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# Temperature and nutrient effects on the relative importance of brown and green pathways for stream ecosystem functioning: A mesocosm approach

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## Abstract

1. In addition to global warming, aquatic ecosystems are currently facing multiple global changes among which include changes in nitrogen (N) loads. While several studies have investigated both temperature and N impacts on aquatic ecosystems independently, knowledge on their interactive effects remains scarce.
2. In forested headwater streams, decomposition of leaf litter represents the main process ensuring the transfer of nutrients and energy to higher trophic levels, followed by autochthonous primary production, mainly ensured by phototrophic biofilms. The main aim of this study was to disentangle the independent and combined effects of temperature increase and nutrient availability on the relative importance of brown and green processes involved in stream functioning. We hypothesised that water temperature and nutrients would lead to a general increase in leaf-litter decomposition and primary production, but that the intensity of these effects would be largely modulated by competitive interactions arising between microorganisms as well as by the top-down control of microorganisms by macro-invertebrates. Macro-invertebrates would, in turn, be bottom-up controlled by microbial resources quality.
3. To test these hypotheses, we conducted a 56-day experiment in artificial streams containing leaf litter, microbial decomposers and biofilm inoculum, and an assemblage of macro-invertebrates. Two water inorganic N:phosphorus (P) ratios (33 and 100, molar ratios) and two temperatures (ambient, +2°C) were manipulated, each treatment being replicated three times. Fungal and biofilm growth as well as leaf-litter decomposition and primary production were quantified. Top-down impacts of invertebrate primary consumers on brown and green compartments were evaluated using exclosures while bottom-up control was evaluated through the measurement of resource stoichiometry and fatty acid profiles, as well as quantification of macro-invertebrate growth and survival.
4. Contrary to expectations, microbial decomposition was not significantly stimulated by nutrient or temperature manipulations, while primary production was only improved under ambient temperature. In the + 2°C treatment with high N:P,

greater biofilm biomass was associated with lower fungal development, which indicates competition for nutrients in these conditions. Temperature increased macro-invertebrate growth and leaf-litter consumption, but this effect was independent of any improvement of basal resource quality, suggesting that temperature mediated changes in consumer metabolism and activity was the main mechanism involved.

5. Most of our hypotheses that were based on simplified laboratory observations have been rejected in our semi-controlled mesocosms. Our study suggests that the complexity of biological communities might greatly affect the response of ecosystems to multiple stressors, and that interactions between organisms must be explicitly taken into account when investigating the impacts of global change on ecosystem functioning.

#### KEYWORDS

decomposition, fatty acids, headwater streams, primary production, top-down versus bottom-up control

## 1 | INTRODUCTION

The functioning of forested headwater streams mostly relies on allochthonous organic matter inputs from the riparian zone (Wallace, Eggert, Meyer, & Webster, 1997). Mainly composed by dead leaves, this organic material represents the major energy source at the base of food webs (commonly qualified as brown food webs), and its decomposition plays a non-negligible role in the global carbon (C) cycle (Benfield, 2006). As they reach streams, leaves are rapidly colonised by aquatic fungi, which convert refractory leaf litter to more bioavailable nutrients and energy that fuel aquatic food webs. Microbial activity initiates leaf-litter decomposition (Bärlocher, 1985), and provides a digestible food resource for numerous detritivorous macro-invertebrates. Hence, both detritivores and microbes contribute significantly to the key process of decomposition in streams (Hieber & Gessner, 2002). In addition to these detritus-based processes, the forested riparian zone of headwater streams strongly reduces light availability and limits the development of aquatic autotrophic organisms (Richardson & Danehy, 2007). In such heavily shaded streams, surface-attached phototrophic biofilms ensure most of the in-stream primary production (Weitzel, 1979). While reduced, this autochthonous production (green pathway) of high quality organic matter can be extremely important for stream functioning, impacting, for example, leaf-litter decomposition (Danger, Cornut, et al., 2013; Halvorson et al., 2018; Kuehn, Francoeur, Findlay, & Neely, 2014) and representing a potentially important input of essential compounds to higher trophic levels (e.g. essential fatty acids; Crenier et al., 2017; Guo, Kainz, Sheldon, & Bunn, 2016). Despite the growing interest in the potential importance of the green pathways in the functioning of detritus-based headwater streams, most studies dealing with stream functioning have omitted this autotrophic compartment. However, interactions between the green and the brown food

webs might significantly complicate our view of stream functioning and stream responses to anthropogenic stressors.

Over the last decade (2006–2015), anthropogenic global warming induced a 0.87°C increase in mean global surface temperature above pre-industrial levels. As it is currently increasing at 0.2°C per decade, the recent Intergovernmental Panel on Climate Change (IPCC) report (IPCC 2018) anticipates that the warming is likely to reach 1.5°C between 2030 and 2052 if it keeps increasing at the current rate. In this context of global warming, aquatic ecosystems are affected by similar trends in increasing temperatures (Langan et al., 2001). In addition to global warming, some ecosystems are also facing changes in nitrogen (N) loading. Since the early 20th century, anthropogenic activities strongly modified N biogeochemical cycle of terrestrial and aquatic ecosystems, adding ever-increasing N inputs (Galloway et al., 2008). Combustion of fossil energy, fertilisation of agricultural soils, animal breeding, and domestic waste induced the emission of reactive N into the atmosphere. Atmospheric emissions are transported over long distances by air masses and deposited on both terrestrial and aquatic ecosystems, regardless of the proximity to the emission source (Elser, 2011). In particular, forested headwater streams, which are often ecosystems located apart from human activities (including agriculture), suffered from large increases in dissolved N concentrations (Elwood & Mulholland, 1989) that might have changed the intensity or the nature of nutrient limitations, as already observed in lakes (shift from N to P limitation, Elser et al., 2009). Current efforts to limit atmospheric deposition are likely to induce further changes on these ecosystems, impacted streams being expected to face future conditions of decreasing N inputs (Gilliam et al., 2019).

In the last decade, an increasing number of studies investigated the effects of temperature and nutrients on freshwater ecosystems, but disentangling their respective and combined

effects remains a challenge (Cross, Hood, Benstead, Huryn, & Nelson, 2015). On the one hand, temperature controls biological processes through its effect on metabolic rates (Woodward, Perkins, & Brown, 2010) and is responsible for species distribution and phenology (Odum, 1949). On the other hand, nutrients are resources required for growth, maintenance, and reproduction of all organisms (Reiners, 1986), and, in turn, consumers are essential for nutrient cycling and play a major role in energy pathways (Wetzel, 1995). One of the difficulties of studying the interactive effects of temperature and nutrients on aquatic ecosystems is to incorporate interactions between species within the system studied, because these interactions can strongly influence the response of both species and ecological processes to environment change (Gilman, Urban, Tewksbury, Gilchrist, & Holt, 2010). Another part of this challenge relies on the difficulty to control both temperature and nutrient concentration while studying complex ecosystems. Differences between study scale and ecosystem complexity certainly explain the large variability observed between experiments. For example, Ferreira and Chauvet (2011b) observed that simultaneous increases in water temperature and dissolved nutrients under simplified laboratory conditions enhanced alder leaf-litter decomposition, as well as fungal growth and reproduction, while inducing changes in fungal community structure. Furthermore, Moghadam and Zimmer (2016) found that the presence of shredders exacerbates the synergistic effects of temperature and nutrients on litter decomposition. However, in a transplantation experiment, Pérez, Martínez, Descals, and Pozo (2018) reported an increase in microbial decomposition rate when litter bags were transferred from colder and N-poorer to warmer and N-richer streams, although their results pointed to a higher efficiency of microbial decomposers under colder conditions. The field studies from Hood et al. (2018) and Myrstener et al. (2018) aiming to predict the effects of climate change on aquatic primary production came to similar conclusions about the overriding importance of nutrient limitations in comparison with climate-related factors, including increased temperature. Given the complexity and variability of the biological responses to the projected global changes in temperature and nutrient concentrations, experiments in realistic and semi-realistic systems (e.g. mesocosms, manipulative experimentations, field studies) with natural communities are necessary in order to successfully predict responses of species and ecosystem functioning to global change (Pérez et al., 2018).

The main objective of this study was to disentangle the individual and combined effects of temperature increase and nutrient availability on the relative importance of brown and green processes involved in stream functioning. Brown and green processes were evaluated through the measurement of the leaf-litter decomposition process and phototrophic biofilm production, respectively, and consequences at higher trophic levels were evaluated through the investigation of invertebrates' life history traits and activity. We conducted a 2-month experiment in artificial channels, and monitored the effects of an increase in water temperature (ambient and +2°C) combined with two levels of nutrient inputs (molar N:P ratios of 33

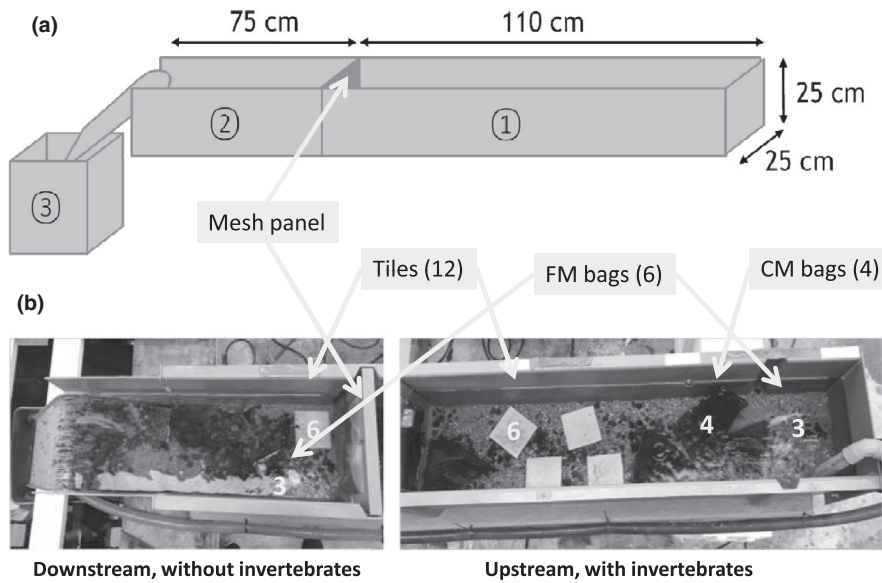
and 100) on: (1) resources (leaf litter and biofilm) quality (C:N:P ratios, poly unsaturated fatty acids [PUFA] contents) and quantity; and (2) on the growth, activity (fungal conidia production, invertebrate resources consumption), and survival of consumers (microorganisms and macro-invertebrates). We hypothesised that increased water temperature and higher N:P level would lead to a stimulation of microbial growth and activity (higher fungal biomass and leaf-litter decomposition, Ferreira & Chauvet, 2011b; higher biofilm biomass and primary production, Díaz-Villanueva, Font, Schwartz, & Romani, 2011). We also expected temperature  $\times$  water N:P interactions: temperature-stimulated microbial growth should sharpen competition between microbial decomposers and biofilm primary producers in the lowest N:P conditions (Danger, Cornut, et al., 2013; Daufresne & Loreau, 2001), hence altering microbial leaf-litter decomposition and primary production. When considering invertebrate consumers, temperature-stimulated metabolism and activity (Hogg & Williams, 1996) should increase resource consumption by invertebrates (higher top-down impact), leading to increased leaf-litter mass loss (Ferreira, Chauvet, & Canhoto, 2014) and stronger top-down control of biofilm biomass. In addition, lower temperature and higher nutrient availability should individually lead to biofilms richer in essential PUFAs, furnishing a potentially higher quality resource for invertebrates that could further increase macro-invertebrate growth (Crenier et al., 2017; Guo et al., 2016).

## 2 | METHODS

### 2.1 | Experimental setting

Experiments were conducted outdoor during 56 days between October and December 2016 in 12 artificial channels situated on a platform covered by a partially transparent roof. Therefore, the channels were exposed to natural variations in both light and temperature. However, only 10–45% of the ambient sunlight, depending on the time of the day, could reach the water surface, which is similar to a forested stream environment (Hill & Knight, 1988). Before filling each channel with 100 L of water, each channel bed was covered with 3 cm of sand and gravel, which were abundantly rinsed with deionised water beforehand. Each channel functioned in closed circuit, and was composed of three main sections (see Figure 1a,b): an *upstream* section (110 cm long), which was separated from a *downstream* section (75 cm long) by a fine-mesh panel (0.25-mm mesh). The upstream section received macro-invertebrates, while the downstream section was exempted of such organisms, allowing us to determine the top-down impact of consumers on phototrophic biofilm. Finally, the overflow of water was collected in 20 L drip trays (Figure 1a) where a submerged pump (Universal 2,400, Eheim GmbH & Co. KG, Germany) was re-injecting the water in the *upstream* section.

To manipulate water temperature, the 12 drip trays (Figure 1a) were placed in water baths with (heated channels) or without (ambient channels) water heaters (Profi Heater 1 kW, Xclear VGE International,



**FIGURE 1** (a) Schematic representation and dimensions of an artificial stream used for this experiment. The upstream section ① receives water from the pump immersed in the drip tray ③. A 250- $\mu\text{m}$  mesh separate the upstream ① and the downstream ② sections to avoid invertebrates drift and keep the downstream section free of invertebrates. Comparison of the upstream ① and the downstream ② sections thus permit to evaluate top-down effects of macro-invertebrates on biofilms. (b) Picture of the two successive sections of an artificial stream: an upstream section with invertebrates separated by a 250- $\mu\text{m}$  nylon mesh from a downstream section without invertebrates. CM, coarse mesh; FM, fine mesh

Netherlands). This permitted us to achieve a mean difference of *c.* 2°C between heated and ambient channels, while following natural diurnal and seasonal temperature variations (see Figure S1). Supplementary water heaters and external insulation of the channels with expanded polystyrene panels prevented the water of all channels from freezing when air temperature dropped below 0°C. Ambient and heated channels received weekly nutrient supplies, in order to attain two different N:P ratios: 33 and 100 (hereafter low and high N:P ratio treatments). Our experiment followed a full factorial design, leading to four different treatments, each treatment being replicated three times: ambient temperature and high N:P; ambient temperature and low N:P; elevated temperature and high N:P; elevated temperature and low N:P.

## 2.2 | Physical and chemical water characterisation

To fill the channels, we used a mix of water sampled in a reference stream (La Maix, Vosges, France: 40%) and a commercial spring water (Laqueuille bottled spring water, Aquamark: 60%) to control water quality. This commercial water was selected to meet several technical and chemical criteria, such as its relatively low conductivity and neutral pH, and the stability of its chemical composition. Spring water analyses revealed a nitrate ( $\text{NO}_3^-$ ) concentration of 0.5 mg/L, a pH of 7.7 and a  $\text{PO}_4^{3-}$  concentration of 0.229 mg/L. Water samples (50 ml) from each channel were collected each week and the concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and  $\text{PO}_4^{3-}$  were immediately determined by ion chromatography (Dionex 1500i). Each week for 7 weeks, N and P additions were adapted to current ion concentrations of each channel just after measuring ion concentrations to attain a N:P molar ratio of 33 or 100

(see Table S1), depending on the subset of channels (named low and high N:P treatments hereafter). Nitrogen was added as potassium nitrate ( $\text{KNO}_3$ ), and P as potassium phosphate ( $\text{KH}_2\text{PO}_4$ ). Due to the high initial  $\text{PO}_4^{3-}$  concentrations in commercial water, reaching the targeted N:P molar ratios of 100 and 33 required to add only  $\text{KNO}_3$  at the beginning of the experiment, without any P addition. Concentrations of  $\text{PO}_4^{3-}$  dropped dramatically after one week (see Figure S2), while  $\text{NO}_3^-$  levels decreased slowly during the 56-day experiment. To avoid excess of nutrients in our closed mesocosms, we chose to let the total amount of N and P naturally decline instead of maintaining the same amount of dissolved N and P throughout the experiment. Thus, during the whole experiment,  $\text{KH}_2\text{PO}_4$  and  $\text{KNO}_3$  were added to the mesocosms weekly to maintain the targeted 100 and 33 N:P ratios while letting N and P amounts decline in the stream water (all data available in Table S1). After 7 weeks of experiment, water samples (500 ml) were collected in each channel for stream pH (pH 3000, WTW), acid-neutralising capacity, and water conductivity measurements (Metrohm Herisau Conductometer E518; Herisau, Switzerland) at 25°C. Concentrations of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ , and  $\text{K}^+$  were also determined on this date by atomic absorption spectrophotometry (AAnalyst 100; Perkin Elmer and Varian SpectraAA-300). Water temperature was recorded every 15 min in each channel with submersed data loggers (Hobo Pendant UA-001-08, Onset Computer Corp.).

## 2.3 | Microbial inoculation in artificial streams

On the first day of the experiment, microbial inoculates were introduced into the channels (natural bacterial, conidial, and biofilm

suspensions from the reference stream). More precisely, about 100 dead leaves (mainly hazel [*Corylus avellana*, L.], alder [*Alnus glutinosa*, L.], and beech [*Fagus sylvatica*, L.] leaves) from the La Maix reference stream were collected, brought back to the laboratory and placed in 40 L of aerated stream water. After 24 hr, 0.5 L of this water was incorporated into each mesocosm in order to add fungal spores and bacteria to the mesocosms. On the same date, natural biofilm was collected from 5 rocks (surface of approximately 100 cm<sup>2</sup> each) from the reference stream using a toothbrush. Biofilm was gently rinsed with stream water, collected in a 1-L glass bottle, and brought back to the laboratory. A volume of 50 ml of this biofilm suspension was added to each mesocosm to permit the initial colonisation of the mesocosms by biofilm species. Volumes of inoculates were chosen to represent small amounts (<1%) of microbial biomass compared to expected final biomass to let the experimental treatments shape microbial communities.

## 2.4 | Leaf conditioning and mass loss

Leaf litter of hazel, a common riparian tree species in Europe, was collected just after abscission in October 2015, air-dried and stored in the dark at ambient temperature. Pre-weighed portions of leaf litter were spray moistened and introduced in coarse (10-mm mesh size; CM) and fine (1-mm mesh size; FM) mesh bags (3.0 and 1.5 ± 0.05 g dry mass in CM and FM bags, respectively). Before being placed in mesocosms, leaf bags were quickly incubated in the reference stream (La Maix, Vosges) to ensure leaching and inoculation by natural microbial decomposer assemblages. After 3 days, the leaf bags were retrieved from the stream, gently rinsed in stream water to remove invertebrates, and transported to the laboratory in a cooler with stream water and kept at 4°C for 1 day. Four CM bags and three FM bags were placed in each upstream section of the channels, and three other FM bags were placed in the downstream section. The leaves from four supplementary bags of each mesh type (CM and FM) were also rinsed with distilled water, oven-dried (60°C, 72 hr) and weighed to determine *initial* dry mass (DM). These leaves were then ground, and a portion (0.25 ± 0.05 g) of the powder obtained was ignited in a muffle furnace at 550°C for 4 hr to determine the initial ash-free DM (AFDM). To analyse the leaf-litter decomposition dynamics, the FM bags in channels were retrieved by pairs (one from the upstream section, one from the downstream section) after 21, 35, and 52 days. In contrast, to avoid a loss of macro-invertebrates enclosed in CM bags during intermediate samplings, the CM bags were only retrieved on a single date, after 56 days. As for initial mass determination, leaves were first oven-dried (60°C, 72 hr), weighed to determine DM and grinded. A portion (0.25 ± 0.05 g) of the leaf powder was ignited (550°C, 4 hr) to determine AFDM. Decomposition rates of hazel tree leaves in FM and CM bags (*k*) were calculated assuming an exponential decomposition rate, by linear regression of the Ln-transformed negative exponential model  $M_t = M_0 \times e^{-kt}$  (Pozo & Colino, 1992).

## 2.5 | Fungal biomass and diversity

At each sampling date, six disks were cut with a cork-borer from the rinsed leaves of the two FM bags collected per channel (three disks coming from the FM bag collected upstream, and three disks from the FM bag collected downstream, each disk coming from different leaves chosen randomly in the leaf bags). The six disks were placed in 100-ml Erlenmeyer flasks filled with 20 ml of filtered stream water (Glass microfibre GF/F, Whatman; nominal cut-off 0.7 µm) and then incubated at 12°C (a temperature close to channel water temperature on the first sampling date) in the dark for 48 hr on an orbital shaker (100 rpm) to determine sporulation rates and species composition of leaf-associated fungal assemblages. After incubation, the disks were removed and the remaining water containing conidial suspensions were poured into a 50-ml Falcon tube, and fixed with 2 ml of 37% formalin. The suspensions were mixed with magnetic stirring bars to ensure uniform distribution of conidia, and an aliquot (1–12 ml) 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 & Webster, 1973), and conidia were identified and counted under a microscope at × 200 magnification (Graça, Bärlocher, & Gessner, 2005). Conidial production was expressed as the number of conidia released per mg leaf AFDM per day. In the meantime, 10 other disks were cut from the leaves of the pairs FM bags (five from each) per channel and frozen at -20°C for future ergosterol content measurement according to Gessner and Newell (2002). Ergosterol was quantified by high-performance liquid chromatography on the disks (Graça et al., 2005), and then converted into fungal biomass using a conversion factor of 5.5 µg ergosterol/mg fungal dry mass (Gessner & Chauvet, 1993).

## 2.6 | Biofilm production

Twelve ceramic tiles (individual upper surface area of 95 cm<sup>2</sup>) were placed on the channel beds (six in the upstream section, i.e. with macro-invertebrates, six in the downstream section, i.e. without invertebrates). After 21, 35, and 52 days, two tiles were randomly retrieved from each channel section. The tiles were gently scraped with a smooth toothbrush in order to remove the attached biofilm while limiting damage to algal cells. Suspensions of biofilm were poured into tubes and the volume was adjusted to 100 ml with distilled water. Two subsamples of 5 ml were filtered through GF/F membrane filters (25 mm diameter, pore size 0.7 µm; Whatman, GE Healthcare, USA) and stored at -80°C until further analysis of pigments. The remaining suspension was freeze-dried, ground and weighed to the nearest 0.01 g. A portion of the powder (0.15 ± 0.05 g) was ignited in a muffle furnace at 550°C for 4 hr to determine AFDM. For pigment extraction, samples were thawed in 90% acetone, sonicated, and then left overnight in the dark at 4°C. After extraction, each acetic extract was centrifuged (10,000 g, 15 min), and the absorbance of the supernatant was measured spectrophotometrically at

664 and 750 nm before and after addition of 10% HCl. Chlorophyll *a* and pheophytin *a* concentrations were determined for each extract using the equations of Lorenzen (1967) and were expressed in  $\mu\text{g}$  pigment per unit of substrate colonised area ( $\mu\text{g}/\text{cm}^2$ ). In addition to the pigment values available on each date, primary production of biofilms (in  $\text{day}^{-1}$ ) were calculated based upon the temporal dynamic of pigment increase in the absence of grazers (in the downstream section), assuming a linear production of pigments in time (linear regressions).

## 2.7 | Stoichiometric and biochemical quality of leaves and biofilm

Subsamples of leaf litter and biofilm powders were used to determine their carbon (C), N, and P contents on the first and the last date of the experiment (i.e. 52 days for leaf litter collected in FM bags and biofilm). The initial CNP content of leaf litter was measured from the bags used to measure initial leaf mass loss. Subsamples were weighed to the nearest 0.001 mg (Perkin Elmer AD6 Autobalance). Carbon and N contents in each resource were quantified using a CHN elemental analyser (Carlo Erba NA2100, Thermo Quest CE International, Milan, Italy). Phosphorous content was quantified after sodium persulfate digestion and spectrophotometry. Leaves and biofilm C:N, N:P, and C:P were expressed as molar ratios.

Fatty acid analyses were performed on biofilm collected in each channel at the end of the experiment, using the protocol described in Crenier et al. (2017). Briefly, lipids were extracted twice using a chloroform/methanol solution (Folch, Lees, & Stanley, 1957). Once extracted, fatty acids were converted into fatty acid methyl-esters by acid-catalysed transesterification and analysed on an Agilent Technologies™ 6850 gas chromatograph (Agilent Technologies, Massy, France). Fatty acid methyl-esters were identified by comparing retention times with those obtained from Supelco and laboratory standards, and were quantified against internal standards (13:0).

## 2.8 | Macro-invertebrate introduction in the channels, survival, and growth

To obtain a functionally diverse macro-invertebrate assemblage, four common species of macro-invertebrates from Vosges mountain streams were introduced in the channels: two species feeding mainly on detritus (*Sericostoma personatum* and *Odontocerum albicorne*), one generalist species (*Gammarus fossarum*), and one grazer species feeding mainly on biofilms (*Epeorus sylvicola*). All macro-invertebrates were collected in four reference headwater streams located in the Vosges Mountains (La Maix, Le Marteau, La Plaine, and Le Tihet). Macro-invertebrates were quickly transported to the laboratory in a cooler with aerated stream water and maintained at 4°C until sorting. Depending on the species, sorting was made directly in the field (for Trichoptera and Ephemeroptera), or

in the laboratory (Gammarids). Indeed, gammarids were sorted by size (three size classes: A,  $4.4 \pm 0.7$  mm; B,  $5.8 \pm 0.8$  mm; and C,  $8.4 \pm 0.6$  mm), in order to obtain a size-distribution representative of natural populations (Felten, 2003) and for accurate estimations of gammarids growth. To limit manipulation of Ephemeropteran and Trichopteran invertebrates, and since it was not possible to precisely evaluate individual or population growth of these taxa during the two-month experiment, we chose not to measure initial size of these taxa. However, for Trichopterans and Ephemeropterans, we initially produced 12 batches of invertebrates (one per channel) with roughly similar size distributions for each species, each batch being then randomly introduced in one channel. Before introduction into the channels, macro-invertebrates were slowly (4–6 hr) acclimated to the mixed water by gently mixing artificial stream water to stream water containing the invertebrates. To remain close to what is generally observed in La Maix stream (Felten, 2003), a total number of 25 Ephemeroptera, 10 Trichoptera (five of each species), and 259 gammarids (60% of A, 30% of B, and 10% of C) were introduced in each channel.

## 2.9 | Data analysis

Temperature differences between channels were evaluated using a general linear model (GLM). We also used GLMs to assess the effects of temperature, water N:P ratio, and the temperature  $\times$  water N:P ratio interactions on several response variables: (a) exponential decomposition rates; (b) log-transformed total sporulation rates; (c) fungal biomass, (d) C:N, C:P and N:P ratios of FM bags leaf litter; (e) biofilm biomass; (f) log-transformed biofilm pigments concentrations; (g) biofilm C:N, C:P, and N:P ratios; (h) biofilm fatty acids contents; (i) primary production; (j) ratio of FM decomposition rate to primary production; and (k) gammarid body length. Prior to those analyses and to avoid complicated three- and four-ways interactions, we investigated the temporal effect on (b), (c), (e), and (f), and we further evaluated the effect of the position in the channel (upstream and downstream; presence and absence of macro-invertebrates, respectively) on biofilm related response variables—i.e. (f), (g), and (h). If the effect of time and/or position in the channel was significant, the effects of temperature, water N:P ratio, and the temperature  $\times$  water N:P ratio interactions were evaluated on response variables—i.e. (b), (c), (e), and (f)—split by time, position, or both. For all GLMs, the significance of predictor variables was evaluated using a Type II or Type III ANOVA (car-package) depending on the presence of significant interactions in the model. We graphically assessed model validation and further evaluated the normality of model residuals using Shapiro tests. Top-down impacts of macro-invertebrates on leaf-litter decomposition and algal biomass in biofilm were investigated through the calculation of Ln-transformed ratios of decomposition in CM (accessible to detritivores) to decomposition in FM (inaccessible to detritivores) litter bags and of Ln-transformed ratios of algal biomass (pigments) measured upstream (section with grazers) to those measured downstream (section

without grazers). Effects of temperature and water N:P ratio were considered as significant when confidence intervals at 95% did not overlap with zero. Finally, to assess the multivariate response of weekly ions concentrations to the four different treatments (i.e. two temperature and two nutrients related treatments), we used a PERMANOVA based on the Bray–Curtis dissimilarity index, and 999 permutations. Similar tests were performed to analyse the effects of temperature, water N:P ratio, and the temperature × water N:P ratio interactions on species-specific sporulation rates. All statistical analyses were performed with the R software (R Core Team 2017, version 3.4.2.).

### 3 | RESULTS

#### 3.1 | Physico-chemical conditions in the mesocosms

The mean temperature difference between ambient and heated channels was 1.9°C and remained quite constant over the 56 days of the experiment (Table 1, Figure S1). On average, the temperature of ambient channels ( $\pm$  SD) was  $9.2 \pm 4.0^\circ\text{C}$  and the temperature of heated channels was  $11.1 \pm 3.5^\circ\text{C}$ . Temperatures significantly differed between ambient and heated channels (ANOVA,  $p < 0.0001$ , Tukey HSD).

With respect to water nutrient concentrations,  $\text{NH}_4^+$  and  $\text{PO}_4^{3-}$  concentrations measured each week before adjustments were similar between channels ( $0.007 \pm 0.007$  mg $\text{NH}_4^+$ /L and  $0.029 \pm 0.057$  mg- $\text{PO}_4^{3-}$ /L in average, respectively). However,  $\text{NO}_3^-$  concentrations significantly differed between high N:P ratio channels ( $4.69 \pm 2.20$  mg $\text{NO}_3^-$ /L) and low N:P ratio channels ( $0.87 \pm 0.61$  mg $\text{NO}_3^-$ /L; PERMANOVA, dissolved N:P ratio:  $p = 0.001$ ; temperature:  $p > 0.05$ ; Table 1, Figure S2).  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ , and  $\text{K}^+$  concentrations remained high throughout the experiment (final concentrations of  $29.9 \pm 1.5$ ,  $3.4 \pm 0.4$ ,  $3.8 \pm 0.4$ , and  $6.4 \pm 1.0$  mg/L for  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ , and  $\text{K}^+$ , respectively) suggesting that these elements were not limiting during the experiment. All other parameters were unaffected by the temperature and dissolved N:P ratio treatments throughout the experiment: water was slightly alkaline (pH =  $8.10 \pm 0.03$ ), conductivity reached  $218 \pm 7.9$   $\mu\text{S}/\text{cm}$ , and acid-neutralising capacity) was  $1.9 \pm 0.1$  meq/L.

#### 3.2 | Effects of temperature and water N:P ratio on aquatic fungi

Fungal biomass, as evaluated by ergosterol quantification in leaf litter (Figure 2a), was significantly affected by the interaction of time, dissolved N:P ratio, and temperature (ANOVA,  $p = 0.02$ ). To better understand this complex interaction, fungal biomass was then analysed date by date, showing a temperature × dissolved N:P ratio interaction ( $p = 0.03$ ) after 21d (lower fungal biomass in the high N:P, heated streams), and only a temperature effect ( $p = 0.01$ ) after 52 days, fungal biomass being significantly higher in the ambient temperature treatment.

Mean total sporulation rates of aquatic hyphomycetes associated with hazel leaves were the highest after 21 days (128–309 conidia mg AFDM $^{-1}$  day $^{-1}$ ), then decreased from 35 (58–97 conidia mg AFDM $^{-1}$  day $^{-1}$ ) to 52 days (29–68 conidia mg AFDM $^{-1}$  day $^{-1}$ ) in all treatments (Figure 2b). Since statistical analyses revealed a significant influence of time (ANOVA;  $p = 0.04$ , other  $p > 0.05$ ), further analyses were performed independently on sporulation rates measured at each sampling time and revealed a positive influence of temperature on sporulation rates measured after 52 days (ANOVA, Temperature,  $p = 0.04$ ).

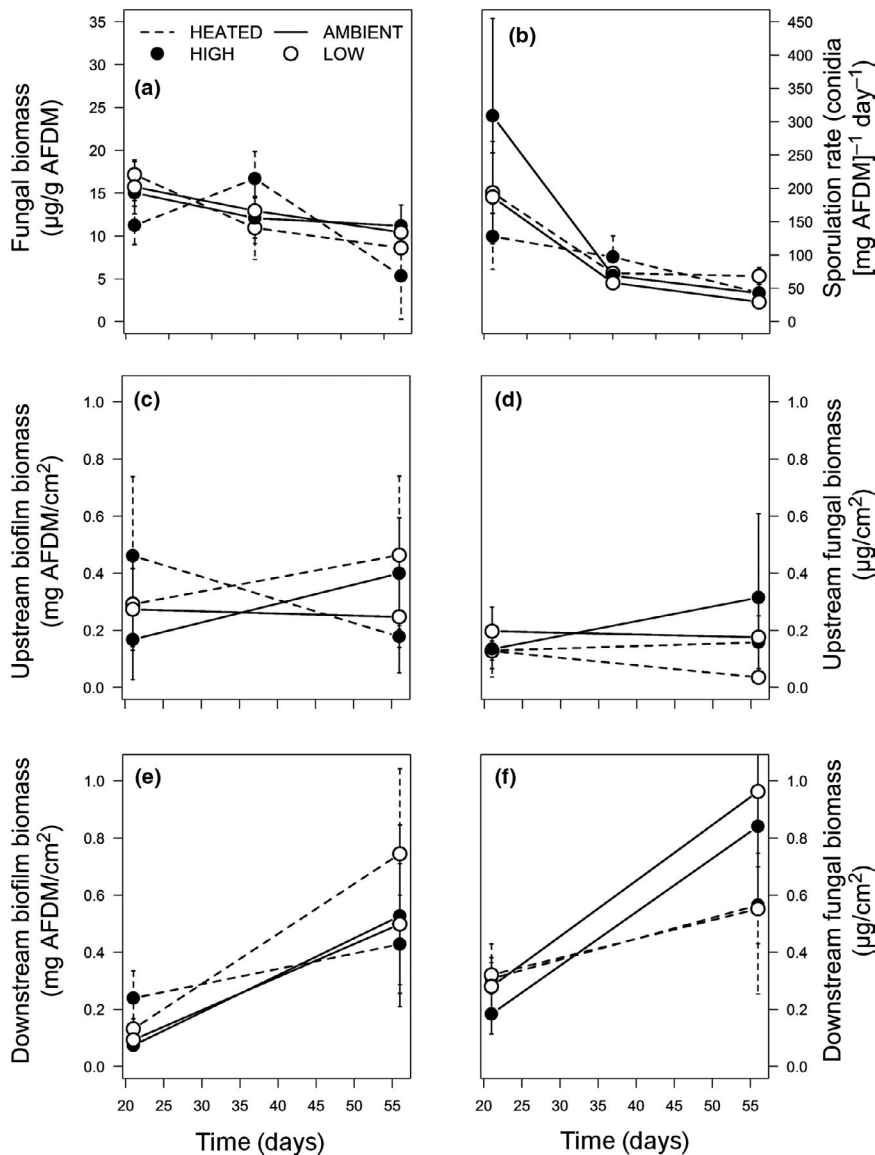
Species richness of aquatic hyphomycete communities was similar between treatments, and reached a total of 16 species. Consortia of aquatic hyphomycetes observed in all channels were mainly represented by four species (*Flagellospora curvula*, *Tetrachaetum elegans*, *Clavariopsis aquatica*, and *Lunulospora curvula*), while the other species (*Anguillospora crassa*, *Articulospora tetracladia*, *Alatospora acuminata*, *Alatospora pulchella*, *Tricladium chaetocladium*, *Tetracladium marchalianum*, *Tripodermum myrti*, *Anguillospora longissima*, *Heliscella stellata*, *Triscelophorus monosporum*, *Heliscus lugdunensis*, other sigmoids) did not exceed 5% of the relative abundance (Figure 3a). *Flagellospora curvula* was dominant in almost all channels (relative abundance:  $53 \pm 15\%$  and  $64\% \pm 17\%$  after 21 and 35 days respectively), and no significant differences between treatments were observed at these two sampling times (PERMANOVA). However, after 52 days, aquatic hyphomycete consortia were significantly different between ambient and heated channels (PERMANOVA, temperature:  $p = 0.004$ ). At this date, although *Flagellospora curvula* remained dominant in heated channels, the dominant species in ambient

**TABLE 1** Main physical and chemical water parameters measured in the four treatment channels during the 56-day experiment ( $n = 2,688$  for temperature;  $n = 8$  for other parameters)

	Temperature (°C)			pH	Conductivity ( $\mu\text{S}/\text{cm}$ )	$\text{NH}_4^+$ (mg/L)	$\text{NO}_3^-$ (mg/L)	$\text{PO}_4^{3-}$ (mg/L)
	Mean $\pm$ SD	Min	Max					
Ambient high	$9.20 \pm 4.01^a$	1.33	19.09	$8.08 \pm 0.01$	$223 \pm 9$	$0.006 \pm 0.007$	$4.80 \pm 2.21^a$	$0.028 \pm 0.057$
Ambient low	$9.18 \pm 4.05^a$	1.22	19.28	$8.10 \pm 0.02$	$217 \pm 5$	$0.006 \pm 0.008$	$0.93 \pm 0.61^b$	$0.028 \pm 0.058$
Heated high	$11.08 \pm 3.47^b$	4.42	19.38	$8.12 \pm 0.01$	$217 \pm 12$	$0.008 \pm 0.008$	$4.58 \pm 2.23^a$	$0.029 \pm 0.059$
Heated low	$11.12 \pm 3.49^b$	4.42	19.47	$8.09 \pm 0.06$	$217 \pm 9$	$0.007 \pm 0.007$	$0.80 \pm 0.61^b$	$0.027 \pm 0.056$

Note: Treatments with the same letter are not significantly different (Tukey HSD test,  $p > 0.05$ ).





**FIGURE 2** Effects of temperature and water N:P ratios on the temporal dynamics of microbial development and activity: Fungal biomass, measured as ergosterol content per unit of leaf-litter biomass (a) and sporulation rates (b) changes through time measured on leaf litter enclosed in fine-mesh bags; Changes in upstream (with grazers, panels c–d) and downstream (i.e. grazers excluded, panels e–f) biofilm biomass (in mg ash-free dry mass [AFDM]/cm<sup>2</sup>, panels c and e) and algal abundance (in µg chlorophyll-*a*/cm<sup>2</sup>, panels d and f) through time. Error bars ( $n = 3$ ) correspond to SD except on panel b (SE)

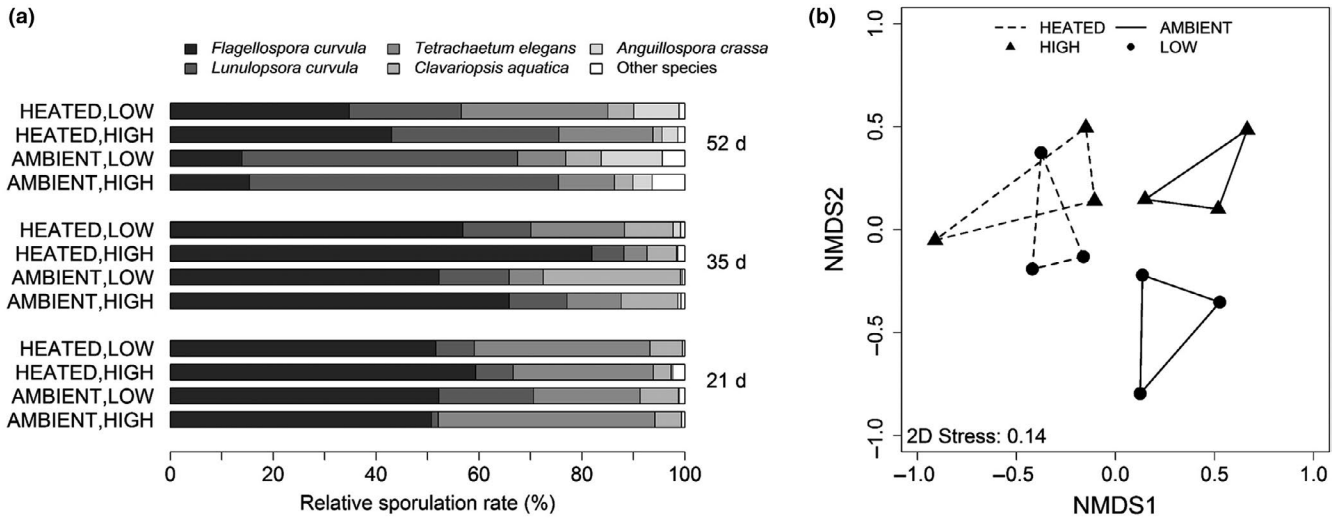
channels was *Lunulospora curvula* (relative abundance: 27% ± 12% and 57% ± 13% in heated and ambient channels, respectively; Figure 3a). This observation was supported by non-metric multidimensional scaling ordination diagram of fungal communities based on conidial production after 52 days of incubation (Figure 3b).

### 3.3 | Effects of temperature and water N:P ratio on biofilm abundance

Biofilm biomass (measured in mg of AFDM per surface unit) was significantly affected by the sampling time (21 and 56 days; ANOVA, time,  $p < 0.001$ ) and the position in the stream channel (upstream versus downstream; ANOVA, position,  $p = 0.002$ ; Figure 2c,e). Thus, the effects of temperature and dissolved N:P ratio were analysed separately on sub-groups of biomass data (e.g. 21d-upstream, 21d-downstream). For all sub-groups, results showed no significant effect of the interaction between temperature and dissolved N:P ratio

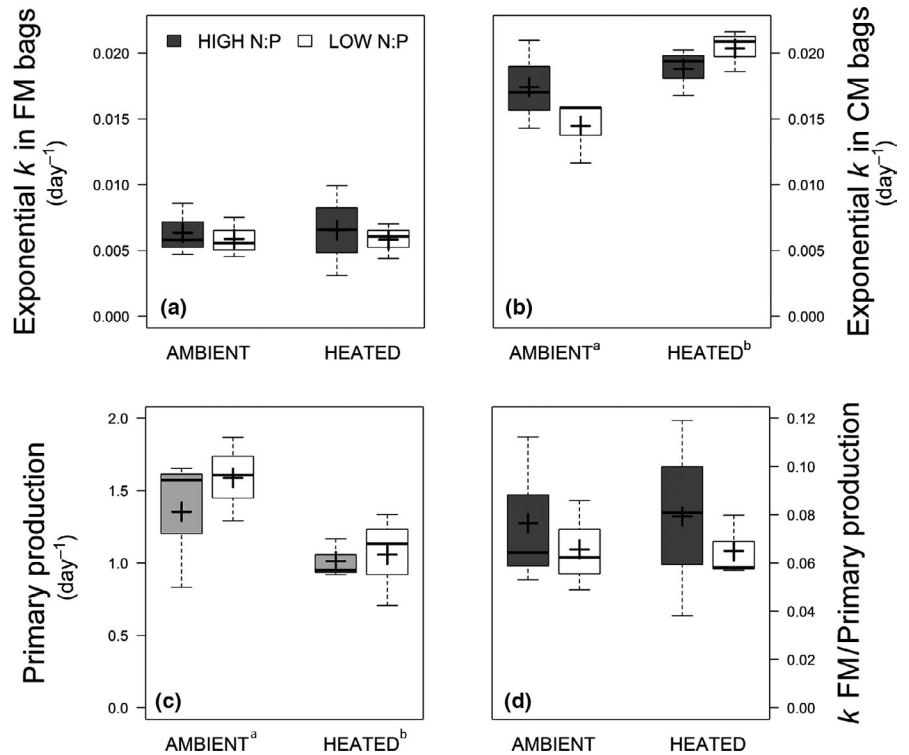
on biofilm biomass (ANOVA, temperature × dissolved N:P ratio, all  $p > 0.05$ ). Furthermore, no individual effects of temperature and dissolved N:P ratio were found on biofilm biomass grown upstream (in presence of invertebrates, Figure 2c). However, concerning biofilm growing downstream (in absence of invertebrates), biomass was positively related to temperature in the high N:P treatment after 21 days ( $0.23 \pm 0.43$  in heated, low N:P channels and  $0.10 \pm 0.03$  mg AFDM/cm<sup>2</sup> on average in the three other treatments, interaction Temperature × dissolved N:P ratio,  $p = 0.04$ ; Figure 2e).

Total pigment concentration (chlorophyll *a* and pheophytin) concentrations in biofilm (Figure 2d,f) were significantly lower in the upstream section ( $0.17 \pm 0.14$  µg/cm<sup>2</sup>) than in the downstream section of the channels ( $0.62 \pm 0.58$  µg/cm<sup>2</sup>; ANOVA, position effect,  $p < 0.0001$ ). No significant differences were found between the two sampling times (21 and 56 days; ANOVA, time,  $p > 0.05$ ). Thus, the effect of temperature and nutrient supply was analysed separately on two sub-groups of pigments data (i.e. upstream and downstream), without considering time effect. For both



**FIGURE 3** Percentage contribution of the main aquatic hyphomycete species to total conidial production (a) in the four treatments channels for each sampling date (21, 35, and 52 days) and non-metric multidimensional scaling (NMDS) ordination diagram based on fungal communities assessed from conidia released from hazel leaf disks after 52 days of incubation (b)

**FIGURE 4** Effects of temperature and water N:P ratios on leaf-litter exponential decomposition rates in fine (a) and coarse (b) mesh bags, primary production (c), and microbial decomposition to primary production ratios (d) measured during the 56-day experiment in the four treatments



sub-groups, the temperature  $\times$  dissolved N:P ratio interaction was not significant (ANOVA,  $p > 0.05$ ). Due to the high variability, no significant effect of temperature and dissolved N:P ratio was evidenced in the downstream section (Figure 2f). In contrast, upstream total pigment concentration was independently influenced by temperature and dissolved N:P ratios (Figure 2d). Warmer temperature was associated with lower total pigments concentrations ( $0.12 \pm 0.05$  and  $0.22 \pm 0.12 \mu\text{g}/\text{cm}^2$  in heated and ambient channels, respectively; ANOVA, temperature,  $p < 0.001$ ; Figure 2d). By contrast, higher N:P ratios in water was positively related to

pigment concentrations ( $0.20 \pm 0.05$  and  $0.13 \pm 0.07 \mu\text{g}/\text{cm}^2$  in high N:P and low N:P ratio channels, respectively; ANOVA, dissolved N:P ratio,  $p = 0.03$ ; Figure 2d).

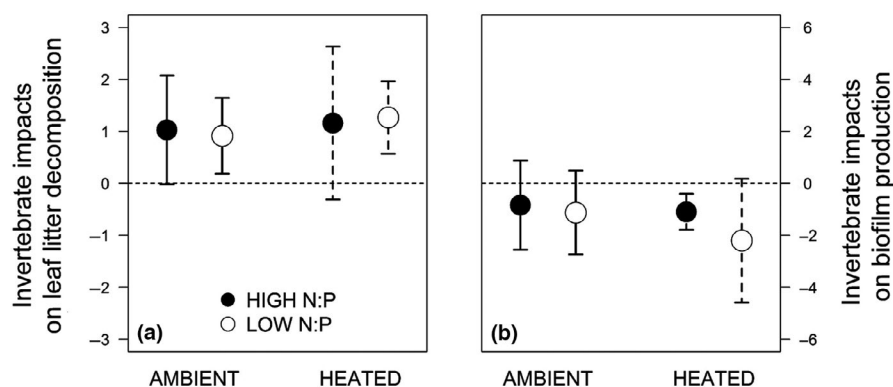
### 3.4 | Effects of temperature and water N:P ratio on leaf-litter decomposition and primary production

Microbial decomposition rates, evaluated from the mass loss of leaf litter enclosed in FM bags, ranged from  $0.006 \pm 0.001 \text{ day}^{-1}$  in low

N:P, heated channels to  $0.007 \pm 0.001 \text{ day}^{-1}$  in high N:P, heated channels (Figure 4a). Decomposition rates measured in FM bags were not significantly affected by temperature or by nutrients, and did not differ significantly between treatments (ANOVA, all  $p > 0.05$ ). Fine mesh decomposition rates were significantly lower than mean overall decomposition rates (including both microbial and macro-invertebrates activities) measured in CM bags (ANOVA, mesh size effect,  $p < 0.0001$ ), with mean overall decomposition rates ranging from  $0.014 \pm 0.002 \text{ day}^{-1}$  in low N:P, ambient channels to  $0.020 \pm 0.002 \text{ day}^{-1}$  in low N:P, heated channels after 56 days of incubation (Figure 4b). Decomposition rates measured in CM bags were not significantly influenced by the interaction between temperature and dissolved N:P ratios or by the different N:P ratios alone (ANOVA, Temperature  $\times$  N:P ratio and N:P ratio alone,  $p > 0.05$ ). However, overall decomposition rates were significantly higher in heated than in ambient channels ( $0.020 \pm 0.003$  and  $0.016 \pm 0.002 \text{ day}^{-1}$  in heated and ambient channels, respectively; ANOVA, Temperature,  $p = 0.02$ ; Figure 4b). Primary production (in  $\text{day}^{-1}$ ), as evaluated by the linear rate of biofilm pigment (in  $\mu\text{g}/\text{cm}^2$ ) increased throughout the 56-day experiment (Figure 4c), and was significantly and negatively impacted by temperature, shifting from  $1.04 \pm 0.22$  in heated conditions to  $1.47 \pm 0.36$  in ambient conditions (ANOVA, Temperature,  $p = 0.03$ ). In contrast, the ratio of microbial decomposition to primary production (kFM/primary production, Figure 4d) was unaffected by the two treatments and their interactions (ANOVA, all  $p > 0.05$ ).

### 3.5 | Effects of temperature and water N:P ratio on top-down impacts of invertebrates

Evaluation of the importance of macro-invertebrates on leaf-litter decomposition rates (measured through the ratio of  $k$  measured in CM bags to  $k$  measured in FM bags, Figure 5a) revealed that the detritivorous invertebrates significantly increased rates of leaf-litter decomposition only in the low N:P treatments, independently of water temperature. In contrast, negative impacts of macro-invertebrates on biofilm biomass after 56 days (evaluated through the ratio of biofilm pigments measured upstream, i.e. with grazer, to biofilm pigments measured downstream, i.e. without grazers, Figure 5b) was only significant in the heated, high N:P ratio treatment. All other ratios did not differ significantly from 0.



**FIGURE 5** Evaluation of the invertebrate consumer impacts on rates of leaf-litter decomposition (a; calculated as the ln-transformed ratio of  $k_{\text{CM}}$  on  $k_{\text{FM}}$ ) and biofilm production (b; calculated as the ln-transformed ratio of biofilm pigments concentration with invertebrates [upstream] on concentration without invertebrates [downstream]) in the four different treatments. Error bars correspond to 95% confidence intervals

### 3.6 | Stoichiometry and PUFA content of leaf litters and biofilms

Initially, hazel leaf litter introduced in FM bags contained 42.8% of C, 1.2% of N, and 0.02% of P, resulting in C:N, N:P, and C:P molar ratios of 42.1, 149.0, and 6,224.5, respectively. After 52 days of conditioning, leaf litters contained 41.2% of C, 1.5% of N and 0.05% of P, resulting in C:N, N:P, and C:P ratios of  $31.5 \pm 2.0$ ,  $68.3 \pm 7.0$ , and  $2,153.0 \pm 227.6$ , respectively (Table 2). Statistical analyses revealed no significant effect of temperature and dissolved N:P ratio (in interaction or as simple additive factors) on leaf litter C:N:P ratios (ANOVA, all  $p > 0.05$ ; Table 2).

After 56 days, biofilm stoichiometry did not differ significantly between treatments (Table 2). In contrast, fatty acid profiles were affected by dissolved N:P ratios. Considering biofilms from the four treatments, about 90% of total fatty acids were identified and these fatty acid were represented in average by 34% of saturated fatty acids, 33% of monounsaturated fatty acids, and 24% of polyunsaturated fatty acids. Biofilms contained predominantly palmitoleic acid (16:1 $\omega$ 7), palmitic acid (16:0), eicosapentaenoic acid (20:5 $\omega$ 3) and myristic acid (14:0), these fatty acids representing respectively  $28.9 \pm 3.5\%$ ,  $19.9 \pm 2.5\%$ ,  $7.7 \pm 1.3\%$ , and  $7.2 \pm 0.9\%$  of total fatty acids. Both concentrations of 18:3 $\omega$ 3 and 20:5 $\omega$ 3 were significantly increased in the high N:P channels when compared to low N:P channels ( $p < 0.001$ ). In contrast, 20:5 $\omega$ 3 were slightly reduced in heated channels when compared to ambient channels ( $p < 0.05$ ), while no interactions between temperature and N:P ratios were evidenced neither on 18:3 $\omega$ 3 nor on 20:5 $\omega$ 3 biofilm content.

### 3.7 | Macro-invertebrate responses to temperature and nutrients

Initially, 259 *Gammarus fossarum* of known length (mean  $\pm$  SD:  $5.07 \pm 1.32$  mm, sorted in three size classes) were introduced in each channel. After 56 days, mean growth rates ( $\pm$  SD) of gammarid population reached  $0.0051 \pm 0.0002 \text{ day}^{-1}$  in heated channels, and  $0.0036 \pm 0.0001 \text{ day}^{-1}$  in ambient channels, for a mean length ( $\pm$  SD) of  $6.70 \pm 1.43$  mm and  $6.23 \pm 1.37$  mm in heated and ambient channels respectively (Table 3). The faster growth of gammarids population was observed for those placed in heated channels (Figure 6), with a larger shift of the associated density curves

to larger sizes in comparison with gammarids grown in ambient channels. Because of the non-normality of the residuals of the GLM model, the effect of temperature and nutrients on gammarid length was assessed using Kruskal-Wallis multi-comparison test. It revealed a significant difference between the length of gammarids found in heated and ambient channels, but not between high and low N:P channels ( $p = 0.05$ , Bonferroni adjusted). Mean mortality rate ( $\pm$  SD) of macro-invertebrates in all channels after 56 days was  $46.30 \pm 13.02\%$  for *Gammarus fossarum*,  $46.33 \pm 9.57\%$  for *Epeorus* spp.,  $13.33 \pm 15.57\%$  for *Odontocerum* spp. and  $16.67 \pm 23.87\%$  for *Sericostoma* spp. (Table 3), these mortalities being independent of the treatments.

## 4 | DISCUSSION

The main objective of this study was to investigate the potential interactive effects of temperature and dissolved N:P ratio in water on the relative importance of brown and green processes occurring in stream ecosystems and, in turn, on their invertebrate consumers. To date, most studies dealing with this question were conducted in highly simplified conditions. However, these effects might largely differ when investigated in more complex systems, representing diverse habitats, resources, and species interactions, as well as different trophic levels (Woodward et al., 2010). Our results revealed complex single and combined effects of the two parameters tested on different functional processes, underlining the importance of including ecological complexity.

### 4.1 | Effects of temperature and water N:P ratio on microbial decomposer–primary producer interactions

Microbial decomposition is a key ecosystem process, especially in forested headwater streams where allochthonous detritus constitutes the main input of nutrients and energy for stream food webs (Wallace et al., 1997). Inorganic nutrients, and N in particular, are directly incorporated in fungal biomass and stimulate mycelial growth and activity (Gulis et al., 2017). Microbial decomposition is also a temperature-dependent process that is expected to benefit from increased temperatures (Follstad Shah et al., 2017). Both parameters were thus expected to speed up microbial decomposition processes, either independently or interactively. However, contrary to expectations, our study did not reveal any significant effect of water N:P ratio and temperature on the microbial decomposition of hazel leaf litter. Similarly, fungal biomass and spore production, an indicator of fungal activity, were only moderately affected by the different treatments. Fungal growth was slightly delayed in the high N:P, heated treatment, while spore production was only stimulated by temperature on the last date of the experiment. These results contrast with those from previous studies that reported an increase in microbially driven decomposition and fungal activities under increased nutrient availability (Gulis & Suberkropp, 2003), temperature (Ferreira & Chauvet, 2011a), or both simultaneously (Fernandes, Seena, Pascoal, & Cássio, 2014; Ferreira & Chauvet, 2011b). Nevertheless, our results are consistent with those of two recent field studies (Gossiaux, Jabiol, Poupin, Chauvet, & Guérol, 2019; Pérez et al., 2018) that suggest that microbial activity can be highly sensitive to other

**TABLE 2** Leaf litter and biofilm C:N, N:P, and C:P molar ratios and biofilm content in the main polyunsaturated fatty acids measured on the last date of the experiment (day 56)

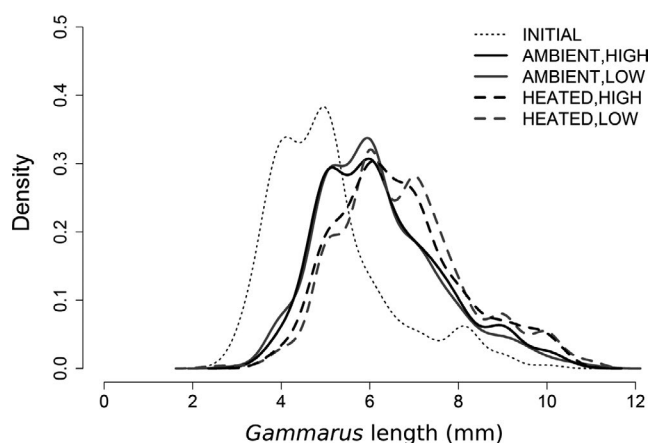
	Ambient, N:P high	Ambient, N:P low	Heated, N:P high	Heated, N:P low
Leaf litter (fine-mesh bags)				
Elemental composition				
%C	40.09 $\pm$ 3.39	39.04 $\pm$ 3.01	36.39 $\pm$ 8.06	38.14 $\pm$ 3.50
C:N	33.21 $\pm$ 2.44	31.52 $\pm$ 2.30	31.29 $\pm$ 2.14	31.17 $\pm$ 2.97
C:P	2,356.89 $\pm$ 278.51	1958.84 $\pm$ 210.89	2,042.24 $\pm$ 416.24	2,219.04 $\pm$ 343.06
N:P	71.15 $\pm$ 8.82	62.16 $\pm$ 5.26	65.00 $\pm$ 11.47	70.98 $\pm$ 6.46
Biofilm (downstream)				
Elemental composition				
%C	15.76 $\pm$ 2.63	12.56 $\pm$ 2.40	14.88 $\pm$ 2.61	14.24 $\pm$ 3.15
C:N	14.56 $\pm$ 1.54	15.71 $\pm$ 2.00	14.92 $\pm$ 2.19	18.14 $\pm$ 4.42
C:P	255.19 $\pm$ 115.04	197.06 $\pm$ 75.82	223.42 $\pm$ 52.80	211.12 $\pm$ 61.90
N:P	17.31 $\pm$ 6.51 <sup>A</sup>	12.29 $\pm$ 3.34 <sup>B</sup>	15.05 $\pm$ 3.12 <sup>A</sup>	11.70 $\pm$ 2.11 <sup>B</sup>
Main PUFA content ( $\mu$ g/mg DW)				
18:3 $\omega$ 3	0.15 $\pm$ 0.03 <sup>A</sup>	0.08 $\pm$ 0.03 <sup>B</sup>	0.13 $\pm$ 0.06 <sup>A</sup>	0.06 $\pm$ 0.02 <sup>B</sup>
20:5 $\omega$ 3	1.97 $\pm$ 0.07 <sup>A*</sup>	1.26 $\pm$ 0.38 <sup>B*</sup>	1.90 $\pm$ 0.37 <sup>A</sup>	1.20 $\pm$ 0.23 <sup>B</sup>
22:6 $\omega$ 3	0.14 $\pm$ 0.01	0.09 $\pm$ 0.03	0.13 $\pm$ 0.04	0.08 $\pm$ 0.01
Sum of PUFAs	6.45 $\pm$ 0.40	4.13 $\pm$ 1.15	5.64 $\pm$ 1.16	3.79 $\pm$ 0.63

Note: Values are mean ( $\pm$  SD,  $n = 3$ ). Letters indicate a significant effect of water N:P level; \* indicates a significant effect of temperature; no interaction was evidenced.

**TABLE 3** Mean  $\pm$  SD ( $n = 3$ ) length, growth, and mortality of Gammarids and mean  $\pm$  SD ( $n = 3$ ) mortality of Ephemeropterans and Trichoptera per treatment

	Gammarids			Ephemeroptera	Trichoptera
	Length (mm)	Growth (mm/day)	Mortality (%)	Mortality (%)	Mortality (%)
Ambient high	6.23 $\pm$ 0.24 <sup>a</sup>	0.0037 $\pm$ 0.0007	51 $\pm$ 12	45 $\pm$ 2	20 $\pm$ 10
Ambient low	6.15 $\pm$ 0.33 <sup>a</sup>	0.0034 $\pm$ 0.0010	45 $\pm$ 9	49 $\pm$ 18	7 $\pm$ 15
Heated high	6.67 $\pm$ 0.25 <sup>b</sup>	0.0049 $\pm$ 0.0006	54 $\pm$ 18	51 $\pm$ 6	3 $\pm$ 6
Heated low	6.77 $\pm$ 0.27 <sup>b</sup>	0.0052 $\pm$ 0.0007	35 $\pm$ 7	40 $\pm$ 4	30 $\pm$ 10

Note: Treatments with the same letter are not significantly different (Kruskal–Wallis multiple comparison test,  $p > 0.05$ ).



**FIGURE 6** Size distribution of gammarids at the beginning of the experiment and after 56-day experiment in the four different treatments. Dotted line is the initial distribution, solid lines are ambient distributions, and dash lines are heated distributions. Dark and light grey represent treatments with high and low nutrients supply

ecological variables (e.g. seasonal variations, interactions with other biological compartments). These contrasted observations emphasise the need to consider more diversified systems when assessing the effects of complex environmental changes.

Leaf-litter quality may have been among the factors that reduced the effect of temperature and nutrients on microbial decomposition. In a meta-analysis on nutrient enrichment effects on leaf-litter decomposition, Ferreira et al. (2015) showed that impacts of nutrient enrichment were maximised on low-quality substrates. The use of a low-refractory resource in our experiment might thus have reduced the magnitude of the response to the nutrient treatment. In addition to this hypothesis, the putative role of competition between microbial decomposers and phototrophic biofilms on leaf-litter decomposition was specifically tested in this experiment. Indeed, both primary producers and decomposers compete for the same inorganic nutrients (Daufresne & Loreau, 2001), and any factor that could favour the development of one biological compartment could, in turn, negatively affect the other one. To date, very few data are available concerning the interactions of primary producers and decomposers in forested headwater streams. Some results suggested that aquatic fungi could control algal growth either through higher

competitive capabilities for nutrients (Danger, Cornut, et al., 2013) or through the production of allelopathic compounds (Allen et al., 2017). In addition, algal exudation, exacerbated by nutrient depletion, could negatively or positively impact leaf-litter decomposition through priming effect mechanisms (Danger, Cornut, et al., 2013; Halvorson et al., 2018; Kuehn et al., 2014). In headwater streams, in addition to low light levels (Hill, Ryon, & Schilling, 1995), phototrophic biofilm development can be constrained by low temperature (Delgado, Almeida, Elias, Ferreira, & Canhoto, 2017) and dissolved nutrient concentrations (Gao et al., 2019; Myrstener et al., 2018). In this context, we expected a simultaneous increase in these two factors would have stimulated phototrophic biofilm biomass and, by extent, pigment concentrations. However, our study revealed that temperature and nutrient effects are not simply additive or synergistic, but can differentially influence biofilm development depending on the stage of substrate colonisation. Indeed, in the absence of invertebrate grazers, higher temperature and high N:P ratios significantly increased biofilm biomass after 21 days. Interestingly, greater biofilm biomass was associated with decreased fungal biomass in decomposing leaf litter. Higher biofilm biomass in high temperature, high N:P conditions could have led to higher competition for nutrients with microbial decomposers and at least partly impeded the expected stimulation of leaf-litter decomposition by temperature elevation and nutrient addition.

To further investigate the interactions between microbial decomposers and primary producers, we calculated primary production based on the temporal dynamic of pigment concentration increase in the absence of grazers. Such a time-integrated indicator was expected to be more comparable to the leaf-litter decomposition rate calculated using the FM leaf mass loss. Results showed a significant negative effect of temperature on primary production, with maximal algal biomass being ultimately higher in the ambient than in the heated channels. Based on the results of microcosm experiments, primary production could have been expected to be higher in heated than in ambient systems, as already suggested in previous laboratory studies (e.g. Díaz-Villanueva et al., 2011). However, results from the literature are far less conclusive when measurements are made in natural ecosystems. For example, using streams representing natural gradients of temperature, no trend was found between temperature and biofilm biomass (Friberg et al., 2009). As underlined by Woodward et al. (2010), the response

of ecological processes to parameters such as temperature might largely differ between highly simplified laboratory conditions and more complex ecosystems, where species interactions might modify the observed responses. Despite the significant effect of temperature on primary production, the relative intensity of microbial decomposition when compared to primary production were not significantly impacted by temperature and dissolved N:P ratio. These last results suggest that even if interactions between primary producers and decomposers occurred throughout the 56-day experiment, these were not strongly impacted by the treatments tested. Nevertheless, searching for such comparisons of green and brown process interactions might be interesting to investigate in more nutrient contrasted ecosystems, these interactions being expected to be exacerbated in the most oligotrophic conditions (Danger, Cornut, et al., 2013).

An alternative hypothesis to explain the absence of a response of microbial decomposition to both temperature and inorganic nutrient level could be that these treatments altered fungal community structure and, in turn, changed the intensity of their decomposition activity. Indeed, temperature and nutrients could have led to significant changes in microbial decomposer community structure. Species replacement of more efficient fungal species in the less favourable conditions (i.e. ambient temperature, lower N:P ratios) by less efficient conditions in the more favourable conditions (i.e. high temperature, higher N:P ratios) could have occurred to maintain decomposition at similar levels. In other words, it could be hypothesised that some species with lower nutrient and thermal requirements could have higher decomposition capabilities. In our experiment, analyses revealed a significant effect of temperature on fungal community structure but only at the latest stage of decomposition. Thus, it seems unlikely for the former hypothesis to occur or, at least, to be sufficient for explaining the absence of response of microbial decomposition. Interestingly, analyses also revealed a positive effect of temperature on the relative contribution of *Lunulospora curvula* in total conidia production in ambient channels in comparison with heated channels. This result is surprising given that this species is classically found in warm waters from tropical ecosystems or in summer assemblages at our latitudes (47–49°N). Changes in assemblages in late decomposition could be due to a lower competitiveness of some aquatic hyphomycete species under higher temperatures. However, this explanation is unlikely given that the occurring shift concerns a species that generally prefers higher temperatures than those observed in all our channels, and particularly at the end of the experiment when air temperatures dropped below zero (°C).

## 4.2 | Effects of temperature and water N:P ratio on the top-down control of higher trophic levels

Although microbially driven decomposition remained similar in all treatments, we observed that even a slight elevation in water temperature (+2°C) was able to significantly enhance the overall decomposition rates (ensured by both microorganisms and macro-invertebrates) in comparison with ambient channels. Thus, temperature might have

stimulated detritivorous invertebrates' activity and leaf-litter consumption rate. Leaf-litter consumption is generally related to the degree of microbial development (Graça, Maltby, & Calow, 1993; Danger et al., 2012). In our experiment, the increase in leaf-litter consumption was certainly independent of leaf-litter microbial conditioning, since microbial biomass was not stimulated by temperature. A stimulation of invertebrate metabolism is thus a more plausible explanation for this positive response of leaf-litter mass loss in heated channels. In contrast to temperature, nutrients supply did not significantly influence overall decomposition rates. However, when investigating the ratio of CM to FM decomposition rates, the presence of invertebrates was shown to significantly change leaf-litter decomposition only in the lower N:P conditions. These observations contrast with the results from Moghadam and Zimmer (2016) who observed that simultaneous increase in nutrient concentration and temperature accelerated litter decomposition as a result of synergistic interactions between those factors. However, as shown by Greig et al. (2012), the effect of nutrient on litter decomposition can vary seasonally, hence depending on several other factors. In our study, which took place in late autumn, the relatively low temperatures (9–11°C) could explain the lack of synergistic effects between temperature and nutrients on decomposition rates.

Concerning the top-down impact of the invertebrate consumers on biofilms, comparing the upstream and downstream sections of our mesocosms permitted us to evaluate the intensity of the biofilm biomass control ensured by grazers. Interestingly, grazers significantly reduced the algal biomass measured at 56 days only in the high N:P, heated treatment. In an in situ experiment carried out in headwater streams, increases in biofilm biomass following experimental nutrient enrichment were almost undetectable due to grazing by macro-invertebrates (Greenwood & Rosemond, 2005). Our results suggest that such a control of biofilm production might also be dependent on the interaction between temperature and nutrient availability. Testing this hypothesis in natural streams exposed to nutrient pollution in different seasons might be interesting to evaluate the susceptibility of benthic algae blooms to occur.

## 4.3 | Effects of temperature and water N:P ratio on the bottom-up control of higher trophic levels

It is henceforth admitted that fungal nutrient immobilisation potentially increased the stoichiometric quality of the leaf litter, in particular in nutrient-enriched conditions (Danger, Arce Funck, et al., 2013; Gulis et al., 2017; Jabiol, Cornut, Tlili, & Gessner, 2018; Manning et al., 2015), whereas regarding temperature effects on stoichiometric quality, the literature has not yet come to an agreement. In our study, differences in leaf-litter elemental contents and ratios were independent of the temperature and of the water N:P ratios. This last result was unexpected since leaf-litter content generally reflects the nutrient availability in the water column (Farrell et al., 2018). Similarly, C:N:P ratios of biofilms collected on the last date of the experiment did not significantly differ between treatments. Gulis et

al. (2017) results suggested that P was more susceptible than N to luxury uptake by aquatic fungi, N being mainly used for immediate organism biomass increase. The fast decrease in water dissolved P observed between each nutrient addition (see Methods, data not shown) could suggest that in these relatively high N:P conditions (molar N:P ratios of 33 and 100, ratios generally above those leading to N limitations, Güsewell & Gessner, 2009), the potentially limiting P was quickly immobilised by microorganisms after each nutrient addition, with this P being then used for microbial growth with the non-limiting N, thus maintaining a low variability in microbial biomass. It must also be noted that results of our experiment might have differed if dissolved N and P amounts had been constant throughout the experiment. Indeed, it has been shown that both nutrient concentrations and ratios are fundamental for determining the nature and the intensity of microbial nutrient limitations (Güsewell & Gessner, 2009). In our experiment, since N:P ratios were maintained constant but N and P availabilities declined through time, one can imagine that nutrient limitations might have changed during the experiment.

Finally, in contrast with elemental ratios, biofilm fatty acid proportions were significantly influenced by water N:P ratios and temperature. In particular, the biofilm contents in 18:3 $\omega$ 3 and the 20:5 $\omega$ 3 were significantly higher in the high N:P channels than in the low N:P channels, and biofilm contents in 20:5 $\omega$ 3 were slightly reduced in heated channels. Temperature increase is largely known to reduce an organisms' PUFA requirements (e.g. Masclaux et al., 2009). In our experiment, these differences, while significant, remained reduced. Differences in temperature might have not been sufficient for inducing strong changes in biofilm PUFA content, especially because mean temperatures in experimental streams were quite low (between 9 and 11°C). In contrast, the higher PUFA content of biofilms grown in high N:P conditions are in agreement with some results showing a higher synthesis of these compounds by benthic algae in higher nutrient conditions (e.g. Hill, Rinchar, & Czesny, 2011). Since some PUFAs can be limiting for macro-invertebrate growth (Crenier et al., 2017; Torres-Ruiz, Wehr, & Perrone, 2007), a stimulation of biofilm consumer growth in the high N:P ratio channels mediated by an increase in resource quality could have been expected. In our experiment, we choose to focus on gammarids to evaluate temperature and nutrient impacts on secondary production. Indeed, gammarids are mostly detritivorous invertebrates that still have substantial capacity for herbivory and could cope with low-quality detrital resources by compensatory feeding on biofilms (Flores, Larrañaga, & Elozegi, 2014; Taylor & Brown, 2006). As such, they provide an important link between brown and green food webs, and can have substantial influence on auto- and heterotrophic microbes (Frost, Elser, & Turner, 2002; Hillebrand, De Montpellier, & Liess, 2004; Jüttner, 2001). In our study, gammarid growth was significantly increased in the heated mesocosms, whatever the N:P ratio tested. Gammarid growth was not related to leaf-litter elemental quality or to resource PUFA content, probably due to the fact that these resources were either not sufficiently limiting or not sufficiently different between

treatments for inducing a stimulation of consumer growth. The increase in gammarid metabolism associated with higher leaf-litter consumption rates in heated channels might explain the fastest growth observed with higher temperature.

## 5 | CONCLUSION

Overall, results from our mesocosm experiment indicate that even small changes in temperature and nutrient supply may have complex and pervasive effects on stream ecosystem processes. Most of our hypotheses that were based upon the results of laboratory experiments with wide gradients of both temperature and nutrients have been rejected in our semi-controlled mesocosms. Contrary to expectations, microbial leaf-litter decomposition remained unchanged under higher temperatures and N:P ratios, and biofilm growth stimulation by nutrients and temperature were limited, and transitory. Most of temperature responses concerned macro-invertebrates that showed increased activity and growth, while these changes were unrelated to changes in resource quality (in terms of resource stoichiometry and PUFA content). Our mesocosm study thus demonstrated that interactive effects of temperature and nutrients were certainly far much more complicated than initially thought. Undoubtedly, our mesocosm study also had limits, particularly concerning the temperature variability in such experimental settings. Indeed, as the water temperature followed air temperature fluctuations, water temperature variations in our experimental channels upon 56 days were certainly wider than actual variations occurring in situ. However, the mean temperature difference between heated and ambient channels (1.91°C) is consistent with current IPCC previsions for the years to come (IPCC 2014). Our experiment also took into account diel temperature oscillations, which have long been neglected in ecological experiments, but might influence ecosystem functioning (Dang, Schindler, Chauvet, & Gessner, 2009). Such semi-controlled experiments, involving a large complexity of biological communities, might be essential to better understand the effects of potential interactions amongst multiple stressors on natural ecosystems and to provide insights into possible mitigation effects through the complex network of species interactions.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data supporting this publication are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.