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Hydrological contingency: drying history affects aquatic microbial decomposition

Maite Arroita^{1,2} · Lorea Flores³ · Aitor Larrañaga¹ · Eric Chauvet⁴ · Arturo Elosegi¹

Abstract

Climate change, land use intensification, and water abstraction magnify the frequency and severity of droughts but also result in totally unnatural hydrological patterns. Longer and more severe droughts slow down organic matter decomposition, whereas the effect of drying history, i.e. the specific sequence of different conditions to which organic matter is exposed, has seldom been addressed. Drying history could have important consequences for microbial communities colonizing and decomposing leaf litter in streams given the rapid fluctuations in microbial composition and processes. We studied whether the effects of drought-related impacts (stagnation, drying and both) on microbial activity and leaf litter decomposition are affected by the timing when peak stress (stagnation or drying) occurs, and whether the effect of the drying history is consistent among the three different stress types. In laboratory microcosms, we recreated areas with flowing water (aerated water), isolated pools with stagnant water (non-aerated water) and dry beds (dry microcosms). Combining these conditions and their sequence, we created nine treatments (ten with the control) differing in stress type (stagnation, drying, both) and timing of peak stress (early, middle, late), and measured fungal biomass, sporulation, microbial respiration, and microbial decomposition of alder leaf disks. The effects of drought-related stress conditions were not consistent among response variables. However, disturbances were systematically more detrimental to decomposition in early stages, resulting in a lower fungal biomass and activity, and reduced microbial litter decomposition. These results suggest that the effects of stress on decomposition-associated variables depend not only on the intensity of the stress, but also on its timing, with early stress and its legacy effects having greatest impact on leaf litter decomposition.

Keywords Aquatic hyphomycetes · Microbial community · Organic matter · Decomposition · Drought · Timing

Introduction

Allochthonous organic matter is the main energy source supporting food webs in streams and rivers (Tank et al. 2010), with leaf litter decomposition being a major ecosystem-level process, especially in systems running through forested

catchments (Webster and Benfield 1986; Abelho 2001). Litter decomposition results from several abiotic and biotic processes, which include leaching, mechanical abrasion, microbial degradation, and fragmentation by invertebrates (Graça 2001; Hieber and Gessner 2002). Within all these processes, the activity of microbial decomposers is among the most important mechanisms (Gessner and Chauvet 1994). In fact, the nutritional value and palatability of freshly fallen leaves of most tree species are usually too low for stream invertebrates, and thus, leaf litter is hardly consumed unless it is first conditioned by microbes (Petersen and Cummins 1974; Gessner et al. 1999; Allan and Castillo 2007). During the conditioning phase, the chemical composition of plant tissue is modified mainly through (1) conversion of plant tissue into microbial materials, (2) breaking of complex leaf molecules into simpler ones by means of microbial extracellular enzymes, (3) mechanical alteration of leaves mainly due to fungal hyphae growth; and (4) microbial nutrient

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incorporation (Bärlocher and Kendrick 1975; Gessner et al. 1999). Within the microbial community involved in litter decomposition, bacteria and aquatic fungi are the dominant groups. These communities also exhibit very marked temporal dynamics during the decomposition process (Bärlocher 2009). Quickly after deposition and germination of fungal spores on leaves, mycelial biomass increases exponentially until it stabilizes, then decreases (Gessner and Chauvet 1994; Suberkropp 2001; Artigas et al. 2011). Similarly, fungal sporulation rates may peak as early as 2 weeks after colonization and decrease afterwards (Bärlocher 2009), and the relative importance of different fungal taxa changes during the decomposition process (Gessner and Chauvet 1994; Duarte et al. 2010; Mora-Gómez et al. 2016).

The structure and activity of these microbial communities and, in consequence, litter decomposition, are sensitive to many stressors, such as point-source pollution (Pascoal et al. 2003), eutrophication (Hladyz et al. 2011; Woodward et al. 2012), river regulation (Mendoza-Lera et al. 2012), changes in riparian vegetation (Lecerf et al. 2005), loss of biodiversity (Gessner et al. 2010), or drought (Romaní et al. 2012). In particular, droughts affect the physiology of trees, reducing the quantity and quality of leaves as well as altering the timing of litter inputs into streams (Farooq et al. 2009) and, thereby, reducing leaf litter decomposition (Hladyz et al. 2009). Additionally, flow reduction has been shown to slow down litter decomposition in the wetted channel (Abril et al. 2015; Arroita et al. 2015). Pool isolation and water stagnation rapidly alter water physico-chemical properties, even resulting in hypoxic conditions, which triggers changes in leaf-associated microbial communities and processes, and reduces macroinvertebrate density (Acuña et al. 2005; Canhoto and Laranjeira 2007; Foulquier et al. 2014), thereby slowing down litter decomposition (Medeiros et al. 2009; Schlieff and Mutz 2011; Mora-Gómez et al. 2015). Leaf litter stranded in emerged sediments also breaks down much more slowly than in the wetted channel (Langhans and Tockner 2006; Romaní et al. 2012). In fact, microbial diversity and activity, macroinvertebrate richness and density, and litter decomposition have been shown to depend on the duration (Langhans and Tockner 2006; Larned et al. 2007; Riedl et al. 2013) and severity (Bruder et al. 2011) of drying events, the cumulative duration of emersion being more important than drought frequency (Corti et al. 2011; Foulquier et al. 2015). Moreover, drying and hypoxia events produce legacy effects that reduce litter decomposition long after the flow has resumed (Datry et al. 2011; Dieter et al. 2011; Martínez et al. 2015).

Given the successional nature of microbial colonization during litter decomposition and the mentioned legacy effects, the impacts of pool isolation, drying and rewetting are likely to differ depending on when they occur (Bruder et al. 2011). In particular, it is likely that the resistance and resilience of

microbial decomposers to stressors differ depending on the maturity stage of the microbial community. Nevertheless, unlike the intensity and frequency of droughts, the effect of drying history has seldom been addressed.

This topic can be increasingly relevant in the context of global environmental change. Indeed, flow dynamics are likely to be seriously altered in the near future, affecting also stream communities and processes. The severity, frequency and occurrence of droughts are increasing in many regions due to ongoing climate change, and intensification of land and water uses are magnifying the relevance of these drying events worldwide (Milly et al. 2005; IPCC 2014). In particular, human activities can affect not only the frequency and severity of drying events, but also result in totally unnatural hydrological patterns, such as those produced by hydropeaking, where large portions of stream channels are periodically immersed and emerged following a schedule that depends on human activity patterns (Jones 2014). All these changes can strongly alter the drying history of organic matter in stream and river ecosystems, which can in turn produce severe impacts on riverine communities and on ecosystem processes.

Here we simulated different drying histories by manipulating aeration and water level under laboratory conditions to test whether (1) the intensity of drought-related impacts on fungal activity and leaf litter decomposition is affected by the timing of the impact during the decomposition process, (2) the effect of the drying history is consistent among different drought-related stress conditions, and (3) these potential differences remain even after flow is resumed. We hypothesized dry conditions to have a more negative effect than the lack of aeration on the microbial community and microbial litter decomposition, as evidence has shown that microbial activity can be dramatically reduced by the drying of the organic materials, while microbes can be active under a wide range of pollution and oxygenation levels. Additionally, we expected the drying history to affect microbial activity irrespective of the stress type, with early impacts being more detrimental than late ones because they would have more opportunities for legacy effects.

Materials and methods

Freshly fallen black alder [*Alnus glutinosa* (L.) Gaertner] leaves were collected in autumn, air-dried to constant mass and stored in the dark at room temperature (20 °C). Leaves were soaked and 2550 disks (12 mm in diameter) were punched out with a cork borer. Disks were arranged in groups of 10, identified, weighed, enclosed in 100 µm-mesh bags and incubated in the headwaters of the Agüera Stream (N. Iberian Peninsula; 43°12'36"N, 3°16'12"W) for one week (13–21 February 2013) to allow fungal colonization.

Previous studies reported the Agüera Stream headwaters to be oligotrophic and rich in aquatic hyphomycetes (Pérez et al. 2012). During field incubation, mean water temperature was 8 °C, pH 7.4, conductivity 70.8 $\mu\text{S}\cdot\text{cm}^{-1}$ and water was oxygen saturated (for more details about the Agüera Stream see Eloise et al. 2002, 2006). After a week, bags were carried to the laboratory and all but 5 groups of 10 disks were extracted from bags and arranged in microcosms consisting of a 300-mL glass beaker filled with 60 cm³ of coarse siliceous sand (2–4 mm) that was previously ashed (500 °C, overnight) and washed with deionized water. In total, there were 250 microcosms with ten disks in each.

In these microcosms we recreated three different conditions that leaves could be subjected to in drying water-courses: areas with flowing water, stagnant water and dry beds. Flowing water was simulated by aerating the water in the microcosms by means of an air pump, stagnant water by maintaining the microcosms without aeration and dry bed by removing the superficial water from the microcosms. Combining these conditions and their sequence, we created ten treatments: one was constantly aerated for 6 weeks, and was considered the non-stress Control (Table 1). The other nine treatments were arranged in three groups of drought-related stress conditions and differed in the timing of peak stress. In the Stagnant stress condition, microcosms were subjected to 2 weeks of non-aeration during the 6-week period (three treatments, with non-aeration in the initial (early-Stagnant, the first 2 weeks), middle (middle-Stagnant, weeks 3 and 4) and late (late-Stagnant, weeks 5 and 6) stages, respectively). In the Dry stress condition, microcosms were subjected to 2 weeks of dry conditions during the 6-week period (three treatments, with dry conditions in the initial (early-Dry), middle (middle-Dry) and late (late-Dry) stages, respectively). The StagDry stress condition combined both stagnation

and drying, and microcosms were subjected to 2 weeks of dry conditions and 4 weeks of non-aeration (three treatments, with dry conditions in the initial (early-StagDry), middle (middle-StagDry) and late (late-StagDry) stages, respectively). After these experimental weeks (6 weeks in total), the study was extended by four recovery weeks, during which all microcosms were kept with aerated water, which simulated flow resumption.

During the 10 weeks, microcosms were kept at 6 °C, with a 12:12 h light regime, common winter conditions in the region. In beakers with water, temperature and dissolved oxygen concentration were measured twice a week and water was changed every week to avoid the accumulation of potentially toxic substances. Water for renewal was brought from the Agüera Stream every week and filtered through 100 μm pore size Nytal mesh (Sefar Nytal PA-13, Sefar Maissa, S.A.U., Cardedeu, Spain) to remove organic matter. It must be noted that this mesh size can exclude the largest conidia, but that the most abundant microbial inocula can go through. Adding new spores to microcosms probably homogenized fungal assemblages and made it difficult to detect the disappearance of sensitive taxa, but we aimed to recreate conditions in streams, where new spores arrive when flow is resumed. Every 2 weeks we sacrificed five replicate microcosms per treatment to measure fungal biomass, sporulation rates of fungi, microbial respiration and microbial decomposition of alder disks (five microcosms per treatment per date \times 10 treatments \times 5 dates = 250 microcosms). On each sampling date, five of the disks in each microcosm (containing a total of ten disks) were used to determine respiration rate and ergosterol content subsequently, and the other five to estimate sporulation rates. All disks were used to measure mass loss of alder leaf disks as proxy for microbial leaf litter decomposition.

Table 1 Experimental treatments as a function of type and timing of stress

Stress type	Timing of peak stress	Treatment	Weeks				
			1–2	3–4	5–6	7–8	9–10
Stagnant		Control	F	F	F	F	F
	Early	Early-Stagnant	S ^a	F	F	F	F
	Middle	Middle-Stagnant	F	S ^a	F	F	F
Dry	Late	Late-Stagnant	F	F	S ^a	F	F
	Early	Early-Dry	D ^a	F	F	F	F
	Middle	Middle-Dry	F	D ^a	F	F	F
StagDry	Late	Late-Dry	F	F	D ^a	F	F
	Early	Early-StagDry	D ^a	S	S	F	F
	Middle	Middle-StagDry	S	D ^a	S	F	F
	Late	Late-StagDry	S	S	D ^a	F	F

All variables were measured after each 2-week period

F flowing water (aerated water), S stagnant water (non-aerated water), D drought (dry)

^aPeak stress. Note that all treatments were subjected to aerated water during the last 4 weeks

Fungal biomass

Fungal biomass was estimated as ergosterol content in disks, which is the main component of fungal cell membrane as well as one of the best descriptors of metabolically active fungal biomass (Gessner and Schmitt 1996; Charcosset and Chauvet 2001; Abelho 2009). From each microcosm, five disks out of ten were frozen at -80°C after being used to measure respiration (see below). Frozen samples were lyophilized and weighed to obtain dry mass (DM). Lipids were extracted by incubating lyophilized samples with 0.14 M KOH in methanol at 80°C for 30 min in a shaking bath. Ergosterol was separated and concentrated by solid-phase extraction (Waters Sep-Pack[®] Vac RC, 500 mg, tC18; Gessner and Schmitt 1996) and quantified through high-pressure liquid chromatography (HPLC, Waters). HPLC system consisted of one pump, injector, column (Licrospher 100 RP-18, 25 cm, Merck), UV detector and a recording unit. Chromatograms were set as follows: 100% mobile phase (100% methanol), flow rate of 1.4 mL min^{-1} , column temperature at 33°C , detection wavelength at 282 nm and injection volume of 10 μL . Ergosterol was quantified based on the comparison with ergosterol standards ($1\text{--}100\text{ }\mu\text{g mL}^{-1}$, 98% purity ergosterol, Fluka). Results were expressed as $\mu\text{g ergosterol g}^{-1}\text{ DM}$.

Sporulation

To measure fungal sporulation (i.e. reproductive activity of spore-producing fungi, mainly aquatic hyphomycetes), the other five disks were incubated in 100-mL Erlenmeyer flasks with 25 mL filtered stream water (0.7 μm pore size glass fiber filters, Whatman GF/F, Whatman International Ltd., Maidstone, England) on an orbital shaker (60 rpm; Multitron II, INFORS HT, Bottmingen, Switzerland) for 48 h at 10°C (Bärlocher 2005). Conidial suspensions were decanted to 50-mL centrifuge tubes and 2 mL of 37% formalin were added to fix conidia. Distilled water was added up to 35 mL. The suspensions were stirred to ensure a uniform distribution, and an aliquot (between 1 and 20 mL) was filtered (5 μm pore size mixed cellulose filters, Millipore SMWP). Filters were stained with Cotton Blue in lactic acid (0.05%) and conidia identified and counted under a bright field Leica microscope (a minimum of 300 conidia counted or 0.09 cm^2 of surface viewed). Disks were lyophilized and weighed, and sporulation rates were expressed as number of conidia released $\mu\text{g}^{-1}\text{ DM day}^{-1}$.

Respiration

Microbial respiration rates were measured using a closed six-channel dissolved oxygen measuring system (Strathkelvin 928 System, Strathkelvin Instruments,

Motherwell, U.K.). Oxygen electrodes were calibrated with a solution of 2% sodium sulfite in 0.01 M sodium borate (0% O_2), and 100% O_2 saturated distilled water. Five disks per microcosm were incubated together in chambers with 3 mL 100% O_2 saturated filtered stream water (0.7 μm pore size glass fiber filters, Whatman GF/F, Whatman International Ltd., Maidstone, England) homogenized with a magnetic stirring bar (10°C , 40 min). Chambers without disks were used as a control for oxygen depletion in the water. Oxygen consumption rates were calculated by subtracting the oxygen consumption in the control chamber over a 20-min interval from the consumption in the chamber with the sample. Results were expressed as $\text{mg O}_2\text{ g}^{-1}\text{ DM h}^{-1}$.

Leaf litter mass remaining

Microbial leaf litter decomposition was expressed as the percentage of leaf mass lost during the microcosm experiment. Five out of the 255 10-disk groups incubated in the stream for 1 week were carried to the laboratory, immediately lyophilized and weighed to correct for leaf mass lost during the colonization period in the stream. Remaining mass in each microcosm was calculated by summing up DM of the ten disks after lyophilization and was expressed as percentage of initial mass.

Data treatment

The total amount of ergosterol (μg), the cumulative spore production (spores) and the cumulative oxygen consumption (mg O_2) were calculated following Gessner and Chauvet (1997): ergosterol concentration ($\mu\text{g g}^{-1}\text{ DM}$) in each beaker was multiplied by the remaining DM (g DM) in that beaker and we analyzed when the maximum amount of ergosterol occurred in each treatment; sporulation (conidia $\mu\text{g}^{-1}\text{ DM day}^{-1}$) and respiration ($\text{mg O}_2\text{ g}^{-1}\text{ DM h}^{-1}$) rates measured in each microcosm were also multiplied by the remaining mass (g DM), and these rates (conidia day^{-1} and $\text{mg O}_2\text{ h}^{-1}$) were scaled to the duration of the experiment, assuming the mean rate per treatment and sampling date remained constant during the previous and posterior 7 days, to obtain, respectively, the total amount of spores produced (conidia) and the total amount of oxygen consumed (mg O_2).

For data analysis, we computed log transformed Treatment/Control ratios (T/C) for all studied variables. The effect of stress type and timing of peak stress were analyzed using data measured at the end of the experimental phase (week 6) and during the recovery phase (weeks 8 and 10) independently, by means of a two-way ANOVA, with both stress type (Stagnant, Dry, StagDry) and timing (early, middle, late) as fixed factors. Interaction between both factors was

not significant in any of the analyses and, therefore, was removed from all two-way ANOVAs. Post-hoc Tukey HSD tests were performed when ANOVAs yielded significant differences. To search for general differences in conidial assemblages, a non-metric multidimensional scaling (nMDS) was also performed based on the Bray-Curtis dissimilarity matrix, followed by a PERMANOVA (10^6 permutations) to test the differences along time and among stress types, timing and treatments. Linear regressions were used to test the relationship between mean ergosterol concentration during the experiment, the cumulative sporulation, the cumulative respiration, and the remaining mass of alder disks at the end of the experiment. All statistical analyses were conducted using R statistical software (version 3.0.2; R Core Team, 2014; Vienna, Austria).

Results

Lack of aeration caused small but statistically significant ($F_{1,449} = 159.9$, $P < 0.0001$) reduction in oxygen concentration: the minimum concentration and saturation measured in non-aerated microcosms were, respectively, 10.3 mg L^{-1} and 85%, whereas they were 12.0 mg L^{-1} and 100% in aerated ones. Overall, the mean concentration and saturation of dissolved oxygen during the whole experiment were, respectively, around 11.5 mg L^{-1} and 95% in all treatments (Table 2). Similarly, temperature of water without aeration was higher ($F_{1,426} = 50.95$, $P < 0.0001$) and the mean temperature during the experiment was slightly higher in treatments within the StagDry stress type (6.3°C) than in the rest of the treatments (5.9°C).

Fungal biomass

Ergosterol concentration in the Control treatment ranged between 87 and $340 \text{ } \mu\text{g g}^{-1} \text{ DM}$, whereas the lowest

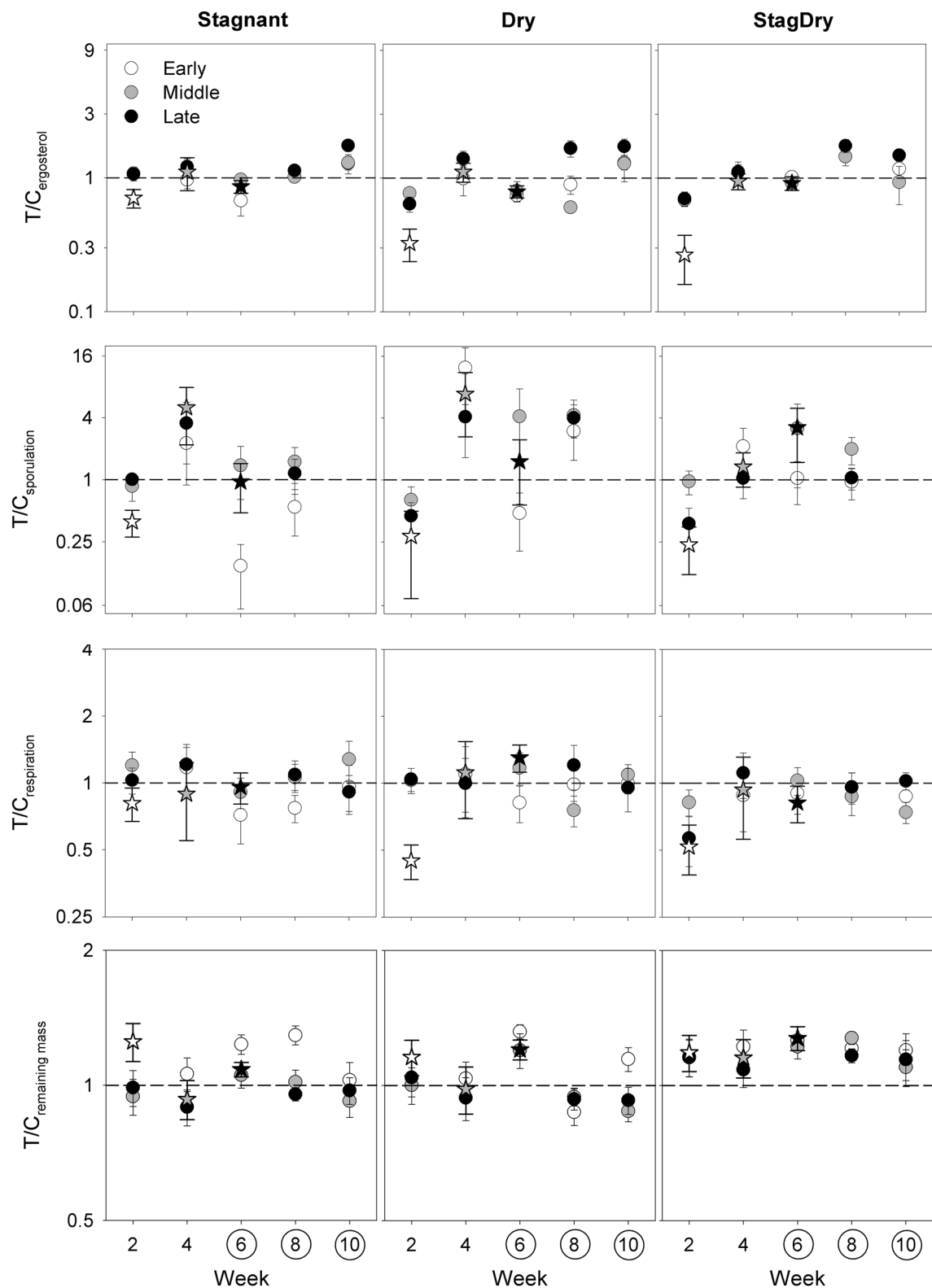
($77 \text{ } \mu\text{g g}^{-1} \text{ DM}$) and highest ($531 \text{ } \mu\text{g g}^{-1} \text{ DM}$) values of ergosterol concentration were measured in week 2 of early-Dry treatment and week 8 of late-StagDry treatment, respectively (Fig. 1). Ergosterol concentration measured at the end of the experimental phase (week 6) was highest in Control microcosms, but these differences were small. However, there were significant differences among stress types ($P = 0.005$; Table 3), even though they did not follow the stress gradient: ergosterol content was lowest in disks that were only subjected to two weeks of dry conditions (Dry stress condition), highest in leaves subjected to a combination of drying and non-aeration (StagDry stress condition) and intermediate in leaves subjected only to non-aeration (Stagnant stress condition). The timing of peak stress did not affect ergosterol concentration measured in week 6 ($P = 0.788$; Table 3).

Subjecting all treatments to aerated water during weeks 7 and 8 stimulated fungal growth in treatments combining dry periods with non-aeration (StagDry; Fig. 1). Therefore, ergosterol content measured in week 8 showed similar patterns to the ones observed in week 6, but greater differences among stress types ($P < 0.001$; Table 3), with ergosterol content in treatments within StagDry stress condition being even higher than in the Control treatment. Flow resumption also revealed significant differences among timings, and treatments with peak stress in the late stage showed a higher ergosterol concentration than the ones with peak stress in earlier stages ($P < 0.001$; Table 3). At the end of the experiment, ergosterol concentration was higher than in Control microcosms in almost all treatments. Differences among timings remained at week 10 ($P = 0.002$), whereas the ones among stress types disappeared ($P = 0.111$; Table 3). The peak of the total amount of ergosterol was highest in the StagDry stress condition ($20.4\text{--}23.9 \text{ } \mu\text{g}$; Table 4) and was measured after subjecting disks to aerated water (week 8), whereas in the Control treatment and in the Stagnant stress condition it only reached $15.6 \text{ } \mu\text{g}$ and occurred at the

Table 2 Mean \pm standard error concentration (mg L^{-1}) and saturation (%) of dissolved oxygen, and water temperature ($^\circ\text{C}$) measured during the experiment for each treatment

Treatment	Oxygen concentration (mg L^{-1})	Oxygen saturation (%)	Temperature ($^\circ\text{C}$)
Control	12.2 ± 0.06	100.0 ± 0.46	6.0 ± 0.06
Early-Stagnant	11.9 ± 0.06	93.3 ± 0.51	5.9 ± 0.06
Middle-Stagnant	11.2 ± 0.10	94.1 ± 0.75	5.9 ± 0.05
Late-Stagnant	11.3 ± 0.12	95.5 ± 0.93	5.9 ± 0.04
Early-Dry	12.3 ± 0.07	100.1 ± 0.55	5.8 ± 0.06
Middle-Dry	12.2 ± 0.11	100.0 ± 0.95	6.0 ± 0.08
Late-Dry	12.3 ± 0.12	101.1 ± 1.03	6.0 ± 0.07
Early-StagDry	10.5 ± 0.08	89.5 ± 0.67	6.2 ± 0.07
Middle-StagDry	10.4 ± 0.09	89.0 ± 0.76	6.2 ± 0.08
Late-StagDry	10.7 ± 0.08	90.4 ± 0.68	6.4 ± 0.06

$n = 54$ for Control and Stagnant treatments and $n = 30$ for Dry and StagDry treatments



beginning of the experiment (week 2). The peak of the total amount of ergosterol, as well as the stage when it occurred, were more variable in the Dry stress condition (Table 4).

Sporulation

In total, 20 hyphomycete taxa were identified, but only 6 appeared in all treatments and sampling dates, accounting

Fig. 1 Treatment/Control (T/C) ratios of ergosterol concentration (row 1), sporulation rates (row 2), respiration rates (row 3) and remaining mass of alder disks (row 4) measured every 2 weeks for treatments with Stagnant (column 1), Dry (column 2) and StagDry (column 3) stress conditions and with peak stress occurring in the early (white), middle (grey) and late (black) experimental stages. Note that the y-axis is log-transformed and the 1 line indicates a T/C ratio of 1 ($T=C$), values above 1 indicating $T>C$ and values below 1 indicating $T<C$. Within each treatment, results obtained after being subjected to peak stress are shown with stars. Circles in the x-axis indicate the results used for statistical analysis and shown in Table 3. Note that all treatments were subjected to aerated water during weeks 7–10. Error bars show the standard error. Differences among stress types were not consistent, but early impacts tended to be more detrimental than impacts at a later stage

for more than 95% of the conidia in every sample: *Alato-*
spora acuminata Ingold, *Anguillospora filiformis* Great-
head, *Anguillospora* sp., *Articulospora tetracladia* Ingold,
Crucella subtilis Marvanová & Suberkropp and *Flagel-*
lospora curvula Ingold. Although the conidial assem-
blage changed significantly over time (PERMANOVA:
 $pseudoF_{3,80} = 0.14$, $P < 0.0001$), these changes were
not consistent among stress types, as shown by statis-
tically significant interactions between stress type and
time (PERMANOVA: $pseudoF_{9,104} = 0.13$, $P < 0.0001$),
and were mainly driven by the occurrence of rare taxa in
isolated samples. Changes in assemblages during non-
aeration or dry periods were minor, and no taxa prolifer-
ated or disappeared in these conditions. Mean sporula-
tion rates in the Control treatment decreased from 0.74
conidia μg^{-1} DM day^{-1} on the first sampling date to 0.10
conidia μg^{-1} DM day^{-1} on the last one. Sporulation also
showed a decreasing trend in the Stagnant stress condi-
tion, decreasing to 0.05 conidia μg^{-1} DM day^{-1} in early-
Stagnant microcosms, whereas rates were more variable
in Dry and StagDry stress conditions and showed mini-
mum and maximum values at different stages. Therefore,
even if sporulation rates differed significantly among
stress types, these differences were not consistent at dif-
ferent sampling dates and did not support our hypotheses:
sporulation in the StagDry stress condition was highest in
week 6, while it was lowest in week 8 (Table 3; Fig. 1).
The total production of spores was not affected by the
stress type ($P = 0.086$), even though fungi in the Dry
stress condition produced 50% more conidia than the ones
in the Stagnant and StagDry stress conditions (Table 4).
On the contrary, the timing of peak stress significantly
influenced sporulation rates and, as hypothesized, treat-
ments subjected to stress in the initial stage showed lower
rates in week 6 ($P = 0.001$), differences being similar in
week 8, after flow resumption ($P = 0.016$; Table 3).

Respiration

Mean respiration rates in the Control treatment decreased
from 0.275 mg O_2 g^{-1} DM h^{-1} on the first sampling date
to 0.183 mg O_2 g^{-1} DM h^{-1} on the last date. Variability in
respiration rates was relatively high and we did not detect
any consistent pattern relative to the stress type or timing
of peak stress (Fig. 1), differences being statistically non-
significant in all cases ($P > 0.05$; Table 3). Stress type did not
affect the total amount of oxygen consumed either by the end
of the experimental phase (week 6; $P = 0.200$) or by the end
of the recovery phase (week 10; $P = 0.698$; Table 3), even
though total oxygen consumption was highest in the Dry
stress condition and lowest in the StagDry stress condition
(Table 4). Nevertheless, microbial communities perturbed in
the late and initial experimental stages consumed the highest
and lowest amount of oxygen, respectively, these differences
being statistically significant by the end of the experiment
(week 10; $P = 0.045$; Table 3).

Leaf litter mass remaining

Alder leaf disks in the Control treatment lost, on average,
25% of their initial DM, decomposition mainly occurring
during the first 6 weeks. Microbial decomposition of alder
disks in treatments within Stagnant stress condition was sim-
ilar to that in the Control treatment (Fig. 1), and significantly
higher than in Dry and StagDry stress conditions ($P = 0.030$;
Table 3). Although microbial decomposition of alder disks
in late-Stagnant and middle-Stagnant treatments tended to
be higher than in early-Stagnant, overall, differences did not
achieve statistical significance ($P = 0.065$), probably because
timing did not affect mass loss in the other stress types, espe-
cially in the StagDry condition (Fig. 1). Flow resumption
accelerated microbial litter decomposition in the Dry stress
condition and by the end of the experiment leaf disks within
Stagnant and Dry stress conditions lost, on average, around
25% of their initial DM, similar to Control microcosms. Sub-
jecting treatments within StagDry stress condition to aerated
water did not counterbalance the reduction of microbial lit-
ter decomposition due to the first 6-week period, and disks
only lost 10–15% of the initial DM ($P = 0.013$; Table 3).
Flow resumption also revealed differences among timings,
microbial decomposition of alder leaf disks being lowest in
treatments disturbed in the initial stage and highest in the
ones disturbed in the medium stage ($P = 0.033$).

The remaining mass of alder leaf disks at the end of the
experiment was significantly related only to the cumulative
respiration ($R^2 = 0.40$, $P = 0.048$), where the higher the oxy-
gen consumption, the lower the remaining mass. Among all
the other possible regressions, only the one between mean
ergosterol concentration and cumulative respiration was sta-
tistically significant ($R^2 = 0.56$, $P = 0.012$).

Table 3 Statistical results of response variables to stress type and timing

Variable	Week	Stress type				Timing of peak stress			
		DF	F	p	Tukey HSD	DF	F	p	Tukey HSD
Ergosterol	6	2.40	6.19	0.005	SD ^a S ^{ab} D ^b	2.40	0.24	0.788	
	8	2.40	11.39	<0.001	SD ^a S ^b D ^b	2.40	5.76	<0.001	l ^a m ^b e ^b
	10	2.40	2.33	0.111		2.40	7.21	0.002	l ^a m ^b e ^b
Sporulation	6	2.22	5.41	0.012	SD ^a D ^{ab} S ^b	2.22	9.11	0.001	l ^a m ^a e ^b
	8	2.22	17.13	<0.0001	D ^a S ^b SD ^b	2.22	5.02	0.016	m ^a l ^{ab} e ^b
	10	–	–	–	–	–	–	–	–
Cumulative sporulation	6	2.4	3.47	0.134		2.4	1.79	0.279	
	8	2.4	3.70	0.123		2.4	3.92	0.114	
	10	–	–	–	–	–	–	–	–
Respiration	6	2.39	1.27	0.292		2.39	2.61	0.086	
	8	2.40	0.15	0.858		2.40	2.71	0.079	
	10	2.40	0.57	0.570		2.40	0.27	0.767	
Cumulative respiration	6	2.4	2.48	0.200		2.4	4.86	0.085	
	8	2.4	1.33	0.362		2.4	6.54	0.055	
	10	2.4	0.39	0.698		2.4	7.41	0.045	l ^a m ^{ab} e ^b
Remaining mass	6	2.40	3.83	0.030	SD ^a D ^{ab} S ^b	2.40	2.94	0.065	
	8	2.40	23.83	<0.0001	SD ^a S ^b D ^c	2.40	2.32	0.111	
	10	2.40	4.84	0.013	SD ^a D ^b S ^b	2.40	3.71	0.033	e ^a l ^{ab} m ^b

S stagnant, D dry, SD StagDry, e early, m middle, l late

Post hoc Tukey HSD tests were only performed when the two-way ANOVA yielded significant differences. Letters are ranked from highest (a) to lowest (b) values

Table 4 The peak of the total amount of ergosterol and when it occurred for each treatment. Mean oxygen consumption and spore production at the end of the experimental phase (week 6) and recovery phase (week 10, week 8 in the case of cumulative sporulation)

Treatment	Peak ergosterol		Cum. sporulation (spores × 10 ⁶)		Cum. respiration (mg O ₂)	
	Week	µg	Week 6	Week 8	Week 6	Week 10
Control	6	15.55	0.89	1.00	11.85	18.88
Early-Stagnant	2	14.83	0.71	0.74	11.43	17.75
Middle-Stagnant	2	15.55	1.08	1.25	11.35	19.25
Late-Stagnant	2	15.55	0.89	1.01	11.93	19.05
Early-Dry	4	14.36	1.34	1.47	10.09	16.79
Middle-Dry	2	15.55	1.42	1.86	12.65	19.45
Late-Dry	8	18.19	0.95	1.20	12.83	21.34
Early-StagDry	8	20.41	0.53	0.67	9.46	17.47
Middle-StagDry	8	20.46	0.92	1.31	10.74	18.42
Late-StagDry	8	23.91	0.90	1.24	11.56	19.65

Min and max values per variable are in bold

Discussion

We hypothesized dry conditions would have a stronger effect than the lack of aeration on the microbial community and microbial leaf litter decomposition. However, differences among drought-related stress types in our experiment had no consistent effects on most measured response variables. Our experimental conditions had little effect on aquatic hyphomycete assemblages and seemed

neither to benefit nor to lead any taxa to extinction, with six taxa always accounting for more than 95% of the conidia. In addition, although microbial variables, both structural (biomass and structure of fungal assemblages) and functional (rates of fungal sporulation and microbial respiration), showed significant differences at the end of the experimental phase, these effects did not follow the expected stress gradient, neither did the different variables show a common ranking among stress types.

These results probably reflect that the stress range we recreated was small. Indeed, pool isolation and water stagnation are known to alter water physico-chemical properties, enhancing the accumulation of organic matter, concentrating nutrients and potentially toxic leachates, increasing water temperature and often resulting in hypoxic conditions (Lake 2003; Canhoto and Laranjeira 2007), which strongly affect microbial communities and processes (Foulquier et al. 2014). The conditions in our experimental treatments were less harsh, as a consequence of weekly water renewal, supply of new conidia and the fact that the experiment was performed in close systems at constant temperature, contrasting with the higher temperature commonly observed in isolated pools (Boulton 2003; Muñoz 2003). The effect of non-aeration in our microcosms was biologically irrelevant even on oxygen concentration, although it can be extremely strong in the field (Acuña et al. 2005) and severely impair aquatic fungi (Medeiros et al. 2009). Other effects of aeration might remain important in our experiment, namely turbulence, which increases the probability of a spore to colonize leaf litter, promotes nutrient renewal and decreases the concentration of potentially toxic exudates close to the microbial community (Schlief and Mutz 2007; Canhoto et al. 2013). Still, results obtained in non-aerated treatments, especially in treatments impacting middle (middle-Stagnant) and late (late-Stagnant) stages, were very similar to those in Control microcosms. It is likely that the effects of non-aeration would be stronger if they had resulted in more severe anoxia, as has often been reported for drying streams (Acuña et al. 2005). The stress level simulated in our experiment might be compared to pools that are constantly disconnecting and reconnecting to the main oxygenated channel.

Drying has also been shown to alter the structure and activity of microbial assemblages, and slow down the decomposition of organic matter stranded in emerged sediments (Romaní et al. 2012; Mora-Gómez et al. 2016). Nevertheless, most studies are focused on summer droughts characterized by high temperatures and very dry air conditions (Ylla et al. 2010; Vázquez et al. 2011), whereas temperature in our chamber was low and the atmosphere very humid. These conditions could lead to underestimation of the effects of natural summer droughts, but could also reflect better human-induced drying events in humid regions (e.g. caused by water abstraction) and, thus, could contribute to better understanding of the impacts of such drying events. As a result, alder leaf disks did not completely dry up and, because aquatic fungi are able to persist in moist substrata (Sanders and Webster 1978; Sridhar and Bärlocher 1993; Chauvet et al. 2015), fungal activity (i.e. sporulation and respiration) in our microcosms recovered quickly after rewetting, as has been described elsewhere (Langhans and Tockner 2006). One of the key factors that help fungi persist under these conditions is the thickness of their cell wall

(Schimel et al. 2007), which could explain why we measured the highest ergosterol concentration in treatments combining stagnation and dry conditions (StagDry). Our results agree with those from Bruder et al. (2011) that showed severe drying (oven-drying) has long-term consequences on fungal communities, whereas the effects of less severe desiccation, more similar to natural conditions in streams, were not significant. It is also likely that our experiment underestimated the effects of pool isolation and drying, as other factors such as abrasion and fragmentation by macroinvertebrates, which are important in organic matter decomposition (Graça 2001; Hieber and Gessner 2002), are strongly affected by flow reduction (Acuña et al. 2005; Arroita et al. 2015).

Concerning the relationship between variables, microbial mass loss of alder leaves was not related to mean ergosterol concentration in our study. Although many studies reported that fungi contributed to more than 98% to the microbial biomass measured as ergosterol concentration and emphasized the greater role of fungi than bacteria in litter decomposition (Baldy et al. 1995; Gulis and Suberkropp 2003; Pascoal et al. 2005), Mille-Lindblom et al. (2004) highlighted that ergosterol should be used cautiously as a biomarker for living fungi because it can be stable even when associated with dead fungi. Some studies also found a strong relationship between sporulation and ergosterol concentration and litter decomposition (Pascoal and Cássio 2004), whereas others reported sporulation not to be related to these variables (Lecerf and Chauvet 2008; Martínez et al. 2014), as in our case. Taxa from several fungal phyla as well as thousands of species of bacteria and archaea are able to colonize leaf litter (Nikolcheva and Bärlocher 2004; Manerkar et al. 2008), all of which do not produce conidia. Moreover, species with high reproductive activity are not always the ones producing more biomass (Duarte et al. 2006) and fungal involvement in leaf decomposition is reflected by the active mycelia, not the conidia. In fact, the remaining mass of alder leaf disks at the end of the experiment was significantly related only to the cumulative respiration, as has been shown elsewhere (Gulis and Suberkropp 2003), because it integrates the activity of the whole microbial community involved in leaf litter decomposition. Therefore, cumulative respiration of the microbial community may be a better proxy of microbial leaf litter decomposition than ergosterol or sporulation, which provide important but partial information.

Overall, the measured variables differed in their sensitivity to stress. Despite the small differences and the lack of consistent patterns in fungal biomass, sporulation or respiration, microbial decomposition of alder disks was significantly reduced in the StagDry stress condition, thus showing a pronounced effect of lack of aeration and dry conditions. In our study, microbial decomposition rate of alder disks was more sensitive than the microbial variables to differences among stress types, likely because it integrated all processes

occurring during the whole experiment, i.e. the last outcome reflects the integration of the entire sequence. In contrast, results obtained with the rest of the variables could be considered instantaneous pictures of microbial assemblages at definite times, because biomass, sporulation and respiration measurements relied on punctual determinations related to the status of microbial assemblages at specific, discrete times and, thus, could be noisier. Although cumulative spore production and oxygen consumption also integrate the whole experiment, these estimates could be biased, as they were calculated from data obtained in six sampling dates, assuming constant rates during 2 weeks. Even though the significant relationship between total oxygen consumption and microbial alder decomposition suggests that cumulative microbial respiration could be an appropriate surrogate, statistical power was lost as averages per sampling were used to estimate cumulative oxygen consumption.

As expected, the drying history affected microbial activity irrespective of the stress type; early impacts tended to be more detrimental than impacts at a later stage, resulting, in general, in a lower fungal biomass and activity, and reduced microbial litter decomposition. The consistency of our results shows that the legacy effects of stress affect the assemblages even after they return to more benign conditions. Therefore, the impacts of stress on leaf decomposition-associated variables depend not only on the duration of stress, but also on that of post-stress periods and, thus, on the timing of the stress. In particular, the effects of drought on leaf litter decomposition can start as early as in the pre-conditioning phase (Dieter et al. 2011, 2013) and persist during the whole process (Datry et al. 2011; Martínez et al. 2015). These results could also apply to other ecological processes showing a clear succession of steps in which the last outcome integrates the processes occurring in the entire sequence. For instance, the biomass produced by an ecosystem would be more affected by disturbances occurring early than late in the year. Therefore, the timing of an impact can be as relevant as its intensity or frequency, emphasizing the hydrological contingency.

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