± 1.69 | 3.55 ± 0.45 | 5.27 ± 0.28 | 1.99 ± 0.15 |

Values are means ± SD

## Fungal biomass and diversity

To determine sporulation rates and species composition of leaf-associated fungal assemblages, five other discs from each bag were placed in 100-mL Erlenmeyer flasks filled with 20 mL of filtered stream water (glass microfibre GF/F, Whatman; pore size 0.7 µm) at each sampling date. They were then incubated for 48 h on an orbital shaker (100 rpm) at 17 °C in the dark. Unfortunately, in spring, remaining masses were insufficient to perform fungal biomass and diversity analyses at 28 days.

After incubation, the discs were removed and frozen at –20 °C before ergosterol content measurement according to Gessner and Newell (2002). Ergosterol was quantified by HPLC (Gessner 2005), and then converted into fungal biomass using a conversion factor of 5.5 µg ergosterol per mg fungal dry mass (Gessner and Chauvet 1993).

Conidial suspensions were poured into 50-mL Falcon tubes, fixed with 2 mL of 37% formalin, and the final volume was adjusted to 40 mL with distilled water. 250 µL TritonX-100 (0.5%) was added to the suspension, mixed with a magnetic stirring bar to ensure uniform distribution of conidia, and an aliquot (1–10 mL) of the suspension was filtered through membrane filters (25 mm diameter, pore size 5 µm; Millipore SMWP, Millipore Corporation, MA, USA). Filters were stained with 0.1% Trypan blue in 60% lactic acid (Iqbal and Webster 1973), and conidia were identified and counted under a microscope at ×200 magnification (Bärlocher 2005). Sporulation rate was expressed as the number of conidia released per mg leaf AFDM per day, and per mg fungal biomass per day.

Moreover, conidial assemblages from stream water were characterized following the same procedure, based on three filtrations of 100 mL of the stream water from each site and for each season (25 mm diameter, pore size 5 µm; Millipore SMWP, Millipore Corporation, MA, USA).

## Chlorophyll-*a*

At each site, six ceramic tiles (individual upper surface area: 5×5 cm) were placed on the stream bed for 28 days. At 7, 14, 21 and 28 days, the overall biomass of benthic algal assemblages colonizing the ceramic tiles was quantified *in situ* using the BenthoTorch (bbe Moldaenke), a fluorometric probe designed for use in the field that has been proven reliable for total chlorophyll-*a* concentration assessments (see Kahlert and McKie 2014). The BenthoTorch was directly applied to the surface of each ceramic tile ensuring that the foam pad around the diodes shaded the biofilms from external light. The overall biomass of benthic algae was expressed as µg chlorophyll-*a* per $cm^2$.

## Data analysis

Decomposition rates of alder leaf discs ($k$) were calculated assuming (a) a linear decay, by regression of the linear model $Mt = M0 - k \times t$ (where M0 is the initial AFDM, Mt is the AFDM at time t and $k$ is the decomposition rate), and (b) an exponential decay, by linear regression of ln(Mt) vs incubation time ($Mt = M0 \times e^{-k \times t}$; Pozo and Colino 1992). Comparison of $R^2$ of linear and exponential models indicated a slightly better fit of the linear model, in particular in spring (Table 2). Although the exponential model is more commonly used in published literature, further analyses thus relied on the linear decomposition rates.

Similar statistical analyses have been performed on fungal biomass and sporulation rates at 21 days, on chlorophyll-*a* concentration at 28 days and on linear decomposition rates (Table 3). The effects of season, temperature and interaction between season and temperature on the four variables studied were evaluated using linear mixed-effects models (LME; *nlme-package* in R) with random effects of mesh bags or ceramic tiles (i.e. blocks) identity nested into site. The significance of independent factors in LMEs was evaluated using type "III" or "II" sum of squares (*car-package*

**Table 2** Linear and exponential decomposition rates of alder leaf discs incubated at each of the four sites characterized by their mean temperature in both seasons (spring and winter), and coefficient of determination of the regression

| Seasons | Sites | Mean temperature (°C) | Linear model ($d^{-1}$) | | Exponential model ($d^{-1}$) | |
|---|---|---|---|---|---|---|
| | | | $k$ | $R^2$ | $k$ | $R^2$ |
| Spring | S1 | 15.3 | $0.031^{b}$ | 0.94 | $0.070^{ab}$ | 0.85 |
| | S2 | 15.0 | $0.033^{b}$ | 0.94 | $0.107^{ac}$ | 0.71 |
| | S3 | 14.7 | $0.031^{bc}$ | 0.96 | $0.084^{ab}$ | 0.78 |
| | S4 | 14.2 | $0.034^{b}$ | 0.92 | $0.100^{ab}$ | 0.76 |
| Winter | S1 | 18.2 | $0.021^{a}$ | 0.91 | $0.033^{b}$ | 0.96 |
| | S2 | 16.2 | $0.021^{ac}$ | 0.89 | $0.034^{b}$ | 0.94 |
| | S3 | 14.9 | $0.024^{ab}$ | 0.94 | $0.043^{ab}$ | 0.96 |
| | S4 | 13.2 | $0.023^{ac}$ | 0.96 | $0.038^{ab}$ | 0.92 |

Treatments with the same letter are not significantly different (TukeyHSD test, $p > 0.05$)

**Table 3** Summary table of mixed-effects models performed on decomposition rates (28 days), fungal biomass (21 days) and [log-transformed] sporulation rates (21 days) and chlorophyll-*a* concentration (28 days), at each of the four sites in both seasons (spring and winter)

| | Decomposition rate | | | Fungal biomass | | | Sporulation rate | | | Chlorophyll-*a* concentration | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n = 24 | | | n = 24 | | | n = 24 | | | n = 48 | | |
| | Estimate ± SE | $X^2$ | *p* value | Estimate ± SE | $X^2$ | *p* value | Estimate ± SE | $X^2$ | *p* value | Estimate ± SE | $X^2$ | *p* value |
| Model 1: lme(X ~ Season + Temperature + Season × Temperature); Type "III" Anova | | | | | | | | | | | | |
| Intercept | 0.055 ± 0.04 | 2.40 | 0.122 | 277 ± 119 | 5.41 | 0.020 | 16.68 ± 5.76 | 8.38 | 0.004 | − 46.21 ± 5.67 | 66.43 | < 0.0001 |
| Season | − 0.025 ± 0.04 | 0.48 | 0.490 | − 246 ± 122 | 4.05 | 0.044 | 8.29 ± 5.55 | 2.23 | 0.135 | 40.82 ± 5.82 | 49.10 | < 0.0001 |
| Temperature | − 0.002 ± 0.00 | 0.42 | 0.517 | − 17 ± 9 | 3.93 | 0.048 | − 0.74 ± 0.42 | 3.17 | 0.075 | 3.17 ± 0.38 | 68.58 | < 0.0001 |
| Season × temperature | 0.001 ± 0.00 | 0.18 | *0.675* | 17 ± 9 | 3.64 | *0.056* | 0.50 ± 0.40 | 1.54 | *0.214* | − 2.89 ± 0.39 | 54.24 | **< 0.0001** |
| Random effect | 0.0012 | | | 0.0001 | | | 0.2896 | | | 0.0004 | | |
| Marginal $R^2$/ conditional $R^2$ | 0.7/0.8 | | | 0.4/0.4 | | | 0.7/0.8 | | | 0.7/0.8 | | |
| Model 2: lme(X ~ Season + Temperature); Type "II" Anova | | | | | | | | | | | | |
| Season | − 0.010 ± 0.00 | 53.25 | **< 0.0001** | − 13.62 ± 5.74 | 5.21 | **0.022** | − 1.63 ± 0.24 | 35.10 | **< 0.0001** | | | |
| Temperature | − 0.001 ± 0.00 | 1.19 | 0.274 | − 0.97 ± 1.83 | 0.28 | 0.597 | − 0.23 ± 0.09 | 7.16 | **0.007** | | | |
| Random effect | 0.0012 | | | 0.0001 | | | 0.0309 | | | | | |
| Marginal $R^2$/ conditional $R^2$ | 0.7/0.8 | | | 0.3/0.3 | | | 0.7/0.8 | | | | | |

Degrees of freedom of each independent variable = 1

Significant *p* values are in bold

in R), depending on the presence of interactions. Indeed, when not significant, the interaction was removed for model simplification. Season, site and block identity were considered as categorical variables, whereas temperature was integrated as a continuous variable in all LME models. The model assumptions (normality and homoscedasticity) were assessed graphically, as well as with Shapiro tests on the residuals. When necessary, dependent variables (sporulation rates and chlorophyll-*a* concentration) were log-transformed in order to achieve normality. Finally, to assess the effect of between-sites differences in both seasons on each variable, linear models were constructed with season and site as categorical variables, then analyzed with ANOVA, and finally followed by Tukey HSD post hoc test ($p < 0.05$).

The possible influence of the distance from the source was analyzed for ambient conidial counts (to represent inocula) before the beginning of the experiment at each site and in each season with Jaccard similarity (J) and Bray-Curtis dissimilarity (BC) coefficients.

During the experiment, variations in fungal assemblages associated with leaf discs were analyzed by nonmetric multidimensional scaling ordination (NMDS) based on Bray-Curtis dissimilarity of each species sporulation rate data. PERMANOVA was performed to test for effects of time, season, temperature and interactions between variables on community assemblages (*RVAideMemoire-package* in R).

Finally, multiple comparison tests after Kruskal–Wallis (*kruskalmc; pgirmess-package* in R) were used to analyze temperature differences between sites.

All statistical analyses were performed with the R software (R Core Team 2017, version 3.4.2.).

## Results

### Stream water characterization

The mean temperature range was wider in winter than in spring ($4.94 \pm 1.27$ °C and $1.10 \pm 0.33$ °C respectively, Fig. 1). Thus, in winter, mean temperature of the warmest site (S1) was higher than in spring (18.2 °C and 15.2 °C, respectively), while it was lower at the coldest site (S4; 13.2 °C in winter and 14.2 °C in spring, respectively). On average, the temperature difference (± SD) between two successive sites (S1–S2; S2–S3 and S3–S4) was $0.37 \pm 0.09$ °C in spring and $1.65 \pm 0.37$ °C in winter during the 28 days of experiment. It resulted in a global 429–398 degree-days gradient in spring and a 508–307 degree-days gradient in winter. Temperatures were significantly different between all site × season combinations except for S2-spring and S3-winter (Fig. 1).

During the experiments, the stream water characteristics remained rather constant through time and did not differ between sites. Water was slightly alkaline and relatively nutrient-poor (Table 1) in comparison with other streams of the area (Dangles et al. 2004). Air temperature was higher in spring than in winter ($8.52 \pm 5.85$ °C and $-0.17 \pm 5.86$ °C on average, respectively). Finally, aquatic hyphomycete inocula before the onset of the experiment were very similar between sites in spring (BC < 0.16 and J > 0.9), with the similarity indices being slightly lower in winter (BC < 0.34 and J > 0.8).

### Decomposition rates

Alder leaf litter lost 83–96% of its initial AFDM over 28 days in spring and 61–69% in winter. Whatever the range of temperature observed (1.1 °C in spring and 4.9 °C in winter between sites 1 and 4), linear decomposition rates were not significantly different among the four sites within a season (Table 2). Overall, we observed a strong seasonal effect (Table 3; Model 2; Season, $p < 0.0001$) with significantly lower decomposition rates in winter (0.021–0.024 $day^{-1}$) than in spring (0.031–0.034 $day^{-1}$). This 1.3- to 1.6-fold lower decomposition in winter occurred independently of temperature differences between winter and spring. Mean water temperature in winter was actually higher for site 1 and 2 (respectively + 2.8 °C and + 1.2 °C than in spring), but lower for site 4 (–1.0 °C than in spring). By contrast, temperature was similar across seasons on site 3 (0.2 °C higher in winter).

### Fungal biomass and diversity

Temporal dynamics of fungal colonization differed between seasons (Fig. S1): while fungal biomass was stationary between 7 and 28 days in winter; it generally reached its highest value at day 21 in spring. After 21 days, average fungal biomass was lower in winter than in spring (26.6 and 41.2 mg $g^{-1}$ AFDM, respectively; Fig. 2). Furthermore, fungal biomass after 21 days decreased with increasing temperature in spring (Fig. 2, dashed line regression, $R^2 = 0.26$, $p = 0.05$), while it was not influenced by temperature in winter (Fig. 2, solid line regression, $R^2 = 0$, $p = 0.88$). Finally, the influence of the interaction of season and temperature was marginally significant (Table 3; Model 1; Season × Temperature $p = 0.06$). However, in the model without interaction, a significantly higher fungal biomass (measured at 21 days) was revealed in spring (Table 3; Model 2; Season, $p = 0.022$).

After 21 days (temporal dynamics provided in Fig. S2), total sporulation rates of aquatic hyphomycetes associated with alder litter showed a similar response pattern to fungal biomass (Fig. 3). Indeed, mean sporulation rate at 21 days was higher in spring than in winter, despite the wider temperature gradient observed in winter (723 and 135 conidia

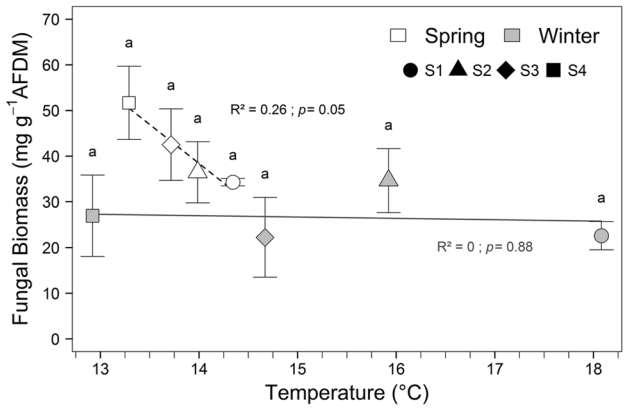


**Fig. 2** Mean fungal biomass ± SE associated with alder leaf discs incubated at the four sites along the temperature gradient across the two study periods after 21 days. Lines represent regressions with mean temperature (dashed for spring, $R^2 = 0.26$; solid for winter, $R^2 = 0$). Points with the same letter do not differ significantly (Tukey's HSD test)

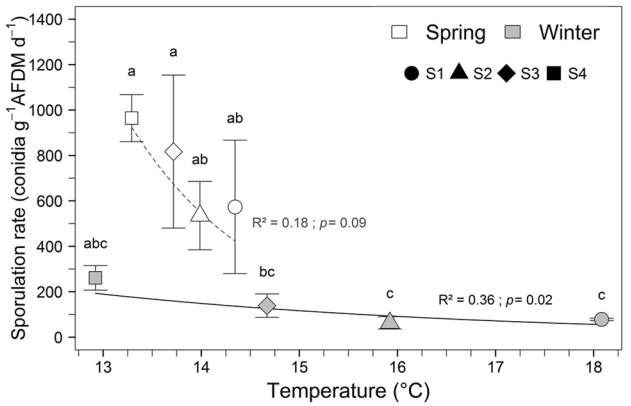


**Fig. 3** Mean sporulation rate ± SE of aquatic hyphomycetes associated with alder leaf discs incubated at the four sites along the temperature gradient across the two study periods after 21 days. Lines represent regressions with mean temperature (dashed for spring, $R^2 = 0.18$; solid for winter, $R^2 = 0.36$). Points with the same letter do not differ significantly (Tukey's HSD test)

$mg^{-1}$ AFDM $day^{-1}$ in spring and winter, respectively). Sporulation rate decreased with increasing temperature, though more slightly in spring (Fig. 3, dashed line regression, $R^2 = 0.18$, $p = 0.09$) than in winter (Fig. 3, solid line regression, $R^2 = 0.36$, $p = 0.02$). Our models revealed significant influence of both season and temperature (Table 3; Model 2; Season, $p < 0.0001$; Temperature, $p = 0.007$). Furthermore, when expressed as conidia per unit of fungal biomass, sporulation rates were similar in sites below a mean temperature of 15 °C (with an average of 541 ± 74 conidia $mg^{-1}$ fungal biomass $day^{-1}$). In sites with higher temperatures, sporulation rates per unit of fungal biomass dropped to 156 ± 51 conidia $mg^{-1}$ fungal biomass $day^{-1}$ (data not shown).

Aquatic hyphomycete species richness was similar between seasons and sites, with a total of 18 species observed (Fig. S3). The dominant species at all sampling times and sites in both seasons was *Lunulospora curvula*, whose relative contribution to total sporulation rate was particularly high during early leaf decomposition stages (Fig. 4a). On the contrary, a shift between spring and winter occurred in the relative contribution of the second dominant species (*Articulospora tetracladia* in spring, *Clavariopsis aquatica* in winter; Fig. 4a). The NMDS ordination of fungal communities based on sporulation rates (Fig. 4b) indicates that time and season had a significant influence on aquatic hyphomycete community structure. PERMANOVA revealed that season (Season, $p = 0.001$) and time (Time, $p = 0.001$), as well as all double interactions of the factors significantly affected community structure (PERMANOVA, Season × Time, $p = 0.013$; Temperature × Time, $p = 0.011$; Season × Temperature, $p = 0.012$).

### Chlorophyll-*a* concentration

During the experiment, chlorophyll-*a* (chl-*a*) concentration in biofilm on the ceramic tiles increased gradually from 0 to 28 days (Fig. S4). After 28 days, mean chl-*a* concentration was almost ninefold higher in spring than in winter (Fig. 5; 4.53 and 0.52 µg $cm^{-2}$, respectively). However, while an increase of 1 °C in spring led to a 24.7-fold increase in mean chl-*a* concentrations (Fig. 5, dashed line regression, $R^2 = 0.67$, $p < 0.0001$), an increase of 5 °C in winter only led to a 5.3-fold increase (Fig. 5, solid line regression, $R^2 = 0.38$, $p < 0.001$). The interaction of season and temperature had a significant influence on chl-*a* concentration after 28 days (Table 3; Season × Temperature, $p < 0.0001$).

## Discussion

Contrasting with the current leading conceptions of temperature effects on biological rates (Brown et al. 2004), our results suggest that those effects are mostly season-dependent, at least in a geothermal spring located in a temperate area. Indeed, while we expected the increase of temperature to stimulate decomposition process and algal production, differences in seasonal factors appeared to be more determining than temperature itself. Our different variables exhibited contrasting variations along the temperature gradient: while decomposition rates did not vary with temperature, high temperature was associated with high biofilm autotrophs biomass, but low fungal biomass and sporulation rate. Interestingly, correcting sporulation data by fungal biomass allowed us to determine that this decreased fungal spore production with increasing temperature was mostly driven by an effect on fungal biomass, but did not reflect any

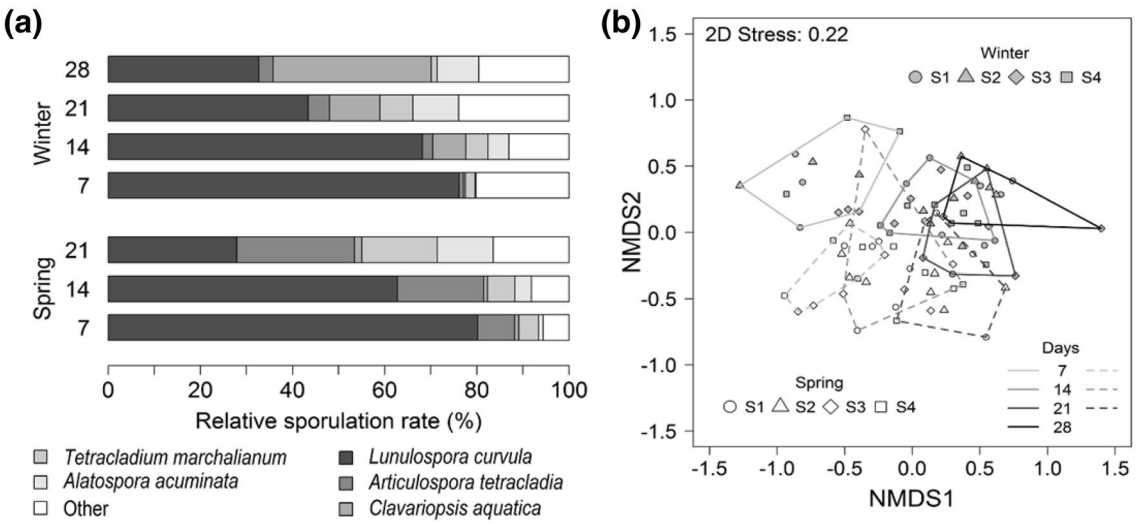


**Fig. 4** Percentage contribution of aquatic hyphomycete species to total sporulation rate (**a**) and NMDS (**b**) ordination diagram based on fungal communities assessed from conidia released from alder leaf discs during 7, 14, 21 and 28 days of immersion at the four sites along the negative temperature gradient in spring 2016 and winter 2017

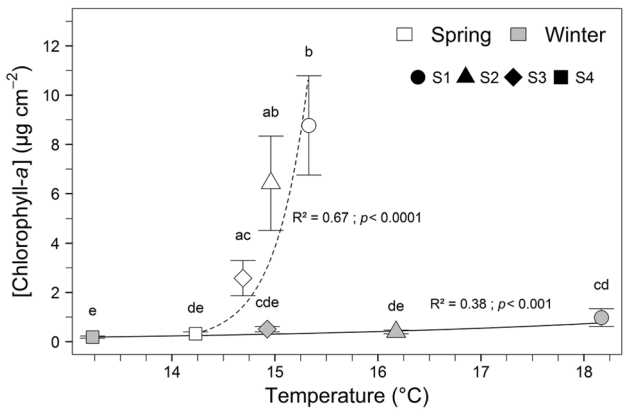


**Fig. 5** Mean chlorophyll-*a* concentration ± SE after 28 days of immersion at the four sites along the temperature gradient across the two study periods. Lines represent regressions with mean temperature (dashed for spring, $R^2 = 0.67$; solid for winter, $R^2 = 0.38$). Points with the same letter do not differ significantly (Tukey's HSD test)

temperature effect on fungal reproductive activity. In contrast, temperature-independent seasonal variations affected all the variables observed, with average values significantly lower in winter than in spring.

The observed temperature independence of microbial decomposition contrasts with results of several previous field and microcosm studies conducted in similar ranges of temperature (Fenoy et al. 2016; Fernandes et al. 2014; Ferreira and Chauvet 2011a, b). However, it echoes results by Ferreira and Canhoto (2014, 2015) who observed no effect of temperature increase on litter decomposition in spring in manipulative experiments under similar temperature ranges. Furthermore, linear decomposition rates of alder leaf-litter observed in this study are consistent with those found by Baudoin (2007) in two reference streams in winter (0.025 and 0.024 $day^{-1}$; mean temperature: 6.5 and 4.8 °C, repectively). Consistent with the decomposition dynamics, fungal biomass and aquatic hyphomycete community composition exhibited no clear pattern along the temperature gradient, as found by Ferreira et al. (2014) and Ferreira and Canhoto (2014, 2015). Several potential mechanisms can explain together the temperature invariance of litter decomposition. First, several studies found that temperature and nutrient availability could have synergistic effects on microbial activity and litter decomposition (Ferreira and Chauvet 2011a; Fernandes et al. 2014 but see; Manning et al. 2018). This interactive influence can explain weak temperature effects in oligotrophic conditions such as in our stream (Ferreira and Chauvet 2011a). Moreover, temperature effects on biological activities typically follow a hump-shaped relationship, with a decrease of performances at higher temperature than typically experienced by organisms (Huey and Stevenson 1979). To date, very little is known about the thermal performance curves of aquatic microbial decomposers, and it is not unlikely that the temperature of our stream exceeded the optimal temperature for microbial decomposition in temperate systems. Finally, temperature can have indirect effects on processes through changes in community structure and trophic interactions among species. For instance, several studies performed in soil ecosystems suggested thad mid- to long-term warming experiments could fail to elicit any temperature effect on microbially driven processes due to quick adjustments of microbial communities (Bradford et al. 2008; Giardina and Ryan 2000; Wei et al. 2014). However, in our study, the lack of temperature effect on fungal community

structure, as well as the proximity between sites (with likely fluxes of conidia between sites) make this latter explanation unlikely. Thus, the adjustment to temperature, if any, might here occur at the intra-specific level (e.g. through physiological response) and does not involve any shift in species composition. Indeed, *Lunulopsora curvula* was the dominant species at each site in both seasons, although it is classically found in warm waters such as from tropical ecosystems, or during summer in temperate ecosystems.

In contrast with the heterotrophic microbes, we observed that even a small increase of temperature (~ 1 °C) in spring had a strong positive effect on biofilm chlorophyll-*a* concentration. This suggests a strong effect of temperature on autotrophic microbial colonization and growth (Delgado et al. 2017; Díaz-Villanueva et al. 2011), which could reflect the kinetic effect of temperature on the enzymes involved in photosynthesis (Allen et al. 2005; Padfield et al. 2017). Alternatively, this could also be due to an increased chlorophyll-*a* concentration in the biofilm grown under warm conditions, as described by Ylla et al. (2014).

In winter, even a large temperature increase (~ 5 °C) did not hasten biofilm production of chlorophyll-*a* to an extent seen in spring, with winter algal biomass accrual remaining low between 13 and 18 °C. This pattern, together with the strong effect of season on decomposition rates, fungal biomass and sporulation rates, suggests that season has an overwhelming importance in determining ecosystem process rates that cannot be explained by temperature seasonal variations only. In the case of autotrophic biofilms, primary production is likely limited by light availability in winter, explaining the lower values and temperature-dependency of chlorophyll-*a* concentrations (Romaní et al. 2014). Concerning fungal decomposers, higher decomposition rates in spring (despite lower temperature) could be due to shifts in community composition, which could reflect among-species variations in phenology (Suberkropp 1984). Alternatively, light availability could influence directly or indirectly fungal decomposers as well. This could be due to a direct effect of light on aquatic hyphomycete activity (Rajashekhar and Kaveriappa 2000) or to interactions with autotrophic biofilms (Halvorson et al. 2016). Indeed, assuming that the increase in algal biomass accrual observed in spring on ceramic tiles occurred on any hard surface of the watercourse (including on leaf discs), it is likely that auto- and heterotrophs interacted. We hypothesized that a priming effect, a mechanism by which a release of labile carbon by autotrophic biofilms stimulates the decomposition of refractory carbon by heterotrophic micro-organisms (Danger et al. 2013; Kuehn et al. 2014) may have led to higher decomposition rates in spring than in winter. Otherwise, the amounts of labile carbon released by phototroph's activity are transported by water and can therefore benefit surrounding fungi, even if physically separated. Finally, other seasonal drivers might have influenced decomposition rates and fungal communities, such as consumer activity or nutrient availability. However, in the present study, nutrient concentrations did not exhibit significant temporal variations.

Using a natural and original ecosystem, our study opens a new perspective on the effect of temperature on microbially-driven processes in streams, despite some obvious limitations. The small size and scarcity of this system limits our ability to study temperature effects over larger and/or colder gradients, and precludes the replication of the experiment in another similar system. Based on the results of our study, we strongly recommend improving the grain (number of intermediate values) and extent (range between extreme values) of temperature gradients, which would probably allow us to determine the optimal temperatures of ecosystem processes in headwater streams. It remains that such springs are natural ecosystems and as such exhibit complex and representative communities and food webs, compared to oversimplified laboratory assemblages. Most importantly, they allow overcoming the technical challenge associated with temperature manipulation in natural streams (Canhoto et al. 2013), and provide a similar water temperature across seasons. It is thus a useful tool for the assessment of temperature effects on ecological processes at the ecosystem scale, as suggested in previous studies on Icelandic tundra geothermal ecosystems (O'Gorman et al. 2014; Woodward et al. 2010) and should allow a better understanding of the influence of short- (e.g. seasonal) to long-term (e.g. climate warming) temperature variations on communities and ecosystem processes under temperate latitudes as well.

**Acknowledgements** We thank P. Rousselle and D. Lambrigot for water and ergosterol analyses, respectively, and P. Wagner, M. Odinot, and F. Louis for valuable help in the field. We are grateful to Christopher Swan, Stuart Findlay and two anonymous reviewers who provided excellent feedback and suggestions to improve this manuscript. The present study was funded by the French National Research Agency (ANR-14-CE01-0009) and the French National Program EC2CO (2016–2017).

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