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Functional Ecology

RESEARCH ARTICLE

Can induced drying modulate the response of leaf litter decomposition and fungal diversity to wastewater effluents in permanent streams?

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Abstract

1. Watercourses are among the most threatened ecosystems globally and face multiple anthropogenic stressors of different origins and intensities that reduce biodiversity and disrupt ecosystem functioning. Among these stressors, both stream drying and wastewater treatment plant (WWTP) effluents are expected to become more intense with global change, reducing water availability and consequently the dilution capacity of nutrients and pollutants. Leaf litter decomposition is a key ecosystem function in which aquatic hyphomycete communities play a crucial role, yet the interactive impacts of drying and consecutive WWTP exposure on both remain poorly understood.
2. We conducted a field experiment in three permanent Mediterranean streams to assess the effects of induced drying and WWTP effluents on leaf litter decomposition and its associated fungal communities. After microbial colonization, leaf litter bags were subjected to two drying intensities by moving the bags into and out of the streambed: pulse (short cycles of drying and rewetting) and press (prolonged drying). The bags were then exposed to WWTP effluents downstream. We analysed the individual and joint effects of both stressors on leaf litter mass loss, fungal biomass, leaf C:N and fungal community structure via DNA sequencing.
3. We found that drying increased fungal species richness and changed community composition through the colonization of terrestrial species but reduced leaf litter decomposition. The differences between drying intensities were minimal. In contrast, the WWTP effluent had no individual effects on leaf litter decomposition, but previously dried leaves exhibited legacy effects. The WWTP effluent also altered the fungal community composition by reducing the number of terrestrial species and promoting their replacement with more tolerant aquatic species.

4. Our results highlight the long-lasting legacy effects of drying on leaf litter decomposition and its associated fungal community when leaves are exposed to WWTP effluent. The increase in the number of dying events limits the dilution capacity of streams and likely increases the interaction between the two stressors, leading to more pronounced changes in fungal assemblages and their decomposition capacity, which is an important threat to ecosystem functioning.

KEYWORDS
aquatic hyphomycetes, drought, ecosystem functioning, global change, multiple stressors, OTU richness, urban pollution, water scarcity

1 | INTRODUCTION

Watercourses are biodiversity hotspots and suppliers of valuable ecosystem services (Dudgeon et al., 2006; Yeakley et al., 2016). However, those services are threatened by human activities (stressors) that also change their ecological quality (Grizzetti et al., 2017; Lemm et al., 2021). These stressors can simultaneously occur in space and time, and their effects depend on stressor intensity and duration, but also on the specific biological response considered (Gutiérrez-Cánovas et al., 2022; Jackson et al., 2016). For example, pulse disturbances generally allow ecosystems to return to their previous state, whereas press disturbances often result in a lasting shift to a different condition (Ratajczak et al., 2017; Ryo et al., 2019). Moreover, stressors can have long-lasting legacy effects that influence how communities and their functions respond to new stressors (Sabater et al., 2019; Smith et al., 2009). Therefore, diverse stressor combinations, varying in origin and intensity, can lead to ecological outcomes that remain largely unexplored.

Permanent watercourses are increasingly shifting to an intermittent flow regime due to global change, especially in low-order streams of arid regions such as Mediterranean areas (Bogan & Lytle, 2011; Carlson et al., 2024; Crabot et al., 2021). Anthropogenic-driven desiccation can lead to the loss of aquatic biodiversity and ecosystem functions, but biological responses can be shaped by the drying regime (Datry et al., 2023). For example, streambed desiccation can be partial, with the formation of isolated pools or complete within hours or days, and several cycles of drying and rewetting can occur before flow resumes (Boulton et al., 2017; Datry, 2017; Vidal-Abarca et al., 2020). Moreover, wastewater treatment plant (WWTP) effluents are the most widespread point sources of urban pollutants, affecting more than 1,200,000 km of the global river network (Ehalt Macedo et al., 2022). The extent of this impact is expected to increase in the coming years as urban areas continue to expand (Kohlhase, 2013). Their effluents contain a high proportion of different pollutants (Martí et al., 2010; Rout et al., 2021), and the impact on aquatic communities and ecosystem functioning depends mainly on the dilution capacity of watercourses (Büttner et al., 2022; Rice & Westerhoff, 2017). Drying events may limit the watercourse's dilution capacity, with low-order streams even receiving just WWTP effluents as a water source during the drought period (Carey & Migliaccio, 2009; Luthy et al., 2015). Therefore, understanding how future potential drying, combined with wastewater discharge, influences ecosystem responses in permanent watercourses is crucial.

In low-order streams, leaf litter from adjacent riparian habitats accounts for more than 60% of the total organic matter input and represents the main carbon source supporting aquatic food webs (Swan et al., 2021; Tonin et al., 2021). Aquatic fungi play a crucial role contributing up to 99% of the total microbial biomass, driving up to 66% of leaf mass loss and enhancing leaf palatability for other consumers (Arias-Real et al., 2018; Arsuffi & Suberkropp, 1984). To date, the duration and intensity of drying are known to impact leaf litter decomposition through reductions in aquatic microbial activity driven by the loss of aquatic fungal species and biomass (Abril et al., 2016; Foulquier et al., 2015; Mora-Gómez et al., 2018). Nevertheless, flash storms during the drying period and rewetting can rapidly reactivate fungal communities and consequently lead to leaf litter decomposition (Arias-Real, Hurtado, et al., 2023; Arias-Real, Menéndez, et al., 2023; Mora-Gómez et al., 2020; Schreckinger et al., 2021). However, their reactivation under WWTP effluents remains uncertain.

The response of leaf litter decomposition to WWTP effluents is difficult to predict, with studies reporting positive (Solagaistua et al., 2018), negative (Bastias et al., 2018) or even no net effects (Martinez-Sanz et al., 2024). This variability could be produced by potential antagonistic interactions between nutrients and pollutants (Burdon et al., 2020). On the one hand, moderate levels of organic nutrients can stimulate leaf litter decomposition, increasing microbial activity and fungal biomass (Ferreira et al., 2015, 2020; Manning et al., 2021). On the other hand, pollutants such as fungicides can negatively impact microbial diversity and litter processing (De Castro-Català et al., 2025; López-Doval et al., 2013). Thus, understanding the impacts of drying and consecutive WWTP exposure on biodiversity and ecosystem function is essential for anticipating ecosystem responses to global change.

In this study, we assessed the effects of different intensities of induced drying and subsequent exposure to WWTP effluents on the decomposition of leaf litter and its associated fungal community. To accomplish this objective, we conducted a field experiment in three permanent Mediterranean streams, simulating two intensities of drying stress: pulse (short cycles of drying and rewetting) and press (prolonged drying), moving bags into and out of the streambed.

Finally, leaf litter bags were exposed to the effluent of a WWTP downstream. We analysed leaf litter mass loss, ergosterol concentration (as a proxy of fungal biomass), leaf C:N (as a proxy of leaf palatability) and fungal diversity (DNA metabarcoding).

Our main hypothesis is that drying strongly impacts the aquatic fungal community, primarily by reducing richness and shifting community composition by favouring terrestrial fungal species. The loss of biodiversity leads to reduced leaf litter decomposition and fungal biomass while increasing or at least maintaining leaf C:N, these effects being more pronounced in leaves exposed to press drying. We hypothesized two possible opposite responses of leaf litter decomposition to WWTP effluents: (1) stimulation by nutrients that enhances leaf litter decomposition and fungal biomass while decreasing the leaf C:N and (2) a negative effect of pollutants resulting in the opposite pattern for the three variables. Independent of these two results, we expect that WWTP effluent changes the fungal community composition by favouring more tolerant aquatic species. Finally, we hypothesized that exposure to drying has legacy effects on leaves that will condition the response of microbial communities to the next stressor, reducing litter decomposition, fungal biomass and leaf quality.

2 | MATERIALS AND METHODS

2.1 | Study site

The three streams are located at low mountain altitudes (320–730 m a.s.l.) and are characterized by dry summers, with precipitation occurring primarily in autumn and winter. Maximum daily air temperature varied from 16.08 to 35.10°C, and the most significant daily precipitation varied from 17.1 to 54.6 mm, depending on the stream (Table S1). Catchments are mainly siliceous bedrock surrounded by deciduous forest (dominated by *Populus* spp., *Alnus glutinosa*, *Fagus sylvatica*, *Quercus petraea* and *Platanus orientalis*; Table S1). The three streams share a similar channel width, channel depth and average air humidity (Table S1). The mean discharge varied from 161.8 to 594.8 cm^3/s, and mean water temperature varied from 11.6 to 17.8°C, depending on the stream (Table S1). See Table S1 for more details. The study spanned from spring to summer (2022: Arbúcies and Llémena; 2023: Riera Major).

2.2 | Experimental design

In each stream, we selected two 50-m long reaches: one downstream of the effluent of a WWTP and one upstream of the effluent. To ensure that the main differences in water physicochemical parameters between the upstream and downstream reaches were due to WWTP effluent, both reaches were located close to each other (between 0.4 and 5.8 km) and had similar land uses (Table S1). In situ environmental characteristics were measured at each stream and reach during the experiment. Methodological details are available in Supporting Information (Appendix S1).

We used a total of 123 leaf litter bags (13 cm × 18 cm), with 0.5 mm mesh size to prevent invertebrate colonization, each filled with 3 g of air-dried *Populus nigra* leaves. The leaves were collected from the bank of one stream just after abscission in October 2022 to minimize local differences in their initial quality. At the start of the experiment, three bags were transported to the field and back to the laboratory to calculate initial dry mass (DMi) using the correction factor between air-dry mass and oven-dry mass (60°C, 48 h) considering handling losses (Bärlocher et al., 2020), while 40 bags were placed at each upstream reach for microbial colonization. After 2 weeks, a set of five bags was removed from each stream and analysed in the laboratory to assess the initial experimental conditions (sampling time 1; Figure 1). Three additional sets (five bags per set) remained in the upstream reaches as a control treatment under natural conditions. Twenty bags (ten per treatment) were subjected to two different intensities of drying: (1) press treatment (prolonged drying), where the leaf litter bags were placed on the streambank for 3 weeks and (2) pulse treatment (short cycles of drying and rewetting), where the bags were left on the streambank for 5 days and then submerged in the water for 2 days, with this cycle repeated over the same three-week period. During drying simulations, bags were carefully attached to the base of tree trunks to prevent their movement. At the end of the drying simulations (sampling time 2; Figure 1), five bags from each treatment (control, press and pulse) were removed for laboratory analysis. The remaining bags (including five from the control treatment) were moved downstream to the WWTP effluent and left for an additional 3 weeks (sampling time 3; Figure 1). A set of controls was also maintained in the upstream reaches until the end of the experiment. Although fieldwork permits were not needed, the experiment was conducted with the approval from the administration of Catalan Water Agency.

2.3 | Leaf litter decomposition and elemental analysis

The remaining leaf litter from each bag was washed with distilled water in the laboratory to remove sediment particles. Five 12-mm leaf discs were obtained from the leaves (randomly selected) of each bag using a cork borer for ergosterol analysis (Bärlocher et al., 2020). The discs were lyophilized and weighed individually to calculate their mean weight. Three additional leaf discs were obtained from the leaves of three bags of each treatment, excluding the colonization control (sampling time 1), from two streams (Arbúcies and Llémena) for DNA extraction. The mean weight of the DNA leaf discs was inferred from the mean weight of the ergosterol leaf discs. The remaining leaves were oven-dried at 60°C to a constant weight (48 h). The remaining mass (RM) of each bag was then calculated as the sum of the remaining leaves plus the corresponding discs. The leaf litter mass loss (ML) of each bag was subsequently calculated as the total remaining biomass of leaves to the initial dry mass and expressed as a percentage: ML = DMi − RM.

The carbon (C) and nitrogen (N) concentrations of the leaf litter were analysed in 2 mg of dried and homogenized leaf mass from each

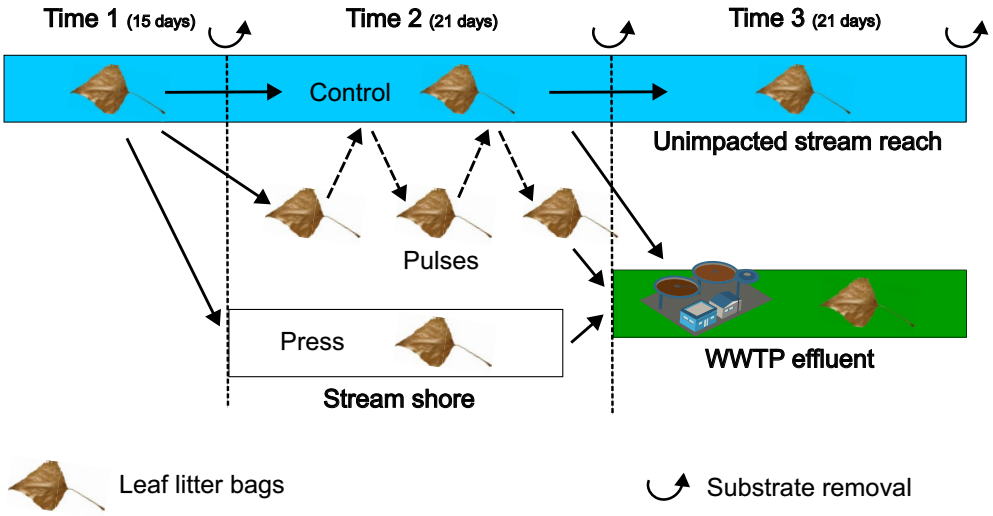

FIGURE 1 Design of the experiment conducted in the three streams. The sequence of the treatments during the three sampling times (separated by dotted lines), the simulation of the two intensities of hydric stress, and the final downstream movement to WWTP effluent exposure are shown. Substrate manipulation and directionality in time are indicated by the straight arrows and dashed arrows (only for pulses of drying), whereas substrate removal is indicated by the curved arrows. WWTP symbols extracted from *Tracey Saxby, Integration and Application Network* (ian.umces.edu/media-library).

sample using an elemental autoanalyser (1108-Thermo Scientific, Milan, Italy). C:N molar ratios were calculated for each sample.

2.4 | Fungal biomass and community characterization

Ergosterol was extracted from the leaf litter discs following the method described in Gessner and Schmitt (1996), and final concentration was determined using high-performance liquid chromatography (HPLC, Jasco HPLC system, USA) at 282 nm with a Gemini-NX 5 µm C18 250×4.6 mm column (Phenomenex, UK). Fungal biomass was subsequently calculated using a conversion factor of 5.5 mg of ergosterol per g of fungal mycelium (Bärlocher et al., 2020). We expressed the results in mg of fungi per g of leaves.

Fungal community of leaf litter was characterized by DNA metabarcoding of the ITS2 region (Blaalid et al., 2013), and taxonomy of the generated OTUs was predicted with UNITE database (Nilsson et al., 2018). Details of DNA quantification, bioinformatics and taxonomy are available in the Supporting Information (Appendix S2).

2.5 | Replication statement

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Leaf litter bags	Steam	3 for leaf litter decomposition and quality, 2 for fungal community structure

2.6 | Statistical analysis

All the statistical analyses were conducted in the R programming environment version 4.2.0 (R Core Team, 2022). To analyse and test the effects of drying treatment and WWTP exposure on leaf litter decomposition, fungal biomass, leaf C:N and fungal richness, we performed generalized linear models (GLMs, using the *glm* function, family 'gaussian'). To reduce the skewness of the distribution, a logarithmic link function was used in the GLM for fungal richness analysis. A four-factor fixed effects design was specified: stream identity (Arbúcies, Llémena or Riera Major), sampling time (1, 2 or 3; Figure 1), drying treatment (control, press or pulse) and WWTP exposure (yes or no). Stream identity was considered a fixed effects factor because of differences in the physical and chemical properties of the water between reaches (Table S2). Automated model selection from the MuMIn package (Barton, 2009) was used to select a parsimonious model on the basis of differences in the Akaike information criterion corrected for small sample size (ΔAICc), resulting in a model with main effects and two interactions: stream × sampling time and drying × WWTP exposure. The analysis of deviance tables was performed through the Anova function in the car package, specifying contrasts=contr.sum and running type III tests. When significant differences were detected in the main tests, Tukey's HSD post hoc tests were performed. To assess normality and homoscedasticity, Shapiro–Wilk and simulation-based dispersion tests were applied using the DHARMa package (Hartig, 2022).

To assess how completely the fungal community was represented by DNA sequencing, coverage (Roswell et al., 2021) was estimated for each treatment and stream with the iNEXT package (Hsieh et al., 2016), using 100 permutations. For each stream, we first performed a nonmetric multidimensional scaling (NMDS) analysis using Bray–Curtis dissimilarity to identify differences in the OTU community

composition among the treatments. The data were previously square root transformed to downweight the influence of the most abundant species (Clarke & Gorley, 2006) and the skewness of the distributions. Second, we conducted a two-way factorial PERMANOVA ($n = 9999$ permutations) to look for significant differences in composition driven by drying (control, pulse or press), WWTP exposure (no or yes) and their interaction. Third, we ran a similarity percentage analysis (SIMPER) to determine which OTUs contributed the most to the observed differences, using a matrix containing only the OTUs assigned to the species level (3344). The three analyses were performed with the functions available in the vegan package (Oksanen et al., 2022). In addition, beta diversity values and their two components were calculated pooling all replicas of each treatment group together (control vs. drying vs. WWTP exposure), using the BAT package (Cardoso et al., 2015). Beta diversity represents the degree of change between the communities of the treatment groups, ranging between 0 (equal communities) and 1 (completely different communities). The values are calculated as the sum of two components: species replacement (turnover), produced by changing the species between communities without altering their total number, and species gain or loss (nestedness), produced by adding or losing a subset of species between the communities (Cardoso et al., 2014, 2015).

3 | RESULTS

3.1 | Water characteristics of the study sites

A comparison of the upstream reaches of the three streams revealed that Arbúcies had the lowest values of N-NO_2^- (<0.003 μg/L), N-NO_3^- (191 μg/L), N-NH_4^+ (0.28 μg/L) and consequently DIN (Table S2). Llémena had the highest values of conductivity (539 μS/cm) and Cl^- (12.66 mg/L; Table S2). Riera Major had the lowest water temperature (10.45°C) and the highest value of P-PO_4^{3-} (3.51 μg/L; Table S2). On the basis of the IBMWP index, Arbúcies and Llémena were classified into the 'very good' ecological category, whereas Riera Major fit the 'good' category (Table S2). The oxygen concentration, pH and TOC were similar among the streams (Table S2).

A comparison of the upstream and downstream reaches of the three streams revealed that the latter had higher values of temperature (Δ 4.6–7.5°C), conductivity (Δ 289–992 μS/cm), P-PO_4^{3-} (Δ 2.40–1209.22 μg/L), Cl^- (Δ 55.64–208.39 mg/L) and TOC (Δ 2.84–6.44 mg/L), but lower values of oxygen concentration (Δ −1.54-3.18 mg/L; Table S2). The DIN increased in Arbúcies (Δ 1438 μg/L) and Riera Major (Δ 230 μg/L) but decreased in the impacted reach of Llémena (Δ −208 μg/L; Table S2). WWTP effluents affected the pH only in Riera Major (Δ 0.8; Table S2). The IBMWP index decreased in Arbúcies, lowering the category of the former to 'good' (Table S2).

3.2 | Effects of the treatments on leaf litter and fungal biomass

Litter mass loss over time significantly interacted with stream identity (Table 1). Arbúcies and Llémena had similar patterns, with no differences in mass loss between sampling times 2 and 3, whereas Riera Major had differences across all sampling times (Figure S1). Drying (sampling time 2) led to lower mass loss than the controls in all streams (27%–29%, depending on the stream; Table 1), but no significant differences were found between the pulse and press treatments in Arbúcies and Llémena (Figure 2). However, in Riera Major, mass loss was 20% greater in the press treatments than in the pulse treatments (Figure 2). In addition, a significant interaction between drying and WWTP effluent was observed (Table 1). In the presence of WWTP effluent (sampling time 3), there were no differences between the exposed and nonexposed controls, and both drying treatments resulted in 10% to 20% lower mass loss than the controls did (Figure 2). After WWTP exposure, the differences between the pulse and press treatments in Riera Major disappeared (Figure 2). The mass loss between sampling times 2 (after drying) and 3 (after WWTP exposure) was greater in both drying treatments (20%–28%, depending on the stream) than in the nonexposed control (1%–18%, depending on the stream), reflecting

TABLE 1 Results of the analysis of deviance tables (Type III tests) for the three response variables of leaf litter decomposition studied.

			Response variable							
Source of variation	Df1	Df2	Chisq	Leaf litter mass loss (%)	Chisq	Fungal biomass (mg/g)	Chisq	C:N	Chisq	OTUs richness
Stream	2	1	4.243	$p = 0.120$	40.235	$p < 0.001$***	66.939	$p < 0.001$***	16.060	$p < 0.001$ ***
Time	2	1	180.477	$p < 0.001$***	16.599	$p < 0.001$***	123.834	$p < 0.001$***	0.311	$p = 0.577$
Stream × Time	4	1	12.735	$p = 0.013$*	18.733	$p < 0.001$***	13.876	$p = 0.008$**	2.020	$p = 0.155$
Drying	2	2	268.975	$p < 0.001$***	1.043	$p = 0.594$	2.690	$p = 0.261$	24.917	$p < 0.001$***
WWTP exposure	1	1	25.689	$p < 0.001$***	1.288	$p = 0.256$	0.672	$p = 0.412$	0.934	$p < 0.334$
Drying × WWTP exposure	2	2	26.331	$p < 0.001$***	1.836	$p = 0.399$	5.897	$p = 0.052$ ·	6.563	$p < 0.038$*

Note: Asterisks indicate the level of significance for each source of variation of the models: · near significant, *p value < 0.05, **p value < 0.01, and ***p value < 0.001. Df1: Degrees of freedom of the models for leaf litter mass loss, fungal biomass and leaf C:N. Df2: Degrees of freedom of the model for OTU richness. Chisq: Chi-square statistic values.

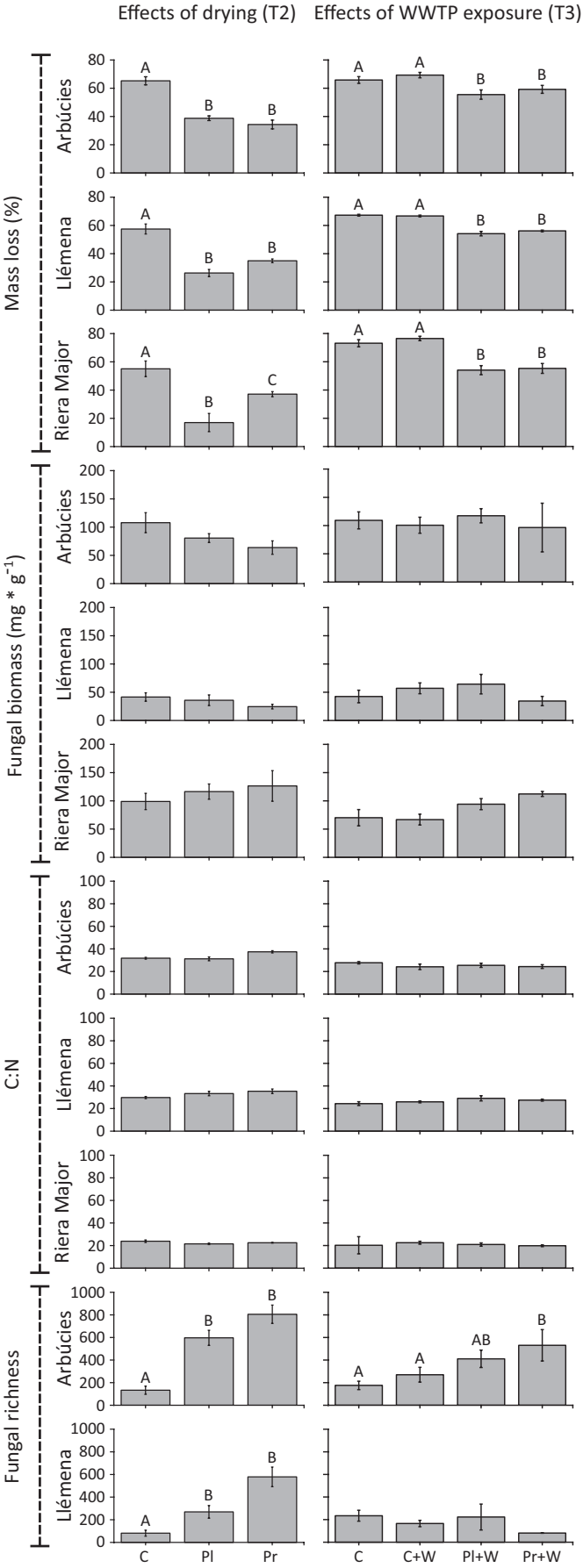

FIGURE 2 Average and SE of litter mass loss, fungal biomass, the C:N ($N = 5$) and fungal richness ($N = 3$) for each treatment per stream. First column: Comparison among both drying treatments and the corresponding control. Second column: Comparison among treatments exposed to WWTP effluents and the corresponding control. Letters over the bars indicate significant differences according to Tukey's (HSD) post hoc tests. T2: Sampling time 2. T3: Sampling time 3. C: Control. Pl: Pulse. Pr: Press. W: WWTP exposure.

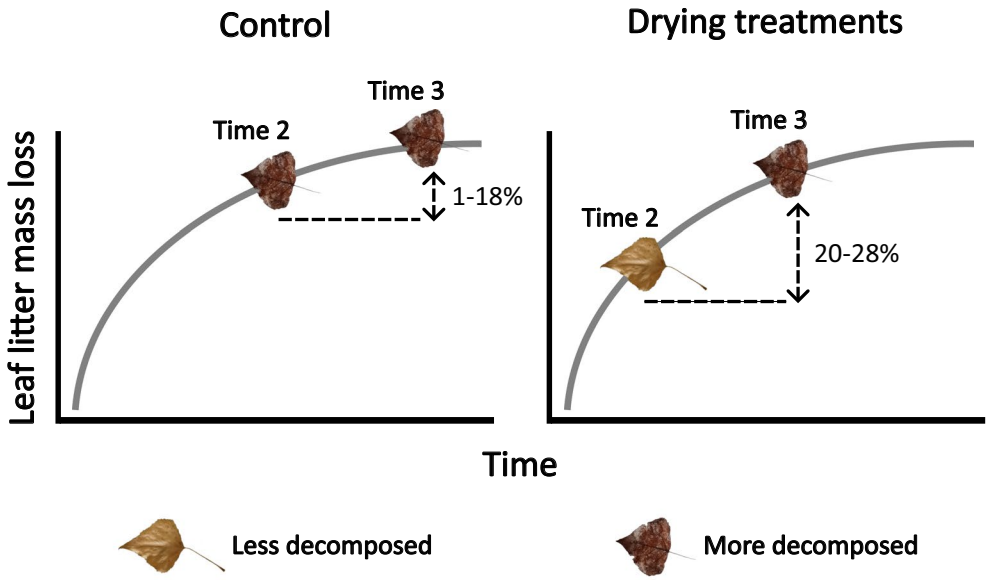

FIGURE 3 Theoretical model explaining leaf litter decomposition dynamics between the control (considering that both controls exposed and not exposed to WWTP did not differ in terms of their mass loss) and the drying treatments according to the exponential model of this process (Petersen & Cummins, 1974; Webster & Benfield, 1986). The dashed vertical lines and the values near them represent the amount of mass loss between times 2 and 3 for each treatment. The values are given as a range due to variability between the streams. The leaf litter of the drying treatments was less decomposed than that of the controls at time 2. At the end of the experiment (time 3), a legacy effect of drying was observed, with a significantly lower total mass loss in the drying treatments (Figure 2). These leaves needed more time to reach the same total mass loss value than the control leaves did. However, the relative mass loss between the two experimental times was greater in the drying treatments than in the control because of the model dynamics.

the exponential dynamics of leaf litter decomposition and the presence of legacy effects (Figure 3).

Fungal biomass increased over time only in Riera Major (Figure S1). The C:N of the leaf litter decreased over time, but this pattern varied across streams (Table 1). In Arbúcies and Riera Major, the C:N decreased only between the first two sampling times, whereas in Llémena, it decreased consistently across all sampling times (Table 1, Figure S1). However, none of the studied stressors affected the fungal biomass or the leaf C:N (Figure 2).

3.3 | Effects of the treatments on fungal diversity

Coverage values were higher than 98% for all the treatments in both streams (Figure S2), indicating that the entire fungal community present in the leaves was determined and, therefore, that treatments were comparable using the original OTUs data. A total of 12,156 OTUs from the fungal kingdom were recorded, with the majority assigned to Ascomycota (45.8%), followed by Basidiomycota (20.1%), Chytridiomycota (8.6%) and other minor phyla (9.1%), including Aphelidiomycota, Basidiobolomycota, Calcarisporiellomycota, Entorrhizomycota, Glomeromycota, Kickxellomycota, Monoblepharomycota, Mortierellomycota, Mucoromycota, Olpidiomycota, Rozellomycota and Zoopagomycota.

Despite some similarities, the global pattern of richness varied among the streams (Table 1; Figure 2). Drying significantly increased richness in both streams, with no differences between the pulse and press treatments (Table 1; Figure 2). In addition, a significant interaction effect between drying and WWTP exposure was observed (Table 1). After WWTP exposure, the richness did not differ between the exposed and nonexposed controls but decreased in both drying treatments compared with the previous conditions (Figure 2). In Llémena, no significant differences were observed due to wastewater exposure. However, in Arbúcies, the press leaves still showed greater richness than the rest of the treatments did (Table 1, Figure 2).

NMDS ordinations for both streams revealed clustering of samples from the control, drying and WWTP exposure treatments (Figure 4), indicating differences in their composition (Table 2). In Llémena, composition differences were driven primarily by the interaction between the two stressors (Table 2), with the treatment groups being more distinctly separated in the NMDS space (Figure 4).

The beta diversity values were high in all pairwise comparisons among the three treatment groups (Table 3). In Arbúcies, species gain or loss was the dominant component in all pairwise comparisons, although species replacement was almost equally important in the comparison between the drying and WWTP exposure groups (Table 3). In Llémena, species gain or loss dominated the comparisons between the controls and drying, as well as between drying and WWTP exposure, whereas species replacement was the main driver in the comparison between the control and WWTP exposure groups (Table 3).

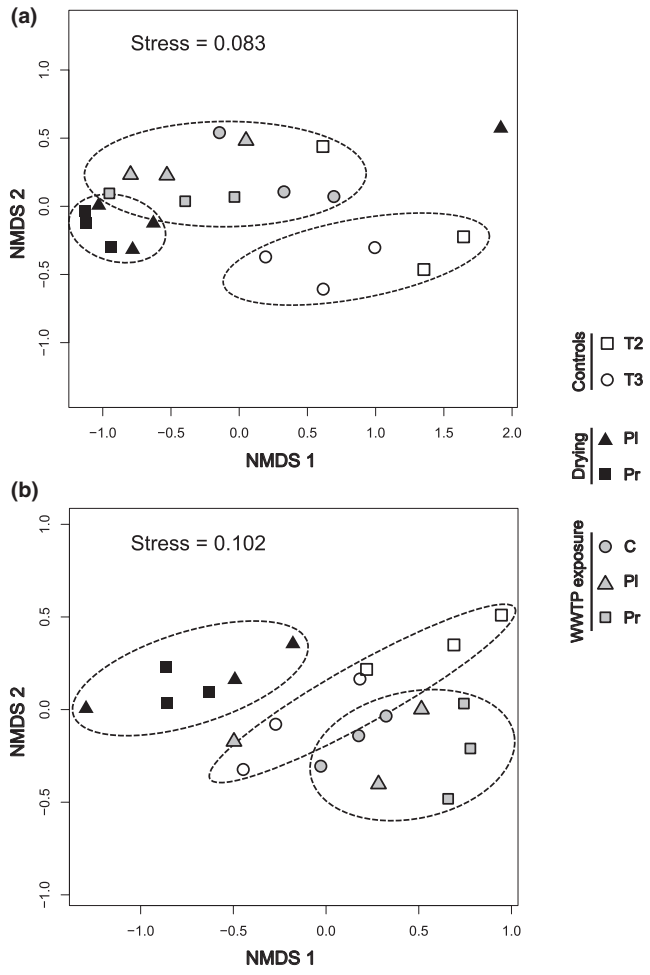

FIGURE 4 NMDS ordination of samples per treatment by fungal (OTU) composition for both the Arbúcies (a) and Llémena (b) streams. T2: Time 2. T3: Time 3. C: Control. Pl: Pulse. Pr: Press.

SIMPER analysis revealed similar species assemblages across all pairwise comparisons among the treatment groups in both streams (Table S3), highlighting the prevalence of aquatic fungal species such as *Alatospora acuminata*, *Alternaria alternata*, *Amniculicola guttulata* and *Xylomyces aquaticus*. However, leaves in the drying treatments were also colonized by a diverse group of terrestrial species, including *Aureobasidium pullulans*, *Calophoma rosae*, *Cladosporium cladosporioides*, *Filobasidium* spp., *Idriella lunata*, *Nectria ramulariae*, *Phallus impudicus* and *Volutella ciliata*, some of which persisted after WWTP exposure (Table S3). In addition, some aquatic species, such as *Dimorphospora foliicola*, were found primarily in the WWTP effluent treatments.

4 | DISCUSSION

In this study, we assessed the response of fungal communities and their associated ecosystem functions to two different intensities of drying and subsequent exposure to WWTP effluent. Overall, our results showed that drying increased fungal richness due to the colonization of terrestrial species but impacted leaf litter decomposition. Drying intensity affected leaf litter decomposition only in one stream but in a different way than expected. The response of leaf litter to WWTP effluent was weak, although mass loss was lower when the leaves were previously exposed to drying and in one stream, fungal richness was greater, indicating legacy effects. In addition, wastewater exposure resulted in the loss of terrestrial species and replacement with aquatic species.

4.1 | Effects of drying

In contrast to our hypothesis, drying significantly increased fungal richness on the leaves by enabling colonization by terrestrial species without the loss of aquatic species. The detection of terrestrial species combined with the high coverage values suggests that DNA sequencing captured the fungal community more comprehensively, aligning with other freshwater studies (Duarte et al., 2017; Fernandes et al., 2015), although the persistence of DNA from dead fungi must be considered (Bärlocher, 2016). Nevertheless, drying remains the main stressor of leaf litter, reducing decomposition, likely due to the decline in microbial activity without flowing water (Abril et al., 2016, 2024).

TABLE 2 PERMANOVA results for the two studied stressors and their interaction with fungal community composition for each stream.

Source of variation	Df	Arbúcies		Llémena	
Drying	2	$F=2.433$	$p<0.001^{***}$	$F=1.853$	$p=0.008^{**}$
WWTP exposure	1	$F=2.789$	$p<0.001^{***}$	$F=2.902$	$p=0.001^{**}$
Drying × WWTP exposure	2	$F=1.162$	$p=0.196$	$F=1.648$	$p=0.019^{*}$

Note: Asterisks indicate the level of significance for each source of variation: $^{*}p$ value < 0.05, $^{**}p$ value < 0.001, and $^{***}p$ value < 0.001.

Abbreviation: Df: degrees of freedom.

TABLE 3 Beta diversity values (β_{total}) and its two components, that is species gain or loss ($\beta_{richness}$) and species replacement ($\beta_{replacement}$), among each pair of treatment groups for both streams.

		Controls-drying	Controls-WWTP exposure	Drying-WWTP exposure
Arbúcies	β_{total}	0.836	0.924	0.810
	$\beta_{richness}$	0.770	0.891	0.438
	$\beta_{replacement}$	0.066	0.033	0.372
Llémena	β_{total}	0.928	0.716	0.913
	$\beta_{richness}$	0.737	0.188	0.824
	$\beta_{replacement}$	0.191	0.528	0.089

In contrast to our expectations, pulses of rewetting did not enhance leaf litter decomposition compared with press drying and even decelerated this function in one stream. In similar experiments, Foulquier et al. (2015) found no differences in leaf litter decomposition among three different frequencies of leaf emersion, and Langhans and Tockner (2006) observed greater decomposition in leaves exposed to one long cycle of emersion than in those subjected to several shorter cycles. In our streams, the similar changes in fungal community composition under both drying intensities may explain the lack of differences in leaf mass loss. However, this finding emphasizes how unpredictable human-driven alterations in flow regimes can be for stream functioning. In addition, terrestrial species may compensate for the loss of aquatic species biomass, explaining the lack of response of fungal biomass to drying stress. Arroita et al. (2018) and Martinez-Sanz et al. (2024) also observed no consistent response of fungal biomass to drying, whereas other studies reported that changes in fungal biomass can be season dependent or may not differ among different drying intensities (Foulquier et al., 2015; Langhans & Tockner, 2006). Finally, it must be considered that ergosterol can remain stable even when fungi are dead (Mille-Lindblom et al., 2004).

Drying can reduce the nitrogen concentration of leaves via microbial biomass loss (Abril et al., 2016; Martinez-Sanz et al., 2024; Palmia et al., 2019), which also decreases leaf palatability for shredders (Arias-Real et al., 2018; Arsuffi & Suberkropp, 1984). However, in our experiment, the leaf C:N stabilized within the first 15 days in two streams, potentially preventing this parameter from being affected by the studied stressors. Similar dynamics have been observed across studies with varying litter species, durations and experimental designs, suggesting that major changes in leaf stoichiometry occur at early stages of the decomposition process (Abril et al., 2016; Datry et al., 2011; Menéndez et al., 2001, 2011).

4.2 | Effects of WWTP effluents

WWTP effluents contain complex mixtures of contaminants (Rout et al., 2021) and high concentrations of organic nutrients (Carey & Migliaccio, 2009; Martí et al., 2010). As a result, their discharge into watercourses typically leads to severe reductions in ecological status (Büttner et al., 2022). In our streams, the downstream reaches had altered water properties, including higher temperatures, conductivity and nutrient concentrations, lower dissolved oxygen levels and, in one stream, a reduced biological quality.

Contrary to our hypothesis, these changes in water properties did not lead to a shift in leaf litter decomposition, fungal biomass or fungal richness in the exposed control treatment. Several studies suggest that leaf litter decomposition responses to WWTP effluents are variable due to the opposing effects of pollutants and organic nutrients (Bastias et al., 2018; Burdon et al., 2020). Our results align with those of Münze et al. (2017), Pereda et al. (2020, 2021) and Martinez-Sanz et al. (2024), who reported no consistent response of leaf litter decomposition or fungal biomass to WWTP exposure. While WWTP effluents can reduce fungal richness in decomposing substrates (Burdon et al., 2020), downstream aquatic microbial communities may tolerate or adapt to these stressors (David et al., 2024), especially when dilution reduces their impact (Pereda et al., 2020).

In contrast, decomposition accelerated when leaves previously subjected to drying were exposed to wastewater, eliminating the differences between the pulse and press treatments observed in Riera Major. The most plausible explanation, considering the lack of differences between the exposed and nonexposed controls, is that rewetting reactivated the exponential decay of dried leaves, as previously reported by Arroita et al. (2018). In addition, WWTP exposure led to a loss of terrestrial species that colonized the dried leaves, a phenomenon similar to the initial stages of decomposition when leaves enter streams from riparian areas (Nikolcheva et al., 2005). However, this loss was more pronounced in Llémena than in Arbúcies, suggesting that Arbúcies maintains more amphibious or facultative species potentially capable of contributing to decomposition (Grossart et al., 2019; Park, 1972). Consequently, fungal community shifts in Arbúcies were driven mainly by species gain or loss, whereas in Llémena, species replacement dominated due to the presence of more tolerant aquatic fungi, as also observed in Swiss streams affected by WWTP effluent (Burdon et al., 2020). Moreover, in Llémena, community shifts were shaped by the interaction between the two studied stressors, aligning with findings by Tolkkinen et al. (2015), which suggest that under harsh conditions (i.e. imposed drying), fungal assemblages become more vulnerable to additional disturbances (i.e. WWTP effluent).

As expected, drying had two legacy effects after WWTP exposure (21 days of submersion). Some terrestrial species persisted on dried leaves, increasing species richness in at least one stream. However, the final leaf litter mass loss was lower than that in the controls. Similar legacy effects of drying have been observed under natural conditions in leaves placed in stream reaches with varying summer drying durations (Datry et al., 2011), in leaves previously

exposed to air for 7 days (Bruder et al., 2011), and in those previously subjected to early drying events (Arroita et al., 2018). These findings highlight a significant threat to ecosystem functioning due to long-lasting shifts in fungal communities and their decomposition capacity.

AUTHOR CONTRIBUTIONS

Isabel Muñoz and Margarita Menéndez conceived the ideas and the experimental design. Manuel Pinilla-Rosa, Itxaso Martinez-Sanz, Isabel Muñoz and Margarita Menéndez conducted fieldwork and direct data collection. Manuel Pinilla-Rosa and Itxaso Martinez-Sanz conducted the laboratory analysis. Rebeca Arias-Real and Jun-Tao Wang were the experts in charge of fungal DNA extraction, sequencing and bioinformatics. Statistical analysis design was conceived by Francesc Oliva and conducted by Manuel Pinilla-Rosa. Manuel Pinilla-Rosa conceptualized and created figures and tables. Original draft writing was led by Manuel Pinilla-Rosa. All authors critically revised the manuscript and approved it for publication. Financial support was acquired by Isabel Muñoz and Margarita Menéndez.

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CONFLICT OF INTEREST STATEMENT

The authors state that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available from the CORA repository: https://doi.org/10.34810/data2163 (Pinilla Rosa et al., 2025).

STATEMENT ON INCLUSION

Our study brings together local scientists from different regions of the country, who participate in the study design and collection of local data, but also authors from different countries who are experts on innovative analytical techniques. Most of the authors participated early in the research and the study design to consider different perspectives. In addition, literature published by local scientists was cited, including relevant works in the local language.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Stream health measurements.

Appendix S2. Detailed description of the fungal community characterization.

Figure S1. Average and SE of litter mass loss, fungal biomass, the leaf C:N ($N = 5$) and fungal.

Figure S2. Coverage-based sampling completeness for each treatment in both the Arbúcies.

Table S1. Main characteristics of the stream reaches. The precipitation, air temperature and air humidity data for the study period were extracted from https://ruralcat.gencat.cat/agrometeo.estacions. Data on the population equivalents of the WWTPs were extracted from https://aca.gencat.cat. Drainage areas and land uses were calculated using QGIS software (QGIS, 2024) with layers downloaded from Institut Cartogràfic i Geològic de Catalunya (https://www.icgc.cat/en).

Table S2. Average (±SD) physical, chemical and ecological characteristics of the water of the stream reaches. $N = 5$ and $N = 2$ for the upstream and downstream reaches, respectively, except for nutrient concentrations: $N = 3$ for the upstream and $N = 1$ for the downstream reaches of Arbúcies and Llémena and $N = 4$ and $N = 2$ for the upstream and downstream reaches of Riera Major, respectively. The limit of detection (LOD) was as follows: $N\text{-}NO_2^-$ (μg/L): 0.003; $P\text{-}PO_4$ (μg/L): 0.774.

Table S3. SIMPER analysis for each pair of treatment groups in both streams. The last column shows the percentage of cumulative contribution (up to 35%) to the total dissimilarity of the selected species for each pair of treatment groups.

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