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Research Article

Fungal richness does not buffer the effects of streams salinization on litter decomposition

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Abstract – Freshwater salinization is a world-wide phenomenon threatening stream communities and ecosystem functioning. In these systems, litter decomposition is a main ecosystem-level process where fungi (aquatic hyphomycetes) play a central role linking basal resource and higher levels of food-web. The current study evaluated the impact of aquatic hyphomycete richness on leaf litter decomposition when subjected to salinization. In a microcosm study, we analysed leaf mass loss, fungal biomass, respiration and sporulation rate by fungal assemblages at three levels of species richness (1, 4, 8 species) and three levels of salinity (0, 8, 16 g NaCl L^{-1}). Mass loss and sporulation rate were depressed at 8 and 16 g NaCl L^{-1}, while fungal biomass and respiration were only negatively affected at 16 g L^{-1}. A richness effect was only observed on sporulation rates, with the maximum values found in assemblages of 4 species. In all cases, the negative effects of high levels of salinization on the four tested variables superimposed the potential buffer capacity of fungal richness. The study suggests functional redundancy among the fungal species even at elevated salt stress conditions which may guarantee stream functioning at extreme levels of salinity. Nonetheless, it also points to the possible importance of salt induced changes on fungal diversity and identity in salinized streams able to induce bottom-up effects in the food webs.

Keywords: Fresh waters / salt contamination / species richness / aquatic hyphomycetes / litter processing

1 Introduction

Decomposition of leaf litter is a key process on the functioning of forested streams due to its importance transferring the energy through the food-web and recycling of nutrients (Chauvet *et al.*, 2016; Gessner and Chauvet, 2002; Webster and Benfield, 1986; Young *et al.*, 2008). Aquatic hyphomycetes – a polyphyletic group of microbial fungi – play a key role in this process (Canhoto *et al.*, 2016; Gessner and Chauvet, 1994), directly metabolizing leaf material of terrestrial origin (Gulis and Suberkropp, 2003) and alleviating the stoichiometric constrains between leaf litter and other consumers such as invertebrate detritivores (France, 2011). Their activity has been proven to be sensitive to a wide array of human-induced alterations (Bärlocher, 2016) with consequences that may propagate across the food web to the whole ecosystem (Gonçalves *et al.*, 2014). This group of aquatic fungi are, consequently, a suitable target group to assess threats to, and consequences for, stream ecosystem functioning.

Freshwater salinization – the increase of ion concentrations in water – is a widespread threat directly related to human activities such as agriculture, irrigation, mining, and the use of salts as defrosting agent (Cañedo-Argüelles *et al.*, 2016; Kaushal *et al.*, 2018). Its intensification is expected to follow human population growth, with associated increasing water demands and urbanization (Vineis *et al.*, 2011). Not surprisingly, this stressor is attracting increasing interest of freshwater ecologists. Studies have mainly focused on structural attributes rather than functional processes such as litter decomposition (Hintz and Relyea, 2019).

Streams salinization is known to depress leaf associated fungal activities such as growth, respiration and sporulation rate (Gonçalves *et al.*, 2019b; Martínez *et al.*, 2020; Tyree *et al.*, 2016), and to slow down litter decomposition rate, particularly at ≥4g salt L^{-1} (Canhoto *et al.*, 2017; Gonçalves *et al.*, 2019a; Sauer *et al.*, 2016; Schäfer *et al.*, 2012). These effects are modulated by factors such as salt ionic composition (Martínez *et al.*, 2020; Sauer *et al.*, 2016), drought regime (Gonçalves *et al.*, 2019b), leaf traits (Almeida Júnior *et al.*, 2020) and fungal species origin (Gonçalves *et al.*, 2019a).

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To date, despite observed changes in species richness with increased salt contamination, a single study considered the relationship between fungal richness (and identity) and fungal-related variables on decaying leaf litter in salinized conditions (Canhoto *et al.*, 2017); but in that study, the various richness levels were not subjected to all experimental salt concentrations. Decomposition rates are influenced by the number of aquatic hyphomycete species (Costantini and Rossi, 2010; Duarte *et al.*, 2006; Gonçalves *et al.*, 2015; Pascoal *et al.*, 2010), the functional efficiency being generally saturated in the presence of 3–5 species. A higher fungal richness has been reported to buffer inhibitory effects of a wide array of environmental stressors on litter decomposition (Duarte *et al.*, 2008; Fernandes *et al.*, 2011; Gonçalves *et al.*, 2016, 2015; Pascoal *et al.*, 2010). Whether the same response occurs with salt contamination is unknown. Greater insight may lead us to anticipate consequences of this worldwide threat on stream functioning in systems with dissimilar species composition.

This study assessed whether species richness of aquatic hyphomycete communities influences fungal responses to salinization by measuring leaf litter mass loss and associated variables (fungal biomass, respiration rate and sporulation rate). We conducted a microcosm study with three levels of fungal diversity (1, 4 and 8 species), under three NaCl concentrations (0, 8 and 16 $g\,L^{-1}$; electric conductivity of 462, 14,610 and 28,883 $\mu S\,cm^{-1}$ respectively). These salt levels, although found in naturally salinized streams (Gómez *et al.*, 2016), were chosen to assess the potential consequences of heavy secondary salinization driven by diverse anthropogenic activities (Cañedo-Argüelles *et al.*, 2012; Cochero *et al.*, 2017; Cooper *et al.*, 2014). We hypothesize (1) a clear inhibitory effect of salt addition on all measured variables, (2) an enhancement effect in assemblages with 4 and 8 species compared to monocultures and (3) that multi-specific assemblages buffer the magnitude of the inhibitory effects driven by salinization.

2 Materials and methods

2.1 Experimental setup

A total of 134 sets of 20 oven-dried (60 °C, 48 h) discs (Ø = 12 mm) of senescent *Populus nigra* L. were weighed (dry mass, DM), autoclaved (121 °C, 15 min) and leached in distilled water during 24 h under shaking (120 rpm). Eight sets were then oven-dried (60 °C, 48 h) and weighed for mass loss correction due to handling and leaching. The other 126 sets were immersed in 100 mL Erlenmeyer flasks (microcosms) containing 40 mL of nutrient solution [75.5 mg $CaCl_2$, 10 mg $MgSO_4 \cdot 7H_2O$, 0.5 g 3-morpholinopropanesulfonic acid – MOPS, 5.5 mg K_2HPO_4 and 100 mg KNO_3 per liter of sterile distilled water; pH 7; salinity 0.22 psu (Dang *et al.*, 2005)] enriched with salt (0, 8 and 16 $g\,L^{-1}$ of NaCl).

Twelve aquatic hyphomycete species common in temperate streams of Portugal (Fernandes *et al.*, 2012; Gonçalves *et al.*, 2019a) were used in the experiment: *Tricladium splendens* Ingold, *Tetracladium marchalianum* de Wild, *Tetrachaetum elegans* Ingold, *Flagellospora curta* Webster, *Tricladium chaetocladium* Ingold, *Lemonniera aquatica* de Wildeman, *Varicosporium elodeae* Kegel, *Clavariopsis aquatica* de Wildeman, *Lemonniera pseudofloscula* Dyko *et al.*, *Anguillospora filiformis* Greath, *Articulospora tetracladia* Ingold, and *Heliscus lugdunensis* Sacc. & Théry. Microcosm were inoculated with plugs (Ø = 12 mm) of malt extract agar (MEA; 20 $g\,L^{-1}$; Cultimed) collected from the edge of 14-day-old colonies of the twelve fungi to obtain three richness levels: single species (3 replicates × 3 salt levels × 12 species), a random combination of four fungal species (3 replicates × 3 salt levels), and a random combination of eight species (3 replicates × 3 salt levels). The species combinations were randomly selected in order to eliminate identity and specific trait effects (Gonçalves *et al.*, 2015). The inoculum size was maintained independently the richness level. All microcosms were incubated on orbital shakers at 120 rpm under a 12 h light: 12 h dark photoperiod. After 6 days, nutrient solution from all microcosms was renewed and the plugs were removed; the medium was subsequently renewed every 2 days. The experiment run for 42 days.

2.2 Leaf mass loss

From each microcosm, 12 discs were oven-dried (60 °C, 48 h), and weighed (DM). Litter mass loss was calculated as the difference between initial and final DM of the 20 discs (see below the DM determination of the remaining 8 discs) and expressed as percentage (%).

2.3 Fungal biomass

Ergosterol concentration, as a proxy of fungal biomass (Gessner and Chauvet, 1993), was estimated using a subset of 3 leaf discs of each microcosm that was freeze dried, and weighed (DM). Ergosterol extraction was performed according to Reis *et al.* (2018) and Gessner (2003): ergosterol concentration was quantified by high performance liquid chromatography (HPLC) using a Merck LiChroCART 250-4 (LiChrospher 100) RP-18 column, by measuring absorbance at 282 nm (Young, 1995). Ergosterol was converted into fungal biomass using a conversion factor of 5.5 μg ergosterol mg^{-1} fungal DM (Gessner and Chauvet, 1993). Results were expressed as mg fungi g^{-1} leaf DM.

2.4 Microbial respiration

To evaluate respiration rate, five discs from each microcosm were immersed in 50 mL Falcon tubes filled with the corresponding oxygen saturated (Jenway 9200 oxygen meter; Jenway, UK) experimental solutions. Tubes were kept in the dark for 16 h. Oxygen consumption was obtained by the difference between the initial and the final oxygen concentrations divided by DM (60 °C, 48 h) of the discs; rates were expressed as mg O_2 mg^{-1} DM h^{-1}.

2.5 Sporulation rate

After 15 days of incubation, the media of the microcosms was collected in order to assess the peak of sporulation rate (based on previous works; Abelho, 2009; Bärlocher *et al.*, 2013). Conidia were fixed with 2 ml of formalin (37%). To ensure even distribution of the conidia, the suspension was

Table 1. Statistical results for the two-way ANOVA for the four measured variables. Statistical significances are highlighted in bold. Small letters denote statistical differences after Tukey post-hoc test.

Variable	Factor	dF	*F* value	*p*	Post-hoc
Leaf mass loss					
	Salt	2,117	52.51	$<$ **0.001**	S0a S8b S16b
	Richness	2,117	0.55	0.576	
	Salt × Richness	4,117	1.58	0.184	
Fungal biomass					
	Salt	2,117	3.69	**0.027**	S0a S8a S16b
	Richness	2,117	0.33	0.716	
	Salt × Richness	4,117	0.57	0.688	
Respiration rate					
	Salt	2,117	22.65	$<$ **0.001**	S0a S8a S16b
	Richness	2,117	0.43	0.650	
	Salt × Richness	4,117	2.03	0.094	
Sporulation rate					
	Salt	2,117	55.42	$<$ **0.001**	S0a S8b S16b
	Richness	2,117	3.95	**0.022**	D1b D4a D8b
	Salt × Richness	4,117	1.31	0.271	

mixed with 100 ml of Triton X-100 (0.5%), an aliquot was filtered (Millipore SMWP filters, 5 μm pore size) and conidia were stained with 0.05% cotton blue in lactic acid (60%). Spores were counted under a microscope (250×). Sporulation rates were expressed as the number of conidia produced per mg^{-1} DM d^{-1}.

2.6 Statistical analyses

The effects of salt level and fungal richness on the four tested variables (leaf mass loss, fungal biomass, respiration rate, and sporulation rate) were tested by two-way ANOVAs. As the experimental design was unbalanced, we used the *Anova* function in the *car* R package for Type III SS, followed by Tukey post-hoc tests performed by the *glht* function in the *multcomp* R package.

Whenever necessary, data were log (x+1) transformed to obtain requirements for parametric analyses (normality and homogeneity of variances). Results of statistical analyses were analysed with R statistical software (version 3.2.5; R Development Core Team, 2016) and were considered significant when $p < 0.05$.

3 Results

3.1 Leaf mass loss

The leaf mass loss ranged between 7.4 ± 0.3% at 16 g NaCl L^{-1} in the 8 species level and 10.1 ± 0.1% at 0 g NaCl L^{-1} in the 4 species level. Overall, the two added concentrations of NaCl depressed leaf mass loss independently the fungal richness (Tab. 1, Fig. 1a).

3.2 Fungal biomass

Fungal biomass varied between 588.7 ± 195.3 mg g^{-1} leaf DM in the 8 species level under 16 g NaCl L^{-1} to

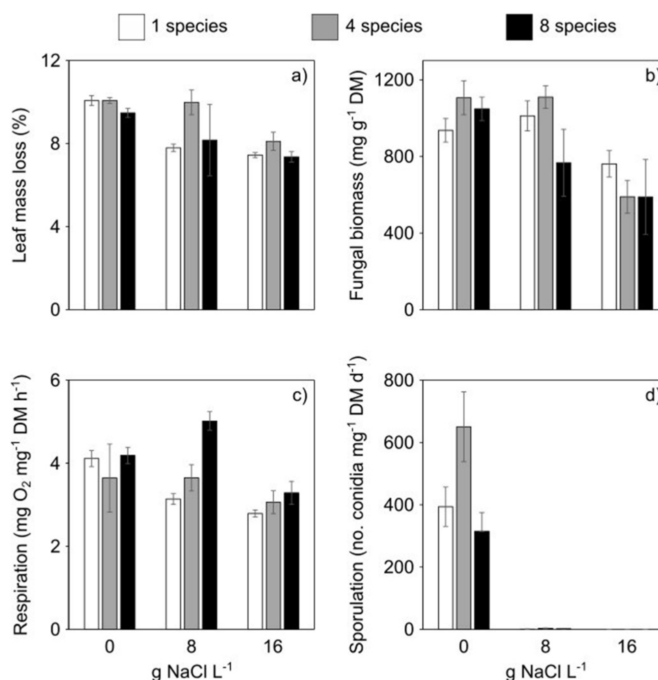

Fig. 1. (a) Leaf mass loss, (b) fungal biomass, (c) microbial respiration rate, and (d) sporulation rate (mean ± SE) for the three salt concentrations and three levels of fungal richness.

1109.6 ± 59.2 mg g^{-1} leaf DM at 8 g NaCl L^{-1} in the 4 species level. Independently of the richness level, the highest salt concentration led to a lower fungal biomass than the other two salt treatments (Tab. 1, Fig. 1b).

3.3 Microbial respiration

The minimum respiration rate –2.8 ± 0.1 mg O_2 mg^{-1} DM h^{-1} –was recorded at 16 g NaCl L^{-1} for single species, and the

maximum one -5.0 ± 0.2 mg O_2 mg^{-1} DM h^{-1}–at 8 g NaCl L^{-1} in the 8 species level. Fungal richness did not affect respiration rate, but the highest salt level reduced the oxygen consumption by microorganisms (Tab. 1, Fig. 1c).

3.4 Sporulation rate

Within the treatment of 0 g NaCl L^{-1}, the values ranged from 314.7 ± 60.1 conidia mg^{-1} DM d^{-1} in the 8 species level to 650.6 ± 112.1 conidia mg^{-1} DM d^{-1} in the 4 species level. When affected by salinization, independently the richness, the sporulation rate was totally inhibited at 16 g NaCl L^{-1}, and extremely low at 8 g NaCl L^{-1} (from 0.5 ± 0.2 to 3.0 ± 1.3 conidia mg^{-1} DM d^{-1}). Sporulation rate was highest when 4 species were incubated together (Tab. 1, Fig. 1d)

4 Discussion

Extreme salinization of fresh waters depressed, but did not eliminate, leaf litter decomposition by aquatic hyphomycetes. Nonetheless, spore production was completely inhibited by salt increases, confirming the deleterious effects of salt-contamination on the reproductive capacity of these decomposers. Contrary to our expectations, our results also showed that higher richness of hyphomycetes species do not buffer the salinization effects.

Overall, the results supported the first hypothesis since salinization depressed all measured variables of microorganisms colonizing leaf litter, which is in agreement with the majority of previous studies (Canhoto *et al.*, 2017; Gonçalves *et al.*, 2019a; Martínez *et al.*, 2020; Sauer *et al.*, 2016; Schäfer *et al.*, 2012; Tyree *et al.*, 2016). Nonetheless, this study also indicates that aquatic hyphomycetes activity still occurs at extreme levels of salinity, potentially guaranteeing leaf litter degradation under extreme conditions. This is surprising considering the fact that the highest salt concentrations tested − namely 16 g NaCl L^{-1}–surpassed by far those used in previous studies. Exception for Gómez *et al.* (2016) that investigated this concentration in natural salinized streams and some reported pulse contaminations (Corsi *et al.*, 2010) where higher values were reached. While leaf mass loss was slowed down by salt contamination, the reduction in relation to control was 1.14- and 1.29-fold at 8 g (14610 μS cm^{-1}) and 16 g (28883 μS cm^{-1}) NaCl L^{-1}, respectively, which is far below the 3-fold reduction between 627 and 13267 μS cm^{-1} reported by Gómez *et al.* (2016). Values at 8 g NaCl L^{-1} are more in the line with those reported by Canhoto *et al.* (2017)) under the same conditions. Other authors (Gonçalves *et al.*, 2019b; Martínez *et al.*, 2020) did not report a significant diminution of microbial decomposer activity below 6 g L^{-1} of salt. Respiration rate and fungal biomass did not mimic the pattern of mass loss, being only depressed at 16 g NaCl L^{-1}. These results contrast with a general inhibitory effect reported below 8 g NaCl L^{-1} in the majority of studies (Almeida Júnior *et al.*, 2020; Canhoto *et al.*, 2017; Martínez *et al.*, 2020) but are in line with results from brackish (Connolly *et al.*, 2014) and estuarine areas (Snronan and Kavr, 1988), reinforcing the fact that freshwater hyphomycetes are highly salt-tolerant, in spite of the potential impairment of the colonization process.

Our second hypothesis was not supported; only the sporulation rate was enhanced at the intermediate richness level. This finding, although not consensual (Costantini and Rossi, 2010; Duarte *et al.*, 2006; Gonçalves *et al.*, 2015; Pascoal *et al.*, 2010), is in line with studies that consider that a greater number of hyphomycete species does not imply an enhancement effect on the tested variables (Dang *et al.*, 2005; Geraldes *et al.*, 2012). The high functional redundancy among freshwater hyphomycetes species seems to allow a few species to maintain litter decomposition efficiency (Gessner *et al.*, 2010), independently of their identity. In this vein, we must also reject the third hypothesis since our findings suggest that the magnitude of the effects of salinization on litter decomposition mediated by aquatic hyphomycetes will not depend on their diversity. This was surprising and contrasts with the general idea (insurance hypothesis; Yachi and Loreau, 1999) that a greater aquatic hyphomycete diversity may buffer the consequences of environmental shifts (Duarte *et al.*, 2008; Fernandes *et al.*, 2011; Gonçalves *et al.*, 2016, 2015; Pascoal *et al.*, 2010).

The different tolerance to salinity shown by aquatic hyphomycete species (Canhoto *et al.*, 2017), combined with distinct feeding preferences by leaf-consumers (Gonçalves *et al.*, 2014) may trigger changes on shredders processing capacity; the consequences, at the ecosystem level difficult to anticipate. Further efforts are needed to understand the possible cascade effects promoted by freshwater salinization on the brown food webs. Assessing salt resistance and resilience of streams, that differ in biodiversity at multiple trophic levels, may constitute an important line of inquiry to properly manage these vital ecosystems.

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