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Universidad del País Vasco

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Functional consequences of biodiversity loss caused by emergent diseases in freshwater ecosystems

Alberto Alonso Blanco

PhD Thesis

March 2024

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Cover image by Alfredo Rodríguez Hernani

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Content

Summary

Biodiversity on Earth is decreasing at alarming rates, with freshwater ecosystems suffering higher extinctions rates than their terrestrial and marine counterparts. Biodiversity loss is known to affect ecosystem functioning and structure, but most research focuses on random species loss, which is unrealistic because species differ in extinction risk based on their traits and sensitivity to stressors. Emergent diseases entail an increasing risk to a wide range of species, with trees and amphibians being two taxonomic groups strongly affected. In this context, this thesis aimed to assess how the loss of several species of trees and amphibians, vulnerable to extinction due to emergent diseases, could impact the functioning and structure of freshwater ecosystems. The first three chapters explored the loss of *Alnus glutinosa* and *Quercus robur* and the replacement of the former by *Robinia pseudoacacia* after infection in headwater streams, finding important effects on the key process of leaf litter decomposition and associated communities of aquatic hyphomycetes and invertebrates. The other three chapters addressed the loss of one anuran (*Alytes obstetricans*) and two urodeles (*Triturus marmoratus* and *Salamandra salamandra*) in montane streams and ponds in the presence and absence of other amphibians, showing different effects on periphyton accrual and leaf litter decomposition through competitive and trophic interactions, as well as changes in algal communities and invertebrate foraging preferences that translated in variations in basal resources. Overall, our results highlighted the importance of considering species traits and the biological and environmental contexts for predicting the impacts of species loss on ecosystems, emphasizing the relevance of studying real-case scenarios if we are to better understand the consequences of extinctions and improve species conservation and ecosystem management.

Resumen

La biodiversidad de la Tierra está disminuyendo a un ritmo alarmante, con los ecosistemas de agua dulce sufriendo tasas de extinción mayores que los ecosistemas terrestres y marinos. Se sabe que la pérdida de biodiversidad afecta al funcionamiento y la estructura de los ecosistemas, pero la mayor parte de las investigaciones se han centrado en la pérdida aleatoria de especies, lo cual carece de realismo, ya que las especies difieren en su riesgo de extinción dependiendo de sus características y su sensibilidad a los estresores. Las enfermedades emergentes suponen un riesgo creciente para una gran variedad de especies, siendo árboles y anfibios dos grupos taxonómicos especialmente afectados. En este contexto, esta tesis pretende evaluar como la pérdida de varias especies de árboles y anfibios, vulnerables a la extinción por enfermedades emergentes, podrían suponer un impacto para el funcionamiento y la estructura de los ecosistemas de agua dulce. Los tres primeros capítulos exploran la pérdida de *Alnus glutinosa* y *Quercus robur*, y la sustitución del primero por *Robinia pseudoacacia* tras su infección, en arroyos de cabecera, y encuentran efectos importantes sobre el proceso clave de la descomposición de hojarasca y las comunidades de hifomicetos acuáticos e invertebrados asociados al proceso. Los otros tres capítulos evalúan la pérdida de un anuro (*Alytes obstetricans*) y dos urodelos (*Triturus marmoratus* y *Salamandra salamandra*) en arroyos y charcas de montaña, en presencia y ausencia de otros anfibios, y muestran diferentes efectos sobre la producción de perifiton y la descomposición de hojarasca a través de interacciones competitivas y tróficas; además, se muestran cambios en las comunidades asociadas de algas y en las preferencias alimenticias de los invertebrados que se traducen en cambios en los recursos basales. En general, nuestros resultados resaltan la importancia de considerar las características de las especies y el contexto biológico y ambiental a la hora de predecir los impactos de la pérdida de especies sobre los ecosistemas, y enfatizan la relevancia de estudiar escenarios realistas para una mejor comprensión de las consecuencias de las extinciones y para mejorar la conservación de especies y la gestión de los ecosistemas.

General Introduction

GLOBAL BIODIVERSITY LOSS AND FRESHWATER ECOSYSTEMS

Biodiversity is currently declining at rates that are comparable to those reported for past mass extinctions, where more than three quarters of Earth's species were lost in geologically short intervals (Barnosky et al., 2011; Ceballos et al., 2017). Unlike those events, which were caused by extreme natural phenomena, species losses at present are being mostly originated by anthropogenic impacts such as habitat loss and fragmentation (Haddad et al., 2015), pollution (Sigmund et al., 2023), climate change (Thomas et al., 2004), or the expansion of invasive species (Bellard et al., 2016) and emergent diseases (Daszak et al., 2000). Importantly, while species extinctions are worrying by themselves due to the intrinsic value of biodiversity (Ghilarov, 2000; IPCC, 2014), they can also bring important consequences for *ecosystem functioning* and *structure* (see glossary of terms in Box 1) that need to be investigated (Cardinale et al., 2006; Cardinale et al., 2011; Hooper et al., 2012; Boyero et al., 2021).

Freshwater ecosystems are especially important for Earth's biodiversity, since they harbour around the 6% of all described species despite the fact that they only occupy the 0.8% of the global surface (Dudgeon et al., 2006). However, these ecosystems are suffering extinction rates considerably higher than those of marine or terrestrial ecosystems (Sala et al., 2000; Reid et al., 2019). Remarkably, freshwater ecosystems are not only affected by the loss of freshwater species, but also by extinctions in their surrounding terrestrial ecosystems. This occurs because of the strong land-water interactions existing in riparian areas, with terrestrial plants providing fundamental resources for aquatic consumers, and many animals such as insects and amphibians having both aquatic and terrestrial life stages (Kominoski et al., 2013).

Headwater streams, in particular, are a type of freshwater ecosystem with very strong links to the terrestrial habitat. Many of these streams run through riparian forests that limit instream primary production as a result of shading and, at the same time, they provide allochthonous material in the form of leaf litter that is the basal resource for a predominantly detrital or *brown* aquatic *food web* (Fig. 1; Vannote et al., 1980; Marcarelli et al., 2011; Tonin et al., 2021). When leaf litter enters the stream, it undergoes a series of changes that result in their *decomposition*, including physicochemical processes –

physical abrasion by stream flow and the leaching of soluble compounds– and others that are mediated by microbial decomposers and animal consumers (Marks, 2019). Microbial decomposers, mainly aquatic hyphomycetes, obtain energy and nutrients from leaf litter while they increase its palatability to consumers, mostly invertebrates known as leaf litter-feeding *detritivores* (Marks, 2019). The feeding activity of these invertebrates produces fine particulate organic matter that is eaten by other detritivorous invertebrates –collector-gatherers and filterers–, with all of these invertebrates sustaining higher trophic levels, from predatory invertebrates to fish and amphibians in the stream, as well as some invertebrates and vertebrates in the riparian area (Wallace et al., 1997).

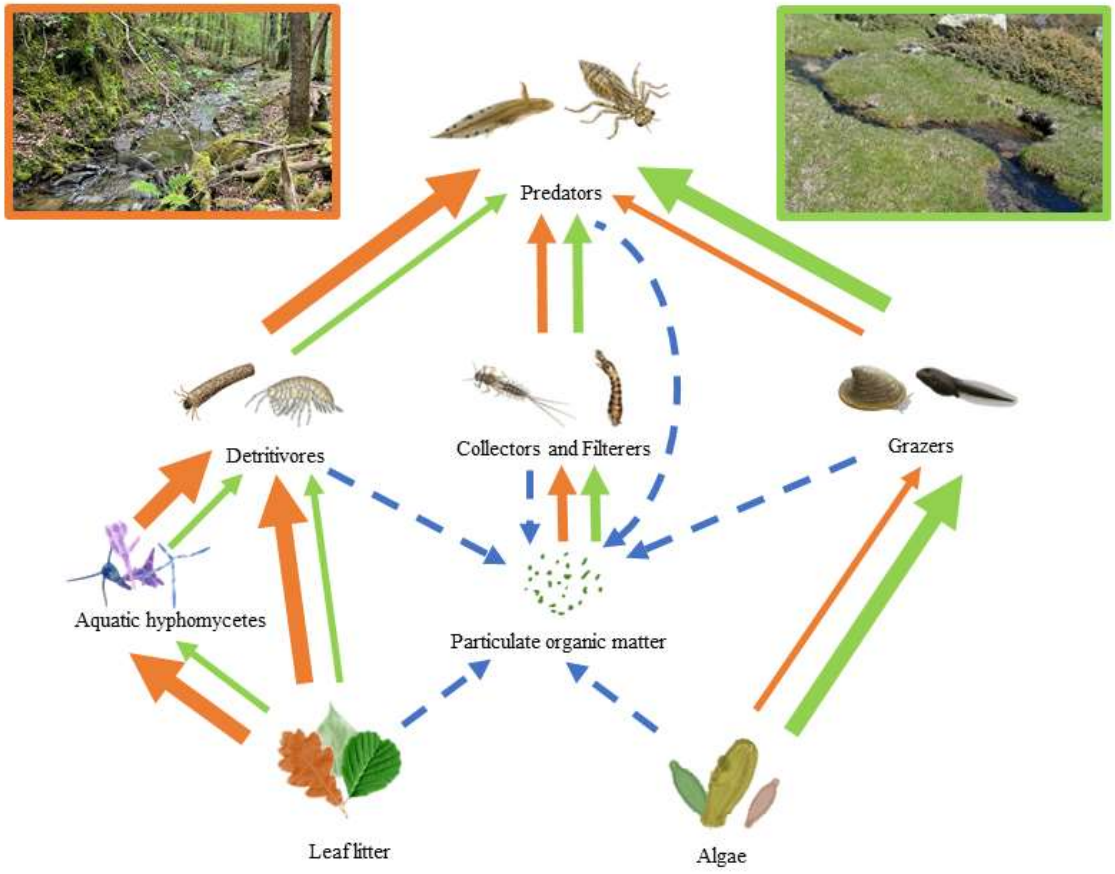

Fig. 1. Main trophic relationships occurring in the predominantly brown and green food webs, respectively, of forested headwater streams (brown arrows) and montane streams (green arrows). Solid arrows represent feeding relationships, and dotted arrows represent the release of substances to the water column. Arrow thickness indicates the importance of the pathway.

Biodiversity: Variety of living organisms. It can refer to species, other taxonomic levels, genes, or species traits (Swingland 2001).

Bottom-up effect: Change in a focal trophic level caused by the change in biomass, species composition or activity of a lower trophic level (Pace et al. 1999).

Brown food web: Set of trophic relationships whose flux of energy proceeds from the processing of dead organic matter (Odum 1969).

Complementarity effect: Partitioning of the net diversity effect caused by synergistic or antagonistic effects among species in a mixture that stimulate or inhibit the performance of other species. It is calculated as the average deviation from the expected decomposition in the mixture, multiplied by the mean decomposition of single species and the number of species in the mixture (Loreau and Hector 2001): $CE = \text{mean}(Value_{Mixture} - Value_{Species}) \times \text{mean } Value_{Species} \times n$.

Decomposition: Set of biological processes that contribute to dead organic matter breaking up into simple inorganic components (Gessner et al. 2010).

Detritivore: Animal that feeds on dead plant material, generally leaf litter, by means of shredding, mining or scraping (Boyero et al. 2020).

Ecosystem functioning: Set of processes that regulate energy and matter fluxes in ecosystems due to the joint activity of organisms (Von Schiller et al. 2017).

Ecosystem structure: Abiotic components and biotic communities that integrate an ecosystem (Sandin and Solimini 2009).

Grazer: Animal that feeds on periphyton by scraping the surface of hard substrata or macrophytes (Tachet et al. 2010).

Green food web: Set of trophic relationships whose flux of energy proceeds from a primary producer such as a plant or an alga (Odum 1969).

Net diversity effect: Deviation between the performance of a process in a species mixture and the expected performance based on the species included in the mixture. It is calculated as the difference between the observed and expected values, the latter calculated from the value of each species in monoculture and pondered by the relative contribution of each species to the mixture. The net diversity effect results from the addition of selection and complementarity effects (Loreau and Hector 2001): $NDE = \Sigma i\ [(Value_{Mixture} - Value_{Species}) \times (Abundance_{Species} / Abundance_{Mixture})]$.

Periphyton: Mature and extended algal growth covering the surface of organic or inorganic substrata (Bellinger and Sigee 2015).

Trophic cascade: Effects among predator and prey that alter the abundance, biomass or productivity of a community across more than one level of a food web (Pace et al. 1999).

Selection effect: Partitioning of the net diversity effect caused by the presence of a species with a high or low performance that can influence the overall process due to its strong influence. It is calculated as the covariance between the deviation from the expected value in the mixture and the value of single species, multiplied by the number of species in the mixture (Loreau and Hector 2001): $SE = \text{cov}\ [(Value_{Mixture} - Value_{Species}), Value_{Species}] \times n$.

Species trait: Morphological, physiological or phenological feature of the phenotype of an organism that determines its effect on the performance of processes and its response to environmental factors (Violle et al. 2007).

Top-down effect: Change in a focal trophic level caused by the change in biomass, species composition or activity of a higher trophic level (Pace et al. 1999).

Box 1. Glossary of main terms used in this thesis.

Nevertheless, not all headwater streams are detritus-based ecosystems: in montane areas that are over the tree line, riparian vegetation is scarce and streams receive low leaf litter inputs and little shading (Atkinson et al., 2018). These circumstances favour algal growth, so *periphyton* is often the main basal resource in this type of stream and a *green food web* prevails (Fig. 1). Periphyton is eaten by invertebrate and vertebrate *grazers*, which feeding activity produces fine particulate organic matter that serves as food for other animals, as described above (Wallace & Webster, 1996; Hillebrand, 2009). In both cases, invertebrate and vertebrate predators at higher levels of the food web control the abundance and activity of consumers at lower levels and can have cascading or *top-down effects* on the processing of basal resources (Hillebrand & Shurin, 2005; Swan, 2021). Besides, different aspects of predator activity (e.g., excretion or bioturbation) can produce

nutrients that stimulate the growth of algae and heterotrophic microbes and thus directly influence ecosystem processes such as primary production and decomposition (Schmitz et al., 2010).

NON-RANDOM SPECIES LOSS AND ITS EFFECTS ON ECOSYSTEM FUNCTIONING

Freshwater ecosystem functioning and structure are known to be affected by biodiversity loss at different trophic levels, both through *bottom-up* and top-down *effects*, in both brown and green food webs (Cardinale et al., 2011). Bottom-up effects are more or less well documented for brown food webs, where higher diversity in a leaf litter mixture often induces faster decomposition than would be expected based on the species present in the mixture (López-Rojo et al., 2018). Still, effects of plant diversity on decomposition are variable and highly dependent on species identities, since species with nutrient-rich leaf litter can accelerate decomposition in the mixture, and recalcitrant species with high contents of tannins and other toxic substances inhibit biological activity and can reduce overall decomposition (Cardinale et al., 2011; Swan, 2021). In green food webs, the reported effects of algal diversity on ecosystems are stronger and more consistent, if not universal, with higher diversity leading to higher rates of primary production or algal biomass accrual (Cardinale et al., 2011). While top-down effects have been less studied, a higher diversity of consumers generally leads to higher consumption rates. Thus, higher detritivore diversity can accelerate decomposition through facilitation and resource partitioning (Gessner et al., 2010), with particularly strong effects when *species* differ in *traits* such as body size or mouthparts (Tonin et al., 2018; Swan, 2021); and higher grazer diversity can more efficiently reduce periphyton biomass accrual (Hillebrand & Shurin, 2005).

Remarkably, most of the above studies and others demonstrating effects of diversity loss on ecosystem functioning have simulated random species loss (Wardle, 2016). This is despite the fact that species traits underly most diversity effects on ecosystem functioning (Hillebrand et al., 2009; Gessner et al., 2010; Cardinale et al., 2011; Swan, 2021), and also that species do not disappear randomly, but rather depending on their traits and the existing environmental conditions (Lepš, 2004). Consequently, the few studies that have focused on non-random species loss have found that the identity of

species lost and the order at which they are lost determines the effects of diversity on ecosystem functioning (Jonsson et al., 2002; Kominoski et al., 2013). Moreover, those studies support mathematical models suggesting that random and non-random species loss can have different consequences for ecosystems (Ives & Cardinale, 2004; Gross & Cardinale, 2005). Despite the difficulty of predicting non-random extinctions, due to a general lack of knowledge of species traits and their sensibility to particular stressors, it seems important that studies use realistic scenarios of species loss. This is particularly evident for species which are known to have high susceptibility to specific stressors, as occurs with species affected by pathogens.

BIODIVERSITY LOSS BY FUNGAL EMERGENT PATHOGENS

In the last decades, global commerce has led to fungal pathogens increasing their distribution area and their virulence through recombination and hybridization with previously allopatric related lineages (Fisher et al., 2012). Thus, invasive fungal pathogens have spread worldwide, infecting native plants and animals and leading to the decline and extinction of more sensitive taxa, and representing an enormous threat for host species (Harvell et al., 2002; Fisher et al., 2012; Almeida et al., 2019; Fisher et al., 2020). While fungal pathogens in plants have been studied mainly in the context of agriculture, there are several wild tree species strongly affected by fungal diseases. For example, elm (*Ulmus* spp.) and chestnut (*Castanea* spp.) have been extirpated from part of their distribution area due to *Ophiostoma novo-ulmi* BRASIER and *Cryphonectria parasitica* (MURRILL) BARR, respectively, which have induced shifts in forest ecosystem structure and functioning (Milgroom et al., 1996; Loo, 2009). More recently, other pathogens have produced declines in several plants, including *Austropuccinia psidii* (G. WINTER) BEENKEN infecting myrtle (*Myrtus* spp.), eucalypts (*Eucalyptus* spp.) and other members of the Myrtaceae family (Berthon et al., 2018); *Hymenoscyphus fraxineus* (T. KOWALSKI) BARAL, QUELOZ & HOSOYA threatening ash (*Fraxinus* spp.) and other members of the Oleaceae family (Landolt et al., 2016); and the different *Phytophthora* species, which are generalist pathogens affecting a wide variety of plant species.

In animals, there are several species declines documented that are related to fungal pathogens (Shearer & Dillon, 1995; Brasier, 1996; Vannini & Vettraino, 2001; Hansen et

al., 2005; Bjelke et al., 2016; Jung et al., 2018), being those of amphibians the most remarkable. Most affected amphibians have been reduced or extinct due to infection by *Batrachochytrium dendrobatidis* LONGCORE, PESSIER & D.K. NICHOLS (Scheele et al., 2019), with European urodeles also threatened by *B. salamandrivorans* A. MARTEL, BLOOI, BOSSUYT & PASMANS (Martel et al., 2014). Also, North American bats are declining due to *Pseudogymnoascus destructans* (BLEHERT & GARGAS) MINNIS & D. L. LINDNER (Blehert et al., 2009; Drees et al., 2017); crayfish distribution has been reduced by *Aphanomyces astaci* Schikora (Holdich et al., 2009); and snakes are starting to decline due to infection of *Ophidiomyces ophiodiicola* SIGLER, HAMBLETON & PARÉ (Allender et al., 2016; Franklinos et al., 2017). Among all of these affected plant and animal taxa (Fig. 2), the most likely to have an impact on freshwater ecosystems are riparian trees and amphibians, given the relevance of riparian leaf litter inputs to many streams and the importance of amphibian larvae as consumers in freshwater habitats, as explained above. Therefore, this thesis will focus on the effects of species loss in these two taxonomic groups.

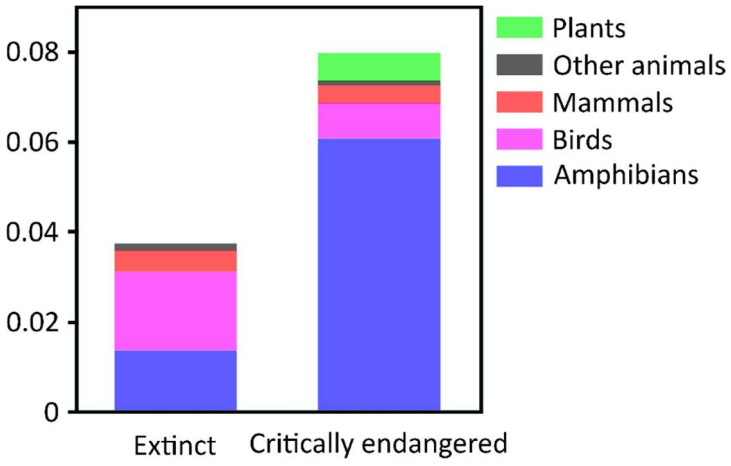

Fig. 2. Proportion of known species extinctions facilitated by disease and critically endangered species threatened by disease, depicted by taxonomic group. "Other animals" include invertebrates, fishes, and reptiles. Adapted from Smith et al. (2006).

LOSS OF RIPARIAN TREES AND INSTREAM LEAF LITTER DECOMPOSITION

Alder (*Alnus* spp.) is a dominant riparian tree species in Eurasia, where it is usually the only nitrogen (N)-fixing tree (Waring & Running, 2010). Its leaf litter is rich in nutrients

and poor in refractory compounds (Waring & Running, 2010), hence highly palatable. When it falls into the stream, these characteristics make it strongly preferred by microbial decomposers and detritivores (Graça et al., 2001), resulting in its fast decomposition (Tonin et al., 2017). Besides, alder leaf litter often causes a positive *net diversity effect* on mixtures, promoting the decomposition of leaf litter through two mechanisms. Firstly, there can be a positive *selection effect*, meaning that preferential feeding on palatable leaf litter results in faster decomposition of the mixture (López-Rojo et al., 2018; Rubio-Ríos et al., 2021). Sometimes it enhances decomposition of less palatable leaf litter by attracting detritivores to the mixture (Ferreira et al., 2012), and it can affect not only stream reaches surrounded by riparian forest but also downstream reaches where alder leaf litter is transported by flow (Piccolo & Wipfli, 2002). Secondly, there can be a positive *complementarity effect*, with alder leaf litter directly increasing the palatability of other leaf litter types by nutrient transfer though fungal hyphae (Handa et al., 2014; López-Rojo et al., 2018; Larrañaga et al., 2020). Overall, due to the fast decomposition of alder leaf litter, the preference of decomposers and detritivores towards it, and its effects on adjacent leaf litter, alder is considered a key species for stream ecosystems (Pérez et al., 2021a).

However, alder trees are suffering widespread dieback due to an infection produced by *Phytophthora alni,* an oomycete species complex composed of *P.* × *alni* (Brasier & S.A. Kirk) Husson, Ioos & Marçais, *P.* × *multiformis* (Brasier & S.A. Kirk) Husson, Ioos & Frey and *P. uniformis* (Brasier & S.A. Kirk) Husson, Ioos & Aguayo (Husson et al., 2015), which has expanded throughout Europe affecting almost half of alder trees in some regions (Bjelke et al., 2016). Tree infection begins when zoospores reach the roots or the trunk during floods, leading to root rot, collar rot, small-size, sparse and often chlorotic foliage, crown dieback and, ultimately, tree mortality (Fig. 3A). Mortality rates reach almost 100%, with young trees usually dying in a few months and old trees dying after several years while losing vitality progressively (Jung & Blaschke, 2004; Bjelke et al., 2016; Jung et al., 2018). Importantly, the potential effects of *P. alni* on stream ecosystems go beyond the disappearance of the species from riparian vegetation, because it produces changes in leaf litter traits as a result of the infection. For example, infected trees show reduced nutrient resorption before leaf senescence, leading to higher nutrient concentrations in leaf litter from infected trees and hence faster decomposition (Ferreira et al., 2022).

Leaf litter inputs to streams are not limited to strictly riparian plants, as some dominant trees of the surrounding forest can also be important sources of leaf litter. Such is the case of oaks (*Quercus* spp.) and other species from the family Fagaceae in Atlantic mixed forests. In contrast with alder, oak leaf litter has lower nutrient concentrations and higher concentrations of recalcitrant components such as tannins and other inhibitory substances, resulting in slower instream decomposition (Ferreira et al., 2012; Pérez et al., 2021b). When present in leaf litter mixtures, its biological traits can slow down overall decomposition through two mechanisms. Firstly, there can be a negative selection effect, as oak leaf litter is avoided by detritivores due to its low palatability (Larrañaga et al., 2020). Secondly, tannins and other inhibitory substances leached from this leaf litter can cause a negative complementary effect, since they can limit microbial colonization and detritivore consumption of contiguous leaves (McArthur et al., 1994; Ferreira et al., 2012). Due to these characteristics, oak leaf litter can persist in the stream for longer than other leaf litter types, and its recalcitrance delay nutrient loss, allowing more efficient assimilation by consumers and hence being also an important resource in streams (Marks, 2019; Siders et al., 2021).

Similarly to alders, oaks are affected by a fungal pathogen, the oomycete *Phytophthora cinnamomi* RANDS. This pathogen is distributed worldwide and it is usually found together with related species such as *P. cambivora* (PETRI) BUISMAN, *P. quercina* JUNG, *P. plurivora* JUNG & BURGESS and *P. gonapodyides* (H.E.PETERSEN) BUISMAN. These species infect oaks and other species of the family Fagaceae, among many other trees, inducing ink disease (Brasier, 1996; Vannini & Vettraino, 2001; Jung et al., 2018). The pathogens infect the host tree when zoospores reach the roots, causing root and crown rot with black exudates, crown thinning, branch dieback, proliferation of epicormic shots, leaf chlorosis and slow tree death (Fig. 3A). Effects of these pathogens vary depending on the species and environmental conditions, but they can lead to high mortality and species declines (Brasier, 1996; Jung et al., 2018).

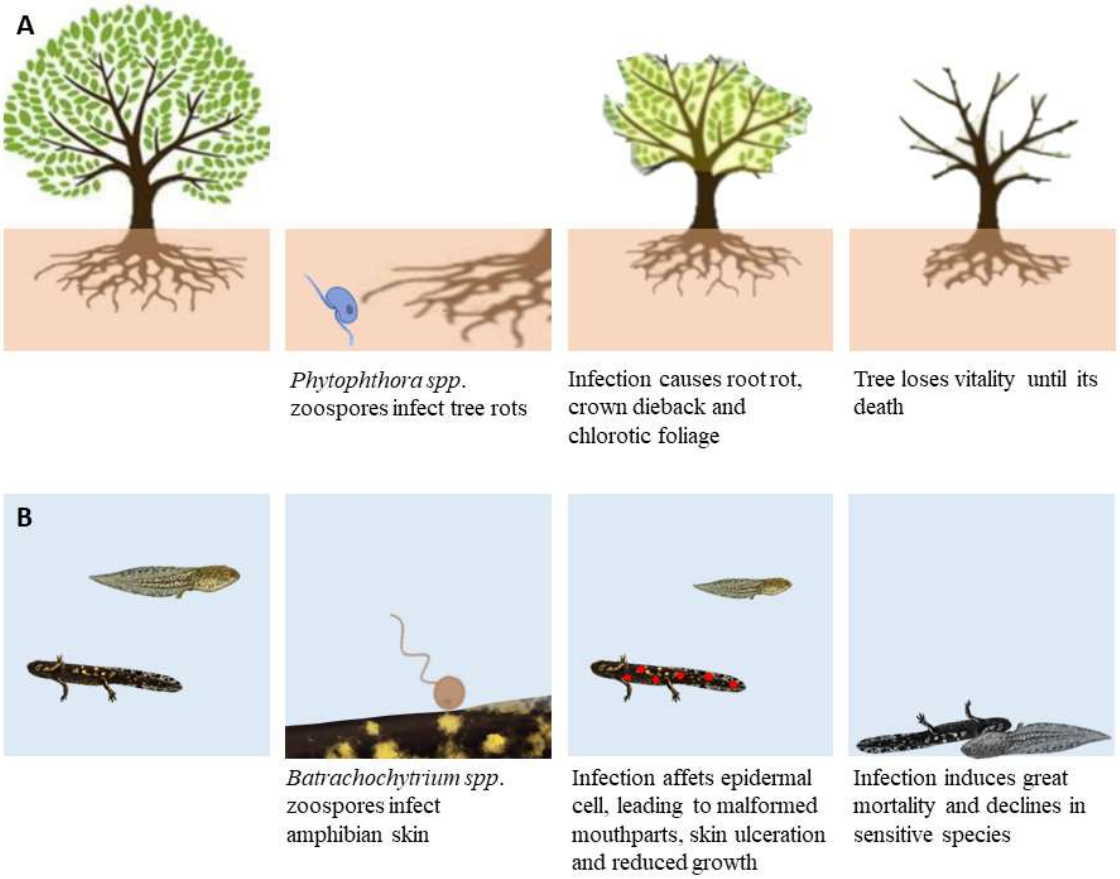

Fig. 3. Scheme of how infection by fungal emergent diseases occurs in trees (A) and amphibian larvae (B) and its consequences.

AMPHIBIAN LOSS AND TROPHIC CASCADES

Amphibians are important consumers in many freshwater ecosystems, where they can influence ecosystem functioning through top-down effects in different ways depending on their trophic level, which can vary from primary consumers to top predators (Davic & Welsh Jr, 2004; Hocking & Babbitt, 2014). Many anuran larvae are periphyton grazers, often more efficient than invertebrates, and they are known to control periphyton biomass accrual and algal communities, although there are few studies exploring their effects on ecosystem functioning, most of them conducted in tropical streams (Kupferberg, 1997; Ranvestel et al., 2004; Mallory & Richardson, 2005; Connelly et al., 2008; Whiles et al., 2013; Rowland et al., 2017; Barnum et al., 2022). Anuran larvae can also influence ecosystem processes through nutrient release due to bioturbation –that is, the removal of sediment and upper periphyton layers– and excretion, which can stimulate primary production and decomposition and control nutrient cycling (Kupferberg, 1997; Iwai &

Kagaya, 2007; Connelly et al., 2008; Iwai et al., 2009; Rugenski et al., 2012; Whiles et al., 2013; Schmidt et al., 2019). In addition, they can influence invertebrate communities in different ways, since they can either compete with them for resources or facilitate their access to underlying food (Kupferberg, 1997; Ranvestel et al., 2004; Colón-Gaud et al., 2009; Colon-Gaud et al., 2010; Barnum et al., 2022).

On the other hand, urodele larvae are predators that feed on zooplankton, macroinvertebrates or other amphibian larvae, depending on their size. Thus, their activity can alter the abundance and community structure of their prey, as observed for some salamanders (Holomuzki et al., 1994; Blaustein et al., 1996; Urban, 2013; Arribas et al., 2014). Besides, their predatory activity not only affects their prey, but can also control lower trophic levels though top-down effects and *trophic cascades*. On the one hand, predators can induce direct effects, with prey consumption leading to reduced prey abundance, which in turn leads to lower consumption of basal resources by this prey. For example, salamander larvae can reduce invertebrate grazer abundance and hence grazing pressure on periphyton or phytoplankton, ultimately provoking an increase in periphyton or phytoplankton biomass (Holomuzki et al., 1994; Blaustein et al., 1996). On the other hand, predators can have indirect effects through nutrient release due to bioturbation and excretion, as observed for crayfish and dragonfly larvae (Costa & Vonesh, 2013; Arribas et al., 2014); or due to changes in prey foraging activity in the presence of predators, as observed for stonefly larvae and fish that reduce their prey grazing activity and therefore increase algal biomass (Peckarsky et al., 1993; Davenport et al., 2020).

Notwithstanding, as previously stated, amphibians are suffering strong declines due to emergent infectious diseases, mainly by chytridiomycosis caused by fungi of the genus *Batrachochytrium* (Daszak et al., 2003; Collins, 2010; Scheele et al., 2019; Fisher & Garner, 2020). *Batrachochytrium dendrobatidis* is a widely generalist pathogen native from Asia which has spread around the globe and infects a wide variety of amphibians (Lips, 2016; O'Hanlon et al., 2018; Scheele et al., 2019; Fisher & Garner, 2020). The zoospores infect the keratinized skin of amphibian larvae and adults, inducing malformation of mouthparts in larvae of sensitive species and inhibiting their feeding and growth, with high mortality at metamorphosis (Fig. 3B). In adults, osmoregulation is impaired, leading in some cases to cardiac arrests. The severity of infection depends on the environmental conditions and species vulnerability, ranging from immune species to several degrees of sensitivity. Some species are tolerant to infection and therefore they

become pathogen reservoirs, and others are highly sensitive and they suffer strong declines and sometimes extinction (Fisher et al., 2009; Lips, 2016; Scheele et al., 2019; Fisher & Garner, 2020). Another species, *B. salamandrivorans*, affects Palearctic urodeles, with its zoospores infecting urodele epidermal cells, causing skin ulceration and mortality in almost all infected individuals of sensitive species and leading to severe declines of those urodeles (Fig. 3B; Martel et al., 2014; Stegen et al., 2017).

OBJECTIVES OF THE THESIS

This thesis intends to improve the knowledge on how non-random biodiversity loss in riparian plants and amphibians affects freshwater ecosystem functioning and structure, using several realistic scenarios of species loss in field and laboratory studies. Specifically, it examines the bottom-up effects of non-random loss, reduction and/or replacement of riparian tree species on key processes and communities of forest headwater streams; and the top-down effects of non-random amphibian species loss and reduction on key processes and communities of montane streams and ponds. In order to asses these general objectives, we divided the thesis in six chapters that addressed several specific objectives. Chapter 1 examined effects of alder loss on leaf litter decomposition and aquatic hyphomycete and macroinvertebrate communities through a stream field experiment. Chapter 2 tested effects of reduction and loss of alder and oak on leaf litter decomposition, detritivore growth and the aquatic hyphomycete assemblage through a stream microcosm experiment. Chapter 3 explored effects of alder infection by *Phytophthora* × *alni* and its replacement by black locust (*Robinia pseudoacacia*) on leaf litter decomposition, fungal biomass and the aquatic hyphomycete assemblage through a stream microcosm experiment. Chapter 4 analysed effects of common midwife toad loss on periphyton biomass, macroinvertebrate growth and the algal community though a stream field experiment. Chapter 5 explored effects of loss and reduction of common midwife toad and marbled newt on periphyton biomass and the algal community through a pond mesocosm experiment. Finally, chapter 6 tested effects of fire salamander loss on periphyton biomass, leaf litter decomposition and the algal, aquatic hyphomycete and macroinvertebrate communities through a stream field experiment. All these chapters allowed a comprehensive understanding of how species loss due to fungal infections could alter the functioning and structure of freshwater ecosystems through changes in

several key ecosystem processes and the biological communities involved, therefore providing a basis for predicting alterations in these ecosystems and for improving their management and conservation.

Fig. 4. Study sites and experimental procedures used in Chapters 1 to 6 (A to F, respectively).

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Chapter 1

LOSS OF KEY RIPARIAN PLANT SPECIES IMPACTS STREAM ECOSYSTEM FUNCTIONING

Alonso, A., Pérez, J., Monroy, S., Lopez-Rojo, N., Basaguren, A., Bosch, J., & Boyero, L. (2021). Loss of key riparian plant species impacts stream ecosystem functioning. *Ecosystems*, 1-14. DOI: https://doi.org/10.1007/s10021-020-00592-7

ABSTRACT

Leaf litter of alder (*Alnus glutinosa*) is a key resource to detrital stream food webs. Due to its high quality and palatability, it is readily colonized by microorganisms and consumed by detritivores, contributing significantly to carbon and nutrient cycling and to ecosystem functioning. Given that this species has declined due to the spread of the pathogen *Phytophthora alni*, we investigated how its loss would alter leaf litter decomposition and associated streams assemblages of aquatic hyphomycetes and invertebrates, in a field experiment conducted in 3 streams. We compared litter mixtures containing alder plus 3 other species (*Corylus avellana*, *Quercus robur* and *Salix atrocinerea*; i.e., 4-species treatments) with mixtures that excluded alder (3-species treatments) and all the monocultures (1-species treatments). The loss of alder reduced decomposition rates, despite the existence of an overall negative diversity effect after 3 weeks of exposure (i.e., monocultures decomposed faster than mixtures) and no diversity effect after 6 weeks. Aquatic hyphomycete and detritivore assemblage structure in the mixture without alder differed from those of the mixture with alder and the monocultures, and the former had lower fungal sporulation rate and taxon richness. Our results suggest that alder loss from the riparian vegetation can significantly slow down the processing of organic matter in streams and produce shifts in stream assemblages, with potential consequences on overall ecosystem functioning. We highlight the importance of assessing the ecological consequences of losing single species, particularly those especially vulnerable to stressors, to complement the multiple studies that have assessed the effects of random species loss.

INTRODUCTION

Biodiversity loss as a result of anthropogenic impact is known to be a major driver of change in the functioning of ecosystems (Hooper et al. 2012). However, most available knowledge has emerged from experiments simulating random species loss (Gross and Cardinale 2005; Wardle 2016), with fewer studies addressing more realistic scenarios of species extinction as a result of particular stressors or their combination (Kominoski et al. 2013). Among these, empirical studies have relied on information about the vulnerability of different species to a given stressor, finding that the consequences of ordered species loss can differ from those of random loss (García-Valdés et al. 2018; Larsen et al. 2005), an outcome supported by mathematical models (Gross and Cardinale 2005; Ives and Cardinale 2004). The relative scarcity of studies considering ordered extinctions could be

due to lack of knowledge on species sensitivity to different stressors, as this requires information about their biological traits that is often unavailable. However, this may not be the case for some stressors such as the spread of pathogens, which usually affect particular taxa and thus facilitate the prediction of species loss scenarios (Harvell et al. 2002).

Plant diseases caused by fungal pathogens are poorly known beyond the context of agriculture (Almeida et al. 2019), but they are widespread and cause declines in species abundance and richness that can alter the functioning of terrestrial and aquatic ecosystems (Bjelke et al. 2016). Streams are among the ecosystems most severely affected by plant diversity loss (Kominoski et al. 2013), as they often rely on the allochthonous organic matter inputs from the riparian forest for their functioning, given that primary production is largely limited by riparian shading and low nutrient availability (Fisher and Likens 1973; Wallace et al. 1997). Leaf litter accounts for a high proportion of plant biomass entering streams, with some species providing litter resources that are particularly nutrient-rich and palatable for consumers, such as alder (i.e., Alnus; Graça et al. 2015). Alder species are nitrogen (N)-fixing trees that produce leaves with high concentration of N and low concentration of refractory carbon (C) compounds (Waring and Running 2010). The loss of key species such as alder could be expected to cause notable impact on the functioning of streams, but this remains unexplored, despite its likelihood as a result of the spread of the pathogen *Phytophthora alni* across Europe (Bjelke et al. 2016; Brasier et al. 2004).

Phytophthora alni is a species complex of oomycetes composed by *Phytophthora* × *alni* (Brasier & S.A. Kirk) Husson, Ioos & Marçais, *Phytophthora uniformis* (Brasier & S.A. Kirk) Husson, Ioos & Aguayo, and *Phytophthora* × *multiformis* (Brasier & S.A. Kirk) Husson, Ioos & P. Frey (Husson et al. 2015). It induces alder dieback, whose main symptom is a decrease in tree vitality until its death (Bjelke et al. 2016; Jung and Blaschke 2004). The *P. alni* dieback can affect nearly 50% of alder trees in some European regions (Bjelke et al. 2016) and, in the Iberian peninsula, *Alnus glutinosa* (L.) Gaertn. is infected by the 3 species of the *P. alni* complex. To date it has been reported in the northwest of the peninsula (Pintos-Varela et al. 2017; Solla et al. 2010), but it is expected to expand its distribution area due to the rise in temperature caused by climate change (Thoirain et al. 2007). This is especially true for *P.* × *alni,* which is the most pathogenic and frost-sensitive species (Bjelke et al. 2016). The expansion of *P. alni* will most likely affect *A. glutinosa* populations in Atlantic areas, where this riparian species is dominant and provides a major resource for stream assemblages (Douda et al. 2016).

Here, we assess how the loss of *A. glutinosa* (hereafter alder) from the riparian vegetation affects a fundamental stream ecosystem process, litter decomposition, as well as the composition and structure of associated macroinvertebrate and microbial assemblages, through a field experiment conducted in 3 streams in northern Spain. We incubated litter mixtures with and without alder for 3- and 6-week periods, as well as monocultures (i.e., single-species treatments) that allowed exploring the mechanisms underlying any diversity effects. For this purpose, we studied the net diversity effect (i.e., the deviation between the observed value in the mixture and that expected from the single-species treatments), which we partitioned into complementarity and selection effects. The complementarity effect arises from synergistic or antagonistic interactions among species, which can facilitate (positive complementarity) or inhibit (negative complementarity) the consumption of the others, for example through the leaching of nutrients, tannins or refractory compounds (López-Rojo et al. 2020; Loreau and Hector 2001). The selection effect is caused by the presence of a species with particularly high or low decomposition rate, which can increase (positive selection) or reduce (negative selection) the overall consumption of the mixture due to its strong influence (Handa et al. 2014; Loreau and Hector 2001). While both effects play a role in diversity-decomposition relationships, complementarity is often dominant when random species loss in litter mixtures is assessed (Handa et al. 2014; Tonin et al. 2017), but selection could be expected to gain importance when dealing with the loss of key species since they are preferred by detritivores and microbial decomposers over other plant species, and in consequence, they decompose faster (López-Rojo et al. 2018).

Given alder declines due to the pathogen *P. alni*, we hypothesised that (i) the loss of alder would reduce the decomposition of litter mixtures; (ii) the selection effect would be the main mechanism for reduced decomposition, because of the strong preference of detritivores and microbial decomposers for this species (Graça et al. 2001; Gulis 2001; López-Rojo et al. 2018); and (iii) the loss of alder would reduce the diversity and abundance of microbial decomposers (aquatic hyphomycetes) and invertebrate detritivores, thus altering the composition and structure of both assemblages.

MATERIALS AND METHODS

Study area

The study was conducted in 3 low-order streams of the Agüera stream catchment in northern Spain (43.20º N, 3.26º W), from January to March 2020. The climate is temperate oceanic with an annual mean temperature of 11º C and annual mean precipitation of 1650 mm regularly distributed (López-Rojo et al. 2019). Streams mostly have siliceous substrate and drain mixed Atlantic forests composed by *Quercus robur* L. (Fagaceae; hereafter oak), *A. glutinosa* L. (Betulaceae), *Castanea sativa* L. (Fagaceae), *Corylus avellana* L. (Betulaceae; hereafter hazel) and *Salix atrocinerea* BROT. (Salicaceae; hereafter willow). The studied streams had well-oxygenated water, circumneutral pH, low conductivity, low nutrient concentrations and temperatures of approximately 7.5 °C at the time of the study (Table 1). Anthropogenic activity in the study area is negligible.

Table 1. Physicochemical characteristics of the selected streams during the study period (mean ± SE; n=6). DIN = dissolved inorganic nitrogen, SRP = soluble reactive phosphorus.

Variable	Stream 1	Stream 2	Stream 3
Latitude (º N)	43.208	43.207	43.213
Longitude (º E)	3.267	3.263	3.271
Altitude (m a.s.l.)	325	350	305
Temperature (ºC)	7.57 ± 0.54	7.80 ± 0.53	7.65 ± 0.54
Conductivity (µS cm^{-1})	143.70 ± 5.70	105.87 ± 2.06	95.90 ± 3.46
pH	7.61 ± 0.06	7.72 ± 0.07	7.40 ± 0.15
Dissolved O_2 (mg l^{-1})	11.83 ± 0.21	11.69 ± 0.21	11.69 ± 0.20
% Saturation O_2	101.45 ± 0.30	100.75 ± 0.40	100.33 ± 0.50
Flow (l s^{-1})	27.85 ± 9.89	20.43 ± 6.75	26.33 ± 10.09
DIN (µg l^{-1})	528.86 ± 46.37	613.58 ± 59.99	289.97 ± 34.39
SRP (µg l^{-1})	3.48 ± 1.01	5.60 ± 1.62	8.51 ± 2.03

Stream water characterization

Water temperature, conductivity, pH, oxygen concentration and saturation, discharge, dissolved inorganic N (DIN = nitrate + nitrite + ammonium) and soluble reactive phosphorus (SRP) were measured 6 times at each stream during the study period. Temperature, conductivity, pH and oxygen were determined with a multiparametric probe (WTW Multi 3630 IDS; WTW, Weilheim, Germany) and discharge was estimated from water velocity obtained with a current meter (MiniAir 20; Schiltknecht Co, Gossau, Switzerland). Nutrients were determined from water samples that were filtered (glass fibre

filters, Whatman GF/F; pore size: 0.7 μm) and frozen until analysis by capillary ion electrophoresis (nitrate; Agilent CE, Agilent Technologies, Waldbronn, Germany), the sulphanilamide method (nitrite), the salicylate method (ammonium) and the molybdate method (SRP; APHA 1998).

Decomposition experiment

In autumn 2019, leaves of alder, hazel, oak and willow were collected from the forest floor immediately after their natural abscission. These species were chosen in order to represent litter inputs in forested streams in the study region, and differed in several leaf traits (Table S1). Leaves of alder, hazel and oak were collected along the Agüera catchment, whereas willow leaves were collected next to the University of the Basque Country (43.32º N, 2.97º W) due to its more gradual abscission and hence lower availability at the study site. Leaves were air-dried in the laboratory, weighed and enclosed in coarse-mesh litterbags (20×25 cm, 5-mm mesh). Each of these litterbags received 4.0 ± 0.2 g of litter in total, comprising 1, 3 or 4 species depending on the treatment. There were 6 treatments: one litter mixture with all species (i.e., including alder; 1 ± 0.05 g per species), one mixture containing hazel, oak and willow (i.e., excluding alder; 1.33 ± 0.07 g per species), and the 4 monocultures (alder, hazel, oak or willow). We also used fine-mesh litterbags (12×15 cm, 0.5-mm mesh) containing the monocultures (1 ± 0.05 g) to examine differences in microbial decomposition among plant species. We used less litter mass in fine-mesh than in coarse-mesh litterbags to facilitate the observation of differences across treatments (because of the lower pace of microbial decomposition), as done elsewhere (e.g., Kreutzweiser 2008; Schindler and Gessner 2009).

Sixty coarse-mesh and 40 fine-mesh litterbags were deployed in each stream, with 10 replicates per treatment. Deployment (and subsequent collection) was done on 2 separate days in order to be able to handle all sporulation analyses upon collection (see below). One replicate per treatment was tied to one of 10 iron bars, which were anchored at random locations within riffle sections of the streambed. An extra set of 20 fine-mesh litterbags (5 per species, containing 2 ± 0.1 g of litter) was incubated for 92 h in one of the streams. Half of this litter was used to estimate the initial leaching of soluble compounds and calculate air-dry mass (DM) to oven DM (72 h at 70 °C) and to ash-free DM (AFDM; 4 h at 500 °C) conversion factors. The other half was used to measure leaf toughness (in fresh), specific leaf area (SLA), N and phosphorus (P) concentrations, which were used as surrogates for litter quality. The proportion of mass loss due to leaching was calculated as the difference

between AFDM of non-incubated and incubated litter divided by AFDM of non-incubated litter. Leaf toughness was determined using a penetrometer with a 1.55-mm diameter steel rod (Boyero et al. 2011), measured in 5 fragments per sample (fresh litter, after leaching). SLA was calculated as leaf area (mm^2) divided by dry mass (mg) measured in five 12-mm diameter leaf discs per sample. N concentration was measured with a Perkin Elmer series II CHNS/O elemental analyser (Perkin Elmer, Norwalk, Connecticut, USA). P concentration was measured spectrophotometrically after autoclave-assisted extraction (APHA, 1998).

Half of the litterbags were collected after 21 d of instream incubation, enclosed individually in zip-lock bags and transported in a refrigerated cooler to the laboratory. The remaining litterbags were retrieved 42 d after their deployment and processed the same way. Litter material from each litterbag was rinsed with filtered (100 µm) stream water on a 500-µm sieve to remove sediment and associated macroinvertebrates. Five 12-mm diameter leaf discs were obtained from each species in each replicate coarse-mesh litterbag collected at day 21 (randomly selected among litter fragments, with no more than one disc per fragment) to induce fungal sporulation (see below); the remaining material was oven-dried (70 °C, 72 h), weighed to determine final DM, incinerated (500 °C, 4 h) and weighed to determine final AFDM.

Aquatic hyphomycetes and macroinvertebrates

To measure fungal sporulation rate, litter discs were placed in Erlenmeyer flasks filled with 25 mL of filtered stream water (glass fibre filters, Whatman GF/F; pore size: 0.7 µm). Flasks were incubated for 48 ± 2 h on a shaker at 80 rpm and 10º C. Conidial suspensions were poured into 50-mL Falcon tubes, pre-stained with 2 drops of 0.05% trypan blue in 60% lactic acid, preserved with 2 mL of 35% formalin and adjusted to 40 mL with distilled water. In order to identify and count conidia, 150 µL of Triton X-100 (0.5%) were added to each sample and mixed with a magnetic stirring bar to ensure a uniform distribution of conidia. Subsequently, an aliquot of 10 mL of the conidial suspension was filtered (25-mm diameter, pore size 5 µm, Millipore SMWP, Millipore Corporation) with gentle vacuum. Finally, filters were stained with trypan blue and spores were identified and counted at 200× magnification (Gulis et al. 2005). Sporulation rate (number of conidia g litter DM^{-1} d^{-1}) and taxon richness were calculated for each replicate.

Macroinvertebrates collected from coarse-mesh litterbags retrieved at day 21 were preserved in 70% ethanol for subsequent identification. They were identified to the lowest

taxonomic level possible (typically genus) and classified into litter-consuming detritivores and other macroinvertebrates using Tachet et al. (2010). For each sample, abundance and taxon richness (number of individuals and taxa per litterbag, respectively) were calculated for detritivores and all macroinvertebrates.

Data analysis

We quantified total decomposition (in litter mixtures and monocultures) and microbial decomposition (in monocultures only) through proportional litter mass loss [LML = (initial AFDM – final AFDM) / initial AFDM]. Initial AFDM was previously corrected for mass loss due to leaching (using the extra fine-mesh litterbags), which is a common procedure that allows removing differences among species in terms of rapid mass loss as a consequence of litter drying (Bärlocher 2020). We examined differences in decomposition between mixtures with and without alder (for total decomposition) and among plant species (alder, hazel, oak and willow; for total and microbial decomposition) using linear mixed-effect models (*lm* function in the "nlme" R package; Pinheiro et al. 2009). Litter mixture or plant species was a as fixed factor in the model, and stream was a random factor (Zuur et al. 2009). When significant differences were found ($\alpha = 0.05$; significance level used for all tests), Tukey tests were used to identify differences among plant species. We first ran an analysis that included sampling date (3 and 6 weeks) as fixed factor and its interaction with mixture or species. As the interaction was significant, (see Results), we then ran separate analyses for both sampling dates to reduce model complexity.

Net diversity, complementarity and selection effects on LML were calculated in mixtures with and without alder following Loreau & Hector (2001). The net diversity effect was calculated as the difference between observed and expected decomposition, the latter based on decomposition of monocultures and the proportion of each of them in the mixture: Net diversity effect = $\Sigma i\ (Value_{Obs} - Value_{Exp})$ = Complementarity effect + Selection effect. The complementarity effect was calculated as the average deviation from the expected decomposition in a mixture, multiplied by the number of species in the mixture and the mean decomposition in monocultures: Complementarity effect = mean $(Value_{Mixture} - Value_{Monoculture}) \times$ mean $Value_{Monoculture} \times n$. The selection effect was calculated as the covariance between decomposition of species in monoculture and the average deviation from expected decomposition of species in the mixture, multiplied by the number of species in the mixture: Selection effect = cov [(ValueMixture – ValueMonoculture), ValueMonoculture] × n. Differences between the mixtures were examined with linear

mixed-effect models using stream as random effect, followed by Tukey tests when significant differences were found, again separately for each sampling date.

We examined differences in fungal sporulation rate and taxon richness separately for each species, depending on whether it was incubated in the mixture with alder (4 species), the mixture without alder (3 species) or in monoculture (1 species). We used a linear mixed-effect model, with litter diversity (4, 3 or 1 species) and plant species identity as fixed factors, and stream as random factor, followed by Tukey tests when significant differences were found (Zar 1999). Fungal sporulation rate was previously log-transformed to meet the requirements of parametric analyses. The net diversity effect on aquatic hyphomycete variables (i.e. sporulation rate and taxon richness) was calculated as the difference between the weighted mean of the observed values of species in each mixture and the expected value based on the respective monocultures, and were tested with linear mixed-effect models, with net diversity effect as fixed factor and stream as random factor. Fungal assemblage structure was analysed with non-metric dimensional scaling (NMDS) based on the Bray Curtis similarity index using conidial abundance data (*metaMDS* function of the "vegan" R package), followed by permutational multivariate analysis of variance (*adonis* function of the "vegan" R package) to test if the assemblage varied depending on plant species (alder, hazel, oak and willow) and on whether they were in mixtures with alder, in mixtures without alder or in monocultures. The most representative taxa of each assemblage were determined using an indicator value index (*multipatt* function of the "indicspecies" R package; De Cáceres 2013).

Differences of macroinvertebrate abundance and richness between mixtures with and without alder and among plant species (alder, hazel, oak and willow) were examined using linear mixed-effect models, with plant species as fixed factor and stream as random factor, followed by Tukey tests to identify the differences among plant species when significant differences were found. We calculated the net diversity effect of macroinvertebrate abundance and richness as the difference between the observed value in each mixture and the expected value based on the respective monocultures. Differences in net diversity between mixtures with and without alder were analysed with linear mixed-effect models, with net diversity effect as fixed factor and stream as random factor. We used NMDS, permutational multivariate analysis of variance and indicator value index (as above) to examine differences among treatments and indicator taxa of macroinvertebrate

assemblage structure. These analyses were performed for detritivores and all macroinvertebrates.

RESULTS

Litter decomposition

LML was significantly affected by litter mixture, but the effect depended on sampling date (significant interaction between litter mixture and sampling date, $F = 13.816$, $p < 0.001$). Therefore, analyses were done separately for each sampling date. Litter mixtures with alder decomposed faster than those without alder at both sampling dates, and decomposition varied among plant species, being lowest for oak, highest for alder and with intermediate mass loss for hazel and willow, again at both sampling dates (Table 2, Figure 1). Microbial decomposition also differed among plant species at both dates, being higher for alder and hazel than for willow and oak (Table 2, Figure 1). We observed differences in diversity effects between sampling dates. At day 21, both mixtures presented negative net and complementarity effects and a positive selection effect, with no differences between mixtures (Table 2, Figure 2). At day 42, the mixture with alder showed positive complementarity and negative selection effects, resulting in the absence of a net diversity effect, whereas the mixture without alder presented negative net, complementarity and selection effects. Net and complementarity effects differed between mixtures, but there were no differences in the selection effect (Table 2, Figure 2).

Aquatic hyphomycetes

Sporulation rate varied with plant species, with higher values for alder and willow, intermediate for hazel and lower for oak and, for each species, it was higher in the monoculture and the mixture with alder than in the mixture without alder (Table 3, Figure 3A). Taxon richness also differed among species, being lower in oak than in the other species, and was again higher in the monoculture and the mixture with alder than in the mixture without alder (Table 3, Figure 3C). The net diversity effect for sporulation rate (Figure 3C) and taxon richness (Figure 3D) was higher in the presence of alder, although differences were only significant for taxon richness.

Aquatic hyphomycete assemblage structure was influenced by plant species, with differences between all species pairs except alder and willow, and mixtures with alder and monocultures were similar but differed from mixtures without alder (Table 3, Table S2).

Mixtures with alder were associated with *Alatospora acuminata* INGOLD, *Alatospora pulchella* MARVANOVÁ, *Lunulospora curvula* INGOLD and *Tetrachaetum elegans* INGOLD, and *Tetracladium marchalianum* DE WILD., which was also characteristic of monocultures. Assemblages found in alder and willow were characterized by *Articulospora tetracladia* INGOLD, *T. elegans*, *Flagellospora curvula* INGOLD, *Heliscus lugdunensis* SACC. & THERRY, *Anguillospora filiformis* GREATH., *A. pulchella* and *A. acuminata*; *L. curvula* was also important in alder. There were no indicator taxa were specific of hazel, which shared *A. filiformis*, *A. pulchella* and *A. acuminata* with alder and willow. Assemblages in oak were characterized by *Taeniospora gracilis* MARVANOVÁ (Table S3).

Table 2. Results of linear models testing the effects of mixture (with and without alder) and plant species (alder, hazel, oak and willow) on decomposition (total and microbial) and net, complementarity and selection effects in each sampling date (21 and 42 d); num df= degrees of freedom of numerator, den df= degrees of freedom of denominator, F= F-statistic; p= p-value.

Sampling date	Response variable	Factor	num df	den df	F	p
21 d	Total decomposition	Mixture	1	26	16.140	<0.001
	Total decomposition	Species	3	51	79.259	<0.001
	Microbial decomposition	Species	3	51	12.438	<0.001
	Net diversity effect	Mixture	1	26	0.490	0.490
	Selection effect	Mixture	1	26	0.410	0.528
	Complementarity effect	Mixture	1	26	1.014	0.323
42 d	Total decomposition	Mixture	1	26	56.730	<0.001
	Total decomposition	Species	3	52	58.973	<0.001
	Microbial decomposition	Species	3	54	26.373	<0.001
	Net diversity effect	Mixture	1	26	11.959	0.002
	Selection effect	Mixture	3	26	2.821	0.105
	Complementarity effect	Mixture	1	36	8.046	0.009

Macroinvertebrates

Detritivore abundance did not differ between mixtures but varied with plant species, being higher in hazel than in oak and willow (Table 4, Figure 4A). Detritivore richness did not vary between mixtures or plant species (Table 4, Figure 7C). The net diversity effect did not differ between mixtures for abundance and richness (Figure 7). Detritivore assemblage structure varied among treatments (Table 4); the mixture with alder differed from that without alder, hazel differed from oak, willow and the mixture without alder, and oak differed from the mixture with alder (Table S4). The assemblage in hazel was characterized

by Leuctridae (Plecoptera), and *Amphinemura* (Plecoptera) was characteristic of alder, hazel and the mixture with alder, both taxa being (litter-consuming) detritivores (Table S5).

For all macroinvertebrates, abundance varied with plant species, being higher in hazel than in alder, oak and willow, but not between mixtures (Table 4, Figure 4A). There were no differences for taxon richness (Table 4, Figure 4C) and net diversity effects (Figure 4). Assemblage structure varied with treatment (Table 4), with differences between alder and all others; between hazel and the other monocultures; and between the mixture with alder and alder and oak monocultures (Table S4). The indicative species were the same as in detritivore assemblages, with the addition of the collector-gatherer *Habroleptoides* (Ephemeroptera), which was important in all assemblages except those of alder (Table S5).

DISCUSSION

Effects of plant litter diversity loss on decomposition have been widely studied in both terrestrial and aquatic ecosystems, but most experiments have focused on random species losses and have rendered contradictory evidence (i.e., positive, negative or no effects). Thus, even if some studies have revealed general patterns and drivers of litter mixing effects on decomposition (e.g., Liu et al. 2020), the fact that random species loss is unrealistic (Wardle 2016) has been seldom taken into account (Berg et al. 2015). Here, we simulated the loss of alder trees as a result of infection by *P. alni* (Bjelke et al. 2016) and thus examined a realistic extinction scenario and its consequences on litter decomposition in streams and the associated assemblages.

The presence of alder increased decomposition despite an overall negative diversity effect

Our experiment, conducted in 3 streams, showed that the presence or absence of alder strongly influenced stream ecosystem functioning. As predicted, litter decomposition was increased in its presence, with mixtures containing alder decomposing faster than mixtures without alder, regardless of incubation time (i.e., 3 or 6 weeks). Importantly, this difference could not be explained by the increase in diversity *per se* in mixtures (from 3 to 4 species), because the examination of monocultures revealed a negative net diversity effect in most cases. Thus, the most likely explanation for the effect of alder was related to its particular characteristics, as discussed below.

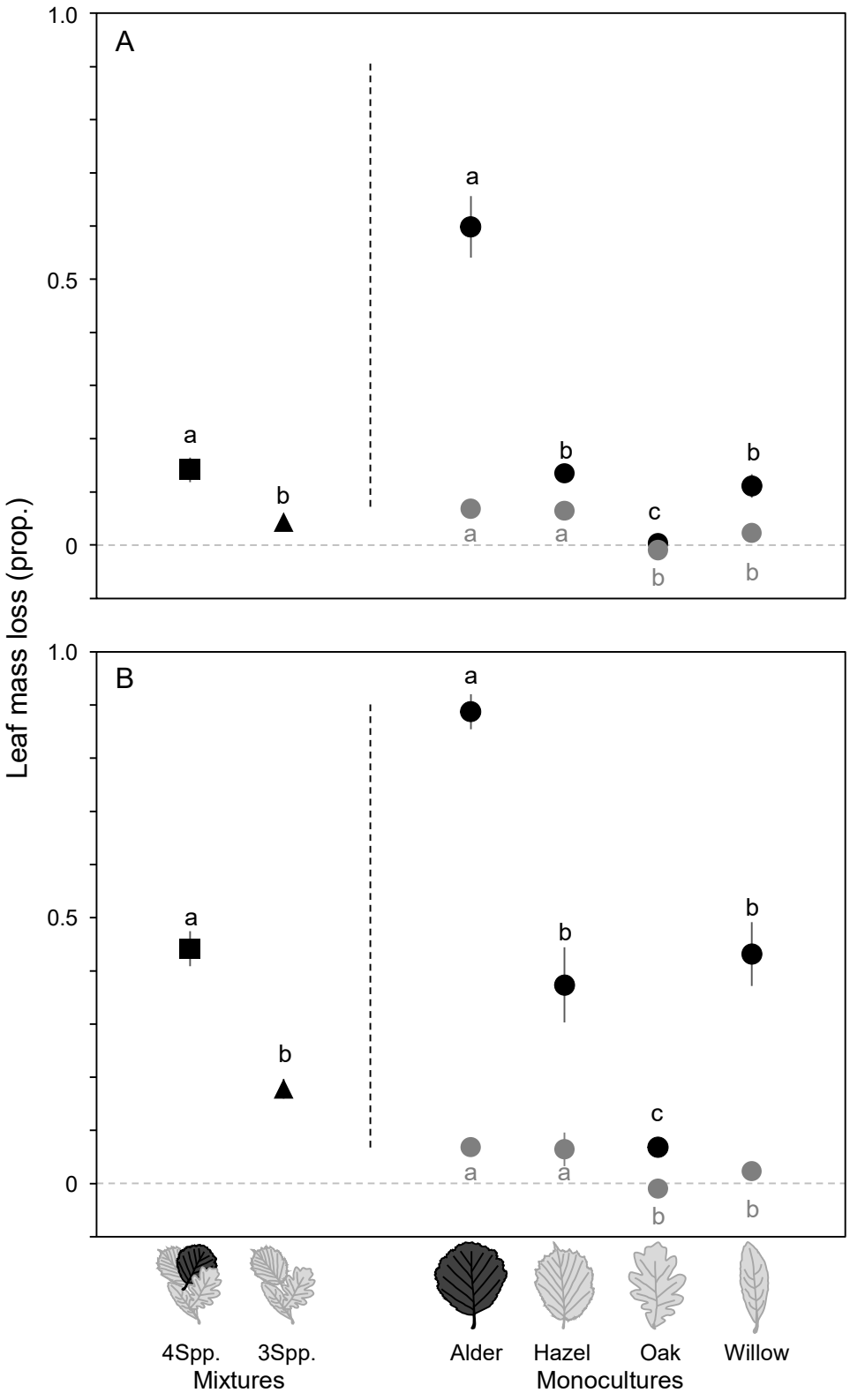

Figure 1. Total (black) and microbial (grey) decomposition (proportion of litter mass loss) at 21 (A) and 42 days (B). Symbols are means (squares represent the mixture with alder, triangles represent the mixture without alder, and circles represent monocultures) and whiskers are standard errors. Different letters indicate significant differences.

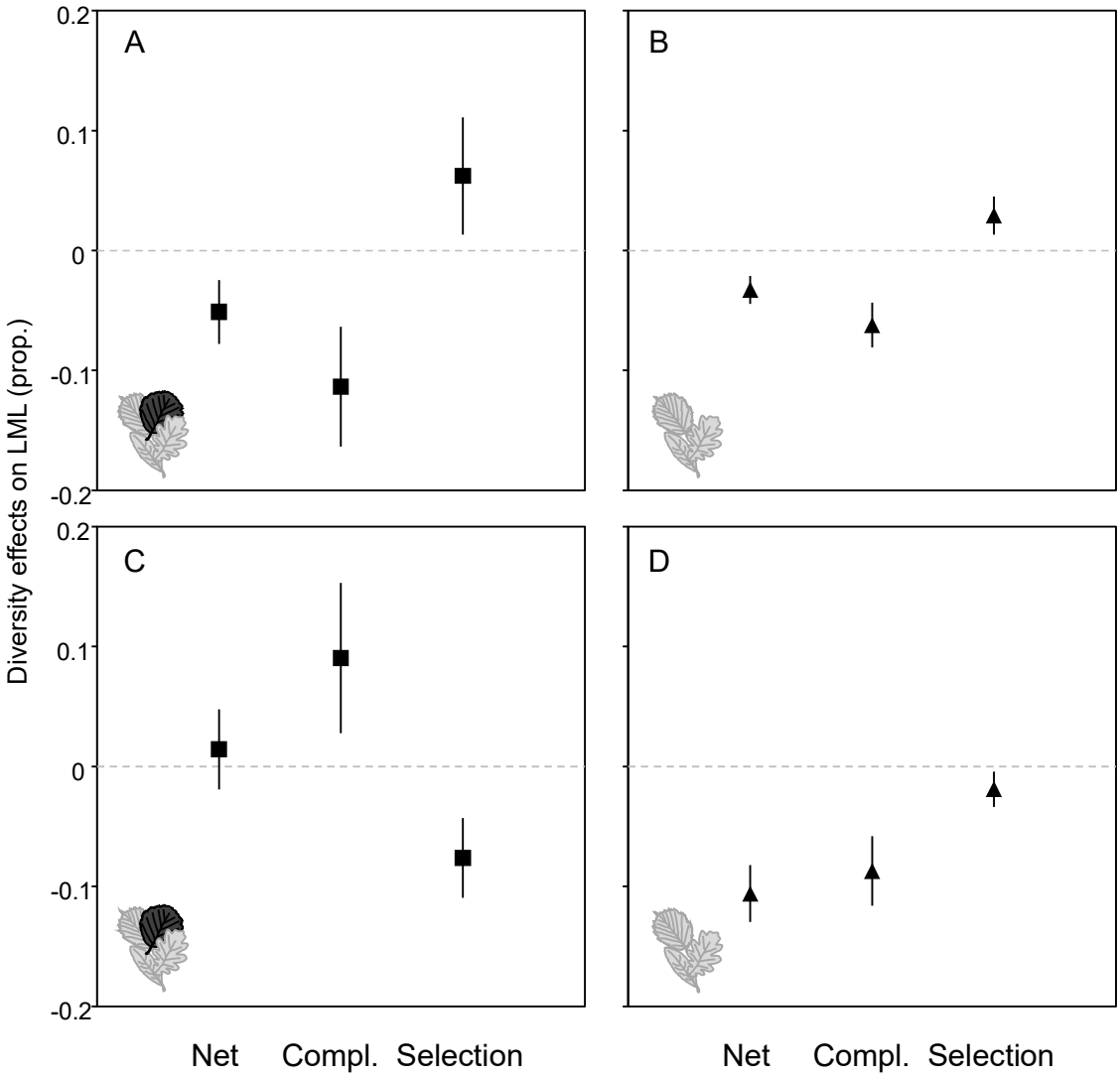

Figure 2. Net diversity, complementarity and selection effects on decomposition (measured as proportion of litter mass loss) at 21 days in the mixture with alder (A) and in the mixture without alder (B), and at 42 days in the mixture with alder (C) and in the mixture without alder (D). Symbols are means (squares represent the mixture with alder and triangles represent the mixture without alder) and whiskers are standard errors.

The negative net diversity effect was mainly explained by a negative complementarity effect, which consisted in a reduction in decomposition due to the interaction among the plant species in the mixture, and possibly indicated the existence of physical or chemical interference among traits of different plant species. For example, the presence of tannins or other inhibitory compounds in one species may limit fungal colonization and/or detritivore consumption of higher-quality species (i.e. high nutrient content), thus reducing overall decomposition in the mixture (Graça et al. 2001; López-Rojo et al. 2020; McArthur et al. 1994). Although we did not measure condensed tannins in litter from this experiment, a previous study found highly variable concentrations in our studied species (17.4% in Salix, 11.4% in Quercus 2.3% in Corylus and 1.7% in Alnus;

L.B., unpubl. data from Boyero et al. 2017). In contrast, the positive selection effect found at week 3 indicated a preference of consumers towards alder (or hazel in the mixture without alder). Preference for alder over other species has been described elsewhere for both microbial decomposers and detritivores and it has been attributed to its high concentrations of nutrients (N and P) and low concentration of secondary compounds, which increase its decomposability and palatability (Friberg and Jacobsen 1994; Graça et al. 2001; Gulis 2001; López-Rojo et al. 2018). The positive selection effect was nevertheless smaller than the negative complementarity effect, and hence not of sufficient magnitude to increase decomposition in the mixture compared to the average monoculture.

Table 3. Results of linear models testing the effects of mixture (monoculture, mixture with and without alder) and plant species (alder, hazel, oak and willow) on aquatic hyphomycete sporulation rate (conidia g-1 DM d-1) and taxon richness, and results of PERMANOVA testing the effects of mixture, plant species and the interaction between them and with stream, on aquatic hyphomycete assemblage structure; df= degrees of freedom; F= F-statistic; R2= adjusted R squared; p= p-value.

Variable	Factor	df	R^2	F	p
Sporulation rate	Species	3, 156		85.068	<0.001
	Mixture	2, 156		20.143	<0.001
Taxon richness	Species	3, 156		15.370	<0.001
	Mixture	2, 156		9.680	<0.001
Community structure	Mixture	2	0.039	4.612	<0.001
	Species	3	0.273	21.319	<0.001
	Mixture: Species	5	0.024	1.115	0.312
	Mixture:Species(Stream)	22	0.103	1.097	0.262

At week 6, the complementarity effect remained negative in the absence of alder but became positive in its presence, with a negative selection effect in both cases. The increase in complementarity effect through time has been observed elsewhere (Cardinale et al. 2007) and could be due to an increase in the palatability of low-quality litter (i.e., litter with low nutrient contents) caused by nutrient transfer from high-quality litter (alder in our study), possibly through fungal hyphae (Handa et al. 2014). The change in the selection effect from positive to negative could be related to the depletion of high-quality litter through time, causing low-quality litter to dominate the mixture, which could make decomposers avoid it (Larrañaga et al. 2020; Loreau and Hector 2001). However, litter traits and detritivore preferences can vary through time (Compson et al. 2018), so this issue merit further attention.

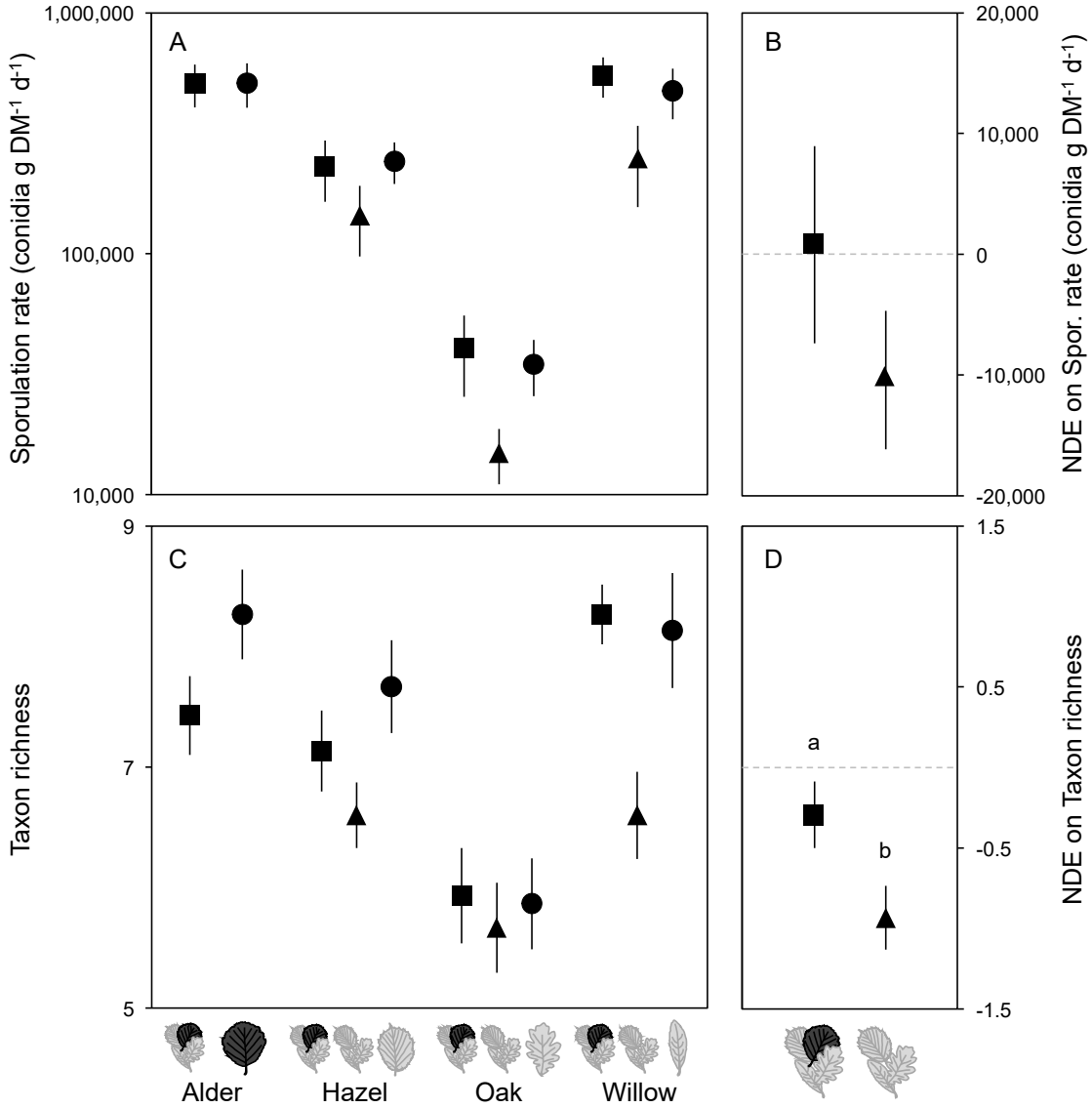

Figure 3. Aquatic hyphomycete sporulation rate (conidia g DM^{-1} d^{-1}; A), net diversity effect on sporulation rate (B), taxon richness (C), and net diversity effect on taxon richness (D), on alder, hazel, oak and willow on mixtures with alder (squares), mixtures without alder (triangles) and monocultures (circles) at 21 days. Symbols are means and whiskers are standard errors.

Aquatic hyphomycete assemblages varied with litter identity and mixture composition

Fungal sporulation rate and taxon richness varied mainly depending on litter identity, in agreement with many other studies (Fernandes et al. 2012; Ferreira et al. 2012; Jabiol and Chauvet 2012; Kominoski et al. 2007). A positive relationship between fungal richness and litter diversity has been reported elsewhere (Laitung and Chauvet 2005), but this was not the case in our experiment. We found the highest sporulation rates and taxon richness (although the latter did not differ statistically from hazel) in alder and willow, and the lowest in oak, which matches the results of other studies (Cornejo et al. 2020; Ferreira et

al. 2012; Gessner and Chauvet 1994; Gulis 2001). This is most likely related to differences in concentrations of nutrients (high in alder and low in oak), which favour the activity of aquatic hyphomycetes, and those of lignin and phenolic compounds (low in alder and high in oak), which inhibit it (Ferreira et al. 2012; McArthur et al. 1994). Willow litter also has high nutrient concentration, similar to alder and other Betulaceae (Gulis 2001; Webster and Benfield 1986), although this species has been seldom used in decomposition stream experiments.

Table 4. Results of linear models testing the effects of mixture (with and without alder) and plant species (alder, hazel, oak and willow) on abundance (ind. bag-1) and taxon richness, and results of PERMANOVA testing the effects of treatment (mixture with alder and mixture without alder, alder, hazel, oak, willow) and their interaction with stream, on detritivore assemblage structure and macroinvertebrate structure, of detritivores and total macroinvertebrates; df= degrees of freedom; F= F-statistic; R2= adjusted R2; p= p-value.

	Variable	Factor	num df	R^2	F	p
Detritivore	Abundance	Mixture	1, 26		1.208	0.282
		Species	3, 54		6.687	<0.001
	Taxon richness	Mixture	1, 26		1.366	0.253
		Species	3, 54		1.300	0.284
	Community structure	Treatment	5	0.113	2.346	<0.001
		Treatment: Stream	12	0.193	1.668	0.001
Macroinvertebrate	Abundance	Mixture	1, 26		0.931	0.344
		Species	3, 54		5.648	0.002
	Taxon richness	Mixture	1, 26		0.470	0.499
		Species	3, 54		1.922	0.137
	Community structure	Treatment	5	0.104	2.197	<0.001
		Treatment: Stream	12	0.215	1.890	<0.001

When fungal sporulation rate and taxon richness were examined for hazel, oak and willow in monocultures vs. mixtures we observed a negative diversity effect when alder was absent (i.e., lower values for the 3-species mixture than for the monoculture in all cases), but no effect when it was present (i.e., similar values in the 4-species mixture and the monocultures). Thus, alder seemed to compensate for any decrease in the fungal assemblage of a given species introduced by the addition of other species, which could possibly occur through increased competition (Jabiol and Chauvet 2012). Competition could be due to harder conditions caused by the presence of condensed tannins or other

inhibitory compounds (Ferreira et al. 2012; Kominoski et al. 2007), and would be indicated by the reduction in taxon richness and sporulation rate in the mixture without alder. Tannin concentrations were 8 and 5 times greater in *Salix* and *Quercus*, respectively, than in *Corylus*, and 10 and 7 times greater than in *Alnus* (L.B., unpubl. data from Boyero et al. 2017). All species of aquatic hyphomycetes decreased in abundance, but the reduction was greater in non-dominant taxa (*Alatospora pulchella, Alatospora acuminata* and *Tetrachaetum elegans*) than in dominant taxa (*Articulospora tetracladia* and *Anguillospora filiformis*). The inclusion of alder in the mixture could increase the nutrient content and hence reduce competition compared to the mixture without alder, allowing fungal assemblages similar to those of monocultures (Kominoski et al. 2007); this may not occur when N is readily available in the water and used by aquatic hyphomycetes (Tonin et al. 2017), but N concentration was low in our study streams (Table 1).

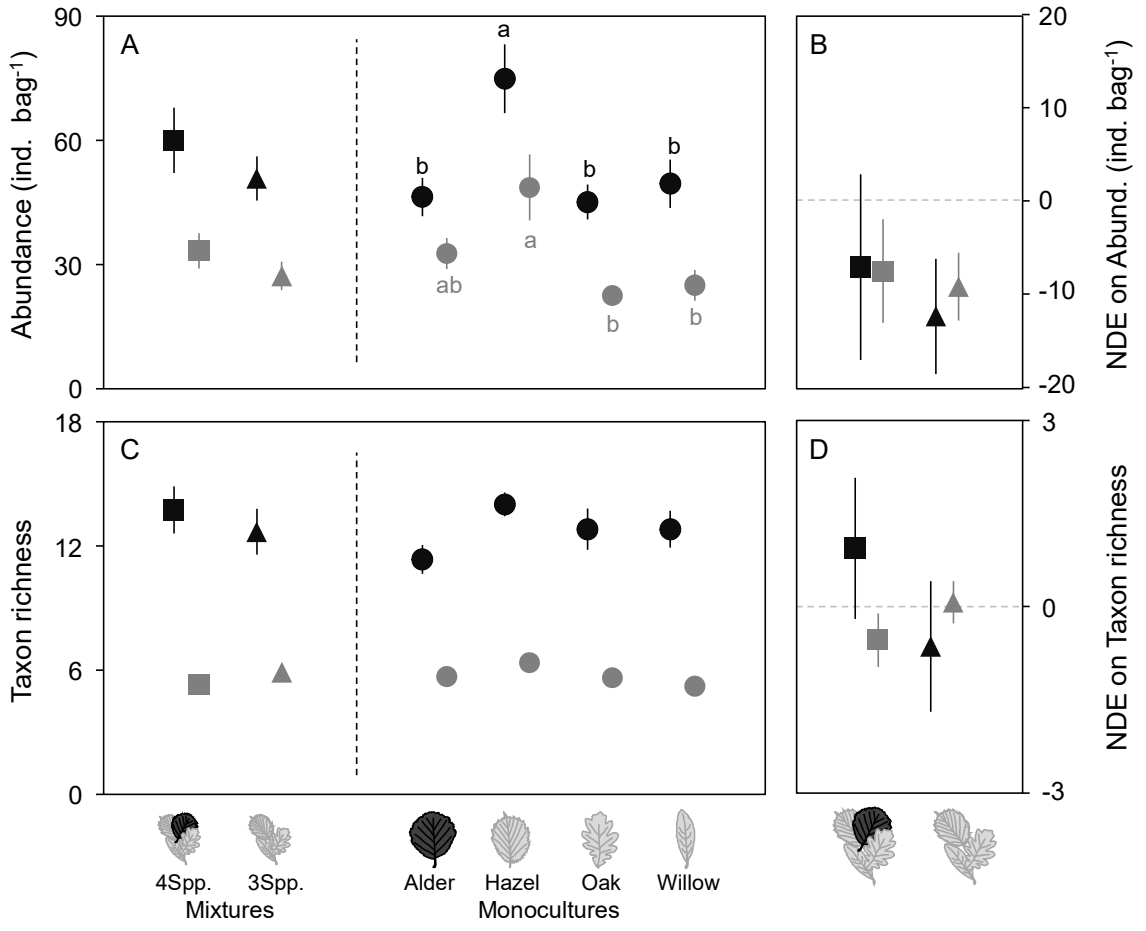

Figure 4. Macroinvertebrate abundance (individuals per bag; A), net diversity effect on abundance (B), taxon richness (C), and net diversity effect on taxon richness (D), for detritivores (grey) and all macroinvertebrates (black) at 21 days. Symbols are means (squares represent the mixture with alder, triangles represent the mixture without alder, and circles represent monocultures) and whiskers re standard error. Different letters indicate significant differences.

The presence of alder in mixtures affected detritivore assemblage structure

Detritivore and total macroinvertebrate abundances were highest in hazel, as observed elsewhere (Sanpera-Calbet et al. 2009). Although higher abundances could be expected in alder, the faster decomposition of this species resulted in large biomass reduction by the end of the experiment, which most likely caused the higher abundances in hazel compared to alder. Hazel litter did not have high nutrient content, but it did have high specific leaf area, thus offering greater surface availability and stability (Dobson 1994; Kominoski and Pringle 2009; Sanpera-Calbet et al. 2009). Hazel and alder shared detritivore indicative taxa (e.g., *Amphinemura*), which suggests that detritivores feeding on alder may shift to hazel when the former is no longer available. Macroinvertebrates that do not feed on leaf litter (e.g., *Habroleptoides*) were less common in alder than in other treatments, possibly because its lower usefulness as substrate due to its faster decomposition (Dobson 1994; Kominoski and Pringle 2009).

CONCLUSIONS

The overall loss of riparian plant diversity is known to have important consequences on stream ecosystem functioning by altering several key processes such us decomposition rates, nutrient dynamics, fine particulate organic matter production and invertebrate growth (López-Rojo et al. 2019). However, not all species are equally vulnerable to extinction, so it is important to establish the most likely scenarios of species loss and assess how losing these particular species will affect the ecosystem. We have shown how the loss of alder, which is caused by the expansion of *P. alni* across Europe (Bjelke et al. 2016), can inhibit litter decomposition and aquatic hyphomycete sporulation and modify stream assemblages, hence seriously altering stream ecosystem functioning and structure. Similar results have been obtained in studies testing the effects of the loss of North American ashes due to the invasive insect *Agrilus planipennis* FAIRMAIRE, where the absence of this species reduced decomposition and modified invertebrate community (Kreutzweiser et al. 2019). Therefore, we suggest that similar studies are conducted with other species such as oak, which is affected by *P. cinnamomi* RAND (Hernández-Lambraño et al. 2019), in order to improve our predictions about how stream ecosystems can be affected by species loss in the near future.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Physicochemical characteristics of the leaf litter of the different plant species used in the study (mean ± SE; n=5) and mixtures (mean weighted by post-leaching dry mass). % N = nitrogen concentration in percentage of dry mass, % P = phosphorus concentration in percentage of dry mass, SLA = specific leaf area. Different letters indicate significant differences among leaf traits.

Species	% N	% P	SLA (mm^2 mg^{-1})	Toughness (kPa)
Alder	2.80 ± 0.06^{a}	0.062 ± 0.002^{a}	18.38 ± 1.33^{b}	2138 ± 254^{b}
Hazel	1.45 ± 0.08^{bc}	0.038 ± 0.002^{c}	35.09 ± 1.61^{a}	1119 ± 128^{a}
Oak	1.39 ± 0.11^{c}	0.035 ± 0.002^{c}	15.37 ± 0.96^{bc}	2865 ± 228^{b}
Willow	1.72 ± 0.05^{b}	0.049 ± 0.002^{b}	13.77 ± 0.67^{c}	2172 ± 94^{b}
Mixture with alder	1.84 ± 0.04	0.046 ± 0.001	20.64 ± 0.64	2073 ± 50
Mixture without alder	1.52 ± 0.05	0.041 ± 0.000	21.39 ± 0.59	2051 ± 50

Table S2: Results of PERMANOVA pair comparisons testing the effects of mixture (monoculture, mixture with and without alder) and plant species (alder, hazel, oak and willow), on aquatic hyphomycete conidial assemblage structure; F= F-statistic; R^2= adjusted R squared; p= p-value.

Pair	F	R^2	p
Mixture with alder:Mixture without alder	5.305	0.049	0.002
Mixture with alder:Monoculture	0.216	0.002	0.984
Mixture without alder:Monoculture	4.555	0.042	0.001
Alder:Hazel	7.910	0.099	0.001
Alder:Oak	41.224	0.364	0.001
Alder:Willow	1.534	0.021	0.164
Hazel:Oak	27.186	0.236	0.001
Hazel:Willow	6.827	0.072	0.001
Oak:Willow	39.871	0.312	0.001

Table S3: Relative contribution (prop.) of each aquatic hyphomycete sporulating taxon to sporulation rate in each litter treatment × stream combination (6-9) for each species (alder, hazel, oak and willow). Mean sporulation rate and accumulated taxon richness are given at the bottom of the table.

		Alder				*A. glutinosa* (L.) GAERTN.	
Species							
Treatment							
Sporulating taxa – ID \ Stream		Stream 1		Stream 2		Stream 3	
Alatospora acuminata INGOLD	AAC	0.001	0.029	0.014	0.034	0.011	0.024
Alatospora pulchella MARVANOVÁ	APU	0.014	0.050	0.008	0.036	0.026	0.050
Anguillospora crassa INGOLD	ACR						
Anguillospora longissima INGOLD	ALO					0.003	0.002
Anguillospora filiformis GREATH.	AFI	0.199	0.315	0.139	0.265	0.394	0.339
Articulospora tetracladia INGOLD	ATE	0.469	0.273	0.446	0.523	0.344	0.377
Clavariopsis aquatica DE WILD	CLAQ			0.001	0.002	0.002	
Clavatospora longibrachiata *	CLO	0.004	0.007		0.002	0.001	0.002
Culicidospora aquatica R.H. PETERSEN	CUAQ		0.001				
Flagellospora curvula INGOLD	FCU	0.183	0.120	0.158	0.054	0.161	0.115
Heliscus lugdunensis SACC. & THERRY	HLU	0.022	0.013	0.027	0.010	0.013	0.030
Isthmotricladia britanica DESCALS	IBR		0.001				
Lambdosporum viridense NAWAWI	LVI		0.001				
Lemonniera terrestres TUBAKI	LTE				0.001	0.001	
Lunulospora curvula INGOLD	LCU	0.002	0.008	0.002	0.002	0.002	0.003
Margaritispora/Goniopila **	M/G						0.003
Taeniospora gracilis MARVANOVÁ	TGR						
Tetrachaetum elegans INGOLD	TEL	0.106	0.181	0.205	0.066	0.042	0.054
Tetracladium marchalianum DE WILD	TMA				0.003		0.001
Tricladium angulatum INGOLD	TAN				0.002	0.001	
Tripospermum camelopardus ***	TCA						
Tripospermum myrti (LIND) S. HUGHES	TMY						
Mean sporulation rate ± SE (Conidia g DM^{-1} d^{-1})×10^4		**57.8 ± 23.2**	**53.7 ± 8.6**	**47.0 ± 16.1**	**73.1 ± 21.8**	**80.1 ± 17.2**	**60.9 ± 18.9**
Taxon richness		**9**	**12**	**9**	**13**	**13**	**12**

*: (INGOLD) SV. NILSSON EX MARVANOVÁ & SV. NILSSON; **: Aggregated forms (Pozo and others 2011)
***: INGOLD, DANN & P.J. MCDOUGALL.

Table S3: Cont. (2/4)

Species	Hazel						*C. avellana* L.		
Treatment									
Stream	Stream 1			Stream 2			Stream 3		
Taxa ID									
AAC	0.014	0.018	0.052	0.062	0.020	0.033	0.022	0.018	0.018
APU	0.101	0.056	0.096	0.028	0.029	0.025	0.026	0.020	0.018
ACR									
ALO				0.001					0.003
AFI	0.320	0.611	0.349	0.375	0.305	0.218	0.464	0.525	0.291
ATE	0.343	0.133	0.267	0.392	0.507	0.556	0.304	0.232	0.464
CLAQ								0.001	0.001
CLO	0.007	0.008	0.006			0.003	0.004		0.001
CUAQ									
FCU	0.126	0.143	0.147	0.095	0.100	0.105	0.154	0.153	0.155
HLU	0.022	0.003	0.002	0.007	0.006	0.013	0.008	0.006	0.013
IBR			0.001						
LVI									
LTE		0.001	0.007			0.001	0.003	0.001	
LCU	0.006	0.001		0.004					0.002
M/G							0.001	0.008	0.001
TGR									
TEL	0.061	0.025	0.066	0.031	0.031	0.044	0.016	0.031	0.032
TMA			0.006		0.002	0.002			0.003
TAN				0.004					
TCA	0.001							0.005	
TMY		0.001				0.001			
MSR *	**20.3 ± 7.4**	**23.0 ± 8.8**	**28.9 ± 5.8**	**47.9 ± 11.2**	**40.0 ± 15.5**	**38.1 ± 4.2**	**29.5 ± 11.0**	**10.0 ± 5.7**	**19.4 ± 6.6**
TR **	**10**	**11**	**11**	**10**	**8**	**11**	**10**	**11**	**13**

***: Mean sporulation rate ± SE (Conidia g DM^{-1} d^{-1})×10^4; **: Taxon richness.**

Table S3: Cont. (3/4)

Litter specie	Oak					*Q. robur* L.			
Litter treatment									
Stream	Stream 1			Stream 2			Stream 3		
Taxa ID									
AAC	0.011	0.009	0.017	0.014	0.011	0.019	0.015	0.015	0.016
APU	0.021	0.011	0.015	0.007	0.080	0.011	0.010	0.015	0.005
ACR									
ALO	0.001	0.003	0.021				0.002	0.003	0.002
AFI	0.286	0.309	0.374	0.603	0.243	0.613	0.503	0.414	0.613
ATE	0.290	0.182	0.153	0.151	0.115	0.198	0.100	0.291	0.092
CLAQ									
CLO	0.005	0.004		0.001					
CUAQ									0.003
FCU	0.188	0.432	0.275	0.129	0.474	0.095	0.266	0.187	0.179
HLU	0.015	0.034	0.082	0.009	0.008	0.008	0.012	0.030	0.009
IBR									
LVI									
LTE									
LCU	0.003					0.001	0.005	0.003	0.005
M/G	0.001		0.002						
TGR				0.005	0.023				0.005
TEL	0.179	0.012	0.060	0.079	0.039	0.050	0.086	0.042	0.067
TMA					0.003	0.005			0.003
TAN									
TCA		0.003			0.005				
TMY				0.002					
MSR *	**10.1 ± 6.2**	**1.7 ± 0.8**	**3.6 ± 0.6**	**9.2 ± 5.1**	**2.7 ± 1.3**	**9.3 ± 4.8**	**4.5 ± 2.0**	**2.0 ± 0.7**	**2.9 ± 0.6**
TR **	**11**	**10**	**9**	**10**	**10**	**9**	**9**	**9**	**12**

***: Mean sporulation rate ± SE (Conidia g DM^{-1} d^{-1})×10^4; **: Taxon richness.**

Table S3: Cont. (4/4)

Species	Willow						*S. atrocinerea* BROT.		
Treatment									
Stream	Stream 1			Stream 2			Stream 3		
Taxa ID									
AAC	0.013	0.005	0.022	0.029	0.011	0.025	0.031	0.018	0.017
APU	0.020	0.006	0.031	0.032	0.013	0.034	0.020	0.013	0.007
ACR						0.012			
ALO				0.002			0.002		0.004
AFI	0.150	0.181	0.171	0.153	0.209	0.129	0.413	0.309	0.249
ATE	0.277	0.462	0.371	0.588	0.528	0.485	0.363	0.315	0.405
CLAQ	0.001	0.002	0.001		0.001				
CLO	0.001	0.005	0.015	0.001		0.001			0.001
CUAQ							0.001		0.002
FCU	0.313	0.183	0.251	0.068	0.130	0.161	0.099	0.195	0.216
HLU	0.050	0.061	0.021	0.030	0.036	0.037	0.016	0.053	0.035
IBR				0.001					0.001
LVI									
LTE				0.001		0.004			
LCU	0.001		0.004		0.001	0.001	0.002		0.001
M/G									
TGR									
TEL	0.173	0.095	0.114	0.096	0.071	0.109	0.050	0.097	0.059
TMA						0.002	0.001		0.004
TAN									
TCA		0.001							
TMY									
MSR *	**60.3 ± 23.2**	**47.2 ± 24.1**	**60.5 ± 22.8**	**79.2 ± 18.3**	**59.2 ± 9.4**	**41.6 ± 7.1**	**62.7 ± 21.2**	**23.3 ± 14.3**	**94.1 ± 36.7**
TR **	**10**	**10**	**10**	**11**	**9**	**12**	**11**	**7**	**13**

***: Mean sporulation rate ± SE (Conidia g DM^{-1} d^{-1})×10^4; **: Taxon richness.**

Table S4. Results of PERMANOVA pair comparisons testing the effects of treatment (mixture with alder, mixture without alder, alder, hazel, oak, willow) on detritivore assemblage structure and macroinvertebrate structure; F= F-statistic; R^2= adjusted R^2 ; p= p-value.

Assemblage	Pair	F	R^2	p
Detritivore	Mixture with alder:Mixture without alder	2.202	0.073	0.049
	Mixture with alder:Alder	0.971	0.034	0.470
	Mixture with alder:Hazel	1.740	0.058	0.128
	Mixture with alder:Oak	3.056	0.098	0.002
	Mixture with alder:Willow	1.874	0.063	0.080
	Mixture without alder:Alder	2.043	0.068	0.057
	Mixture without alder:Hazel	2.915	0.094	0.033
	Mixture without alder:Oak	1.537	0.052	0.161
	Mixture without alder:Willow	1.070	0.037	0.373
	Alder:Hazel	1.859	0.062	0.075
	Alder:Oak	1.988	0.066	0.055
	Alder:Willow	1.563	0.053	0.147
	Hazel:Oak	4.362	0.135	0.001
	Hazel:Willow	3.225	0.103	0.011
	Oak:Willow	1.663	0.056	0.114
Macroinvertebrate	Mixture with alder:Mixture without alder	1.855	0.062	0.067
	Mixture with alder:Alder	1.852	0.062	0.046
	Mixture with alder:Hazel	1.362	0.046	0.174
	Mixture with alder:Oak	2.185	0.072	0.015
	Mixture with alder:Willow	0.966	0.033	0.480
	Mixture without alder:Alder	2.877	0.093	0.006
	Mixture without alder:Hazel	1.877	0.063	0.067
	Mixture without alder:Oak	1.614	0.054	0.124
	Mixture without alder:Willow	0.852	0.030	0.564
	Alder:Hazel	2.487	0.082	0.009
	Alder:Oak	2.269	0.075	0.013
	Alder:Willow	2.187	0.072	0.016
	Hazel:Oak	3.295	0.105	0.002
	Hazel:Willow	2.050	0.068	0.041
	Oak:Willow	1.314	0.045	0.212

Table S5. (Stream 1) Macroinvertebrate taxa found in each litter treatment (mean ± SE) and their functional feeding group (FFG) following Tachet and others (2010); Sh: Shredder, FC: Filters, GC: Gatherers, Sc: Scrapers, P: Predators.

			Litter treatment					
FFG	Class/Order	Taxa						
Sh	O. Amphipoda	*Echinogammarus*	1.0 ± 0.6	3.0 ± 0.8	1.8 ± 1.1	0.4 ± 0.2	8.8 ± 3.3	2.2 ± 1.5
Sh	O. Coleoptera	*Dryops* (a)				0.2 ± 0.2		
Sh	O. Coleoptera	*Pomatinus* (a)						
Sh	O. Diptera	Tipulidae						
Sh	O. Ephemeroptera	*Ephemerella*	0.2 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2		0.2 ± 0.2
Sh	O. Plecoptera	*Amphinemura*	19.2 ± 4.5	10.2 ± 2.7	16.6 ± 4.0	35.4 ± 9.1	4.4 ± 1.5	12.8 ± 5.4
Sh	O. Plecoptera	Capniidae		0.2 ± 0.2		0.4 ± 0.2	0.2 ± 0.2	1.0 ± 0.8
Sh	O. Plecoptera	Leuctridae	10.8 ± 3.0	15.2 ± 2.2	8.4 ± 2.7	23.2 ± 6.1	7.6 ± 2.6	6.0 ± 2.0
Sh	O. Plecoptera	*Nemoura*		0.6 ± 0.4	0.2 ± 0.2	1.2 ± 0.5	0.4 ± 0.2	0.4 ± 0.2
Sh	O. Plecoptera	*Protonemura*	2.4 ± 1.9		1.2 ± 0.6	2.4 ± 1.7	0.4 ± 0.2	2.4 ± 2.4
Sh	O. Trichoptera	*Lepidostoma*			0.6 ± 0.6	0.2 ± 0.2	1.0 ± 0.8	
Sh	O. Trichoptera	Leptoceridae				0.2 ± 0.2		
Sh	O. Trichoptera	Limnephilinae	2.6 ± 0.7	5.0 ± 1.6	3.8 ± 1.2	12.0 ± 5.2	3.0 ± 2.0	4.0 ± 1.8
Sh	O. Trichoptera	*Odontocerum*						
Sh	O. Trichoptera	*Sericostoma*	0.2 ± 0.2		0.8 ± 0.4	0.4 ± 0.2	0.6 ± 0.4	0.4 ± 0.4
FC	O. Diptera	Dasyheleinae	0.2 ± 0.2		0.4 ± 0.4		0.2 ± 0.2	
FC	O. Diptera	Simuliidae	9.8 ± 8.8	0.2 ± 0.2	0.6 ± 0.6	0.6 ± 0.2	0.4 ± 0.2	0.8 ± 0.8
FC	O. Trichoptera	*Diplectrona*		0.4 ± 0.4		0.6 ± 0.6		0.2 ± 0.2
FC	O. Trichoptera	*Ecnomus*						
FC	O. Trichoptera	*Hydropsyche*	1.6 ± 1.2		1.8 ± 1.4	1.4 ± 0.7		0.2 ± 0.2
FC	O. Trichoptera	*Neureclipsis* *						
FC	O. Trichoptera	Philopotamidae						
GC	O. Diptera	Chironomini	0.8 ± 0.4			0.4 ± 0.2		
GC	O. Diptera	*Dixa*						
GC	O. Diptera	Orthocladiinae	1.4 ± 0.5	1.2 ± 0.5	0.8 ± 0.8	2.2 ± 0.7	1.6 ± 1.0	0.6 ± 0.2
GC	O. Diptera	Tanytarsini	1.0 ± 0.4			0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
GC	O. Ephemeroptera	*Habroleptoides*	5.4 ± 2.1	7.8 ± 2.1	2.6 ± 1.6	12.8 ± 3.5	6.2 ± 2.7	7.2 ± 2.3
GC	O. Ephemeroptera	Heptageniidae		0.8 ± 0.8		0.2 ± 0.2	0.2 ± 0.2	
GC	C. Oligochaeta			0.2 ± 0.2	0.4 ± 0.2		0.8 ± 0.6	
GC	O. Trichoptera	Brachycentridae **						
GC-Sc	O. Coleoptera	*Elmis* (a)	0.4 ± 0.4		0.2 ± 0.2	0.4 ± 0.2		1.2 ± 0.7
GC-Sc	O. Coleoptera	*Elmis* (l)			0.2 ± 0.2		0.2 ± 0.2	
GC-Sc	O. Coleoptera	*Limnius* (l)				0.2 ± 0.2		
GC-Sc	O. Ephemeroptera	*Baetis*	2.4 ± 0.9	3.2 ± 1.3	2.6 ± 1.0	6.6 ± 1.6	1.0 ± 0.6	2.2 ± 0.9
Sc	O. Coleoptera	*Cyphon* (l)						
Sc	O. Coleoptera	*Elodes* (l)						
Sc	O. Coleoptera	*Hydraena* (a)						
Sc	O. Coleoptera	*Hydrocyphon* (l)				0.2 ± 0.2		
Sc	O. Trichoptera	Agapetinae						0.2 ± 0.2
Sc	O. Basommatophora	Hydrobiidae						
Sc	O. Littorinimorpha	Planorbidae						
P	O. Diptera	*Atherix*	2.0 ± 0.8	0.6 ± 0.2	1.4 ± 0.7	2.0 ± 0.8	0.8 ± 0.5	1.8 ± 0.9
P	O. Diptera	*Ceratopogoninae*						0.2 ± 0.2
P	O. Diptera	*Hemerodromiinae*			0.2 ± 0.2			0.2 ± 0.2
P	O. Diptera	*Pediciini*	0.2 ± 0.2					
P	O. Diptera	*Tanypodinae*		0.2 ± 0.2				
P	O. Odonata	*Boyeria*						
P	O. Odonata	*Calopteryx*						
P	O. Plecoptera	*Chloroperla*	0.2 ± 0.2		0.2 ± 0.2		0.2 ± 0.2	0.6 ± 0.4
P	O. Plecoptera	Perlodidae	0.2 ± 0.2	0.6 ± 0.2	0.6 ± 0.6	1.4 ± 0.7		0.8 ± 0.6
P	O. Plecoptera	*Siphonoperla*	0.4 ± 0.2	0.2 ± 0.2		0.2 ± 0.2		
P	O. Plecoptera	*Xanthoperla*		0.2 ± 0.2	0.2 ± 0.2		0.4 ± 0.4	0.4 ± 0.4
P	O. Trichoptera	*Hyporhyacophila*						
P	O. Trichoptera	*Parahyacophila*						
P	O. Trichoptera	*Polycentropus*						
P	O. Trichoptera	*Rhyacophila*						
P	C. Arachnida	Acari		0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	

(a): adult; (l):larva; FC-P; ** GC-FC

Table S5. (Cont. Stream 2)

FFG	Class/Order	Taxa	Litter treatment 					
Sh	O. Amphipoda	*Echinogammarus*	0.8 ± 0.2	2.0 ± 1.3	5.4 ± 5.2	0.4 ± 0.2	0.4 ± 0.2	0.8 ± 0.4
Sh	O. Coleoptera	*Dryops* (a)					0.2 ± 0.2	
Sh	O. Coleoptera	*Pomatinus* (a)		0.2 ± 0.2				
Sh	O. Diptera	Tipulidae		0.2 ± 0.2				
Sh	O. Ephemeroptera	*Ephemerella*	0.8 ± 0.4	1.4 ± 0.6	0.4 ± 0.2	3.2 ± 0.7	1.4 ± 1.0	0.4 ± 0.2
Sh	O. Plecoptera	*Amphinemura*	10.2 ± 2.2	6.6 ± 2.0	7.6 ± 2.6	8.4 ± 2.4	3.2 ± 1.2	5.6 ± 1.4
Sh	O. Plecoptera	Capniidae	0.2 ± 0.2	0.2 ± 0.2	0.4 ± 0.4	0.4 ± 0.2	0.2 ± 0.2	0.4 ± 0.2
Sh	O. Plecoptera	Leuctridae	3.2 ± 0.2	5.0 ± 1.1	2.2 ± 0.7	7.8 ± 1.5	3.0 ± 1.4	4.6 ± 0.9
Sh	O. Plecoptera	*Nemoura*	0.6 ± 0.4		0.2 ± 0.2	0.6 ± 0.4	1.6 ± 1.1	0.6 ± 0.2
Sh	O. Plecoptera	*Protonemura*	13.8 ± 10.4	3.2 ± 2.2	3.2 ± 2.0	4.6 ± 2.0	5.4 ± 4.7	0.2 ± 0.2
Sh	O. Trichoptera	*Lepidostoma*	1.4 ± 1.0	0.4 ± 0.2	1.0 ± 0.8	0.6 ± 0.4	0.6 ± 0.4	1.0 ± 0.3
Sh	O. Trichoptera	Leptoceridae						
Sh	O. Trichoptera	Limnephilinae	2.4 ± 0.8	2.0 ± 0.3	4.0 ± 1.9	3.2 ± 1.0	0.8 ± 0.4	3.4 ± 0.2
Sh	O. Trichoptera	*Odontocerum*			0.2 ± 0.2			
Sh	O. Trichoptera	*Sericostoma*	0.6 ± 0.6	0.6 ± 0.4	0.2 ± 0.2	0.6 ± 0.4	0.8 ± 0.4	0.6 ± 0.4
FC	O. Diptera	Dasyheleinae	0.2 ± 0.2			0.4 ± 0.4	0.4 ± 0.4	0.2 ± 0.2
FC	O. Diptera	Simuliidae	1.0 ± 0.8		0.2 ± 0.2	0.4 ± 0.4	0.8 ± 0.4	0.2 ± 0.2
FC	O. Trichoptera	*Diplectrona*			0.2 ± 0.2			
FC	O. Trichoptera	*Ecnomus*				0.2 ± 0.2		
FC	O. Trichoptera	*Hydropsyche*	0.4 ± 0.4	0.2 ± 0.2	0.4 ± 0.2			
FC	O. Trichoptera	*Neureclipsis* *	0.2 ± 0.2	0.4 ± 0.2				
FC	O. Trichoptera	Philopotamidae						
GC	O. Diptera	Chironomini	0.4 ± 0.2	0.4 ± 0.4	0.6 ± 0.4	0.8 ± 0.5	0.8 ± 0.4	1.4 ± 0.7
GC	O. Diptera	*Dixa*						
GC	O. Diptera	Orthocladiinae	3.8 ± 3.1	0.8 ± 0.6	2.2 ± 0.6	2.8 ± 0.6	3.0 ± 1.7	2.0 ± 0.6
GC	O. Diptera	Tanytarsini	0.8 ± 0.4	0.2 ± 0.2		1.8 ± 0.9	0.6 ± 0.4	0.2 ± 0.2
GC	O. Ephemeroptera	*Habroleptoides*	10.2 ± 3.2	12.2 ± 2.5	0.8 ± 0.4	8.0 ± 2.1	7.6 ± 1.4	12.6 ± 2.5
GC	O. Ephemeroptera	Heptageniidae	0.4 ± 0.2	0.2 ± 0.2	0.4 ± 0.4	0.6 ± 0.4		0.8 ± 0.4
GC	C. Oligochaeta		0.2 ± 0.2	1.2 ± 0.4	0.6 ± 0.6	2.0 ± 0.3	0.8 ± 0.5	0.2 ± 0.2
GC	O. Trichoptera	Brachycentridae **	0.2 ± 0.2					
GC-Sc	O. Coleoptera	*Elmis* (a)						
GC-Sc	O. Coleoptera	*Elmis* (l)						
GC-Sc	O. Coleoptera	*Limnius* (l)						
GC-Sc	O. Ephemeroptera	*Baetis*	4.2 ± 1.2	9.6 ± 2.2	5.0 ± 1.0	7.4 ± 3.2	9.6 ± 1.5	5.0 ± 1.1
Sc	O. Coleoptera	*Cyphon* (l)		0.2 ± 0.2			0.2 ± 0.2	
Sc	O. Coleoptera	*Elodes* (l)	0.2 ± 0.2					0.2 ± 0.2
Sc	O. Coleoptera	*Hydraena* (a)		0.2 ± 0.2		0.6 ± 0.4		
Sc	O. Coleoptera	*Hydrocyphon* (l)						0.4 ± 0.2
Sc	O. Trichoptera	Agapetinae						
Sc	O. Basommatophora	Hydrobiidae						
Sc	O. Littorinimorpha	Planorbidae						
P	O. Diptera	*Atherix*	1.2 ± 0.6	0.8 ± 0.4	0.2 ± 0.2	1.8 ± 1.1	1.2 ± 0.4	0.6 ± 0.4
P	O. Diptera	*Ceratopogoninae*						
P	O. Diptera	*Hemerodromiinae*						
P	O. Diptera	*Pediciini*						
P	O. Diptera	*Tanypodinae*				0.2 ± 0.2		0.4 ± 0.4
P	O. Odonata	*Boyeria*						
P	O. Odonata	*Calopteryx*	0.2 ± 0.2	0.2 ± 0.2				
P	O. Plecoptera	*Chloroperla*		0.2 ± 0.2				
P	O. Plecoptera	Perlodidae	2.6 ± 0.9	2.2 ± 1.0	1.6 ± 1.0	0.8 ± 0.6	1.8 ± 1	1.0 ± 0.8
P	O. Plecoptera	*Siphonoperla*	0.4 ± 0.4	1.6 ± 0.6	0.2 ± 0.2		1.6 ± 0.7	2.4 ± 1.0
P	O. Plecoptera	*Xanthoperla*				0.6 ± 0.6		
P	O. Trichoptera	*Hyporhyacophila*						
P	O. Trichoptera	*Pararhyacophila*						
P	O. Trichoptera	*Polycentropus*						
P	O. Trichoptera	*Rhyacophila*						
P	C. Arachnida	Acari	0.6 ± 0.4	0.6 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.4 ± 0.2	0.2 ± 0.2

(a): adult; (l):larva; FC-P; ** GC-FC

Table S5. (Cont. Stream 3)

FFG	Class/Order	Taxa	Litter treatment					
Sh	O. Amphipoda	*Echinogammarus*	0.4 ± 0.2	0.6 ± 0.2		0.2 ± 0.2	0.4 ± 0.2	
Sh	O. Coleoptera	*Dryops* (a)						
Sh	O. Coleoptera	*Pomatinus* (a)						
Sh	O. Diptera	Tipulidae		0.2 ± 0.2				
Sh	O. Ephemeroptera	*Ephemerella*	2.4 ± 0.9	1.4 ± 0.5	3.2 ± 1.7	3.0 ± 1.4	2.0 ± 0.9	1.4 ± 0.7
Sh	O. Plecoptera	*Amphinemura*	10.6 ± 3.0	6.0 ± 3.8	13.0 ± 7.1	10.2 ± 2.2	6.8 ± 1.6	7.0 ± 2.7
Sh	O. Plecoptera	Capniidae	0.6 ± 0.2	0.2 ± 0.2	1.2 ± 0.8	0.4 ± 0.2	0.4 ± 0.2	0.6 ± 0.4
Sh	O. Plecoptera	Leuctridae	10.6 ± 3.0	8.6 ± 1.0	10.8 ± 3.3	16.6 ± 4.7	6.0 ± 1.3	7.6 ± 1.6
Sh	O. Plecoptera	*Nemoura*	0.6 ± 0.4	2.4 ± 1.7	2 ± 0.9	0.8 ± 0.4	1.4 ± 0.6	2.6 ± 1.7
Sh	O. Plecoptera	*Protonemura*	2.2 ± 1.7	2.4 ± 1.7	2.2 ± 2.0	4.2 ± 2.8	3.2 ± 2.1	4.6 ± 4.6
Sh	O. Trichoptera	*Lepidostoma*		0.2 ± 0.2	1.0 ± 0.4	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
Sh	O. Trichoptera	Leptoceridae		0.2 ± 0.2				
Sh	O. Trichoptera	Limnephilinae	1.6 ± 0.7	2.6 ± 0.8	5.2 ± 2.0	3.2 ± 1.2	2.0 ± 1.1	3.2 ± 1.2
Sh	O. Trichoptera	*Odontocerum*						
Sh	O. Trichoptera	*Sericostoma*	0.2 ± 0.2	0.2 ± 0.2	0.4 ± 0.2	0.6 ± 0.4	0.6 ± 0.2	0.4 ± 0.4
FC	O. Diptera	Dasyheleinae			0.4 ± 0.4		0.2 ± 0.2	0.2 ± 0.2
FC	O. Diptera	Simuliidae	3.4 ± 2.2	2.4 ± 2.4	1.2 ± 0.7	1.4 ± 0.7	3.8 ± 2.0	6.8 ± 4.4
FC	O. Trichoptera	*Diplectrona*	0.2 ± 0.2					
FC	O. Trichoptera	*Ecnomus*						0.2 ± 0.2
FC	O. Trichoptera	*Hydropsyche*					0.2 ± 0.2	
FC	O. Trichoptera	*Neureclipsis* *						
FC	O. Trichoptera	Philopotamidae		0.2 ± 0.2			0.2 ± 0.2	
GC	O. Diptera	Chironomini	0.6 ± 0.4		0.8 ± 0.4	0.2 ± 0.2	1.2 ± 0.6	0.6 ± 0.4
GC	O. Diptera	*Dixa*				0.2 ± 0.2		
GC	O. Diptera	Orthocladiinae	4 ± 1.4	3.4 ± 1.5	3.8 ± 1.5	3.4 ± 1.8	4.8 ± 1.8	5.8 ± 3.1
GC	O. Diptera	Tanytarsini		0.8 ± 0.8		0.4 ± 0.2	0.2 ± 0.2	
GC	O. Ephemeroptera	*Habroleptoides*	7.0 ± 0.5	6.8 ± 2.6	3.2 ± 1.6	6.0 ± 1.8	3.4 ± 1.6	5.6 ± 1.7
GC	O. Ephemeroptera	Heptageniidae	0.4 ± 0.2		0.2 ± 0.2			0.2 ± 0.2
GC	C. Oligochaeta		0.4 ± 0.4	0.4 ± 0.2	0.2 ± 0.2	0.6 ± 0.4	0.2 ± 0.2	0.8 ± 0.4
GC	O. Trichoptera	Brachycentridae **			0.2 ± 0.2			
GC-Sc	O. Coleoptera	*Elmis* (a)	0.2 ± 0.2		0.2 ± 0.2			0.2 ± 0.2
GC-Sc	O. Coleoptera	*Elmis* (l)					0.2 ± 0.2	
GC-Sc	O. Coleoptera	*Limnius* (l)						
GC-Sc	O. Ephemeroptera	*Baetis*	1.6 ± 0.7	1.8 ± 1.1	1.0 ± 0.6	1.8 ± 1.1	5.0 ± 1.3	1.2 ± 0.6
Sc	O. Coleoptera	*Cyphon* (l)				0.2 ± 0.2		
Sc	O. Coleoptera	*Elodes* (l)	0.2 ± 0.2					0.2 ± 0.2
Sc	O. Coleoptera	*Hydraena* (a)				0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
Sc	O. Coleoptera	*Hydrocyphon* (l)	0.2 ± 0.2	0.2 ± 0.2				
Sc	O. Trichoptera	Agapetinae						
Sc	O. Basommatophora	Hydrobiidae		0.2 ± 0.2			0.2 ± 0.2	0.2 ± 0.2
Sc	O. Littorinimorpha	Planorbidae	0.2 ± 0.2					0.2 ± 0.2
P	O. Diptera	*Atherix*	0.6 ± 0.2		0.6 ± 0.6	0.8 ± 0.4	0.2 ± 0.2	0.2 ± 0.2
P	O. Diptera	*Ceratopogoninae*						
P	O. Diptera	*Hemerodromiinae*					0.2 ± 0.2	
P	O. Diptera	*Pediciini*						
P	O. Diptera	*Tanypodinae*	0.2 ± 0.2	0.4 ± 0.4	0.4 ± 0.4	0.4 ± 0.4		0.2 ± 0.2
P	O. Odonata	*Boyeria*	0.2 ± 0.2					
P	O. Odonata	*Calopteryx*	0.4 ± 0.2					
P	O. Plecoptera	*Chloroperla*		0.6 ± 0.4		0.2 ± 0.2		
P	O. Plecoptera	Perlodidae	1.2 ± 1.0	1.4 ± 0.8	1.4 ± 0.7	2.0 ± 1.1	2.0 ± 0.8	3.2 ± 1.6
P	O. Plecoptera	*Siphonoperla*	0.6 ± 0.6	0.6 ± 0.6	0.8 ± 0.4		0.4 ± 0.2	0.2 ± 0.2
P	O. Plecoptera	*Xanthoperla*						
P	O. Trichoptera	*Hyporhyacophila*		0.2 ± 0.2				0.2 ± 0.2
P	O. Trichoptera	*Pararhyacophila*					0.2 ± 0.2	0.2 ± 0.2
P	O. Trichoptera	*Polycentropus*	0.2 ± 0.2					
P	O. Trichoptera	*Rhyacophila*	0.2 ± 0.2			0.2 ± 0.2		
P	C. Arachnida	Acari	0.8 ± 0.2	0.2 ± 0.2	0.6 ± 0.4	0.2 ± 0.2	0.6 ± 0.2	0.8 ± 0.5

(a): adult; (l):larva; FC-P; ** GC-FC

Chapter 2

FUNCTIONAL CONSEQUENCES OF ALDER AND OAK LOSS IN STREAM ECOSYSTEMS

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ABSTRACT

Alder (*Alnus glutinosa*) and oak (*Quercus robur*) are dominant tree species in European Atlantic mixed forests, and their leaf litter is a key resource for stream ecosystems. While alder litter has higher nutrient content and palatability than other species and is rapidly processed in the stream by detritivores and microorganisms, oak litter is a tougher and less nutritious but more persistent resource. Given that both species are declining due to the spread of the fungal pathogens *Phytophthora alni* and *P. cinnamomi*, respectively, we investigated how their reduction or loss might alter stream ecosystem functioning through changes in litter decomposition, invertebrate detritivore (*Sericostoma pyrenaicum*) growth and stoichiometry, and fungal decomposer assemblage characteristics. We conducted a microcosm experiment where we incubated litter mixtures representing different scenarios of alder and oak reduction or loss (and a concomitant increase in the other species), compared to a control that contained the 4 most common species in the study area [alder, oak, hazel (*Corylus avellana*) and willow (*Salix atrocinerea*)] in the same proportions as found in nature. The experiment lasted for 9 weeks, with the above variables measured every 3 weeks. Decomposition rates changed depending on which species was lost. Rates decreased as a result of alder loss and increased following oak loss. *Sericostoma* nutrient assimilation also responded to species loss, increasing and decreasing following alder and oak loss, respectively, possibly due to compensatory assimilation. Differences in *Sericostoma* nutrient concentrations among treatments decreased with time in the case of nitrogen, whereas they increased for phosphorus, likely due to microbial colonization. The presence of oak also constrained microbial activity at the end of the experiment, reducing sporulation rates and causing differences in assemblage structure, likely due to inhibitory traits such as tannins or phenolic compounds. Treatments examining the loss of both species did not differ from the control, either in decomposition or sporulation rate, since loss of both alder and oak counteracted their effects. However, sporulation rates were higher for the scenario with loss of both species than for treatments with either alder reduction or loss, whereas sporulation rate and assemblage structure in the treatment with loss of both species were similar to the scenarios with oak reduction and loss, indicating that oak loss is more important for microbial activity. Changes in nutrient assimilation throughout the experiment suggested that effects of plant species reduction and loss can alter ecosystem functioning depending not only on litter palatability, but also on detritivore

life stage. Overall, our results provide evidence for the importance of maintaining native riparian vegetation to preserve various ecosystem functions.

INTRODUCTION

Biodiversity is currently decreasing at alarming rates, comparable to the rates reported for past mass extinctions (Barnosky et al. 2011; Ceballos et al. 2017). There is ample evidence that biodiversity loss can alter ecosystem functioning (Boyero et al. 2021; Hooper et al. 2012), but most experimental studies have simulated random species loss (Wardle 2016). This often represents unrealistic situations, since species in nature do not disappear randomly, but rather suffer reductions in populations depending on their functional traits and influences of particular environmental change drivers (Lepš 2004). Therefore, considering the composition of biological communities and the vulnerability of different species to particular perturbations allows a more realistic estimation of ecosystem-level effects of biodiversity loss (Alonso et al. 2021; Jonsson et al. 2002).

Freshwater ecosystems are not only affected by the disappearance of aquatic biodiversity (Reid et al. 2018; Sala et al. 2000), but they also suffer the consequences of terrestrial plant loss (Ellison et al. 2005; Kominoski et al. 2013). Streams, in particular, are strongly influenced by their surrounding forests (mostly in the riparian zone but also elsewhere in the catchment), which provide an important energy source in the form of leaf litter (Vannote et al. 1980). Leaf litter is then processed by stream detritivores and microorganisms, fueling brown aquatic food webs, mostly in headwaters (Wallace et al. 1997) but also further downstream (Wipfli and Musslewhite 2004).

An important cause of plant species loss is a suite of emergent fungal diseases, which have been long recognised as a main threat to plant diversity, and can cause widespread population declines and extinctions (Bjelke et al. 2016; Boyd et al. 2013; Fisher et al. 2012). In European Atlantic mixed forests, oomycetes of the genus *Phytophthora* affect several key riparian species. One of these is *Alnus glutinosa* (L.) GAERTN. (family Betulaceae; hereafter 'alder'), a dominant riparian species that provides high-quality litter (i.e., rich in nutrients and poor in refractory carbon compounds; Waring and Running 2010). It is highly favoured by consumers (Graça et al. 2001; López-Rojo et al. 2018) and readily processed in streams, and therefore considered a key species for the maintenance of ecosystem functioning (Pérez et al. 2021a). In mixtures, alder speeds up decomposition through a positive selection effect due to its high palatability (Alonso et al. 2021; López-

Rojo et al. 2018; Rubio-Ríos et al. 2021), which also attracts detritivores to these mixtures (Ferreira et al. 2012). Alder litter also enhances the decomposition and fungal colonization of the other species in the mixture (Alonso et al. 2021; Larrañaga et al. 2020; López-Rojo et al. 2018) by increasing overall palatability of the mixture by nutrient transfer through fungal hyphae (Handa et al. 2014). Alder can be infected by the species complex *Phytophthora alni,* composed of *Phytophthora* × *alni* (BRASIER & S.A. KIRK) HUSSON, IOOS & MARÇAIS, *Phytophthora uniformis* (BRASIER & S.A. KIRK) HUSSON, IOOS & AGUAYO, and *Phytophthora* × *multiformis* (Brasier & S.A. Kirk) HUSSON, IOOS & P. FREY (Husson et al. 2015). These fungi reduce tree vitality until death and have caused dieback and local extinctions across Europe (Bjelke et al. 2016; Jung and Blaschke 2004).

Another species affected by fungal disease is *Quercus robur* L. (hereafter 'oak'), a dominant forest tree in European Atlantic mixed forests that provides litter of lower quality but which remains longer in the stream and thus provides a more persistent resource than alder (Compson et al. 2015; Pérez et al. 2021a). It also slows down decomposition of leaf litter mixtures when dominant, maybe due to a negative selection effect, as it is avoided by detritivores due to its low palatability (Alonso et al. 2021; Larrañaga et al. 2020). Another possible pathway is a negative complementarity effect caused by tannins and other inhibitory substances leached by oak leaves, which limit detritivore consumption and fungal colonization of mixtures including oak litter (Alonso et al. 2021; Ferreira et al. 2012; McArthur et al. 1994). Oak can be infected by *Phytophthora cinnamomi* RANDS, which produces ink disease, characterized by collar and root rot, causing widespread declines in this species and other members of the Fagaceae (Brasier et al. 1993; Vannini and Vettraino 2001). Both pathogens have been reported on the Iberian peninsula (Hernández-Lambraño et al. 2018; Solla et al. 2010) and are expected to increase their distribution area due to climate warming (Aguayo et al. 2014; Bergot et al. 2004; Ireland et al. 2013; Thoirain et al. 2007).

In this study, we assessed how the reduction or loss of alder and oak would affect key stream ecosystem processes and associated organisms [i.e., litter decomposition, detritivore growth, changes in detritivore stoichiometry, and aquatic hyphomycete (AH) sporulation, fungal taxon richness and assemblage structure] over time (with observations at weeks 3, 6 and 9) using a microcosm experiment. A previous field study showed that alder loss in European Atlantic mixed forests reduced decomposition of mixtures and sporulation and modified fungal and detritivore assemblages (Alonso et al. 2021). Here, we further examine these effects in the laboratory, as well as those of oak loss, using the same

species, which constitute the majority of litter mixtures in streams of the study area: alder, oak, *Corylus avellana* L. (family Betulaceae; hereafter 'hazel') and *Salix atrocinerea* BROT. (family Salicaceae; hereafter 'willow'). We simulated five scenarios of reduction or loss of alder and/or oak (S1-5; Fig. S1), with total litter mass constant, and species proportions based on the potential effects of infections by *P. alni* and *P. cinnamomi* (Aguayo et al. 2014; Brasier et al. 1993; Jung and Blaschke 2004; Thoirain et al. 2007) compared to the natural, current situation in the study area (control). In addition, we compared those scenarios among themselves to observe if the effects varied depending on whether species were reduced or lost, and to examine differences between the loss of one or both species. Finally, we used the four monocultures (i.e., single-species treatments) to examine the net diversity effect (NDE) on the above processes (i.e., the difference between the observed value in the mixture and the expected value estimated from monocultures) and, when possible, its partitioning into a complementarity effect (CE; caused by synergistic or antagonistic interactions between the species in the mixture) and a selection effect (SE; caused by the presence of a species with high contribution to decomposition; Loreau and Hector 2001).

Based on the main characteristics of litter from alder (high-quality, ephemeral resource preferred by detritivores and microbial decomposers) and oak (low-quality, persistent resource), we hypothesized that:

(i) alder reduction (S1) and loss (S2) **(i-a)** would result in lower process rates of mixtures (i.e., decomposition, growth, changes in stoichiometry and sporulation) compared to the control, due to the overall decrease in litter quality and loss of key litter traits [e.g., the high nitrogen (N) concentration of alder]; **(i-b)** with effects being stronger for S2 than for S1, increasing with time and being greater when detritivores are present (Rubio-Ríos et al. 2021);

(ii) oak reduction (S3) and loss (S4) **(ii-a)** would result in higher process rates of mixtures, due to an overall increase in litter quality (that boosts microbial activity, detritivore feeding and nutrient cycling in the short term) and loss of inhibitory litter traits (e.g., recalcitrant compounds and tannins, which are high in oak litter); **(ii-b)** with effects being stronger for S4 than for S3, and increasing with time and in the presence of detritivores;

(iii) the simultaneous loss of alder and oak (S5) would potentially counteract each other and render similar process rates to those in the control, although the loss of 2 species

(and hence loss of key traits) makes predictions difficult (López-Rojo et al. 2018); and

(iv) aquatic hyphomycete (AH) assemblages would be altered in all cases, but more strongly when species are completely lost (i.e., S2, S4 and S5) due to the often marked substrate preferences shown by AHs (Alonso et al. 2021; Gulis 2001).

MATERIALS AND METHODS

Experimental design

The experimental design included six treatments (i.e., the control and scenarios S1-S5), with 16 microcosms per treatment. All microcosms contained the same total litter mass, but the presence and proportion of different species varied across treatments (Fig. S1). The control represented the current situation in forests of the study area, with dominance of alder (35% of total DM) and oak (35%) followed by hazel (15%) and willow (15%). The first scenario (S1) represented the emergence of *P. alni* and hence a reduction in alder relative abundance (down to 15%), with a concomitant increase in the other species (oak 45%, hazel 20%, willow 20%). The second scenario (S2) represented a more advanced stage of the alder disease, with its total disappearance from the area and thus a further increase in the relative abundance of the other species (oak 50%, hazel 25%, willow 25%). The third scenario (S3) simulated the emergence of *P. cinnamomi* and hence a reduction in oak relative abundance (down to 15%), with a concomitant increase in other species (alder 45%, hazel 20%, willow 20%). The fourth scenario (S4) represented a more advanced stage of the oak disease, its total disappearance from the area and thus a further increase in the relative abundance of the other species (alder 50%, hazel 25%, willow 25%). The fifth scenario (S5) represented the loss of both alder and oak, and thus included litter mixtures composed solely of hazel (50%) and willow (50%). In addition to the above treatments, there were microcosms containing monocultures of each of the four species (15 microcosms each), which contained the same amount of total litter mass as the mixture treatments.

Leaf litter

The four plant species used in the experiment (alder, oak, hazel and willow) are all broadleaf senescent trees that range widely in litter traits (Table 1) and represented natural litter inputs to headwater streams in the study area. Leaves were collected in autumn 2019

from the forest floor immediately after their natural abscission, in the Agüera stream catchment (43.20º N, 3.26º W; alder, oak and hazel) or near the University of the Basque Country (43.32º N, 2.97º W; willow), and air-dried in the laboratory.

Table 1. Physicochemical characteristics of leaf litter of the different plant species used in the study (mean ± s.e.; n=5) and the mixtures (mean weighted by proportion of each species and post-leaching dry mass). N and P, nitrogen and phosphorus concentrations (% dry mass); C:N, C:P and N:P, nutrient elemental ratios; SLA, specific leaf area (mm^2 mg^{-1}); Toughness, leaf toughness (kPa). Different letters indicate significant differences among leaf traits, examined with linear models.

Species	N	P	C:N	C:P	N:P	SLA	Toughness
Alder	3.31 ± 0.09^a	0.038 ± 0.002^a	17.64 ± 0.62^c	3403 ± 189^b	193.5 ± 11.6^a	14.94 ± 0.81^b	1447 ± 81^b
Hazel	1.75 ± 0.05^b	0.028 ± 0.002^b	32.38 ± 0.70^b	4584 ± 307^b	142.4 ± 11.7^b	20.77 ± 1.46^a	1270 ± 21^b
Oak	1.53 ± 0.08^b	0.016 ± 0.001^b	38.71 ± 1.99^a	8430 ± 741^a	218.1 ± 17.3^a	36.76 ± 3.24^b	2571 ± 118^a
Willow	1.47 ± 0.04^b	0.028 ± 0.002^b	38.39 ± 0.42^a	4567 ± 261^b	118.9 ± 6.6^b	20.63 ± 1.09^b	2436 ± 148^a
Control	2.12 ± 0.06	0.027 ± 0.001	30.96 ± 0.92	5664 ± 304	184.0 ± 9.2	24.96 ± 1.27	1986 ± 67
S1	1.80 ± 0.06	0.024 ± 0.001	34.55 ± 1.09	6248 ± 381	180.7 ± 10.1	27.55 ± 1.59	2127 ± 70
S2	1.57 ± 0.05	0.022 ± 0.001	37.02 ± 1.18	6569 ± 424	176.2 ± 10.6	29.01 ± 1.75	2208 ± 71
S3	2.31 ± 0.06	0.030 ± 0.001	28.44 ± 0.59	4727 ± 168	171.8 ± 7.7	20.97 ± 0.67	1789 ± 68
S4	2.42 ± 0.06	0.033 ± 0.001	26.91 ± 0.40	4021 ± 105	160.7 ± 7.3	17.98 ± 0.44	1644 ± 76
S5	1.62 ± 0.04	0.028 ± 0.001	35.21 ± 0.39	4576 ± 188	131.3 ± 6.3	20.70 ± 0.43	1821 ± 76

Aquatic hyphomycetes

We prepared a microbial inoculum for the experiment using mixed litter with different degrees of decomposition collected from the benthos of the Perea stream (43.29º N, 3.24º W), a tributary of the Agüera stream, in September 2020. The mixture (37.1% *A. glutinosa*, 22.9% *Platanus* × *hispanica* MILL. EX MÜNCH., 13% *Q. robur*, 5% *C. avellana,* 2.9% *Castanea sativa* MILL., 0.7% *S. atrocinerea*; dry mass, DM = 49 g) was incubated in a glass jar with 4 L of filtered (100 µm) stream water under aeration at 10º C, for 7 d with water renewal every 24 h. Each microcosm received 20 mL of microbial inoculum, containing *ca*. 13 taxa and 1000 conidia. The AH assemblage of the inoculum was dominated by *Flagellospora curvula* INGOLD, *Alatospora pulchella* MARVANOVÁ and *Articulospora tetracladia* INGOLD (Table S1).

To characterize the initial AH conidial assemblage, six 20-mL samples of this solution were stored in 45-mL centrifuge tubes, pre-stained with 2 drops of trypan blue, preserved with 2 mL of 35% formalin and adjusted to 40 mL with distilled water. We added 150 µL of Triton X-100 (0.5%) to each sample and mixed them with a magnetic stirrer to obtain a uniform distribution of conidia. Then 10 mL were filtered (25 mm diameter, pore size 5 µm, Millipore SMWP, Millipore Corporation) with gentle vacuum (Descals 2020)

and stained with trypan blue. Conidia were identified and counted with a microscope at 200× magnification (Gulis et al. 2020).

Detritivores

Detritivores used in the experiment were larvae of the cased caddisfly *Sericostoma pyrenaicum* PICTET (order Trichoptera, family Sericostomatidae; hereafter '*Sericostoma*'). This species is a common detritivore in the study area and has been successfully used in previous microcosm experiments due to its high survival and litter consumption rates (Cornejo et al. 2020; López-Rojo et al. 2018; Tonin et al. 2017). Larvae of similar size (case opening length ± s.e.: 2.685 ± 0.026 mm) were collected individually from the Perea stream in September 2020. They were acclimated to experimental conditions for 5 d (fed *ad libitum* with mixed litter from the same stream) and then starved for 48 h. Just before the beginning of the experiment, case opening length (COL) was individually measured (0.001 mm precision) using ImageJ software (v. 1.46r), and initial DM (mg) was estimated using a COL-DM relationship ($DM = 0.1238e^{1.2929 \times COL}$, $r^2 = 0.90$) obtained from 16 extra larvae (Cornejo et al. 2020; López-Rojo et al. 2018).

Experimental procedure

The experiment was carried out between October and December 2020, using 156 microcosms in a temperature-controlled room at 10º C (which mimicked natural conditions and minimized evaporation), with a light:dark regime of 12:12 h and constant aeration. Microcosms were 580-mL glass jars containing 400 mL of filtered (100 µm) stream water [mean soluble reactive phosphorus (SRP) ± s.e.: 34.23 ± 3.30 µg L^{-1}; mean dissolved inorganic N (DIN) ± s.e.: 552.95 ± 36.73 µg L^{-1}] and 20 mL of fine sediment (200 µm – 2 mm; collected in the Perea stream, incinerated and washed). They also contained 1.6 ± 0.08 g of air-dried litter accessible to detritivores (which allowed estimating total decomposition; hereafter 'free litter') and 0.2 ± 0.01 g of air-dried litter enclosed in a 3×3-cm, 0.4-mm mesh bag (which prevented access of detritivores and served to estimate microbial decomposition; hereafter 'enclosed litter'). Finally, each microcosm had one larva of *Sericostoma*. Litter fragments of the same species were connected by stainless steel safety pins to avoid floating and facilitate species identification at the time of collection.

Before starting the experiment, litter was submerged in the 156 experimental microcosms plus 20 extra microcosms (5 per species; litter mass, mean ± s.e. DM = 0.8048 ± 0.0055 g) that were used to estimate mass loss due to the leaching of soluble compounds.

After 48 h, the extra microcosms were removed and sampled (as explained below for experimental microcosms). In experimental microcosms, the water was replaced, and each microcosm received 20 mL of the microbial inoculum (*ca*. 1000 conidia) and one larva of *Sericostoma* (mean ± s.e. DM = 4.35 mg ± 0.15). For the duration of the experiment (63 d), the water was renewed every 7 d, and one third of the microcosms were removed and sampled every 3 weeks [i.e., on days 21 (n = 5), 42 (n = 5) and 63 (n = 6)]. Sampling was done as follows: free litter was separated by species, oven-dried (70º C, 48 h) and weighed to calculate DM, and afterwards incinerated (500º C, 4 h) and weighed to calculate ash-free dry mass (AFDM); mesh-enclosed litter was placed in Erlenmeyer flasks filled with 25 mL of filtered stream water (glass fibre filters, Whatman GF/F; pore size: 0.7 µm,) and incubated for 48 ± 2 h on a shaker at 100 rpm and 10º C, then litter was collected, separated by species, and processed in the same way as the free litter. Conidial suspensions obtained from the incubation were poured into 50-mL falcon tubes and examined as described above for the initial inoculum.

At each sampling date, *Sericostoma* larvae were left starving for another 48 h after litter sampling, within the same microcosms filled with new, filtered (100 µm) stream water, and subsequently freeze-dried. We calculated their DM, N concentration (% DM; PerkinElmer series II CHNS/O elemental analyser) and phosphorus (P) concentration (% DM; spectrometer after autoclave-assisted extraction, APHA 1998). The extra microcosms were used to estimate leaching mass loss in experimental microcosms, and therefore initial (post-leaching) litter AFDM, as well as N and P concentrations (measured as above), specific leaf area [SLA, calculated as leaf area (mm^2) divided by DM (mg) obtained from five 12-mm diameter leaf discs per microcosm] and leaf toughness [measured in five fragments per microcosm using a penetrometer with a 1.55 mm diameter steel rod (Boyero et al. 2011)].

Response variables

Total and microbial decomposition were quantified through proportional litter mass loss (LML), calculated as the difference between initial and final AFDM divided by initial AFDM (with initial AFDM previously corrected for leaching losses). Total decomposition was standardized using mean initial *Sericostoma* mass to avoid variability due to differences in larval size. The net diversity effect (NDE), complementarity effect (CE) and selection effect (SE) on decomposition were calculated following Loreau and Hector (2001): the NDE was the difference between observed and expected LML, with expected

LML calculated based on monoculture values taking into account the proportion of each species in the mixture; the CE was calculated as the average deviation from the expected decomposition in a mixture multiplied by mean decomposition in monocultures and the number of species in the mixture; and the SE was calculated as the covariance between decomposition of species in monoculture and the average deviation from expected decomposition of species in the mixture, multiplied by the number of species in the mixture.

Sericostoma growth was calculated as the difference between final and initial DM divided by initial DM. *Sericostoma* N and P final concentrations were used as proxies for changes in stoichiometry, given that they all started with the same conditions and hence with similar initial stoichiometry (C = 47.4 ± 0.5, N = 9.4 ± 0.2, P = 0.53 ± 0.02). AH sporulation rate was calculated as the number of conidia per mg of litter and day, and AH taxon richness was the number of species per sample. We calculated the NDE on *Sericostoma* growth, N and P concentrations, and on AH sporulation rate and taxon richness, as above. The CE and SE were not calculated for these variables due the lack of separate data for each plant species.

Data analyses

Differences in the above variables (i.e., microbial and total decomposition, *Sericostoma* growth, N and P content, AH sporulation rate and taxon richness, and their net diversity, complementarity and selection effects) among treatments (i.e., the control and scenarios S1-S5) and through time (3, 6 and 9 weeks) were examined with linear models (*gls* function, "nlme" R package), with treatment, time and their interaction as fixed factors. Given that the interaction was significant for some variables (see Results), we examined each time separately (using linear models with treatment as fixed factor) to facilitate the interpretation of results. Significant differences among treatments ($\alpha = 0.05$) were further explored with Tukey tests (*ghlt* function of the "multcomp" R package; Zar 1999). The existence of a significant NDE, CE or SE was assessed with ordinary nonparametric bootstrapped 95% confidence intervals (BCa method; *boot* function, "boot" package), based on 999 bootstrap replicates (Canty and Ripley 2016; Davison and Hinkley 1997); intervals that did not contain the value of zero indicated the existence of the examined effect. We explored differences in AH assemblage structure among treatments and times with permutational multivariate analysis of variance (*adonis* function of the "vegan" R package) based on a Bray-Curtis similarity index; again, the interaction between time and treatment was significant, so each time was examined separately. An indicator value index

(*multipatt* function of the "indicspecies" R package) was used to identify the most representative species of each assemblage (De Cáceres 2013).

RESULTS

Litter decomposition

Total decomposition ranged between 12% (S3) and 20% (S5) mass loss at the end of the experiment, and varied among treatments and sampling times (Table 2, Table S3). When each time was examined separately, there were differences among scenarios only at week 9: the treatment with loss of oak (S4) differed from the treatments with reduction and loss of alder (S1 and S2) (Fig. 1A, Table S2, Table S3); however, no scenario differed from the control. Diversity effects on total decomposition again varied with treatment and time. The net diversity effect decreased throughout the experiment, resulting in a negative NDE at week 9 with the exception of the treatment with loss of both species (S5), where the NDE was null and higher than in the control and other scenarios (Fig. S2A, Table S3). The NDE was mainly driven by a complementarity effect, which varied similarly to the NDE, being null and higher than the control in the treatment with loss of both species (S5) and negative in the control and treatments with reduction of alder and oak (S1 and S3; Fig. S2B, Table S3), and was higher in magnitude than the selection effect; the latter was mostly null and there were no differences between the control and the other scenarios (Fig. S2C, Table S3).

The microbial contribution to total decomposition at the end of the experiment was on average 11%, being negligible in the control and the treatment with alder reduction (S1) and greatest (*ca*. 20%) in the treatment with loss of oak and both species (S4 and S5); however, it was not statistically different from zero in most cases. Moreover, microbial decomposition was, in most cases, negative at week 3 and null at the end of the experiment, indicating that there was microbial biomass accrual. It was significantly affected by treatment and time, but at week 9, there were no significant differences among treatments or with the control (Fig. 1B, Table 2, Table S3). Nevertheless, the NDE on microbial decomposition showed similar patterns to the NDE on total decomposition, becoming negative in all treatments with the exception of the treatment with loss of both species (S5; Fig. S2D, Table S3), which was also the only scenario which differed from the control. The partitioning of the NDE into CE and SE did not show a clear pattern due to the abundance of negative data (Fig. S2E, Fig. S2F, Table S3).

Table 2. Results of linear models exploring the effects of scenario (control and species loss scenarios S1-S5), time and their interaction on total (prop. litter mass loss) and microbial decomposition (prop. litter mass loss); *Sericostoma* growth (prop. mass), N (prop. mass) and P concentrations (prop. mass); and aquatic hyphomycete (AH) sporulation rate (conidia mg^{-1} DM d^{-1}) and taxon richness (taxa microcosm-1); significant p-values are indicated in bold; df= degrees of freedom; F= F-statistic; p= p-value.

Variable	Factor	df	F	p
Total decomposition	Scenario	5, 75	3.39	**0.008**
	Time	2, 75	102.32	**<0.001**
	Scenario × time	10, 75	1.92	0.056
Microbial decomposition	Scenario	5, 76	3.13	**0.013**
	Time	2, 76	24.12	**<0.001**
	Scenario × time	10, 76	1.95	0.051
Sericostoma growth	Scenario	5, 77	2.31	0.052
	Time	2, 77	26.46	**<0.001**
	Scenario × time	10, 77	0.92	0.524
Sericostoma N	Scenario	5, 76	14.07	**<0.001**
	Time	2, 76	22.04	**<0.001**
	Scenario × time	10, 76	1.31	0.238
Sericostoma P	Scenario	5, 66	33.68	**<0.001**
	Time	2, 66	18.84	**<0.001**
	Scenario × time	10, 66	5.05	**<0.001**
AH sporulation	Scenario	5, 78	3.05	**0.014**
	Time	2, 78	6.87	**0.002**
	Scenario × time	10, 78	3.27	**0.004**
AH richness	Scenario	5, 78	0.84	0.524
	Time	2, 78	2.31	0.106
	Scenario × time	10, 78	0.69	0.729

Detritivores

Sericostoma growth increased with time, but it did not differ among any scenario or the control (Table 2, Table S3). There was no NDE on growth at any time or treatment (Table S2, Table S3). In contrast, *Sericostoma* N and P contents varied depending on the treatment and the patterns changed throughout the experiment. N concentration was higher in the treatments with loss of alder and both species (S2 and S5) than in the control at week 3, and higher in S2 than in the control at week 6; but these differences disappeared by week 9 (Fig. 2A, Table S2, Table S3). The NDE on N concentration was positive at week 3 (and higher in S5 than in the control) and decreased with time, becoming negative at week 9 (Fig. S3A, Table S3). P concentration increased with time and was significantly higher in the treatment with loss of alder (S2) than in the control at week 6, and in all treatments

except alder reduction (S2-S5) than in the control at week 9 (Fig. 2B, Table S2, Table S3). The NDE on P concentration was positive at week 6 in treatments with loss of alder and both species (S2 and S5) and at week 9 in all treatments except alder reduction (S2-S5), contrasting with the control, where it was negative at week 6 and null by week 9 (Fig. S3B, Table S3).

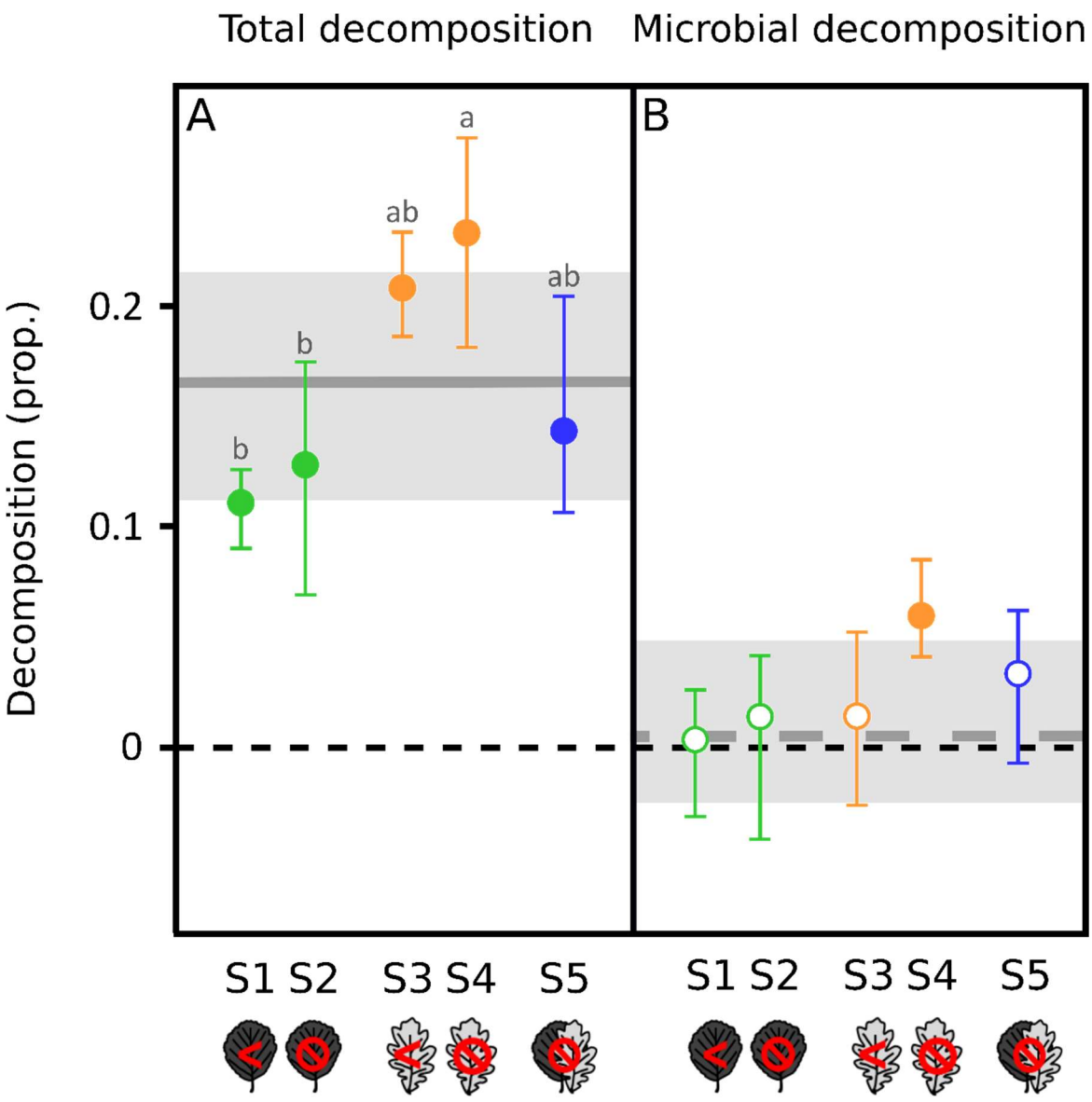

Fig. 1. Total (A) and microbial (B) decomposition (proportion of litter mass loss) at week 9. The mean value for the control is represented by a grey bar; mean values of scenarios by circles (green: S1 and S2; orange: S3 and S4; blue: S5); and upper and lower bounds of 95% nonparametric bootstrapped confidence intervals by grey shadowing (control) and whiskers (scenarios S1-S5). Open and closed circles (solid and dotted line in the control) represent intervals that do (null decomposition) and do not contain the value of zero (positive decomposition), respectively; and different letters indicate significant differences among scenarios.

Aquatic hyphomycetes

Sporulation rates ranged between 50 and 100 conidia mg^{-1} d^{-1} (Table S1), generally increasing with time (Table 2) and being higher in treatments with loss and reduction of oak and loss of both species (S3-S5) than in treatment with loss of alder (S2) at week 9, but without differences from the control (Fig. 3A, Table S3). At that time, only the scenarios S3-S5 showed a sporulation peak, with mean sporulation rates between 150 and 200 conidia mg^{-1} d^{-1}. The net diversity effect on sporulation was highly variable and did not show any clear pattern (Fig. S4A). Neither taxon richness nor its NDE varied with treatment or time (Fig. 3B, Fig. S4B, Table 2).

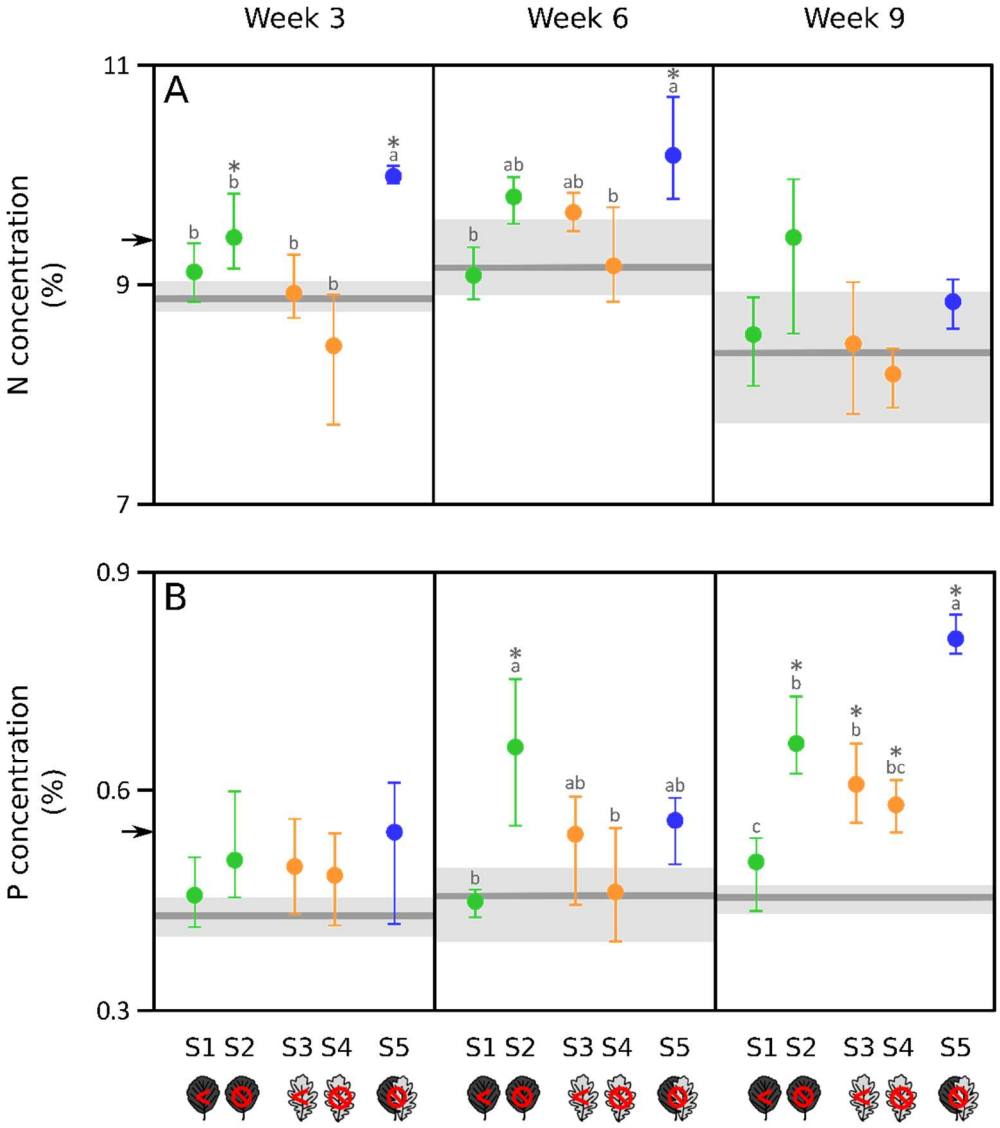

Fig. 2. *Sericostoma* N concentration (% DM; A) and P concentration (% DM; B) at weeks 3, 6 and 9. The mean value for the control is represented by a grey bar; mean values of scenarios by circles (green: S1 and S2; orange: S3 and S4; blue: S5); and upper and lower bounds of 95% nonparametric bootstrapped confidence intervals by grey shadowing (control) and whiskers (scenarios S1-S5). Asterisks indicate significant differences between a given scenario and the control; and different letters indicate significant differences among scenarios. Arrows represent initial *Sericostoma* N and P concentrations.

The AH assemblage structure varied with treatment and time, and the interaction between both factors was significant (Table 3). *Flagellospora curvula* was dominant in all cases (64-94%), and accompanying species (i.e., those with >5% of mean sporulation rate in a given treatment and sampling; Table S1) were *Articulospora tetracladia, Tetracladium marchalianum* DE WILD. and an unidentified hexaradiate. At week 3, the three species-loss scenarios (S2, S4 and S5) differed from the control, but the reduction scenarios (S1 and S3) did not. The control was characterized by *Heliscus lugdunensis* SACC. & THERRY (shared with S2) and *Articulospora tetracladia*. At week 6, there were no differences in assemblage structure among treatments. At week 9, assemblages in treatments with reduction and loss of oak and loss of both species (S3, S4 and S5) differed from the ones in the control and treatments with reduction and loss of alder (S1 and S2); however, assemblages in the treatment with loss of both species (S5) did not differ from the control. S4 was characterised by *Alatospora pulchella, A. tetracladia* and *Tetracladium marchalianum*, and it shared *Tetrachaetum elegans* INGOLD with the control and S3; and S5 presented as indicator species *Flagellospora curvula* (shared with S3*)* and unidentified hexaradiate (shared with control; Table 3, Table S1).

DISCUSSION

Alder and oak litter were key drivers of decomposition at the end of the experiment

Our comparison of five scenarios of reduction and loss of alder and oak rendered differences in total decomposition, although they only occurred at the latest stage of the experiment (i.e., week 9). Differences were evident between scenarios representing alder reduction or loss and that of oak loss, the latter showing faster decomposition. However, contrary to our expectations, none of these scenarios differed from the control. This may have been related to the duration of the experiment, which was probably short compared to certain conditions in highly retentive and flow-stable streams. Another explanation may be the simplified conditions of the experiment, as the presence of only one detritivore avoids interactions among species, which usually increase decomposition rates (Tonin et al. 2018). Some field studies conducted for 6-12 weeks have found lower decomposition rates in the absence of alder or higher in the absence of oak (Ferreira et al. 2012; McArthur et al. 1994), supporting our results.

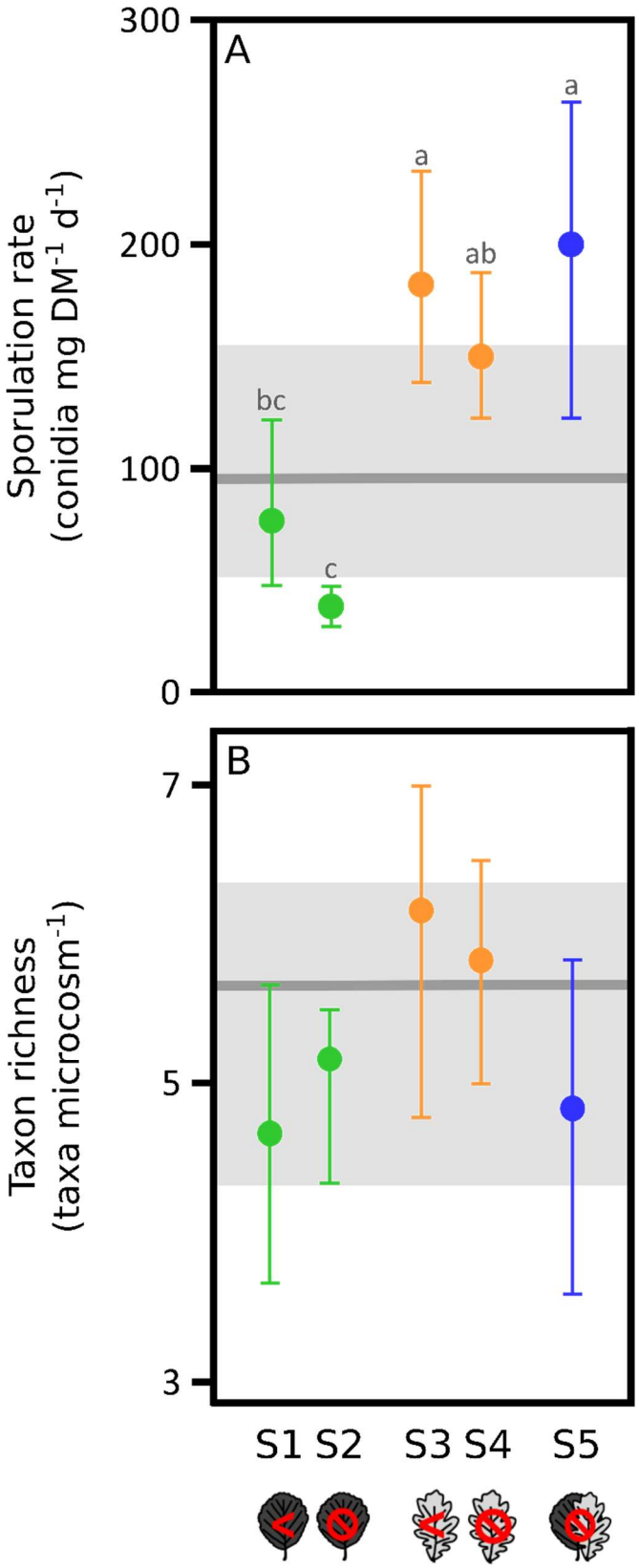

Fig. 3. Aquatic hyphomycete sporulation rate (conidia mg DM^{-1} d^{-1}; A) and taxon richness (taxa $microcosm^{-1}$; B) at week 9. The mean value for the control is represented by a grey bar; mean values of scenarios by circles (green: S1 and S2; orange: S3 and S4; blue: S5); and upper and lower bounds of 95% nonparametric bootstrapped confidence intervals by grey shadowing (control) and whiskers (scenarios S1-S5). Different letters indicate significant differences among scenarios.

Table 3. Results of PERMANOVAs exploring the effects of scenario (control and species loss scenarios S1-S5), time and their interaction, as well as the effects of scenario and significant paired comparisons at each sampling time, on aquatic hyphomycete assemblage structure; significant p-values are indicated in bold; df= degrees of freedom; F= F-statistic; R^2 = adjusted R^2; p= p-value; significant paired comparisons are the pairs of scenarios with significant differences obtained from a multilevel pairwise comparison.

Factor	df	F	R^2	p	Sign. paired comparisons
Scenario	5	2,76	0.1014	**0.003**	
Time	2	7.73	0.1136	**<0.001**	
Scenario × time	10	2.88	0.2119	**<0.001**	
Scenario week 3	5	2.00	0.1886	**0.036**	Control vs. S2, S4, S5; S1 vs. S4
Scenario week 6	5	1.14	0.1922	0.323	
Scenario week 9	5	5.70	0.4870	**<0.001**	Control vs. S3-S4; S1 vs. S3-S5; S2 vs. S3-S5

Remarkably, we found that differences in decomposition among scenarios did not arise from species loss per se, as the net diversity effect on decomposition in those scenarios was null by the end of the experiment; therefore, the most likely explanation for these differences was related to the traits of the species lost (alder and oak). The NDE on decomposition was mostly null or negative and decreased throughout the experiment (especially in the control and reduction scenarios), being mainly driven by a complementarity effect; this agrees with field studies (e.g., Handa et al. 2014), including one conducted with the same species used in our experiment (Alonso et al. 2021). Thus, the CE was negative in the control and reduction scenarios (S1 and S3) at week 9, indicating that physical or chemical interference among traits of different species occurred and reduced decomposition. The exception was the scenario where both alder and oak were lost, where the CE was positive at week 3 and then became null. This seems contradictory, as this scenario did not have the highest nutrient concentrations or SLA, which have been suggested as main causes for a positive CE (Handa et al. 2014; Larrañaga et al. 2020; López-Rojo et al. 2018; Santschi et al. 2018); however, it was the scenario with the lowest N:P ratio, which could have promoted a positive CE. The fact that the CE decreased with time also contradicted previous evidence (Alonso et al. 2021; Cardinale et al. 2007) and requires further exploration. The absence of differences in total decomposition between the treatments with reduction of oak or alder and the control may be due to experimental constrains (i.e., the reduced resources did not become limiting at any time along the experiment). It could also show that the presence of key species even in low amounts can affect decomposition rate, as it has been previously observed with alder (Larrañaga et al. 2020).

The contribution of microorganisms to decomposition was in general low or even negative, especially in the presence of oak, which is known to reduce microbial decomposition (Pérez et al. 2021b). The low values were possibly caused by the compensation of litter mass loss with microbial growth, especially early in the experiment. In contrast with other studies, both in field and laboratory, showing that plant diversity-decomposition relationships are mainly driven by detritivores (López-Rojo et al. 2018; López-Rojo et al. 2019; Sanpera-Calbet et al. 2009), we found negative diversity effects with patterns similar to those of total decomposition at the end of the experiment, showing that microorganisms can also be important drivers of diversity effects on decomposition (as suggested by Tonin et al. 2017). These NDEs were negligible at the first stages of the decomposition, suggesting that microbial responses are observed in longer studies, and thus they are not found in most short-term microcosm experiments. However, under usual conditions a great portion of leaf litter inputs only undergoes initial decomposition in the stream benthos before being transported downstream, incorporated into the sediments during periods of high sediment movement (e.g., spates), or emerged if the stream dries out (e.g., intermittent streams) (Graça et al. 2015; Tonin et al. 2021), making the initial litter decomposition phases the most relevant to assess (Pérez et al. 2021c).

Sericostoma used scarcer nutrients more efficiently

Sericostoma growth was highly variable within treatments and did not differ between the control and the scenarios, which contradicted our hypothesis but agreed with other studies in which detritivore growth did not respond to treatments of litter diversity (Lopez-Rojo et al. 2020; López-Rojo et al. 2019; Tonin et al. 2017) or quality (Larrañaga et al. 2020). It is possible that all the combinations provided enough resources for *Sericostoma* to achieve their maximum growth (Boersma and Elser 2006), which was within the range reported elsewhere for *Sericostoma* spp. (our experiment: 1.52% d-1 on average; other studies: 0.75-2.99% d-1; Friberg and Jacobsen 1999; Lopez-Rojo et al. 2020). *Sericostoma* growth might have been affected by species loss at higher detritivore densities or with lower litter availability, but this needs further exploration.

Despite the lack of differences in *Sericostoma* growth, we found that their stoichiometry varied between treatments and changed with time. Thus, at week 3, N concentration was higher in scenarios where alder or both species were lost than in the control. This sounds counterintuitive, given that alder litter had the highest N concentration.

This pattern indicated that *Sericostoma* N accumulation was favoured when this nutrient was less abundant and litter contained more recalcitrant compounds (Compson et al. 2015; Compson et al. 2018; Marks 2019; Siders et al. 2021), suggesting the existence of compensatory nutrient assimilation (Raubenheimer et al. 2007); this was likely achieved through the modification of the excretion of N compounds depending on its concentration on their food (Balseiro and Albariño 2006). Another explanation is that soluble compounds have leached slower in leaves with higher concentrations of recalcitrant compounds, as nutrients bound with them, allowing better nitrogen assimilation of nitrogen by invertebrates (Compson et al. 2015; Compson et al. 2018; Siders et al. 2021). However, these differences among treatments decreased throughout the experiment and disappeared at week 9, possibly because N demands are greater for larger individuals that are closer to moulting and pupating due to increased silk production (Frainer et al. 2016; Lopez-Rojo et al. 2020).

Patterns were similar for *Sericostoma* P concentration (i.e., P was higher in scenarios where alder or both species were lost than in the control), but in this case differences among treatments increased with time, as it has been observed in previous studies (Lopez-Rojo et al. 2020; López-Rojo et al. 2019). Despite the importance of P for ribosomal RNA production (Gillooly et al. 2005), this nutrient is needed in much lower concentrations than N (Frainer et al. 2016). The higher P concentrations in scenarios of species loss could be related to the necessity of maintaining N:P ratios constant (Raubenheimer et al. 2007), given that P concentrations in these scenarios was not particularly low or high.

There was an overall NDE on P concentration that increased throughout the experiment in some scenarios, suggesting that the higher P concentrations in these scenarios was caused by the interaction of different species in the mixture. It could be caused by nutrient transference among different species through the absorption of leached nutrients by fungi colonizing other species, allowing nutrient assimilation from leaves initially more palatable but scarcer in nutrients (Gessner et al. 2010). It is especially notable in the scenario with loss of both species, where N and P concentrations and their NDE showed the highest values, and was likely caused by complementarity among traits of hazel and willow (soft leaves with high SLA and low nutrient content in hazel, and recalcitrant leaves with medium nutrient content in willow), as this scenario also showed the highest complementarity effect in total decomposition.

Oak litter limited fungal sporulation

Regardless of the treatment, AH assemblages were structured around *Flagellospora curvula*, with few other taxa contributing to total sporulation rate. This matches previous work in our study area (Chauvet et al. 1997; Mendoza–Lera et al. 2012; Pérez et al. 2012), where *F. curvula* is an early colonizer of decomposing litter (Treton et al. 2004). Despite the absence of differences with the control, sporulation rate was lower for the scenario with alder loss than for scenarios where oak was reduced or lost, suggesting a limitation to sporulation (or a later sporulation peak) in the former scenario due to lower nutrient availability and/or higher presence of lignin and tannins (Ferreira et al. 2012; Kominoski et al. 2007; Pérez et al. 2021c).

AH assemblage structure was affected by treatment, but this effect changed throughout the experiment. At week 3, the AH assemblage structure differed between the control and scenarios with loss of one or both species, but not among these scenarios, suggesting an important role of litter diversity (Laitung and Chauvet 2005). However, differences among treatments were evident at week 9: assemblages in the control and the scenarios with reduction and loss of oak differed from the scenarios with reduction and loss of alder and the one with loss of both species, with one exception (no differences between the control and the scenario with loss of both species). This supports previous indications that litter traits are main drivers of AH assemblage structure (Alonso et al. 2021; Gulis et al. 2020) and suggests that these effects on microbial variables are more evident in the longer term, as we observed for the NDE on microbial decomposition.

Aquatic hyphomycetes were more influenced by the presence of oak, as assemblage structure in control was similar to the ones in scenarios with loss and reduction of alder, and the scenario where both species were lost was similar to those in the scenarios of oak loss or reduction. It is likely caused by a greater inhibitory effect of the tannins present in oak leaves, than the stimulation induced by the higher nutrient amount of alder leaves. In contrast, the mixture containing only willow and hazel possibly contained enough nutrients to produce the maximum sporulation and maintained a conidial assemblage similar to the control even in the absence of alder (Alonso et al. 2021).

The importance of real-case studies

Previous studies allow the elaboration of likely predictions for current species loss outcomes, as it has been observed on this study, where results of decomposition and aquatic hyphomycete assemblages were consistent with the hypotheses predicted from more

general studies regarding random species loss and effects of specific leaf traits (Ferreira et al. 2012; Kominoski et al. 2007; Larrañaga et al. 2020; López-Rojo et al. 2018; McArthur et al. 1994; Pérez et al. 2021c), despite doing so in the form of non-significant trends. However, in the cases when the loss of more than one species is considered, predictions based on more general research are more difficult, due to the loss of different traits (López-Rojo et al. 2018). Thus, here we observed that the loss of alder and oak (which strongly differ in litter traits) counteracted their effects on total decomposition, as was expected based on hypotheses derived from previous studies of random species loss. In contrast, fungal sporulation rate responded to the loss of both species similarly to the loss of oak, highlighting the key role of this species for fungal assemblages. Therefore, we can conclude that, despite the usefulness of general research to predict species loss effects, real case studies are important due to their higher accuracy for scenarios where more than one species is lost or when species are affected by other stressors.

CONCLUSIONS

We showed that plausible scenarios of alder and/or oak loss caused by fungal infections could alter stream ecosystem functioning through changes in rates of litter decomposition, detritivore nutrient assimilation and fungal sporulation. These changes were most likely related to the specific traits of alder and oak litter: the loss of the highly nutritious and palatable alder litter (and the concomitant increase in the relative abundance of other species) resulted in reduced total decomposition and sporulation compared to the scenario with loss of oak. One of our most remarkable results was the effect of the different scenarios on the assimilation efficiency of N and P by *Sericostoma*, which were higher in scenarios with alder loss or reduction; this suggested the existence of compensatory nutrient assimilation in conditions with scarcer nutrients and higher concentrations of refractory compounds. Importantly, the consequences of alder and oak loss changed with time, mostly in relation to detritivores, which usually meet different nutritional requirements at different developmental stages (Back and King 2013) and here adapted their rates of nutrient assimilation to those requirements and the different types of litter available. Such changes are likely to translate into different rates of nutrient excretion and hence to be important for nutrient cycling at the ecosystem level (Atkinson et al. 2017). Overall, our results provide evidence that preservation of native riparian vegetation diversity would be advisable for maintaining normal stream function.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL

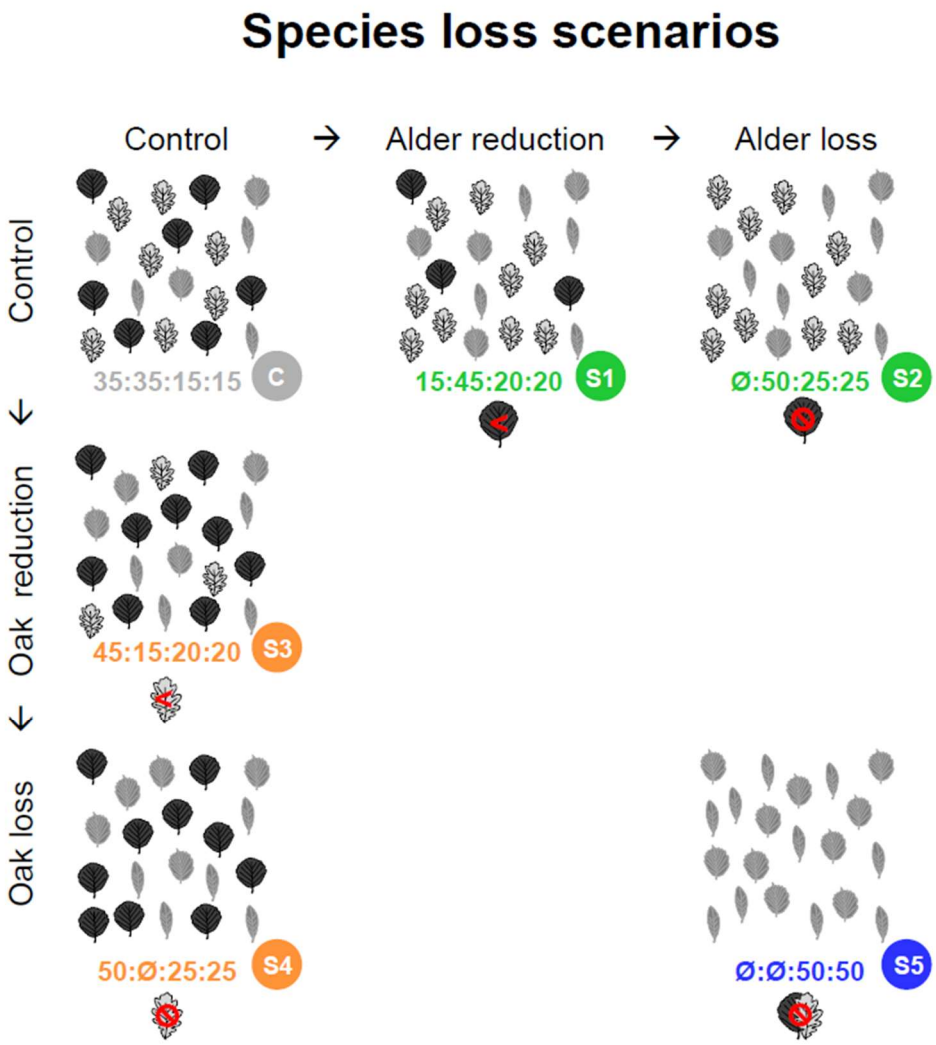

Fig. S1. Schematic diagram of the experimental design showing the proportion of each species (alder, oak, hazel and willow) in each scenario (control and species loss scenarios S1-S5), bottom numbers represent percentage contribution of each litter (Alder:Oak:Hazel:Willow) in terms of DM.

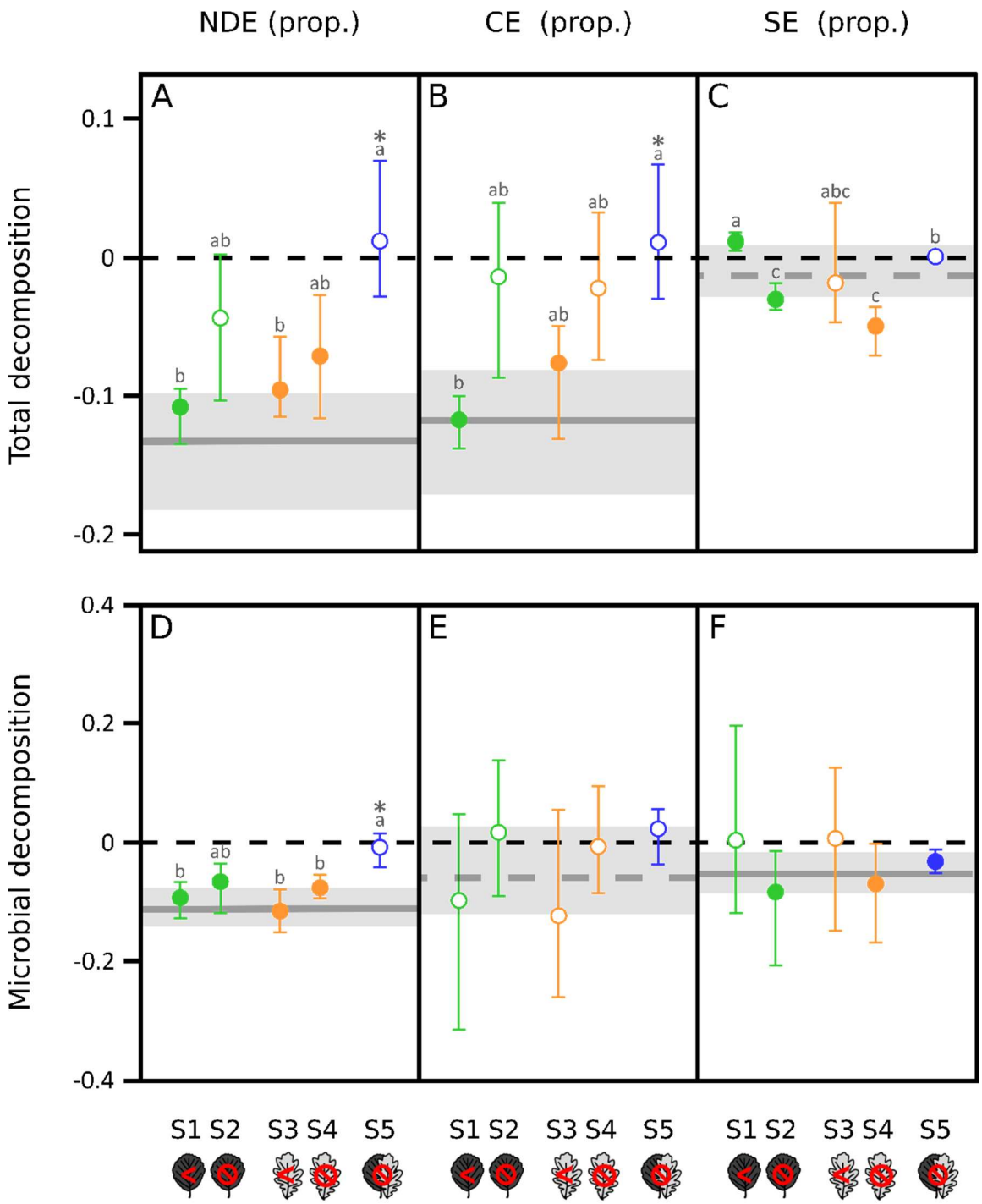

Fig. S2. Net diversity, complementarity and selection effects on total (A, B, C) and microbial (D, E, F) decomposition (proportion of litter mass loss) at week 9. The mean value for the control is represented by a grey bar; mean values of scenarios by circles (green: S1 and S2; orange: S3 and S4; blue: S5); and upper and lower bounds of 95% nonparametric bootstrapped confidence intervals by grey shadowing (control) and whiskers (scenarios S1-S5). Open and closed circles represent intervals that do (no net, complementarity or selection effect) and do not contain the value of zero (net, complementarity or selection effect), respectively; asterisks indicate significant differences between a given scenario and with control; and different letters indicate significant differences among scenarios.

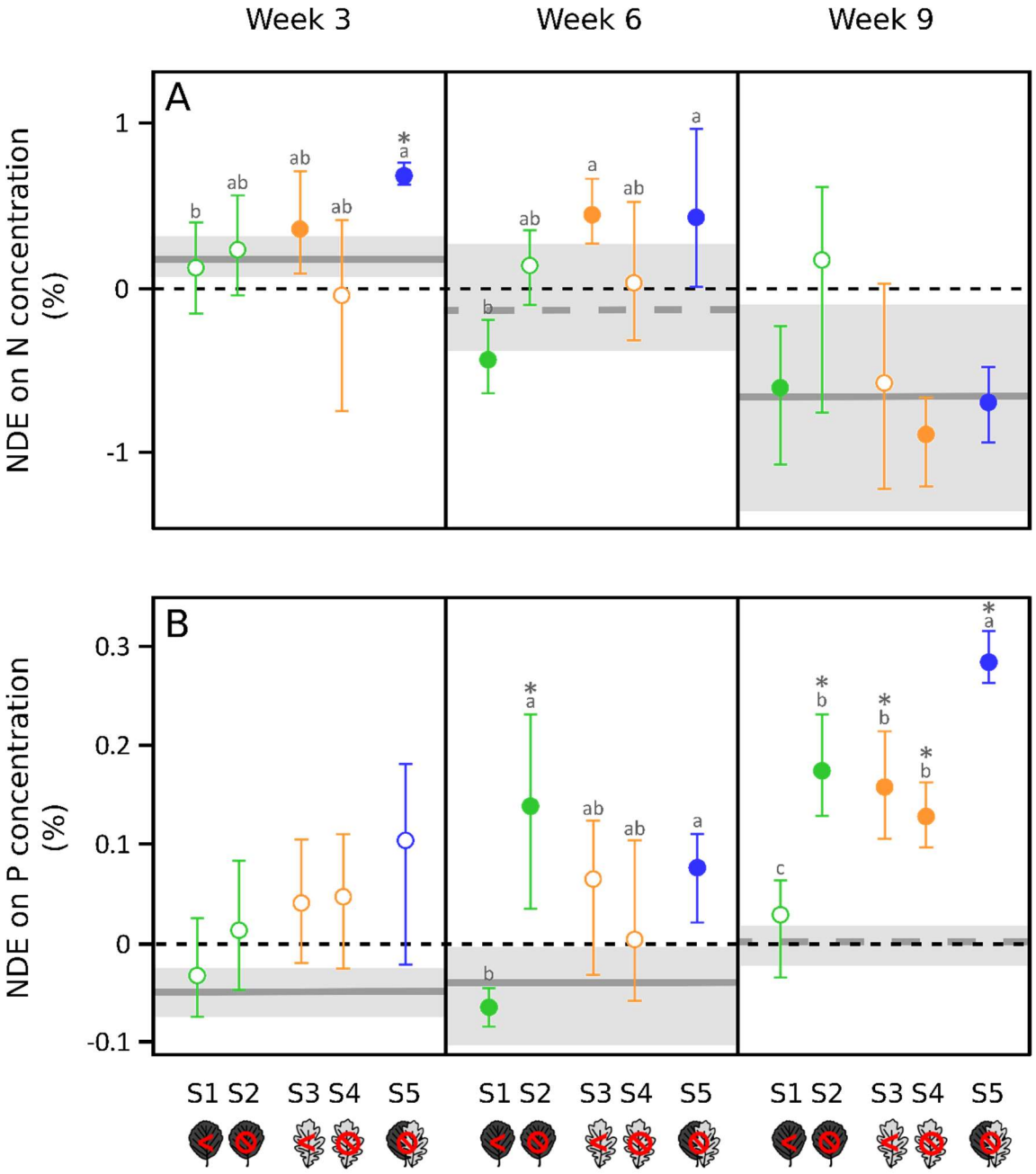

Fig. S3. Net diversity effect on *Sericostoma* N (A) and P concentration (% DM; B) at weeks 3, 6 and 9. The mean value for the control is represented by a grey bar; mean values of scenarios by circles (green: S1 and S2; orange: S3 and S4; blue: S5); and upper and lower bounds of 95% nonparametric bootstrapped confidence intervals by grey shadowing (control) and whiskers (scenarios S1-S5). Open and closed circles represent intervals that do (no net, complementarity or selection effect) and do not contain the value of zero (net, complementarity or selection effect), respectively; asterisks indicate significant differences between a given scenario and with control; and different letters indicate significant differences among scenarios.

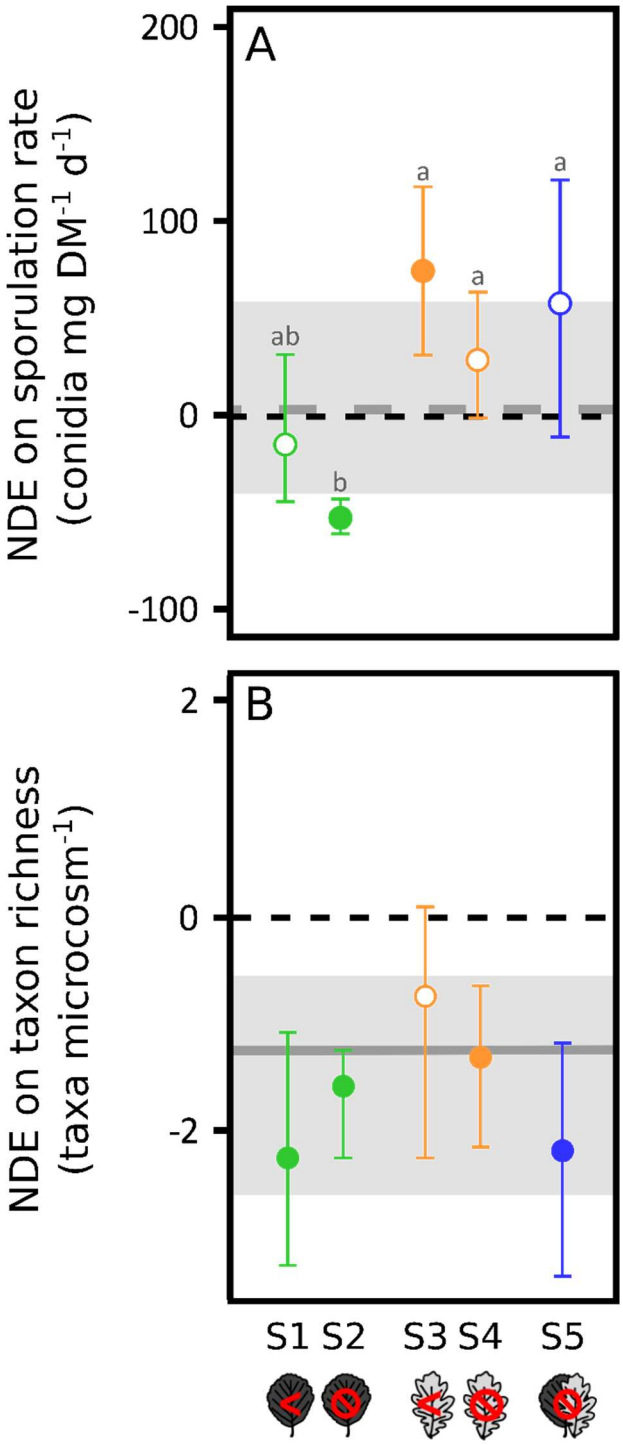

Fig. S4. Net diversity effect on aquatic hyphomycete sporulation rate (conidia mg DM^{-1} d^{-1}; A) and on taxon richness (taxa $microcosm^{-1}$; B) at week 9. The mean value for the control is represented by a grey bar; mean values of scenarios by circles (green: S1 and S2; orange: S3 and S4; blue: S5); and upper and lower bounds of 95% nonparametric bootstrapped confidence intervals by grey shadowing (control) and whiskers (scenarios S1-S5). Open and closed circles represent intervals that do (no net, complementarity or selection effect) and do not contain the value of zero (net, complementarity or selection effect), respectively; and different letters indicate significant differences among scenarios.

Table S1: Relative contribution (prop.) of each aquatic hyphomycete sporulating taxon to sporulation rate in each scenario (control and species loss scenarios S1-S5) × time combination (3, 6 and 9 weeks). Mean sporulation rate and accumulated taxon richness are given at the bottom of the table. Bold type represents species with a contribution to total sporulation higher than 5%. The second column (Inoc.) represents the mean composition of the initial microbial inoculum (% of ca. 1000 conidia added to each microcosm on day 0).

Scenario	Inoc.	Control			S1			S2		
Time	Day 0	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9
Alatospora acuminata INGOLD	11.8		0.1					0.1	0.1	0.1
Alatospora pulchella MARVANOVÁ	**26.2**			0.2		0.1	0.2			0.2
Anguillospora filiformis GREATH.		2.6		0.7	1.5	0.2	1.4	1.4	0.2	
Articulospora tetracladia INGOLD	**16.2**	**6.4**	4.1	3.5	1.3	4.0	3.1	3.7	4.1	4.4
Clavariopsis aquatica DE WILD.	0.4	0.1		0.1						0.1
Flagellospora curvula INGOLD	**34.5**	**70.9**	**91.4**	**90.1**	**77.9**	**89.0**	**91.9**	**81.0**	**87.7**	**90.6**
Heliscus lugdunensis SACC. & THERRY		0.6	0.4	0.2			0.1	0.4	0.7	0.2
Lemonniera terrestris TUBAKI	0.9				0.2					0.4
Lunulospora curvula INGOLD	1.3	0.1								
Tetrachaetum elegans INGOLD	2.2	0.9	1.4	3.6	1.0	1.2	0.5	1.3	0.4	0.7
Tetracladium marchalianum DE WILD.	3.9	**13.4**	2.5	1.5	2.7	4.5	2.8	9.8	5.5	3.3
Tripospermum camelopardus †	0.9	0.2			0.3					
Tripospermum myrti (LIND) S. HUGHES	0.9		0.1			0.1			0.1	0.1
Unidentified hexaradiate		4.9	0.1	0.2	**15.1**	0.9		2.3	1.2	
Sporulation rate (conidia mg DM^{-1} d^{-1})		98.8 ± 11.2	114.9 ± 23.7	95.8 ± 28.2	77.7 ± 15.4	126.8 ± 30.2	77.1 ± 20.4	81.5 ± 51.5	78.6 ± 22.8	38.9 ± 5.0
Accumulated taxon richness	11	10	8	9	8	8	7	8	9	10

† INGOLD, DANN & P.J. MCDOUGALL

Table S1. Cont.

Scenario	S3			S4			S5		
Time	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9
A. acuminata			0.1			0.1			
A. pulchella			0.1			0.8	0.2		0.1
A. filiformis	2.0		0.1	0.4			0.3	0.4	
A. tetracladia	2.1	3.0	**7.5**	2.6	1	**18.6**	2.5	3.9	**5.4**
C. aquatica									
F. curvula	**77.8**	**89.8**	**85.5**	**63.7**	**94.3**	**72.0**	**81.5**	**79.2**	**91.6**
H. lugdunensis		0.8	0.8		0.4	0.5	0.2	0.2	0.1
L. terrestris							0.2		
L. curvula	0.1			0.1					
T. elegans	2.1	1.2	3.7	2.4	1	2.4	0.5	1.3	0.3
T. marchalianum	**11.6**	4.7	2.1	7.4	3.1	**5.6**	**11.1**	**13.5**	2.4
T. camelopardus	0.1	0.2		0.5			0.1		
T. myrti	0.2			0.1	0.1		0.3	0.1	
Unidentified hexaradiate	4.0	0.3		**22.8**	0.1		3.1	1.5	0.1
Sporulation rate	70.8 ± 15.7	85.0 ± 12.8	182.5 ± 26.0	43.8 ± 11.4	79.8 ± 16.8	150.3 ± 18.0	48.9 ± 14.2	67.0 ± 6.2	200.3 ± 37.2
Accumulated taxon richness	9	7	8	9	7	7	11	8	7

Table S2. Results of linear models exploring the effects of scenario (control and species loss scenarios S1-S5) on the net diversity, complementarity and selection effects (NDE, CE and SE, respectively) for each variable (see Table 2) at each sampling time (3, 6 and 9 weeks).; df= degrees of freedom; F= F-statistic; p= p-value.

	Week 3			Week 6			Week 9		
Variable	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Total decomposition	5, 23	2.21	0.088	5, 24	1.34	0.281	5, 28	3.57	0.013
NDE	5, 23	9.08	<0.001	5, 24	12.83	<0.001	5, 26	14.22	<0.001
CE	5, 23	15.89	<0.001	5, 24	3.02	0.030	5, 26	4.08	0.007
SE	5, 23	9.47	<0.001	5, 24	4.20	0.007	5, 26	4.54	0.004
Microbial decomposition	5, 24	6.46	<0.001	5, 22	2.67	0.049	5, 30	1.38	0.259
NDE	5, 22	6.18	0.001	5, 20	1.02	0.005	5, 27	0.56	0.001
CE	5, 22	4.04	0.009	5, 20	0.67	0.642	5, 27	1.42	0.250
SE	5, 22	5.10	0.003	5, 20	4.86	0.431	5, 27	5.67	0.727
Sericostoma growth	5, 24	1.45	0.244	5, 24	0.19	0.965	5, 29	2.93	0.029
NDE	5, 24	1.61	0.197	5, 24	0.21	0.954	5, 30	0.12	0.988
Sericostoma N	5, 24	43.38	<0.001	5, 23	5.66	0.002	5, 29	2.47	0.056
NDE	5, 24	11.44	<0.001	5, 23	3.27	0.022	5, 29	1.62	0.187
Sericostoma P	5, 19	1.23	0.334	5, 19	5.00	0.004	5, 28	34.68	<0.001
NDE	5, 19	2.39	0.076	5, 19	4.30	0.009	5, 28	23.39	<0.001
AH sporulation rate	5, 24	3.42	0.018	5, 24	0.94	0.474	5, 30	8.15	0.001
NDE	5, 24	5.00	0.005	5, 24	3.20	0.024	5, 30	11.00	<0.001
AH taxon richness	5, 24	0.77	0.579	5, 24	0.17	0.971	5, 30	1.28	0.325
NDE	5, 24	1.02	0.426	5, 24	0.34	0.882	5, 30	1.16	0.351

Table S3. Results of Tukey test exploring the differences among scenarios (control and species loss scenarios S1-S5) for each variable (see table S2) at each sampling time (3, 6 and 9 week) when scenario was significant (see Table S2). Z= Z-value, p= p-value.

	Week 3			Week 6			Week 9		
Variable	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**
Total decomposition							Control:S1	-1.443	0.699
							Control:S2	-1.104	0.879
							Control:S3	1.261	0.805
							Control:S4	1.999	0.341
							Control:S5	-0.651	0.987
							S1:S2	0.456	0.998
							S1:S3	2.572	0.104
							S1:S4	3.231	0.016
							S1:S5	0.861	0.956
							S2:S3	2.365	0.168
							S2:S4	3.103	0.024
							S2:S5	0.453	0.998
							S3:S4	0.737	0.977
							S3:S5	-1.913	0.393
							S4:S5	-2.650	0.085
NDE on total decomposition	Control:S1	-1.882	0.413	Control:S1	0.767	0.973	Control:S1	0.652	0.987
	Control:S2	-1.433	0.706	Control:S2	2.670	0.081	Control:S2	2.589	0.099
	Control:S3	0.424	0.998	Control:S3	1.442	0.701	Control:S3	1.037	0.905
	Control:S4	2.088	0.293	Control:S4	2.669	0.082	Control:S4	1.795	0.468
	Control:S5	3.853	0.002	Control:S5	3.953	0.001	Control:S5	4.212	<0.001
	S1:S2	0.531	0.995	S1:S2	1.902	0.400	S1:S2	1.751	0.497
	S1:S3	2.282	0.201	S1:S3	0.675	0.985	S1:S3	0.325	1.000
	S1:S4	3.851	0.002	S1:S4	1.901	0.401	S1:S4	1.006	0.916
	S1:S5	5.515	<0.001	S1:S5	3.186	0.018	S1:S5	3.274	0.014
	S2:S3	1.858	0.428	S2:S3	-1.228	0.823	S2:S3	-1.506	0.659
	S2:S4	3.522	0.006	S2:S4	-0.001	1.000	S2:S4	-0.833	0.961
	S2:S5	5.286	<0.001	S2:S5	1.283	0.794	S2:S5	1.703	0.529
	S3:S4	1.664	0.555	S3:S4	1.226	0.824	S3:S4	0.712	0.981
	S3:S5	3.429	0.008	S3:S5	2.511	0.121	S3:S5	3.130	0.022
	S4:S5	1.765	0.488	S4:S5	1.285	0.794	S4:S5	2.536	0.113

Table S3. Cont.

	Week 3			Week 6			Week 9		
Variable	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**
CE on total decomposition	Control:S1	0.192	1.000	Control:S1	0.768	0.973	Control:S1	0.033	1.000
	Control:S2	3.343	0.008	Control:S2	3.285	0.013	Control:S2	2.759	0.064
	Control:S3	-0.238	1.000	Control:S3	0.972	0.927	Control:S3	1.114	0.876
	Control:S4	0.182	1.000	Control:S4	1.760	0.492	Control:S4	2.572	0.104
	Control:S5	7.606	< 0.001	Control:S5	2.620	0.092	Control:S5	3.426	0.008
	S1:S2	3.977	< 0.001	S1:S2	2.517	0.119	S1:S2	2.554	0.108
	S1:S3	-0.973	0.904	S1:S3	0.205	1.000	S1:S3	1.017	0.912
	S1:S4	0.127	1.000	S1:S4	0.992	0.921	S1:S4	2.379	0.163
	S1:S5	7.804	< 0.001	S1:S5	1.852	0.432	S1:S5	3.180	0.018
	S2:S3	-4.270	< 0.001	S2:S3	-2.312	0.189	S2:S3	-1.596	0.600
	S2:S4	-1.422	0.657	S2:S4	-1.525	0.648	S2:S4	-0.196	1.000
	S2:S5	6.037	< 0.001	S2:S5	-0.664	0.986	S2:S5	0.700	0.982
	S3:S4	0.269	1.000	S3:S4	0.788	0.970	S3:S4	1.409	0.721
	S3:S5	7.915	< 0.001	S3:S5	1.648	0.567	S3:S5	2.263	0.209
	S4:S5	5.851	< 0.001	S4:S5	0.860	0.956	S4:S5	0.896	0.947
SE on total decomposition	Control:S1	-1.590	0.565	Control:S1	-0.072	1.000	Control:S1	2.455	0.112
	Control:S2	-1.588	0.567	Control:S2	-2.055	0.273	Control:S2	-1.560	0.571
	Control:S3	0.423	0.998	Control:S3	0.746	0.970	Control:S3	-0.250	1.000
	Control:S4	1.141	0.843	Control:S4	1.334	0.730	Control:S4	-2.761	0.050
	Control:S5	-1.506	0.622	Control:S5	1.799	0.421	Control:S5	1.479	0.628
	S1:S2	-0.422	0.998	S1:S2	-2.641	0.073	S1:S2	-6.362	<0.001
	S1:S3	4.636	<0.001	S1:S3	1.178	0.820	S1:S3	-1.408	0.675
	S1:S4	3.052	0.022	S1:S4	2.112	0.245	S1:S4	-6.020	<0.001
	S1:S5	0.316	1.000	S1:S5	2.966	0.029	S1:S5	-2.775	0.048
	S2:S3	2.791	0.049	S2:S3	4.344	<0.001	S2:S3	0.502	0.995
	S2:S4	2.779	0.050	S2:S4	5.601	<0.001	S2:S4	-1.826	0.395
	S2:S5	0.606	0.989	S2:S5	6.865	<0.001	S2:S5	5.539	<0.001
	S3:S4	1.141	0.843	S3:S4	1.131	0.844	S3:S4	-1.331	0.725
	S3:S5	-5.550	<0.001	S3:S5	2.334	0.154	S3:S5	0.926	0.924
	S4:S5	-3.024	0.024	S4:S5	1.348	0.721	S4:S5	5.277	<0.001

Table S3. Cont.

	Week 3			Week 6			Week 9		
Variable	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**
Microbial decomposition	Control:S1	0.081	1.000	Control:S1	-1.050	0.901			
	Control:S2	-2.337	0.160	Control:S2	-0.454	0.998			
	Control:S3	0.286	1.000	Control:S3	0.768	0.973			
	Control:S4	0.256	1.000	Control:S4	-1.362	0.749			
	Control:S5	0.738	0.974	Control:S5	1.552	0.630			
	S1:S2	-4.743	<0.001	S1:S2	0.632	0.987			
	S1:S3	0.298	1.000	S1:S3	1.928	0.384			
	S1:S4	0.401	0.998	S1:S4	-0.386	0.989			
	S1:S5	1.153	0.843	S1:S5	2.759	0.064			
	S2:S3	2.502	0.109	S2:S3	1.296	0.787			
	S2:S4	4.918	<0.001	S2:S4	-0.982	0.924			
	S2:S5	4.557	<0.001	S2:S5	2.127	0.273			
	S3:S4	-0.135	1.000	S3:S4	-2.204	0.235			
	S3:S5	0.342	0.999	S3:S5	0.831	0.962			
	S4:S5	0.856	0.951	S4:S5	2.988	0.033			
NDE on microbial decomposition	Control:S1	-0.143	1.000	Control:S1	-0.732	0.978	Control:S1	0.756	0.975
	Control:S2	-2.779	0.060	Control:S2	-0.318	1.000	Control:S2	1.806	0.461
	Control:S3	1.355	0.753	Control:S3	1.247	0.812	Control:S3	-0.148	1.000
	Control:S4	0.673	0.985	Control:S4	-0.408	0.999	Control:S4	1.471	0.683
	Control:S5	2.012	0.335	Control:S5	3.278	0.013	Control:S5	4.289	<0.001
	S1:S2	-2.796	0.058	S1:S2	0.474	0.997	S1:S2	1.050	0.901
	S1:S3	1.572	0.617	S1:S3	1.911	0.394	S1:S3	-0.937	0.937
	S1:S4	0.866	0.955	S1:S4	0.354	0.999	S1:S4	0.682	0.984
	S1:S5	2.286	0.199	S1:S5	3.777	0.002	S1:S5	3.500	0.006
	S2:S3	4.208	<0.001	S2:S3	1.660	0.557	S2:S3	-2.034	0.323
	S2:S4	3.661	0.003	S2:S4	-0.112	1.000	S2:S4	-0.415	0.998
	S2:S5	5.082	<0.001	S2:S5	3.814	0.002	S2:S5	2.403	0.155
	S3:S4	-0.756	0.975	S3:S4	-1.677	0.545	S3:S4	1.698	0.533
	S3:S5	0.584	0.992	S3:S5	2.154	0.258	S3:S5	4.654	<0.001
	S4:S5	1.421	0.714	S4:S5	3.708	0.003	S4:S5	2.956	0.037

Table S3. Cont.

	Week 3			Week 6			Week 9		
Variable	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**
CE on microbial decomposition	Control:S1	-0.298	1.000						
	Control:S2	-2.938	0.033						
	Control:S3	-1.728	0.482						
	Control:S4	1.810	0.428						
	Control:S5	-2.023	0.301						
	S1:S2	-2.787	0.052						
	S1:S3	-1.479	0.650						
	S1:S4	1.993	0.317						
	S1:S5	-1.908	0.367						
	S2:S3	1.918	0.361						
	S2:S4	3.604	0.004						
	S2:S5	-0.143	1.000						
	S3:S4	2.762	0.055						
	S3:S5	-1.343	0.738						
	S4:S5	-2.896	0.038						
SE on microbial decomposition	Control:S1	0.065	1.000						
	Control:S2	0.479	0.997						
	Control:S3	1.436	0.704						
	Control:S4	-1.356	0.753						
	Control:S5	3.099	0.024						
	S1:S2	0.439	0.998						
	S1:S3	1.449	0.697						
	S1:S4	-1.507	0.659						
	S1:S5	3.217	0.017						
	S2:S3	1.035	0.906						
	S2:S4	-1.946	0.373						
	S2:S5	2.779	0.061						
	S3:S4	-2.870	0.047						
	S3:S5	1.585	0.608						
	S4:S5	4.725	<0.001						

Table S3. Cont.

	Week 3			Week 6			Week 9		
Variable	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**
Sericostoma growth							Control:S1	-1.226	0.824
							Control:S2	-1.155	0.858
							Control:S3	0.072	1.000
							Control:S4	0.701	0.982
							Control:S5	-2.642	0.087
							S1:S2	0.014	1.000
							S1:S3	1.298	0.786
							S1:S4	1.927	0.385
							S1:S5	-1.416	0.717
							S2:S3	1.224	0.825
							S2:S4	1.823	0.451
							S2:S5	-1.365	0.748
							S3:S4	0.629	0.989
							S3:S5	-2.715	0.072
							S4:S5	-3.343	0.011
Sericostoma N	Control:S1	1.399	0.692	Control:S1	-0.258	1.000			
	Control:S2	2.808	0.047	Control:S2	2.347	0.175			
	Control:S3	0.297	1.000	Control:S3	1.831	0.445			
	Control:S4	-1.248	0.784	Control:S4	0.055	1.000			
	Control:S5	13.255	<0.001	Control:S5	3.723	0.003			
	S1:S2	1.276	0.768	S1:S2	2.764	0.063			
	S1:S3	-0.828	0.955	S1:S3	2.216	0.230			
	S1:S4	-1.812	0.416	S1:S4	0.332	0.999			
	S1:S5	5.222	<0.001	S1:S5	4.223	<0.001			
	S2:S3	-2.021	0.292	S2:S3	-0.548	0.994			
	S2:S4	-2.575	0.087	S2:S4	-2.432	0.145			
	S2:S5	2.911	0.035	S2:S5	1.459	0.690			
	S3:S4	-1.286	0.762	S3:S4	-1.884	0.412			
	S3:S5	6.141	<0.001	S3:S5	2.007	0.338			
	S4:S5	4.567	<0.001	S4:S5	3.891	0.001			

Table S3. Cont.

	Week 3			Week 6			Week 9		
Variable	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**
NDE on *Sericostoma* N	Control:S1	-0.281	1.000	Control:S1	-1.086	0.887			
	Control:S2	0.304	1.000	Control:S2	0.970	0.928			
	Control:S3	1.022	0.893	Control:S3	2.078	0.299			
	Control:S4	-0.648	0.984	Control:S4	0.593	0.992			
	Control:S5	6.646	<0.001	Control:S5	2.026	0.327			
	S1:S2	0.443	0.997	S1:S2	2.180	0.247			
	S1:S3	0.996	0.903	S1:S3	3.356	0.010			
	S1:S4	-0.456	0.997	S1:S4	1.780	0.478			
	S1:S5	3.295	0.010	S1:S5	3.300	0.013			
	S2:S3	0.511	0.995	S2:S3	1.175	0.849			
	S2:S4	-0.735	0.972	S2:S4	-0.400	0.999			
	S2:S5	2.425	0.124	S2:S5	1.120	0.873			
	S3:S4	-1.088	0.865	S3:S4	-1.575	0.615			
	S3:S5	1.866	0.379	S3:S5	-0.056	1.000			
	S4:S5	2.187	0.210	S4:S5	1.520	0.651			
Sericostoma P				Control:S1	-0.142	1.000	Control:S1	1.595	0.601
				Control:S2	3.537	0.005	Control:S2	6.695	<0.001
				Control:S3	1.359	0.750	Control:S3	4.889	<0.001
				Control:S4	0.093	1.000	Control:S4	4.184	<0.001
				Control:S5	1.865	0.422	Control:S5	11.789	<0.001
				S1:S2	4.182	<0.001	S1:S2	5.174	<0.001
				S1:S3	1.661	0.555	S1:S3	3.368	0.010
				S1:S4	0.271	1.000	S1:S4	2.589	0.100
				S1:S5	2.318	0.185	S1:S5	10.194	<0.001
				S2:S3	-2.084	0.293	S2:S3	-1.729	0.512
				S2:S4	-3.926	0.001	S2:S4	-2.706	0.074
				S2:S5	-1.996	0.342	S2:S5	4.545	<0.001
				S3:S4	-1.426	0.709	S3:S4	-0.900	0.947
				S3:S5	0.346	0.999	S3:S5	6.351	<0.001
				S4:S5	2.046	0.314	S4:S5	7.605	<0.001

Table S3. Cont.

	Week 3			Week 6			Week 9		
Variable	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**
NDE on *Sericostoma* P				Control:S1	-0.452	0.998	Control:S1	0.906	0.945
				Control:S2	3.068	0.026	Control:S2	5.482	<0.001
				Control:S3	1.685	0.539	Control:S3	4.971	<0.001
				Control:S4	0.780	0.971	Control:S4	4.224	<0.001
				Control:S5	2.087	0.292	Control:S5	9.410	<0.001
				S1:S2	3.986	<0.001	S1:S2	4.618	<0.001
				S1:S3	2.336	0.178	S1:S3	4.107	<0.001
				S1:S4	1.423	0.711	S1:S4	3.318	0.012
				S1:S5	2.932	0.039	S1:S5	8.504	<0.001
				S2:S3	-1.267	0.802	S2:S3	-0.489	0.997
				S2:S4	-2.644	0.086	S2:S4	-1.455	0.693
				S2:S5	-1.221	0.825	S2:S5	3.490	0.006
				S3:S4	-1.103	0.879	S3:S4	-0.944	0.935
				S3:S5	0.203	1.000	S3:S5	4.000	<0.001
				S4:S5	1.509	0.657	S4:S5	5.186	<0.001
AH sporulation rate	Control:S1	-1.321	0.753				Control:S1	-0.600	0.991
	Control:S2	-1.513	0.629				Control:S2	-2.272	0.205
	Control:S3	-1.633	0.548				Control:S3	2.590	0.100
	Control:S4	-3.342	0.009				Control:S4	2.046	0.317
	Control:S5	-2.342	0.160				Control:S5	2.698	0.076
	S1:S2	-0.881	0.945				S1:S2	-1.672	0.550
	S1:S3	-0.400	0.998				S1:S3	3.189	0.018
	S1:S4	-1.976	0.330				S1:S4	2.645	0.087
	S1:S5	-1.472	0.657				S1:S5	3.298	0.013
	S2:S3	0.636	0.987				S2:S3	4.862	<0.001
	S2:S4	-0.310	1.000				S2:S4	4.318	<0.001
	S2:S5	-0.259	1.000				S2:S5	4.970	<0.001
	S3:S4	-1.498	0.640				S3:S4	-0.544	0.994
	S3:S5	-1.142	0.849				S3:S5	0.109	1.000
	S4:S5	0.025	1.000				S4:S5	0.652	0.987

Table S3. Cont.

	Week 3			Week 6			Week 9		
Variable	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**	**Pair**	**Z**	**p**
NDE on AH sporulation rate	Control:S1	-0.644	0.986	Control:S1	0.663	0.986	Control:S1	-0.503	0.995
	Control:S2	-0.150	1.000	Control:S2	-0.910	0.944	Control:S2	-1.967	0.332
	Control:S3	-1.940	0.353	Control:S3	-1.548	0.633	Control:S3	1.876	0.386
	Control:S4	-4.421	<0.001	Control:S4	-2.190	0.243	Control:S4	0.799	0.962
	Control:S5	-2.630	0.080	Control:S5	-2.585	0.101	Control:S5	1.148	0.843
	S1:S2	0.093	1.000	S1:S2	-1.572	0.617	S1:S2	-1.838	0.409
	S1:S3	-1.156	0.844	S1:S3	-2.211	0.233	S1:S3	2.741	0.060
	S1:S4	-3.112	0.020	S1:S4	-2.852	0.049	S1:S4	1.633	0.544
	S1:S5	-1.719	0.494	S1:S5	-3.248	0.015	S1:S5	1.689	0.506
	S2:S3	-0.592	0.991	S2:S3	-0.638	0.988	S2:S3	5.047	<0.001
	S2:S4	-1.310	0.762	S2:S4	-1.280	0.796	S2:S4	4.542	<0.001
	S2:S5	-0.814	0.961	S2:S5	-1.675	0.548	S2:S5	2.992	0.029
	S3:S4	-1.760	0.466	S3:S4	-0.641	0.988	S3:S4	-1.446	0.669
	S3:S5	-0.509	0.995	S3:S5	-1.037	0.906	S3:S5	-0.404	0.998
	S4:S5	1.262	0.789	S4:S5	-0.395	0.999	S4:S5	0.649	0.985

Chapter 3

DIEBACK AND REPLACEMENT OF RIPARIAN TREES MAY IMPACT STREAM ECOSYSTEM FUNCTIONING

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ABSTRACT

Alders are nitrogen (N)-fixing riparian trees that promote leaf litter decomposition in streams through their high-nutrient leaf litter inputs. While alders are widespread across Europe, their populations are at risk due to infection by the oomycete *Phytophthora ×alni*, which causes alder dieback. Moreover, alder death opens space for the establishment of an aggressive N-fixing invasive species, the black locust (*Robinia pseudoacacia*). Shifts from riparian vegetation containing healthy to infected alder and, eventually, alder loss and replacement with black locust, may alter the key process of leaf litter decomposition and associated microbial decomposer assemblages. We examined this question in a microcosm experiment comparing three types of leaf litter mixtures: one representing an original riparian forest composed of healthy alder (*Alnus lusitanica*), ash (*Fraxinus angustifolia*) and poplar (*Populus nigra*); one with the same species composition where alder had been infected by *P. ×alni*; and one where alder had been replaced with black locust. The experiment lasted six weeks and every two weeks microbially-driven decomposition, fungal biomass, reproduction and assemblage structure were measured. Decomposition was highest in mixtures with infected alder and lowest in mixtures with black locust, reflecting differences in leaf nutrient concentrations. Mixtures with alder showed distinct fungal assemblages and higher sporulation rates than mixtures with black locust. Our results indicate that alder loss and its replacement with black locust may alter key stream ecosystem processes and assemblages, with important changes already occurring during alder infection. This highlights the importance of maintaining heathy riparian forests to preserve proper stream ecosystem functioning.

INTRODUCTION

The functioning of stream ecosystems can be highly influenced by changes in the riparian forest (Ellison et al. 2005; Kominoski et al. 2013). This applies especially to headwater streams, where the major basal resource is the allochthonous organic material provided by the riparian vegetation (Wallace et al. 1997), mainly in the form of leaf litter (Molinero and Pozo 2004; Pereira and Ferreira 2021; Pereira et al. 2021). Given that different tree species produce leaf litter with different traits (Jabiol et al. 2019; Ostrofsky 1997; Ramos et al. 2021), species composition of the riparian forest can determine how this leaf litter is processed in the stream, and therefore partially regulate its decomposition rates, the transfer

of energy and nutrients between ecosystem compartments (Marks 2019), and the characteristics of stream assemblages involved in these processes (Alonso et al. 2021; Gulis 2001; Kominoski and Pringle 2009). In consequence, changes in riparian species composition, which can occur through species loss (caused, for example, by pathogenic disease or the long term result of biological invasions; Alonso et al. 2022; Molinero and Pozo 2004), species gain or species replacement (as a result of biological invasions; Ferreira et al. 2021a), are expected to be highly relevant to predict changes in stream ecosystem functioning.

Alders (*Alnus* spp.) are key riparian trees across Europe and often the only nitrogen (N)-fixing native tree species present (Waring and Running 2010). Their leaves are soft and high in N concentration (Waring and Running 2010), which makes them highly attractive to microbial decomposers and detritivores, hence allowing rapid leaf decomposition in streams (Graça et al. 2001). However, alders are suffering widespread mortality due to the *Alnus*-specific species complex *Phytophthora alni* (Bjelke et al. 2016; Husson et al. 2015; Jung and Blaschke 2004; Jung et al. 2018). In Spain and Portugal, alder mortality has been observed since the mid-2000s and *P.* ×*alni* was first isolated in 2009 and 2010, respectively (Jung et al. 2016; Solla et al. 2010). In the northern regions, disease severity has been high, leading to the disappearance of most of the trees in Asturias and northern Galicia. At the southern limit of *P.* ×*alni* distribution, occurring in Jerte river (Plasencia, Spain; Ferreira et al. 2022) and Ceira river (Arouce, Portugal; Kanoun-Boulé et al. 2016), disease severity is lower than in the north. Tree infection is produced by zoospores in the roots or in the trunk during floods, leading to root rot, collar rot, small-size, sparse and often chlorotic foliage, crown dieback and tree mortality. Mortality rates reach almost 100%, with young trees usually dying in few months and old trees dying in years while losing vitality progressively (Bjelke et al. 2016; Jung et al. 2018). Leaf litter of *Alnus lusitanica* VÍT, DOUDA & MANDÁK infected trees has higher nutrient concentration than that from healthy trees due to reduced nutrient resorption before senescence, leading to a fast decomposition of leaf litter (Ferreira et al. 2022).

When key tree species disappear from forests, exotic species can readily occupy their niche (Vítková et al. 2020). Riparian forests are particularly prone to invasions because streams act as corridors favoring propagule transport, cause natural disturbances (i.e., floods) that open canopy gaps and alter micro-climatic conditions thus favoring plant establishment (Dyderski et al. 2015; Hood and Naiman 2000; Naiman and Decamps 1997). Occurrence of invasion is higher in riparian areas affected by human activities (e.g.,

forestry, agriculture or urbanization), where propagule pressure is higher and the colonization of shade sensitive invasive species is favored by large forest gaps (Ferreira et al. 2021b; Pyšek et al. 2010). A likely species to replace alder after its disappearance is the black locust (*Robinia pseudoacacia* L.), an N-fixing tree species native to North America that has become a major invasive species in European riparian forests due to its fast growth, high resistance to disturbance, and low nutrient requirements (Castro-Díez et al. 2011; Köhl and Rametsteiner 2007; Vítková et al. 2020). Despite the high N concentration of black locust leaf litter, it decomposes more slowly than leaf litter of many native tree species (e.g., *Salix atrocinerea* BROT., *Fraxinus angustifolia* VAHL. or *Populus alba* L.), possibly because its high concentration of secondary compounds and lignin negatively affect microbial colonization and macroinvertebrate assemblages (Alonso et al. 2010; Medina-Villar et al. 2015; Tonin et al. 2017).

We explored whether alder infection by *P. ×alni* (BRASIER & S.A. KIRK) HUSSON, IOOS & MARÇAIS and subsequent alder replacement by black locust affected stream ecosystem functioning. Microbially-driven leaf litter decomposition and the characteristics of associated fungal assemblages were assessed. With this aim, we conducted a microcosm experiment using leaf litter and simulated the following scenarios: (1) a native riparian forest containing ash (*F. angustifolia*), poplar (*Populus nigra* L.), and healthy alder (*A. lusitanica*, previously *Alnus glutinosa* (L.) GAERTN.); (2) the same forest but with alder infected by *P. ×alni*, representing an early stage of an epidemic; (3) and the same forest but with alder replaced by black locust, representing a post-epidemic stage. To explore differences among native ash, native poplar, healthy and infected native alder, and invasive black locust and facilitate the understanding of interactions in leaf mixtures, including diversity effects on decomposition and fungal biomass, monocultures of all leaf litter types were also assessed. Based on the main traits of different leaf litter types, we hypothesized that:

(i) scenario 2, with infected alder, would show higher leaf litter decomposition rate, fungal biomass and sporulation rate than scenario 1, with healthy alder, since leaf litter of infected alder is richer in nutrients and more labile (Ferreira et al. 2022), thus possibly enhancing decomposition and fungal activity in mixtures (Alonso et al. 2021);

(ii) scenario 3, with black locust, would show lower leaf litter decomposition rate, fungal biomass and sporulation rate than scenarios 1 and 2 (with healthy and infected alder, respectively), because of the higher lignin and polyphenol concentration in black

locust leaf litter, which potentially slows down decomposition and fungal activity in mixtures (Alonso et al. 2021), despite both black locust and alder having high concentration of nutrients (Alonso et al. 2010; Medina-Villar et al. 2015);

(iii) fungal assemblages would be altered in scenarios 2 and 3 compared with scenario 1, due to strong substrate preferences of fungal microorganisms (Gulis 2001), the change being higher under scenario 3 than under scenario 2 because fungi would be more affected by species composition change (Alonso et al. 2021) than by alder infection (Ferreira et al. 2022).

MATERIALS AND METHODS

Leaf litter collection

The four plant species used in the experiment (ash, poplar, alder, and black locust) are broadleaf deciduous trees that range widely in leaf litter traits (Table 1). Leaves of poplar, ash and black locust were collected immediately after natural abscission from the floor in the floodplain of the Mondego river (Coimbra, central Portugal), at Choupalinho (40°12'4.7''N, 8°25'42.9''W, in autumn 2020), Parque Verde (40°12'3.2''N, 8°25'29.7''W, in autumn 2022), and Mata National do Choupal (40°13'4.3''N, 8°26'28.4''W, in autumn 2022), respectively. Senescent alder leaves were gently detached from healthy trees (≤ 5% crown transparency estimated visually) and trees infected by *P.* ×*alni* (≥ 60% crown transparency) located in the floodplain of the Jerte river (Plasencia, Spain; 40°1'51.2''N, 6°4'46.0''W, in autumn 2017; Ferreira et al. 2022). Isolations of *P.* ×*alni* from bark samples, including the cambium (Jung and Blaschke 2004), confirmed infection of the trees, whereas no pathogen was isolated from bark samples of healthy trees. Leaf litter was air-dried at room temperature in the laboratory and stored in the dark until needed.

Leaf litter characterization

Initial characterization was performed using three replicates per leaf litter type. Air-dried litter was milled into fine powder (< 0.5 mm; Retsch MM 400, Haan, Germany), oven-dried at 60°C for 48 h, and used for chemical determinations. Carbon (C) and N concentrations were assessed by isotope ratio mass spectrophotometry (IRMS Thermo Delta V advantage with a Flash EA-1112 series; Thermo Fisher Scientific Inc., Waltham, U.S.A.). Phosphorous (P) concentration was assessed by the ascorbic acid method after

basic digestion with sodium persulfate and sodium hydroxide (Bärlocher et al. 2020). Total polyphenol concentration was obtained by the Folin-Ciocalteu method (Bärlocher et al. 2020), and lignin concentration by the Goering-van Soest method (Goering and Van Soest 1970). Concentrations were expressed as % dry mass (DM). Initial litter toughness (kPa) was estimated with a penetrometer (diameter 1.55 mm) after one hour of soaking in distilled water (Bärlocher et al. 2020).

Table 1. Initial traits of the different leaf litter types used in the study (mean ± s.e.; n=3): carbon, nitrogen, phosphorus, polyphenol, and lignin concentrations, elemental molar ratios and leaf toughness. For each trait, different letters indicate significant differences among leaf litter types, analyzed with linear models; *p < 0.05, **p < 0.01, ***p < 0.001.

Leaf litter traits	*Fraxinus angustifolia*	*Populus nigra*	*Alnus lusitanica* healthy	*Alnus lusitanica* infected	*Robinia pseudoacacia*
Carbon (C; % DM)***	44.16 ± 0.10^{c}	42.00 ± 0.20^{e}	47.62 ± 0.09^{a}	46.48 ± 0.19^{b}	43.44 ± 0.21^{d}
Nitrogen (N; % DM)***	0.75 ± 0.02^{d}	0.87 ± 0.02^{c}	3.80 ± 0.73^{a}	2.52 ± 0.08^{a}	1.33 ± 0.01^{b}
Phosphorus (P; % DM)***	0.17 ± 0.01^{a}	0.14 ± 0.01^{b}	0.06 ± 0.00^{cd}	0.14 ± 0.05^{abc}	0.04 ± 0.01^{d}
Polyphenol (% DM)**	9.62 ± 0.28^{b}	16.30 ± 4.13^{ab}	8.50 ± 0.83^{b}	9.83 ± 0.33^{b}	11.86 ± 0.43^{a}
Lignin (% DM)***	17.99 ± 0.35^{c}	35.88 ± 0.11^{a}	37.06 ± 1.99^{ab}	36.74 ± 1.56^{ab}	34.06 ±0.43^{b}
C:N***	59.25 ± 1.92^{a}	48.52 ± 1.30^{b}	13.69 ± 3.00^{d}	18.49 ± 0.53^{d}	32.70 ± 0.26^{c}
C:P***	253.69 ± 8.04^{c}	304.56 ± 14.39^{b}	845.74 ± 54.69^{a}	422.71 ± 114.52^{bc}	1092.26 ± 165.87^{a}
N:P***	4.29 ± 0.22^{d}	6.28 ± 0.23^{c}	65.85 ± 9.35^{a}	23.22 ± 6.66^{b}	33.38 ± 5.00^{b}
Toughness (kPa)***	801 ± 96^{ab}	898 ± 77^{a}	609 ± 52^{b}	551 ± 111^{abc}	382 ± 9^{c}

Experimental procedure

The experimental design included eight treatments: five of monocultures (ash, poplar, healthy and infected alder, and black locust), and three of mixtures, called scenarios, with three species each: ash, poplar, and healthy alder (scenario 1); ash, poplar, and infected alder (scenario 2); and ash, poplar, and black locust (scenario 3). Each scenario included three replicates collected at each of four sampling dates, thus the experiment comprised 96 microcosms in total. Before the beginning of the experiment, leaf litter was moistened with distilled water and 6-mm diameter discs were cut avoiding central veins (except for the narrow ash leaves, where discs included the vein in the center). Discs were air-dried at room temperature for 72 h and weighed (± 0.1 mg) in groups of 12, either of the same species (monoculture) or 4 discs per species (mixtures, with each species weighed individually). Discs were then distributed in 100-mL Erlenmeyer flasks (microcosms), which were assembled on an orbital shaker (100 rpm; GFL 3017, ProfiLab24 GmbH, Berlin, Germany) and kept under controlled conditions (21°C and 12 h light:12 h dark photoperiod).

For the first seven days, microcosms were supplied daily with 40 mL of a microbial inoculum <24 h old, to allow for leaching and microbial colonization of leaf litter discs.

The inoculum was prepared by incubating a diverse mixture of leaf litter at different decomposition degrees in a glass jar with 4 L of filtered (100 µm) stream water and aeration, kept at 21°C, with water renewal every 24 h. The litter and water were collected in October 2022 from Candal stream (Lousã mountain, central Portugal; 40°4'44.7''N, 8°12'11.3''W), an oligotrophic stream with riparian vegetation at the sampling site dominated by European chestnut trees (*Castanea sativa* Mill.), and from where the tree species used in this study were absent (Pereira et al., 2021). A set of three microcosms per treatment (i.e., 24 microcosms) was sacrificed after the conditioning period (day 0) and processed as the experimental microcosms (see below), to obtain a correction factor to estimate initial, post-leaching, litter DM.

At day 0, all other experimental microcosms were supplied with 40 mL of filtered stream water (Candal stream), which was renewed every 3.5 days. Three replicates of each treatment were sacrificed at days 14, 28 and 42 to assess remaining litter mass, fungal biomass and conidial production. All litter discs were frozen at –20°C, lyophilized overnight (Lablyo Mini, Frozen in Time, North Yorkshire, U.K.), and weighed for determination of DM remaining, with litter species in mixtures weighed individually.

Fungal conidial production

At each sampling date (days 14, 28, and 42), conidial suspensions from the sacrificed microcosms (40 mL) were poured into 50-mL centrifuge tubes, preserved with 2 mL of 37% formalin, adjusted to a volume of 45 mL with distilled water, and stored in the dark until processed. Samples were processed to determine sporulation rates and assemblage structure of aquatic hyphomycetes, a polyphyletic group of aquatic fungi assumed to be major microbial decomposers (Gessner and Chauvet 1994; Hieber and Gessner 2002). Each conidial suspension received 100 µL of 0.5 % Triton X-100 and was homogenized with a magnetic stirrer. Then, 10 mL were filtered through nitro-cellulose filters (25-mm diameter, 5-µm pore size; Sartorius Stedim Biotech GmbH, Goettingen, Germany) and filters were stained with 0.05% cotton blue in 60% lactic acid. Conidia were identified and counted with a microscope (Leica, DM1000, Wetzlar, Germany) at ×200 magnification (Bärlocher et al. 2020). Sporulation rates were expressed as number of conidia mg^{-1} DM d^{-1}, and species richness was expressed as number of species per sample.

Fungal biomass

At days 14 and 42, four discs from each species per microcosm (i.e., 4 discs in monocultures or the 4 discs of each species in mixtures) were used to determine fungal biomass from ergosterol concentration (Bärlocher et al. 2020). Discs were weighed to determine DM and ergosterol was extracted in 10 mL of alkaline methanol (8 g KOH/L) in a hot bath (80°C, 30 min), purified by solid phase extraction (Waters Sep-Pak Vac RC, 500 mg, Tc18 cartridges; Waters Corp., Milford, MA, U.S.A.), and quantified with high-performance liquid chromatography (Dionex DX-120; Sunnyvale, CA, U.S.A.) by measuring absorbance at 282 nm (Bärlocher et al. 2020). Ergosterol concentration was converted into fungal biomass assuming 5.5 µg ergosterol mg^{-1} fungal DM (Gessner and Chauvet 1993), and results were expressed as mg fungal DM.

Data analyses

The fraction of leaf litter DM remaining per species and mixture was calculated by dividing DM remaining by initial DM. Decomposition rates (k, day^{-1}) were calculated for each species and mixture assuming an exponential decay model, through linear regression of the ln-transformed fraction of DM remaining over time, considering the intercept fixed at ln(1)=0. Net diversity, complementarity, and selection effects on decomposition were also calculated (Loreau and Hector 2001); the net diversity effect was the difference between observed and expected litter mass loss (LML, calculated as the difference between initial DM and DM remaining divided by initial DM), with expected fraction LML calculated as the mean of monoculture values taking into account the proportion of each species in the mixture; the complementarity effect was the average deviation from the expected fraction LML in a mixture multiplied by mean fraction LML in monocultures and the number of species in the mixture; and the selection effect was the covariance between fraction LML of species in monoculture and the average deviation from expected fraction LML of species in the mixture, multiplied by the number of species in the mixture. Net diversity, complementarity, and selection effects on fungal biomass was calculated in the same way using fungal biomass of each species in monocultures and mixtures.

Initial leaf litter traits were compared among leaf litter types (ash, poplar, healthy alder, infected alder, and black locust) with linear models and ANOVA (*gls* function of the "nlme" R package), with species as fixed factor. Effects of leaf litter type (five types) and scenario (1, 2, or 3) on different response variables were analyzed separately. To assess leaf litter decomposition, we used analysis of covariance (ANCOVA, *aov* function of the "stats" R package), with fraction DM remaining as a dependent variable, treatment (leaf

litter type or scenario) as a categorical factor and time as a covariate. To assess fungal biomass, sporulation rate and species richness, and net, complementarity, and selection effects on decomposition and fungal biomass, we used linear models and ANOVA, with treatment (litter type or scenario) and time as fixed factors. Significant differences between treatments ($\alpha = 0.05$) were analyzed with Tukey tests, or Fisher's LSD tests when Tukey tests did not identify differences among treatments (i..e., for species richness and net complementarity and selection effects; *ghlt* function of the "multcomp" R package; Zar 1999). Differences in fungal assemblages among treatments and sampling dates were explored with non-metric multidimensional scaling (NMDS) based on the Bray-Curtis similarity index applied to an abundance matrix (*metaMDS* function of the "vegan" R package), followed by permutational multivariate analysis of variance (PERMANOVA; *adonis* function of the "vegan" R package). An indicator value index (*multipatt* function of the "indicspecies" R package) was used to identify the most representative taxa of each assemblage. Normal distribution of residuals was assessed by Shapiro-Wilk test and data were log-transformed when non-normal distribution was detected. All analyses were performed in R software (RCoreTeam 2022).

RESULTS

Leaf litter traits

Initial traits varied with the type of leaf litter (Table 1, Table S1). Native species (ash, poplar, and healthy alder) showed a gradient, with N and lignin concentrations, and C:P and N:P ratios being highest in healthy alder and lowest in ash, while P concentration and C:N showed the opposite pattern; C concentration was lowest in poplar and highest in healthy alder. Healthy and infected alders differed in C concentration and C:P and N:P ratios, being higher in healthy alder. Black locust had higher polyphenol concentration than the other species except poplar; the lowest toughness, although not significantly different from infected alder; and higher lignin concentration than ash and lower than poplar. Black locust had lower N concentration than alder, but higher than ash and poplar; the lowest P concentration, but not significantly different from healthy alder; and the second lowest C concentration, after poplar (Table 1).

Leaf litter decomposition

Leaf litter types significantly differed in their decomposition rates (p-value < 0.001; Table S2, Fig. 1A), with (i) native species showing a gradient from lowest in ash to highest in alder; (ii) infected alder tending to decompose faster than healthy alder, with the difference being non-significant; (iii) and black locust decomposing more slowly than alder (Fig. 1A). Leaf litter decomposition varied with the scenario (p-value = 0.008), being significantly higher (56%) in scenario 2 than in scenario 3, and 23% higher in scenario 2 than in scenario 1, and 21% lower in scenario 3 than in scenario 1, although the last two differences were non-significant (Table S2, Fig. 1B). All scenarios had higher decomposition rates than expected from monocultures (19-43%), with a positive net diversity effect in all of them (Fig. 1B). The net diversity effect increased with time (p-value = 0.001) and it was mainly driven by a positive complementarity effect, with a much lower and negative selection effect (Table S3). Diversity effects changed with scenario (net diversity effect, p-value = 0.014; complementarity effect, p-value = 0.007; selection effect, p-value = 0.010), with net diversity and complementarity effects being higher in scenario 2 than in scenario 3 (Table S3, Figure S1).

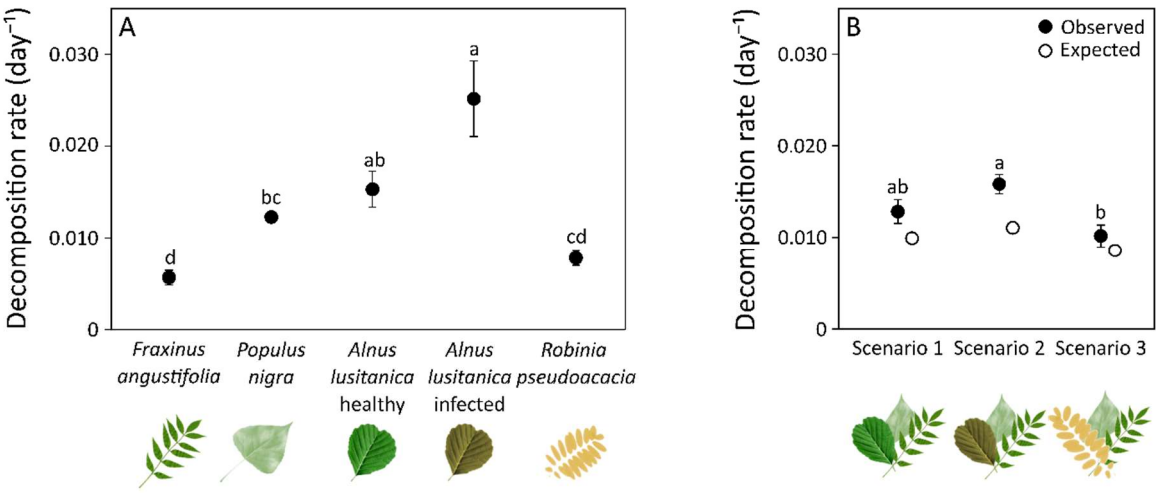

Fig. 1. Decomposition rate (mean ± s.e., n = 9) of leaf litter of five monocultures (A) and three mixtures (scenarios; B). Different letters indicate significant differences among leaf litter types or scenarios, analyzed with Tukey tests.

Fungal biomass

Fungal biomass was not significantly affected by leaf litter type (p-value = 0.422), although it tended to be higher in healthy alder and poplar at the later stages of the experiment (Table S3, Fig 2A); and it did not vary among scenarios (p-value = 0.384). However, fungal biomass decreased with time in all scenarios (p-value = 0.050), with a sharper decrease in scenarios 2 and 3 than in scenario 1 (Table S3, Fig. 2B), although it increased or did not change with time in monocultures. The net diversity effect on fungal biomass was mainly driven by a complementarity effect, with no differences among scenarios either for the net diversity effect (p-value = 0.280) or the complementary effect (p-value = 0.436), but a weak difference for the selection effect (p-value = 0.014) (Table S3, Fig. S2). The net diversity effect changed with time, from null or slightly positive at the first sampling date (10–16% higher than expected; Fig. S2A), to negative at the end of the experiment (Fig. S2D) (p-value < 0.001; Table S3). Despite the non-significant differences among scenarios at the end of the experiment, it was 41% lower than expected in scenario 3 and 9% lower than expected in scenario 1 (Fig. S2D). The net diversity effect was driven by a complementarity effect, which presented the same pattern, shifting with time from slightly positive (Fig. S2B) to negative values (Fig. S2E) (p-value < 0.001; Table S3). The selection effect was also affected by time (p-value = 0.032), remaining null in scenario 1 and negative in scenarios 2 and 3 (Table S3, Fig. S2C, Fig. S2F).

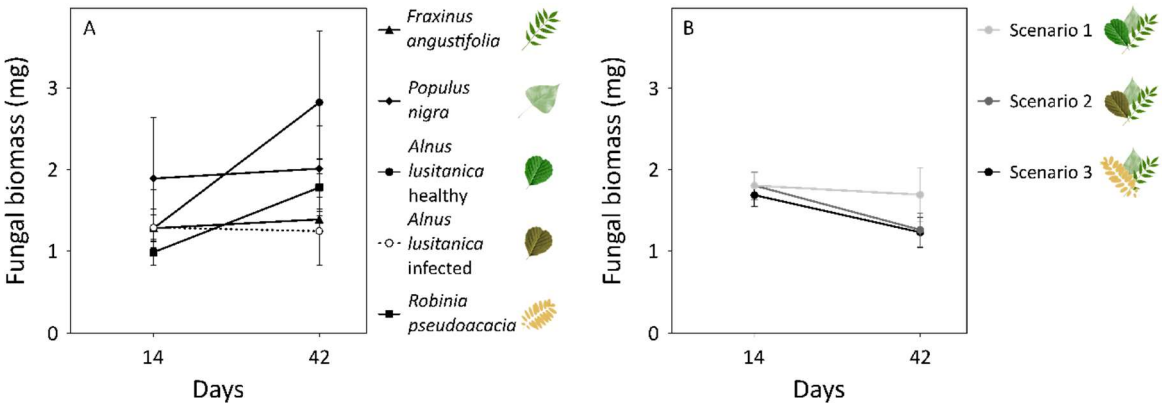

Fig. 2. Fungal biomass (mean ± s.e., n = 3) associated with leaf litter of monocultures (A) and mixtures (scenarios; B) at days 14 and 42.

Fungal conidial production and assemblage structure

Sporulation rates varied with leaf litter type (p-value < 0.001), time (p-value < 0.001) and their interaction (p-value < 0.001) (Table S3); they were significantly higher in infected alder than in ash, poplar, and black locust, and lowest in ash (Fig. 3A). The scenarios also differed in sporulation rates (p-value = 0.001; Table S3), being higher in scenarios 1 and 2 than in scenario 3 (Fig. 3B). Species richness was affected by leaf litter type (p-value = 0.005), time (p-value = 0.038), and their interaction (p-value = 0.008) (Table S3), being lower at day 42 than at day 28. However, differences among leaf litter types were obscured by the interaction with time. Species richness was also affected by the scenario (p-value = 0.017, Table S3), but post hoc tests did not identify differences, only a trend of higher richness in scenario 2 (11 species) compared to scenarios 1 and 3 (9 species in both) (Table 2).

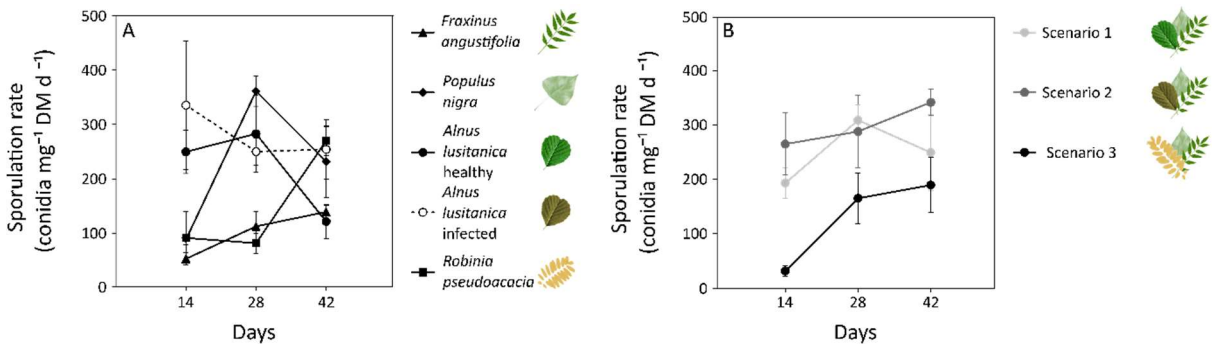

Fig. 3. Fungal sporulation rates (mean ± s.e., n = 3) associated with leaf litter of monocultures (A) and mixtures (scenarios; B) at days 14, 28 and 42.

Fungal assemblage structure varied depending on the leaf litter type, time and their interaction (p-values < 0.001; Table S4). Each leaf litter type showed a different response to time and assemblages in the third sampling date significantly differed from those in the first and second dates, e.g., *Triscelophorus acuminatus* NAWAWI increased (p-value < 0.010). Despite variations due to the interaction with time, post hoc tests showed significant differences among all leaf litter types, except between healthy and infected alder and between healthy alder and poplar. Assemblages in poplar and healthy and infected alder leaf litter were dominated by *Articulospora tetracladia* INGOLD (p-value < 0.001), followed by *Alatospora acuminata* INGOLD (p-value = 0.003), and differed by the high relative contribution of *Tetrachaetum elegans* INGOLD (p-value = 0.007) in poplar, the high relative contribution of *Lunulospora curvula* INGOLD (p-value = 0.021) in healthy alder, and a high relative contribution of *T. acuminatus* (p-value = 0.005) in infected alder. Infected alder

was also characterized by the occurrence of *Flagellospora curvula* INGOLD (p-value = 0.006) and *Alatospora pulchella* MARVANOVÁ (p-value = 0.013), two to five times more abundant than in the other leaf litter types (Table 2). In black locust, *T. acuminatus* (p-value = 0.005) was also dominant. Ash had lower total sporulation, included *T. elegans* as the dominant species (p-value = 0.007) and showed high abundance of *Anguillospora filiformis* GREATH. (p-value < 0.001), and *A. tetracladia* (Fig. 4A, Table 2).

Table 2. Specific fungal sporulation rates (mean across sampling dates, n = 3) in leaf litter of five monocultures and three mixtures (scenarios). Accumulated species richness is given at the bottom of the table. Scenario 1: *Fraxinus angustifolia* + *Populus nigra* + *Alnus lusitanica* healthy; scenario 2: *Fraxinus angustifolia* + *Populus nigra* + *Alnus lusitanica* infected; scenario 3: *Fraxinus angustifolia* + *Populus nigra* + *Robinia pseudoacacia*.

Fungal species	*Fraxinus angustifolia*	*Populus nigra*	*Alnus lusitanica* healthy	*Alnus lusitanica* infected	*Robinia pseudoacacia*	Scenario 1	Scenario 2	Scenario 3
Alatospora acuminata INGOLD	3.35	24.67	26.76	44.81	6.66	10.08	13.77	5.98
Alatospora pulchella MARVANOVÁ			0.25	1.27	0.28		0.28	
Anguillospora filiformis GREATH.	10.96	3.89	2.90	3.61	0.69	4.54	9.14	4.11
Articulospora Tetracladia INGOLD	25.77	149.64	141.99	121.37	47.12	147.36	125.31	67.70
Flagellospora curvula INGOLD	0.62	0.84	1.39	5.28	2.08	2.52	3.49	0.55
Hydrocina chaetocladia SCHEUER	0.12	0.91	0.30	0.59	0.21	2.40	3.20	0.32
Lunuslospora curvula INGOLD	0.20	6.11	5.20	2.71	0.40	13.78	28.27	4.94
Neonectria lugdunensis (SACC. & THERRY) L. LOMBARD & CROUS	0.03				0.31			
Stenocladiella neglecta (MARVANOVÁ & DESCALS) MARVANOVÁ & DESCALS	0.11	0.22		0.73		1.26	0.95	0.10
Taeniospora gracilis MARVANOVÁ			0.10	0.48			0.07	
Tetrachaetum Elegans INGOLD	49.92	31.85	16.80	7.05	6.11	23.16	47.57	24.85
Triscelophorus Acuminatus NAWAWI	11.13	9.60	22.93	92.60	84.36	45.49	66.34	20.29
Accumulated species richness	10	9	10	11	10	9	11	9

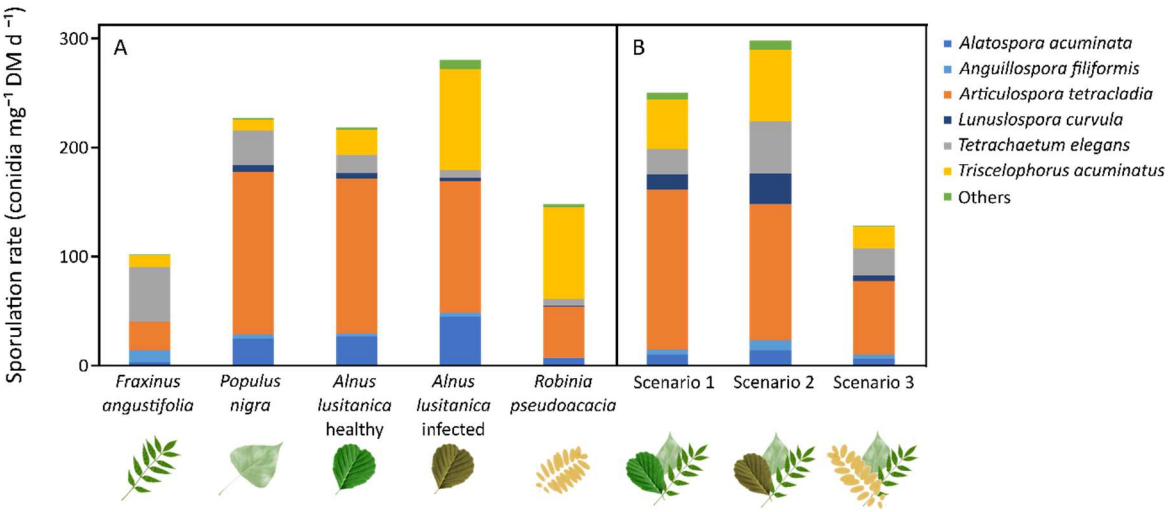

Fig. 4. Fungal sporulation rates (average across sampling dates, n = 3) associated with leaf litter of monocultures (A) and mixtures (scenarios; B). 'Others' include species contributing less than 5% to total conidial production.

Assemblage structure also varied with scenario, time and their interaction (p-values ≤ 0.002; Table S4). Assemblages in the last sampling date differed significantly from those in the first and second dates and was characterized by a higher proportion of *T. acuminatus* (p-value < 0.001). Assemblages in scenarios 1 and 2 differed from those in scenario 3 (Table S4), because total sporulation was higher and *A. tetracladia*, the dominant species, also presented a higher sporulation in scenarios 1 and 2 (p-value = 0.046), with *A. filiformis* showing a higher relative contribution in scenario 3 (p-value = 0.046) (Fig. 4B, Table 2).

DISCUSSION

Our results revealed changes in stream ecosystem functioning and associated fungal assemblages following infection of riparian alder by *P. ×alni* and subsequent replacement of diseased trees by the invasive black locust. Rates of leaf litter decomposition, a fundamental process often used to assess ecological impacts of stressors on ecosystem functioning (Gessner and Chauvet 2002; Pazianoto et al. 2019), experienced first a weak increase after alder infection and subsequently a strong decrease after replacement of infected alder with black locust, when measured in litter mixtures also containing other native species (i.e., ash and poplar). These changes had the expected direction, confirmed hypotheses i and ii, and reflected differences in leaf litter chemistry. Leaf litter of infected alder showed higher nutrient concentrations and lability than that of healthy alder, as reported before (Ferreira et al. 2022), which has been attributed to reduced nutrient resorption efficiency of infected trees (Cao et al. 2015) or to accumulation of nutrients in

leaves due to stress induced by root damage (Milanović et al. 2015). In contrast, black locust leaf litter had lower N concentration than that of alder and the lowest P concentration, as well as high polyphenol concentrations, which reduced its palatability and hence its decomposition rate, as shown also in other studies (Alonso et al. 2010; Castro-Díez et al. 2009; Medina-Villar et al. 2015; Tonin et al. 2017).

Despite the clear differences in leaf litter chemistry and the fact that trends in leaf litter decomposition followed the expected pattern, the reduction in decomposition rates when comparing mixtures with healthy alder (scenario 1) with mixtures with black locust (scenario 3) was not statistically significant during our experiment, which lasted 42 days. In contrast, fungal sporulation rates were significantly lower in mixtures with black locust than in those with healthy alder, which was likely caused by the high concentration of polyphenols in black locust; polyphenols are known to have anti-microbial activity (Alonso et al. 2021; López-Rojo et al. 2020; McArthur et al. 1994; Medina-Villar et al. 2015). The lower reproduction rate of microbial decomposers in mixtures with black locust suggests that decomposition rates might be significantly reduced if measured further in the longer term. This is supported by the reduction in fungal biomass that occurred with time in mixtures with black locust, but not in mixtures with healthy alder. It should be considered that the disappearance of alder generally occurs after a period when healthy alder is infected. Thus, while decomposition and fungal sporulation rates of infected alder leaf litter were slightly higher than those of healthy alder leaf litter (in monocultures and in scenario 1 vs. 2), this increase was large enough to obtain a significant reduction in decomposition and sporulation rates when infected trees (scenario 2) were further replaced by black locust (scenario 3).

All leaf litter mixtures showed a positive net diversity effect on decomposition, that is, they decomposed faster than expected based on the decomposition of individual species. The net diversity effect was mainly driven by a complementarity effect, as shown for the decomposition of other leaf litter mixtures (López-Rojo et al. 2018; Tonin et al. 2017), and it increased with time, also as observed previously (Cardinale et al. 2007). Alder leaf litter has been reported to enhance decomposition of more recalcitrant litter in mixtures (Alonso et al. 2021; López-Rojo et al. 2018; Rubio-Ríos et al. 2021), either by attracting detritivores (Ferreira et al. 2012) or by increasing the leaf nutrient concentration of other species through nutrient transfer by fungal hyphae (Handa et al. 2014). In our experiment, there could have been transfer of N from alder or black locust to the other species, and also a transfer of P in the opposite direction. This could possibly occur through uptake of leached

nutrients (Gessner et al. 2010; López-Rojo et al. 2019), since leaf discs were individually in constant movement in the microcosms, most likely precluding nutrient transfer by fungal hyphae.

In contrast to the pattern revealed for decomposition, we found no significant differences among leaf litter mixtures for fungal biomass, with only a weak trend towards higher biomass in healthy alder. This contrasts with previous studies, where infected alder showed higher fungal biomass than healthy alder (Ferreira et al. 2022). Here, infected alder showed similar values to those of black locust, which usually presents low fungal biomass due to their low nutrient and high polyphenol concentrations (Medina-Villar et al. 2015). Our observed pattern could be caused by nutrient transfer among leaf litter types, which is supported by the positive complementarity effect on fungal biomass found at the early stages of the experiment. Towards later stages, however, there was a negative complementarity effect, suggesting that inhibitory compounds present in different leaf litter types could have reduced fungal growth (Ferreira et al. 2012; Rubio-Ríos et al. 2021), maybe in parallel to a depletion of nutrients (Compson et al. 2018). The greater reduction in mixtures with black locust and infected alder may be caused by the higher polyphenol and lower nutrient concentration in black locust, which make them more prone to negative effects (Medina-Villar et al. 2015), and the enhancement of decomposition caused by infected alder, which may have caused a faster consumption of the nutrients (Compson et al. 2018), which therefore may not have compensated the negative effects of tannins and other compounds (Alonso et al. 2021).

Leaf litter of different species showed different fungal assemblages, according to the different substrate preferences shown by fungal species (Gulis 2001). As previously observed (Ferreira et al. 2022), healthy and infected alder showed similar assemblages, but mixtures with alder had different assemblages compared to mixtures with black locust. This difference was mainly driven by *A. tetracladia*, which was the most abundant hyphomycete in all mixtures but it was reduced in the presence of black locust. This probably occurred because its sporulation was limited due to lower nutrient and higher polyphenol concentrations (Alonso et al. 2022; Ferreira et al. 2012; Kominoski et al. 2007; Pérez et al. 2021).

CONCLUSIONS

Our study reveals changes in the key stream ecosystem process of leaf litter decomposition and associated fungal assemblages, following a sequence of alterations in the species composition of the riparian forest. The most striking changes occur when alder is infected by *P. ×alni*, which slightly accelerates the process of leaf litter decomposition if compared to non-infected alder trees; and after the replacement of alder with the exotic black locust, which induces a significant reduction in the decomposition process and a substantial change in the characteristics of the microbial decomposer assemblages. Our results also suggest that changes detected 42 days after the experiment started would be intensified in the longer term, as proposed elsewhere (Ferreira et al. 2022). Impacts to the riparian forest, such as infectious diseases –which drive changes in the traits of native species and can ultimately lead to their loss– and species invasions –which introduce species with different traits from those of native species– have the capacity to alter the functioning and structure of the stream ecosystem. Our study shows how this occurs even if the native and invasive species belong to the same functional group, in this case N-fixing species. Overall, our study highlights the importance of preserving native and healthy riparian vegetation in order to maintain proper ecosystem functioning.

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COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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SUPPLEMENTARY MATERIAL

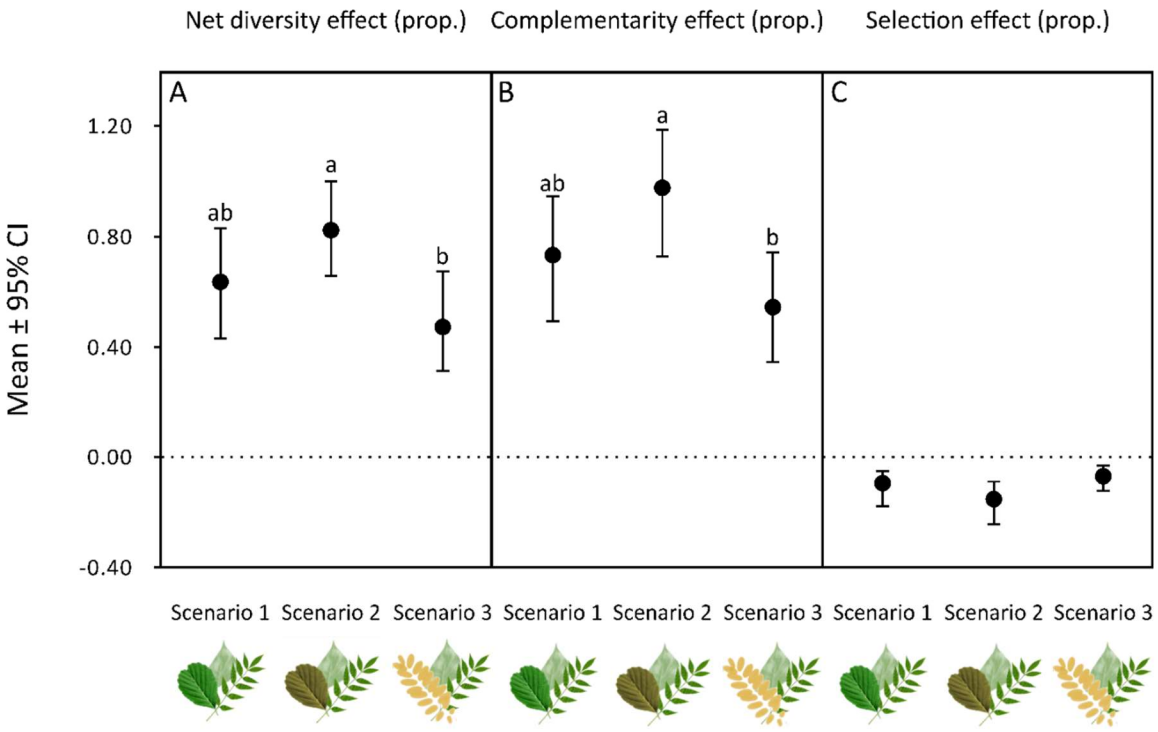

Fig. S1. Net diversity (A), complementarity (B) and selection (C) effects on leaf litter decomposition (measured as fraction of litter mass loss) in scenarios 1, 2 and 3 using the mean of the three sampling dates. Symbols are means, whiskers are upper and lower bounds of 95% nonparametric bootstrapped confidence intervals and different letters indicate significant differences among scenarios.

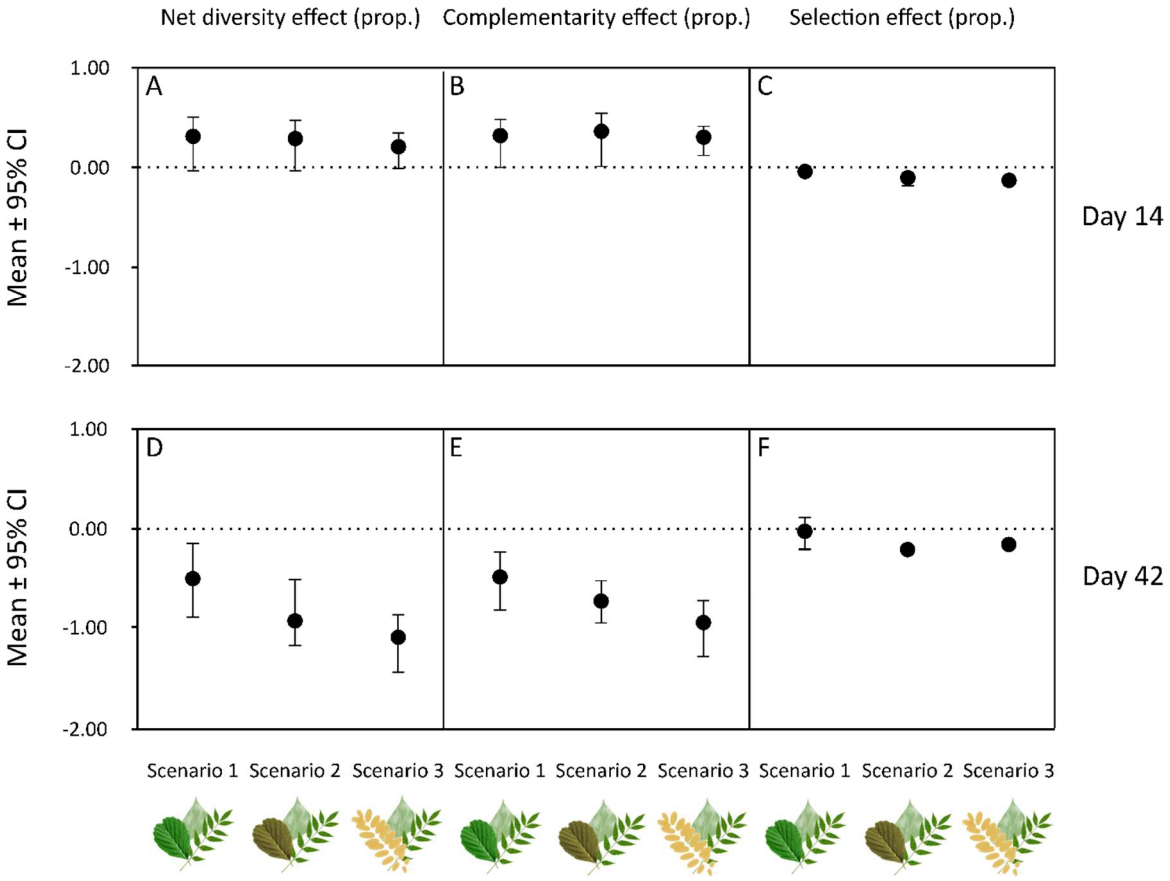

Fig. S2. Net diversity (A, D), complementarity (B, E) and selection (C, F) effects on fungal biomass (mg) in scenarios 1, 2 and 3 at days 14 and 42. Symbols are means, whiskers are upper and lower bounds of 95% nonparametric bootstrapped confidence intervals.

Table S1. Results of ANOVA exploring the effects of leaf litter types (*Fraxinus angustifolia, Populus nigra, Alnus lusitanica* healthy, *A. lusitanica* infected and *Robinia pseudoacacia*) on leaf litter physico-chemical traits: C (carbon), N (nitrogen), P (phosphorus), polyphenol and lignin concentrations (% dry mass), elemental ratios (C:N, C:P and N:P) and leaf toughness (kPa). df = degrees of freedom, F = F-statistic, p = p-value.

Variable	df	F	p
C	4, 10	189.10	<0.001
N	4, 10	271.32	<0.001
P	4, 10	72.87	<0.001
Polyphenol	4, 10	7.19	0.005
Lignin	4, 10	610.02	<0.001
C:N	4, 10	254.25	<0.001
C:P	4, 10	36.43	<0.001
N:P	4, 10	30.21	<0.001
Toughness	4, 10	20.41	<0.001

Table S2. Results of ANCOVA exploring the effects of leaf litter types (*Fraxinus angustifolia*, *Populus nigra*, *Alnus lusitanica* healthy, *A. lusitanica* infected and *Robinia pseudoacacia*) and scenarios (scenarios 1, 2 and 3), with time as a covariate, on fraction leaf mass remaining. df = degrees of freedom, F = F-statistic, p = p-value.

Treatment	Factor	df	F	p
Species	Species	4	14.57	<0.001
	Time	2	11.84	<0.001
Scenario	Scenario	1	5.99	0.008
	Time	2	21.82	<0.001

Table S3. Results of ANOVA exploring the effects of leaf litter types (*Fraxinus angustifolia*, *Populus nigra*, *Alnus lusitanica* healthy, *A. lusitanica* infected and *Robinia pseudoacacia*) or scenarios (scenarios 1, 2 and 3), time and their interaction, on net diversity effect, complementarity effect and selection effect on leaf litter mass loss, sporulation rate (number of conidia mg^{-1} DM d^{-1}), species richness (number of species $sample^{-1}$), fungal biomass (mg fungal DM) and net diversity effect, complementarity effect and selection effect on fungal biomass. df = degrees of freedom, F = F-statistic, p = p-value.

Variable	Treatment	Factor	df	F	p
Net diversity effect on leaf mass loss	Scenario	Scenario	2, 18	5.41	0.014
		Time	2, 18	12.03	0.001
		Scenario×Time	4, 18	0.23	0.920
Complementarity effect on leaf mass loss	Scenario	Scenario	2, 18	6.55	0.007
		Time	2, 18	13.67	<0.001
		Scenario×Time	4, 18	0.16	0.958
Selection effect on leaf mass loss	Scenario	Scenario	2, 18	5.97	0.010
		Time	2, 18	25.85	<0.001
		Scenario×Time	4, 18	1.13	0.372
Fungal biomass	Species	Species	4, 20	1.02	0.422
		Time	1, 20	3.21	0.088
		Species×Time	4, 20	1.27	0.317
	Scenario	Scenario	2, 12	1.04	0.384
		Time	1, 12	4.76	0.050
		Scenario×Time	2, 12	0.60	0.566
Net diversity effect on fungal biomass	Scenario	Scenario	2, 12	1.42	0.280
		Time	1, 12	41.74	<0.001
		Scenario×Time	2, 12	0.79	0.476
Complementarity effect on fungal biomass	Scenario	Scenario	2, 12	0.89	0.436
		Time	1, 12	51.91	<0.001
		Scenario×Time	2, 12	0.81	0.467
Selection effect on fungal biomass	Scenario	Scenario	2, 12	6.22	0.014
		Time	1, 12	5.87	0.032
		Scenario×Time	2, 12	1.14	0.353
Sporulation rate	Species	Species	4, 30	10.83	<0.001
		Time	2, 30	10.01	<0.001
		Species×Time	8, 30	4.86	<0.001
	Scenario	Scenario	2, 18	10.53	0.001
		Time	2, 18	4.07	0.035
		Scenario×Time	4, 18	0.76	0.566
Species richness	Species	Species	4, 30	4.62	0.005
		Time	2, 30	3.67	0.038
		Species×Time	8, 30	3.29	0.008
	Scenario	Scenario	2, 18	5.18	0.017
		Time	2, 18	0.93	0.414
		Scenario×Time	4, 18	1.20	0.343

Table S4. Results of PERMANOVA exploring the effects of leaf litter types (*Fraxinus angustifolia*, *Populus nigra*, *Alnus lusitanica* healthy, *A. lusitanica* infected and *Robinia pseudoacacia*) and scenarios (scenarios 1, 2 and 3), time and their interaction on fungal assemblages. df = degrees of freedom, F = F-statistic, R^2 = R squared, p = p-value.

Treatment	Factor	df	F	R^2	p
Species	Species	4	9.13	0.36	<0.001
	Time	2	5.73	0.11	<0.001
	Species×Time	8	2.74	0.22	<0.001
Scenario	Scenario	2	4.89	0.19	<0.001
	Time	2	6.05	0.23	<0.001
	Scenario×Time	4	2.91	0.23	0.002

Chapter 4

AMPHIBIAN LOSS ALTERS PERIPHYTON STRUCTURE AND INVERTEBRATE GROWTH IN MONTANE STREAMS

Alonso, A., Monroy, S., Bosch, J., Pérez, J., & Boyero, L. (2022). Amphibian loss alters periphyton structure and invertebrate growth in montane streams. *Journal of Animal Ecology*, *91*(11), 2329-2337. DOI: https://doi.org/10.1111/1365-2656.13818

ABSTRACT

Amphibians are declining worldwide due to a combination of stressors such as climate change, invasive species, habitat loss, pollution and emergent diseases. Although their losses are likely to have important ecological consequences on the structure and functioning of freshwater ecosystems, this issue has been scarcely explored. We conducted an experiment in three montane streams – where primary production is the main source of energy and carbon – to assess the effects of amphibian disappearance (i.e., presence or absence of the common midwife toad *Alytes obstetricans*, a common species found in pools of these streams) on several aspects of ecosystem functioning and structure: periphyton biomass and chlorophyll a concentration, algal assemblage structure, and growth of macroinvertebrate grazers. We compared four types of experimental enclosures: (i) without macroinvertebrates or amphibians; (ii) with larvae of the caddisfly *Allogamus laureatus*; (iii) with *A. obstetricans* tadpoles; and (iv) with both *A. laureatus* larvae and *A. obstetricans* tadpoles. The absence of tadpoles increased periphyton biomass, but did not cause differences on inorganic sediment accrual. The algal assemblage had a higher diversity in the absence of tadpoles, and their characteristic taxa differed from the assemblages in presence of tadpoles. *A. laureatus* presented higher mass in presence of tadpoles; however, tadpole length was not affected by presence of macroinvertebrates. Our results suggest that presence of tadpoles is a driver of periphyton accrual and assemblage structure, acting as top-down control and with key potential consequences on the functioning of montane stream ecosystems.

INTRODUCTION

Biodiversity is experiencing a dramatic decline, with current extinction rates being equivalent to or higher than those of past mass extinctions (Barnosky et al. 2011; Ceballos et al. 2017). Among the most affected groups of organisms are the amphibians (Collins 2010; Wake and Vredenburg 2008), whose dramatic declines worldwide are driven by several factors such as climate change (Blaustein et al. 2010), pollution (Blaustein et al. 2003), habitat loss and fragmentation (Becker et al. 2007; Gallant et al. 2007), and invasive species (Falaschi et al. 2020), including pathogens that are responsible for emergent infectious diseases (Fisher and Garner 2020; Scheele et al. 2019).

One of the most significant threats to amphibian conservation is the pathogenic fungus *Batrachochytrium dendrobatidis* LONGCORE, PESSIER & D.K. NICHOLS (Fisher and Garner 2020; Fisher et al. 2009; Scheele et al. 2019). This fungus (hereafter referred to as Bd) is a generalist pathogen that causes chytridiomycosis, a highly infective disease. Bd infects amphibian epidermal cells and causes great mortality after metamorphosis in sensitive species (Fisher et al. 2009; Garner et al. 2009). The fungus originated in Asia and has recently spread globally due to human activities (O'Hanlon et al. 2018), causing amphibian declines and extinctions in all continents where amphibians are found (Lips 2016).

Although the loss of amphibian species from freshwater ecosystems can be expected to cause changes in ecosystem structure and functioning, this consequence has been little explored. Only a few studies have been conducted, mainly in tropical streams, where larval amphibian losses have been shown to cause changes in algal (Barnum et al. 2022; Connelly et al. 2008) and macroinvertebrate assemblages (Barnum et al. 2022; Colón-Gaud et al. 2009; Ranvestel et al. 2004), with a resulting simplification of freshwater food webs (Schmidt et al. 2017), a reduction of primary production (Connelly et al. 2008), an increase of periphyton biomass accrual (Connelly et al. 2008; Mallory and Richardson 2005; Ranvestel et al. 2004) and an alteration of nutrient cycling (Whiles et al. 2013). There are, however, very few relevant studies from freshwater ecosystems in temperate areas, with only one reporting that periphyton accrual was limited by amphibian presence and density (Mallory and Richardson 2005). Differences in the functioning of freshwater ecosystems at different latitudes is plausible due to often different assemblage composition and diversity, which influence key ecosystem processes (Boyero et al. 2021).

Amphibians play a key ecological role in low-order streams of montane areas, where primary production is the main source of energy and carbon (as opposed to streams flowing through forested areas, which are fuelled by allochthonous leaf litter; Swan et al. 2021). In temperate regions, many anuran tadpoles are herbivores that graze on the periphyton growing on mineral or soft substrates, thus tadpoles often interact with grazing macroinvertebrates such as many caddisflies (Cummins and Klug 1979). Tadpoles can compete with caddisflies for food resources (Colón-Gaud et al. 2010; Kupferberg 1997), but they can also facilitate the access of algae by smaller caddisflies through the tadpole's removal of sediment (Colón-Gaud et al. 2009; Colón-Gaud et al. 2010; Ranvestel et al. 2004). The loss of amphibians from these habitats have indirect effects on invertebrates,

with possible impacts across the food web (Hocking and Babbitt 2014) and on fundamental ecosystem processes and structure.

Here, we experimentally quantified how the loss of an amphibian species (*Alytes obstetricans* LAURENTI) from montane streams where it was common before its decline due to Bd infection (Bosch et al. 2018; Bosch et al. 2001), affected the rate of periphyton accrual, the algal assemblage structure and the growth rates of grazing macroinvertebrates. We conducted an outdoor field experiment using enclosures placed in nine stream pools, which contained tiles previously colonized by periphyton and (i) tadpoles only, (ii) macroinvertebrates only, (iii) both or (iv) none. We hypothesized that (1) amphibian losses would enhance periphyton accrual and sediment accumulation, due to the absence of tadpole consumption and removal through bioturbation (Connelly et al. 2008; Ranvestel et al. 2004); (2) the algal assemblage structure would change toward a higher proportion of larger diatoms as a result of amphibian loss, since large algae are more affected by grazing as they are less likely to intact passage through tadpole gut and therefore to recolonize the substrate after grazing (Connelly et al. 2008; Ranvestel et al. 2004); and (3) caddisfly growth rate would increase in the absence of tadpoles, since tadpoles compete with large macroinvertebrate grazers (Colón-Gaud et al. 2010). Additionally, we expected that (4) the combined effects of grazing by tadpoles and caddisflies on periphyton growth would be lower than expected by their isolated effects (i.e., antagonistic) because competition between them would reduce their grazing efficiency.

MATERIALS AND METHODS

Study site and species

The study was conducted in three low-order streams located in the Peñalara Massif (Guadarrama Mountains National Park, Central Spain; Table 1, Fig. 1). This is a montane habitat (1800 - 2430 m), with low mean annual precipitation (694 mm) and a marked seasonality (temperature annual range: 28 ºC). Annual mean temperature is 6.6 ºC, with snowy and cold winters (mean temperature 0.1 ºC) and warm and dry summers (mean temperature 15 ºC and precipitation of 109 mm) [WorldClim database (Fick and Hijmans 2017)]. The substrate is mostly granitic and covered by heathlands dominated by *Cytisus oromediterraneus* RIV. MART. and *Juniperus communis nana* SYME, with isolated *Pinus sylvestris* L. The study sites were close to the treeline and had well oxygenated water, pH

ca. 6.5, low conductivity, low nutrient concentration and temperatures ca. 10 ºC at the time of the study (Table 1). The streams were chosen as they are the two largest streams in the area and the main tributary of one of them, being less likely to dry during the summer than other nearby streams (Bosch et al. 2019).

Table 1. Physicochemical characteristics of the study streams during the experimental period (mean ± SE; n=6). DIN = dissolved inorganic nitrogen, SRP = soluble reactive phosphorus.

Variable	Stream 1	Stream 2	Stream 3
Latitude (ºN)	40.84306	40.83668	40.83563
Longitude (ºW)	3.94333	3.95362	3.95333
Altitude (m a. s. l.)	1869	1942	1949
Temperature (ºC)	10.01 ± 0.07	11.73 ± 0.09	8.16 ± 0.09
Conductivity (µS cm^{-1})	11.86 ± 0.66	9.92 ± 0.33	11.90 ± 0.49
pH	6.29 ± 0.12	6.62 ± 0.17	6.43 ± 0.10
Dissolved O_2 (mg l^{-1})	8.55 ± 0.17	8.29 ± 0.24	8.89 ± 0.29
% Saturation O_2	100.69 ± 0.41	102.57 ± 0.89	100.64 ± 0.56
Flow (l s^{-1})	24.93 ± 17.44	13.55 ± 7.54	6.77 ± 1.68
DIN (µg l^{-1})	30.84 ± 3.28	28.39 ± 2.84	25.88 ± 3.05
SRP (µg l^{-1})	5.31 ± 0.57	4.74 ± 1.72	11.34 ± 1.21

The amphibian study species was chosen based on its previous abundance and ecological relevance in the study area. *Alytes obstetricans* (Family Alytidae, Order Anura) used to be a frequent species in permanent waters of both ponds and stream pools of the study area, but suffered a dramatic reduction since 1997 due to Bd without recovery to the present (Bosch et al. 2018; Bosch et al. 2001). The species has a long-term larval stage that, at high altitudes such as the study area, often prevents metamorphosis during the first year and forces the larvae to remain in the water for several years, reaching large sizes and high numbers in the order of thousands. Other amphibian species at the study area using stream pools are *Salamandra salamandra* LINNAEUS and *Rana iberica* BOULENGER, both under decline due to Bd and global warming (Bosch et al. 2018).

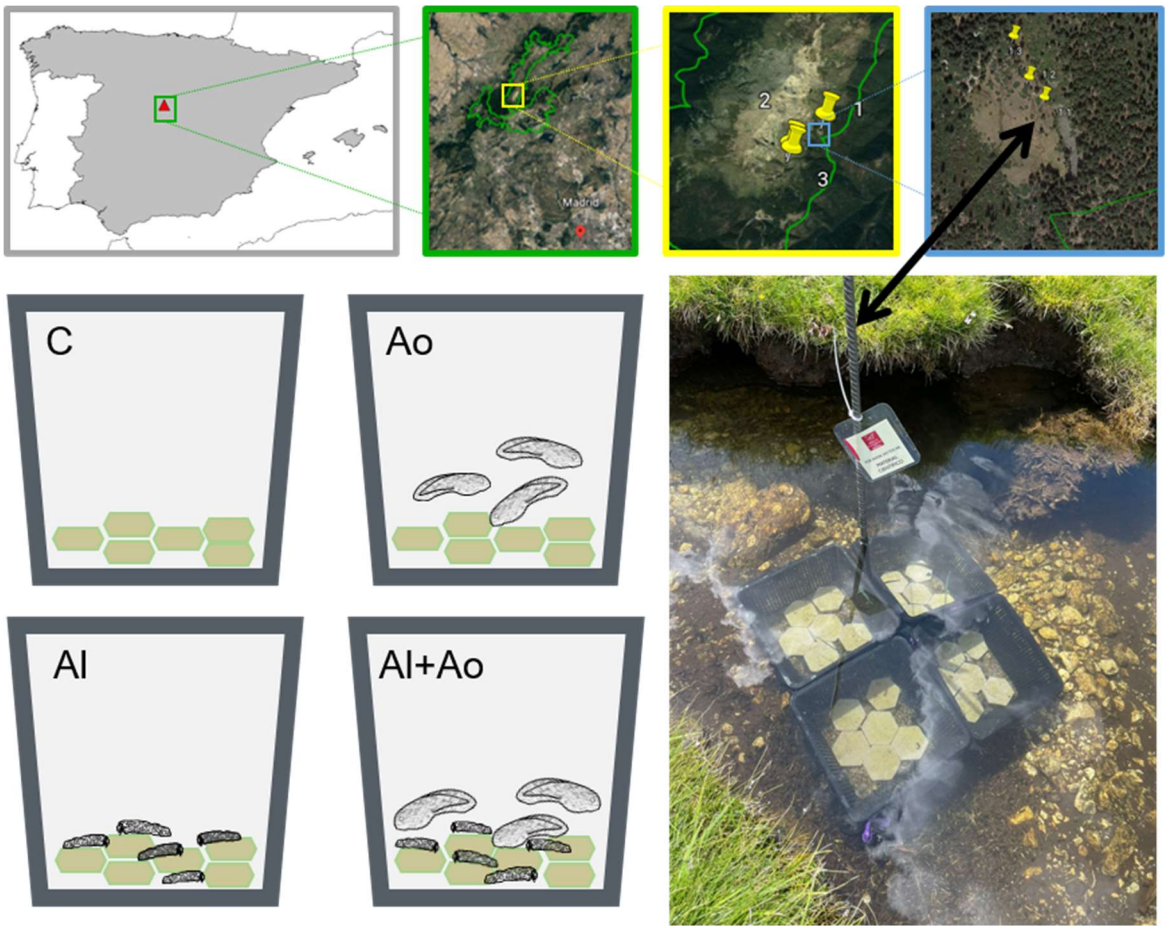

Figure 1. Location of Guadarrama Mountains National Park (yellow) in central Spain and the studied streams (green), with an example of pool location within one of the streams (blue) and a diagram of the experimental design of the different treatments: control (C), presence of caddisflies only (Al), presence of tadpoles only (Ao) and presence of both species (Al+Ao).

The macroinvertebrate used was the species *Allogamus laureatus* NAVÁS (Family Limnephilidae, Order Trichoptera), which is a common shredder and grazer in ponds and streams of the study area (Toro et al. 2020). *A. laureatus* present one generation each year, with aerial adults that lay eggs on streams and ponds. Larvae prefer areas with pebbles or organic material accumulations and feed on particulate organic matter and periphyton (Tachet et al. 2010; Toro et al. 2020).

Experimental procedure

A 14-day experiment was conducted in June 2021. In each stream, we located twelve enclosures, which were installed in 3 pools per stream, where *Alytes obstetricans* larvae used to be found before their decline due to Bd (J. Bosch pers. obs.). Enclosures consisted

of plastic baskets (0.17 x 0.17 x 0.13 m) closed by a 2-mm glass fibre mesh that allowed water and small invertebrate access but precluded tadpoles and bigger macroinvertebrates from moving in or out. The enclosures had the bottom covered by sediment from the corresponding stream and 6 marble tiles (33.6 cm^2 surface area) used as standardized substrata for periphyton growth (Fig. 1). Tiles were colonized by periphyton, by incubating them for 29 days within a pond located nearby (40.88694º N, 3.88613º W). Control enclosures (C) only contained tiles, and the other treatments contained either 5 caddisfly larvae (*Allogamus laureatus*; Al), 3 tadpoles (*A. obstetricans*; Ao), or both caddisflies and tadpoles (Al+Ao). Each treatment was replicated 3 times in each stream (9 replicates in total). In each one of the 3 pool sections of the stream, we tied 1 replicate per treatment to an iron bar and anchored it to the stream bottom (Fig. 1). Field work and experimental protocols were approved by Consejería de Medio Ambiente de la Comunidad de Madrid (ref. 10/045182.9/21).

We collected larvae of *Allogamus laureatus* (hereafter 'caddisflies') of similar size (3.9 ± 0.5 mg) from a nearby stream (40.84167° N, 3.94306° W) in June 2021, and randomly assigned them to the enclosures. We used tadpoles of *Alytes obstetricans* (hereafter 'tadpoles') in development Gosner stage 26 (except for 7 individuals, which ranged from stages 32 to 37) with similar size (snout-to-vent length, SVL ± SE: 20.07 ± 0.361 mm). Tadpoles had been reared in captivity at the "Centro de Investigación, Seguimiento y Evaluación" (Guadarrama Mountains National Park, Spain). Just before introducing tadpoles and caddisflies into the enclosures, we measured tadpole SVL (0.001 mm precision) using ImageJ software (v. 1.46r).

At the end of the experiment, tiles were collected manually from each enclosure. The surface of all tiles from each treatment was scrubbed with a soft toothbrush in ca. 100 mL of distilled water to collect the associated periphyton. Periphyton was frozen until analyses. In the laboratory, the solution was divided in 3 subsamples. One subsample was vacuum filtered through pre-incinerated and pre-weighed GF/F glass fibre filters (0.7-µm); filters were dried (70 ºC) for 72 hours and weighed to quantify dry mass (DM). Afterwards, filters were incinerated (500 ºC) for 5 hours and weighed to estimate periphyton ash-free dry mass (AFDM) and inorganic mass per area unit (g m^{-2}, Steinman 2006). The second subsample was filtered (GF/F, 0.7-µm) and used to quantify chlorophyll concentration. Chlorophyll was extracted from filters using a standard method with acetone 90% as solvent (12 h in darkness at 4 ºC), and samples were sonicated (60 Hz) and centrifuged

(2000 rpm) to ensure complete extraction. Then, chlorophyll *a*, *b* and *c* (hereafter Chl-*a*, Chl-*b* and Chl-*c*) concentrations (μg Chl m^{-2}) were assessed spectrophotometrically, by measuring absorbance at 750, 665, 647 and 630 nm, respectively (Steinman 2006). The three types of Chl were assessed because they are indicative of the biomass of total algae, green algae and diatoms, respectively. The biofilm autotrophic index, which shows the ratio between autotrophic and heterotrophic organisms in biofilm, was calculated as AFDM divided by Chl-*a* concentration (Steinman 2006). The third subsample was preserved with acidic Lugol's solution (0.4 %) and used to characterize the periphyton algal assemblage. Taxonomic identification to the lowest level possible (genus) and cell counting was performed using an optical microscope and a Neubauer chamber at ×200 magnification following Bellinger and Sigee (2015). Due to the difficulty of counting cells of *Coelosphaerium*, cell number per colony was determined by counting the cells of 8 colonies from different samples at ×1000 magnification (19.875 ± 2.240 cells per colony), and all counted colonies were multiplied by the average cell number (Godo et al. 2011). For each sample, abundance (cell m^{-2}), taxon richness (number of taxa per enclosure) and Shannon's diversity index were calculated.

At the end of the experiment, the caddisflies that we had previously introduced were recollected and preserved in ethanol 70%. In the laboratory, they were dried (72 h, 70 ºC) and weighed to quantify final DM (mg). Initial DM was estimated based on the mean DM of each species obtained from 30 extra larvae which were collected at the beginning of the experiment. Tadpoles were collected and measured using ImageJ. Tadpole growth was calculated as the difference between final and initial SVL divided by initial SVL.

Data analysis

Differences in algal biomass, Chl-*a*, Chl-*b* and Chl-*c* concentrations, the autotrophic index, inorganic mass, algal abundance, richness and Shannon's diversity index, caddisfly mass and tadpole growth among treatments (C, Al, Ao and Al+Ao) were examined with linear models (*lmer* function, "lmerTest" R package, Kuznetsova et al. 2015), with treatment as fixed factor, stream as random factor and pool as random factor nested within stream. Significant differences among treatments ($\alpha = 0.05$) were analysed with marginal means and effect sizes (*emmeans* and *eff_size* functions of the "emmeans" R package, Lenth et al. 2018). The influence of water flow on those variables was examined with likelihood ratio

tests to show the significance of the random factors (*ranova* function of the "lmerTest" R package, Kuznetsova et al. 2015), since flow was the main factor differing among streams. Differences in algal assemblage structure among treatments and streams were explored with permutational multivariate analysis of variance based on the Bray-Curtis similarity index (*adonis* function of the "vegan" R package, Oksanen et al. 2007). In order to identify the most representative taxa of each assemblage, an indicator value index (*multipatt* function of the "indicspecies" R package, De Cáceres 2013) was used. All statistical analyses were performed with R version 4.1.2.

RESULTS

Periphyton biomass varied significantly depending on treatment, with higher values in control (C, mean ± SE: 0.43 ± 0.07) than in presence of tadpoles (Ao, 0.29 ± 0.02) or both species (Al+Ao, 0.28 ± 0.03; Table S1, Fig. 2A, Fig S1A). Chl-*a* concentration showed a similar pattern (Ao, 6234.83 ± 1865.77 µg m^{-2}; C, 10526.06 ± 3058.30 µg m^{-2}; Table S1, Fig. 2B, Fig. S1D) but, in this case, it did not present significant differences. Chl-*b* and Chl-*c* concentrations were not significantly different across treatment, although there seemed to be a trend in Chl-*c* concentration, which tended to be higher in Al (232.47 ± 84.58 µg m^{-2}) than in the other treatments (Table S1, Fig. 2C, Fig. 2D, Fig. S1E, Fig. S1F). The autotrophic index (Table S1, Fig. 2E, Fig. S1B) and inorganic mass (Table S1, Fig. 2F, Fig. S1C) did not vary among treatments. All of these variables, except Chl-*c* concentration, were significantly affected by stream but not by pool within stream (Table S2).

Algal abundance did not present statistical differences; however, it tended to be higher in Al (682738.2 ± 167284.7 cell m^{-2}) than in Ao (297532.9 ± 91568.5 cell m^{-2}) and Al+Ao (337028.9 ± 43036.8 cell m^{-2}; Table S1, Fig. 2G, Fig. S1G). Taxon richness did not differ significantly either, but it also tended to be higher in treatments where tadpoles were absent (C, 11.88 ± 0.68 taxa; Al, 12.13 ± 0.42 taxa) than in the ones where they were present (Ao, 9.88 ± 1.06 taxa; Al+Ao, 10.5 ± 0.51 taxa; Table S1, Fig. S1H). Shannon's diversity showed a similar pattern but in this case: being significantly higher in C (0.97 ± 0.03) and Al (0.97 ± 0.05) than in Ao (0.81 ± 0.05; Table S1, Fig. 2H, Fig. S1I). Algal abundance and richness were significantly affected by stream, but not Shannon's diversity (Table S2). Algal assemblage structure varied among streams but it was not significantly affected by treatment (Table 2). However, the indicator value index, which indicates which taxa

characterize a treatment by presenting higher abundance than in other treatments, showed that *Synedra* spp. EHRENBERG was characteristic of C, and *Monoraphidium* spp. KOMÁRKOVÁ-LEGNEROVÁ was common in C and Al. The assemblage in all treatments was, in general, dominated by *Navicula* spp. BORY, *Scenedesmus* spp. MEYEN and *Gomphonema* spp. EHRENBERG (Table S3), with *Oscillatoria* spp. VAUCHER EX GOMONT also being common in the smallest and coldest site (stream 3).

Caddisfly mass was significantly higher in the presence of tadpoles (Al, 0.70 ± 0.26 mg; Al+Ao, 1.20 ± 0.39 mg; Table S1, Fig. 3A). However, tadpole length was not significantly affected by the presence of caddisflies in the enclosures (Ao, 6.11 ± 0.97; Al+Ao, 8.61 ± 1.3; Table S1, Fig. 3B). Neither caddisfly mass nor tadpole length were affected by stream or pond (Table S2).

DISCUSSION

Our experiment showed that presence or absence of tadpoles can alter stream ecosystem structure through changes in primary producer assemblages. Periphyton biomass was higher in the absence of tadpoles than in their presence, and Chl-*a* concentration showed a similar but nonsignificant trend. This agrees with results from other studies in tropical and temperate streams, where tadpole presence has been shown to reduce periphyton accrual due to grazing and bioturbation (Connelly et al. 2008; Flecker et al. 1999; Mallory and Richardson 2005; Ranvestel et al. 2004). In our study, the reduction in periphyton biomass was more likely caused by consumption, since we did not find any differences in sediment accrual among treatments without consumers and the ones containing tadpoles or caddisflies, and the effect of stream (mainly driven by flow) affected most periphyton related variables. This suggests that stream flow was low but enough to overshadow any potential effect of tadpoles on sediment accumulation, and hence any change in bioturbation when tadpoles disappeared, contrasting with other studies where sediment accrual was reduced by tadpole presence (Barnum et al. 2022; Mallory and Richardson 2005; Ranvestel et al. 2004). These differences can be due to the different conditions of neotropical streams and montane temperate streams, since differences in flow and tadpole densities between riffles and pools within the same stream have can determined the effects of tadpole loss on bioturbation (Barnum et al. 2022).

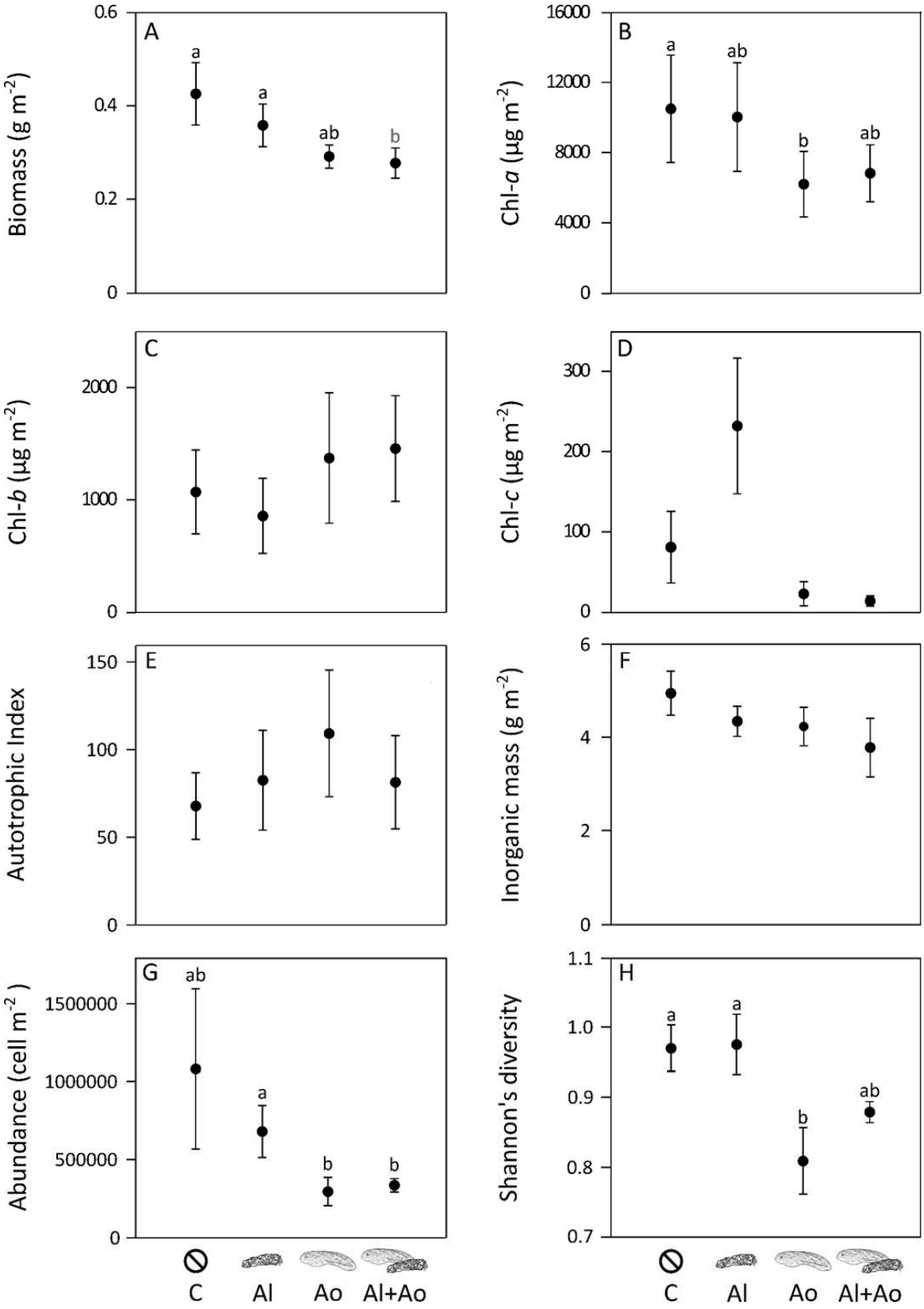

Figure 2. Periphyton biomass (g m^{-2}; A), Chl-*a* (B), Chl-*b* (C) and Chl-*c* (D) concentrations (µg m^{-2}), autotrophic index (E), inorganic mass (g m^{-2}; F), algal abundance (cell m^{-2}; G) and Shannon's diversity index (F) in the control (C), presence of caddisflies (Al), presence of tadpoles (Ao) and presence of both species (Al+Ao). Circles represent means and whiskers standard errors. Different letters indicate significant differences among treatments.

Table 2. Results of PERMANOVAs exploring the effects of treatment (control, presence of caddisflies, presence of tadpoles and presence of both species), stream and their interaction, as well as significant paired comparisons, on algal assemblage structure; df = degrees of freedom; F = F-statistic; R^2 = adjusted R^2; p = p-value. Stress = 0.1471433.

Factor	df	F	R^2	p	Sign. paired comparisons
Treatment	3	1.46	0.1314	0.085	
Stream	1	3.55	0.1068	0.002	Stream 1 vs. Stream 2; Stream 1 vs. Stream 3; Stream 2 vs. Stream 3
Treatment x Stream	3	1.12	0.1011	0.313	

The analysis of algal assemblage structure showed significant differences among streams independently of experimental treatments, possibly in relation to differences in physicochemistry or other stream characteristics (Mallory and Richardson 2005). Despite the fact that neither algal assemblage structure nor abundance nor richness presented statistically significant differences, they showed strong trends. Abundance and richness tended to be higher in presence of only caddisflies than in treatments with tadpoles, possibly due to the more efficient grazing activity of tadpoles compared to macroinvertebrates (Colón-Gaud et al. 2010). The trend of higher Chl-*c* concentration in presence of caddisflies alone suggests that tadpole presence mostly reduced the abundance of diatoms, for several reasons: (1) these algae are more easily removed by bioturbation (Barnum et al. 2022; Ranvestel et al. 2004); (2) they are preferred over filamentous green algae (Kupferberg 1997); and (3), due to their tendency to present a larger size than other algae such as green algae or cyanobacteria, they are better assimilated by tadpoles and less likely to be intact after passage though tadpole gut, and therefore, less prone to recolonize the grazed area (Connelly et al. 2008; Peterson and Boulton 1999; Steinman 1996). This view is supported by the differences in Shannon's diversity and the indicator taxa that we found in both treatments without tadpoles. The control was characterized by *Synedra*, which is a large diatom, known to be a preferred resource for tadpoles and to be efficiently digested by them (Connelly et al. 2008; Peterson and Boulton 1999; Peterson and Jones 2003); and both treatments without tadpoles (control and only caddisflies) were characterized by *Monoraphidium*, which is a green alga whose abundance has previously been found to be affected by grazing (Tarkowska-Kukuryk et al. 2020) and, therefore, potentially more affected by tadpole presence. Unlike other studies showing that grazer presence increased periphyton diversity by opening patches where new colonizers can settle, reducing

dominance of stronger competitive species and allowing the persistence of rarer taxa (Dunck et al. 2018; Hillebrand 2009), we found the highest diversity in absence of tadpoles.

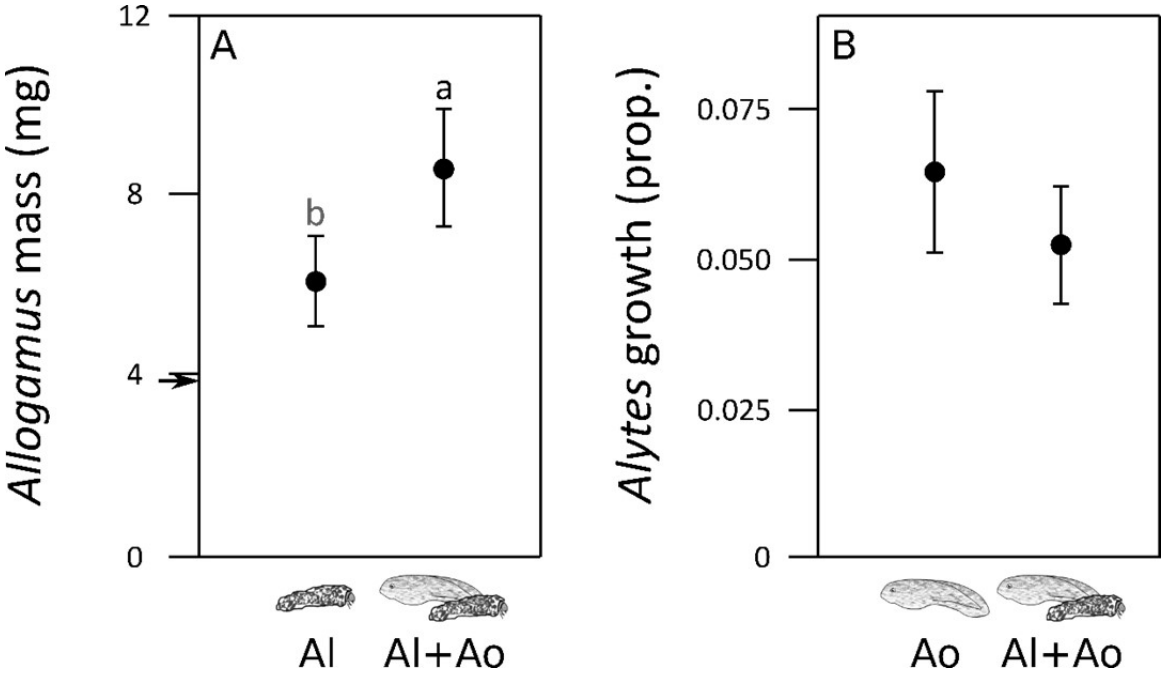

Figure 3. Caddisfly mass (mg; A) and tadpole increase in length (proportion of SVL; B) in treatments with caddisflies (Al), tadpoles (Ao) and both species (Al+Ao). Circles represent means, whiskers standard errors and arrows estimated initial caddisfly mass. Different letters indicate significant differences among treatments.

Tadpoles did not increase their length much during the experiment, regardless of the presence or absence of caddisflies, with no variation among treatments, as it could be expected due to the short duration of the experiment relative to the life cycle of *Alytes obstetricans* (Garriga et al. 2017; Scheidt and Uthleb 2005). On the other hand, caddisflies almost doubled their body mass in both treatments, with greater growth in presence of tadpoles. This all suggests that large grazers such as *Allogamus laureatus* do not directly compete with tadpoles, contrasting with studies in tropical streams where larger grazers were benefited by tadpole losses (Colón-Gaud et al. 2010). Instead, caddisflies seemed to benefit from tadpole activity, as has been observed for small grazers in tropical streams (Colón-Gaud et al. 2010; Ranvestel et al. 2004). This may be due to facilitating access to underlying algal resources caused by tadpole grazing, and to the more generalist feeding of *A. laureatus*, which allows them to feed also on fine particulate organic matter produced by tadpoles or drawn by the current (Kupferberg 1997; Ranvestel et al. 2004).

CONCLUSIONS

Amphibian losses have been shown to alter ecosystem functioning through changes in periphyton and invertebrate assemblages in tropical streams (Colón-Gaud et al. 2010; Connelly et al. 2008; Ranvestel et al. 2004). Here, we have shown that ongoing amphibian declines have similar effects in temperate montane streams, causing an increase in periphyton biomass, a reduction in macroinvertebrate biomass and changes in algal assemblage structure in the short term, effects that might be expected to become more apparent over larger temporal scales (Connelly et al. 2008). Our results are highly relevant, since amphibians are suffering dramatic declines globally (Collins 2010), and that could lead to further alterations of ecosystem structure and functioning in those ecosystems presenting stable amphibian populations. Therefore, it is important to continue studying the role that amphibians play in ecosystems in order to better predict the ecological effects of their disappearance, and to improve protection measures that avoid their extinction.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIAL

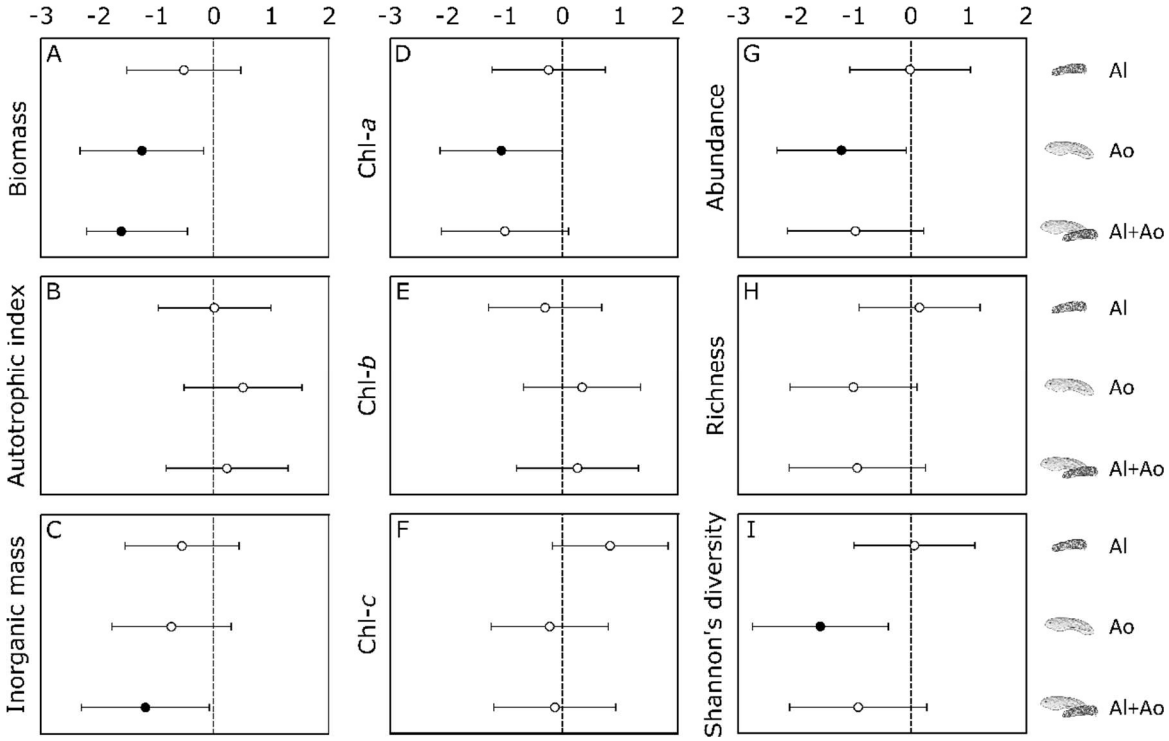

Figure S1. Effect sizes of periphyton biomass (A), autotrophic index (B), inorganic mass (C), Chl-*a* (D), Chl-*b* (E) and Chl-*c* (F) concentrations, algal abundance (G), taxon richness (H) and Shannon's diversity index (I) in presence of caddisflies (Al), tadpoles (Ao) and both species (Al+Ao) compared to the control. Circles represent effect sizes and whiskers 95% confidence intervals. Open and closed circles represent intervals that do and do not contain the value of zero, respectively.

Table S1. Results of linear models exploring the effects of treatment (control, presence of caddisflies, presence of tadpoles and presence of both species) on periphyton biomass accrual (g m^{-2}); chlorophyll *a*, *b* and *c* concentrations (μg m^{-2}); autotrophic index; inorganic mass (g m^{-2}); algal abundance (cell m^{-2}), taxon richness (taxa per enclosure) and Shannon's diversity index; macroinvertebrate mass (mg) and tadpole growth (prop.); df = degrees of freedom; F = F-statistic; η_p^2 = partial eta squared; p = p-value.

Variable	df	F	η_p^2	p
Biomass	3, 21	3.78	0.34	0.025
Chlorophyll *a*	3, 21	2.37	0.25	0.099
Chlorophyll *b*	3, 21	0.69	0.09	0.566
Chlorophyll *c*	3, 21	1.97	0.21	0.147
Autotrophic index	3, 21	0.47	0.06	0.707
Inorganic mass	3, 21	1.91	0.21	0.157
Algal abundance	3, 18	3.04	0.32	0.053
Algal richness	3, 18	2.66	0.29	0.077
Shannon's diversity index	3, 18	4.52	0.41	0.015
Macroinvertebrate mass	1, 5	8.04	0.61	0.035
Tadpole growth	1, 4	0.26	0.04	0.627

Table S2. Results of likelihood ratio tests exploring the effects of stream and pool nested within stream on periphyton biomass accrual (g m^{-2}); chlorophyll *a*, *b* and *c* concentrations (µg m^{-2}); autotrophic index; inorganic mass (g m^{-2}); algal abundance (cell m^{-2}), taxon richness (taxa per enclosure) and Shannon's diversity index; detritivore mass (mg) and tadpole growth (prop.); df = degrees of freedom; LRT = likelihood ratio test statistic; p = p-value.

Variable	Factor	df	LRT	p
Biomass	Stream	1	0.00	1.000
	Pool:Stream	1	5.11	0.024
Chlorophyll *a*	Stream	1	10.04	0.002
	Pool:Stream	1	0.24	0.624
Chlorophyll *b*	Stream	1	12.57	<0.001
	Pool:Stream	1	0.00	1.000
Chlorophyll *c*	Stream	1	0.00	1.000
	Pool:Stream	1	0.00	1.000
Autotrophic index	Stream	1	8.93	0.003
	Pool:Stream	1	0.04	0.839
Inorganic mass	Stream	1	4.61	0.032
	Pool:Stream	1	0.00	1.000
Algal abundance	Stream	1	5.02	0.025
	Pool:Stream	1	0.00	1.000
Algal richness	Stream	1	4.46	0.035
	Pool:Stream	1	0.00	1.000
Shannon's diversity index	Stream	1	0.95	0.330
	Pool:Stream	1	0.00	1.000
Macroinvertebrate mass	Stream	1	2.39	0.122
	Pool:Stream	1	1.54	0.215
Tadpole growth	Stream	1	0.00	1.000
	Pool:Stream	1	0.00	1.000

Table S3. Relative contribution (%) of each algal taxon to algal abundance in each treatment [control (C), presence of caddisflies only (Al), presence of tadpoles only (Ao) and presence of both species (Al+Ao)] × stream combination (stream 1, 2 and 3). Mean abundance and accumulated taxon richness are given at the bottom of the table. Bold type represents species with a contribution to total abundance higher than 10 % and asterisks represent representative taxa of the stream.

Stream		Stream 1				Stream 2				Stream 3			
Treatment		C	Al	Ao	Al+Ao	C	Al	Ao	Al+Ao	C	Al	Ao	Al+Ao
Phylum	Genus	⊘				⊘				⊘			
Bacillariophyta	*Amphora* EHRENBERG EX KÜTZING					0.08*	0.43*	0.89*	0.29*		0.19		
Cyanobacteria	*Anabaena* BORY EX BORNET & FLAHAULT		8.72	5.39		0.71	1.63		2.82				
Chlorophyta	*Chlorella* BEIJERINCK	1.28	7.96	**25.98**	**10.14**	4.32	1.34	2.66	2.92	2.99	2.26	3.97	1.50
Bacillariophyta	*Cocconeis* EHRENBERG							0.15					
Cyanobacteria	*Coelosphaerium* NÄGELI	**12.76**	6.50			**15.86**	5.48			4.79		**18.73**	**10.37**
Charophyta	*Cosmarium* CORDA EX RALFS	4.49			1.45	0.24	1.07	2.07		0.66	0.45	1.75	0.88
Bacillariophyta	*Cymbella* C.AGARDH	2.85	1.56	2.94			0.15	1.19	0.29	0.39		0.71	0.53
Bacillariophyta	*Fragilaria* LYNGBYE					1.66		0.73		0.32	2.60	3.07	
Bacillariophyta	*Gomphonema* EHRENBERG	6.70	**12.65**	2.45	5.80	**10.19***	**19.01***	**11.37***	**26.45***	**12.70**	9.09	**16.69**	**15.55**
Miozoa	*Gymnodimium* F.STEIN			0.49									
Bacillariophyta	*Hannaea* R.M.PATRICK										0.24		
Bacillariophyta	*Melosira* C.AGARDH	3.85	2.62			2.53	0.34	2.08	1.16	0.93	3.31	2.93	3.25
Chlorophyta	*Monoraphidium* KOMÁRKOVÁ-LEGNEROVÁ		3.56	1.96	5.80	1.75*	3.98*	1.79*	4.36*	5.43*	4.40*	0.91*	1.85*
Charophyta	*Mougeotia* C.AGARDH									0.52			
Bacillariophyta	*Navicula* BORY	**39.49**	**30.52**	**30.88**	**15.94**	**23.38***	**28.01***	**44.88***	**35.69***	**11.40**	**19.02**	**20.57**	**28.32**
Cyanobacteria	*Oscillatoria* VAUCHER EX GOMONT	3.13		7.84	**10.14**		6.40			**10.55**	**28.03**	**15.17**	**14.13**
Charophyta	*Penium* BRÉBISSON EX RALFS	6.25	1.89							0.26		1.78	0.35
Bacillariophyta	*Pinnularia* EHRENBERG	1.56					0.14			0.16		0.44	
Chlorophyta	*Scenedesmus* MEYEN		6.07	**10.78**	**40.60**	**20.02**	**10.82**	**19.98**	**15.97**	**21.46**	**15.90**	8.06	**14.10**
Chlorophyta	*Selenastrum* REINSCH	1.56	3.89	0.98		6.87*	8.01*	3.39*	4.42*	5.32	6.18	0.47	1.95
Bacillariophyta	*Stenopterobia* BRÉBISSON EX VAN HEURCK	0.64											
Chlorophyta	*Stigeoclonium* KÜTZING									4.71			
Bacillariophyta	*Synedra* EHRENBERG	8.45	7.67	6.37	5.80	6.89	4.68	6.79	5.29	**14.20**	7.02	2.51	5.04
Bacillariophyta	*Tabellaria* EHRENBERG EX KÜTZING	0.64					0.14	0.15			0.24		

Table S3. Cont.

Stream		Stream 1				Stream 2				Stream 3			
Treatment		C	Al	Ao	Al+Ao	C	Al	Ao	Al+Ao	C	Al	Ao	Al+Ao
Phylum	Genus	⊘				⊘				⊘			
Bacillariophyta	*Tetracyclus* RALFS					0.08		0.15		0.73*	0.30*	0.70*	1.14*
Chlorophyta	*Tetraedron* KÜTZING	6.34	2.33	3.92	4.35	5.25*	4.60*	1.76*	0.35*	0.47	0.79	1.55	1.05
Ochrophyta	*Tribonema* DERBÈS & SOLIER		4.07			0.17	3.78			2.01			
Abundance (cell m^{-2})		133035 ± 53670	312681 ± 87538	101190 ± 39322	113798 ± 61136	2064886 ± 1374795	786803 ± 228239	496252 ± 185596	418972 ± 23616	738452 ± 201549	825378 ± 421842	304444 ± 57894	344041 ± 65688
Accumulated taxon richness		15	14	12	9	16	18	16	12	20	16	17	15

Chapter 5

LOSS OF AMPHIBIAN SPECIES ALTERS PERIPHYTON COMMUNITIES IN MONTANE PONDS

Alonso, A., Bosch, J., & Boyero, L. Loss of amphibian species alters periphyton communities in montane ponds. *Hydrobiologia*, *under review.*

ABSTRACT

Amphibian larvae can affect the structure and functioning of freshwater ecosystems, but their effects have been little explored although amphibian biodiversity is rapidly declining. Given that larvae of different amphibian species belong to different trophic levels, their effects on freshwater communities and processes can be expected to differ, with herbivores likely having direct effects on algae and predators having indirect effects through trophic cascades. We explored this question through a mesocosm experiment conducted in montane ponds, using an anuran and a urodele species affected by emergent diseases. We used different scenarios of reduction and loss of one or both species, and compared them to a control scenario representing a typical amphibian community in the study area composed of four species, with total larval density maintained. Loss of the anuran resulted in lower chlorophyll concentration and algal density, likely due to replacement by more efficient grazers. Loss of the urodele produced similar trends but weaker, possibly due to an increase of invertebrate grazing activity in the absence of this predator. Our study shows how the loss of amphibian species can alter the structure of montane ponds, but also how the mechanisms involved and the intensity of effects differ for different species.

INTRODUCTION

Biodiversity is currently declining at rates comparable to those reported for past mass extinctions (Barnosky et al. 2011; Ceballos et al. 2017) and fresh waters are among the most affected ecosystems, with extinction rates considerably higher than those of marine or terrestrial ecosystems (Reid et al. 2019; Sala et al. 2000). While the loss of species is worrying in itself, due to the intrinsic value of biodiversity (Ghilarov 2000; IPCC 2018), effects go far beyond this value, as demonstrated by decades of experimental work showing that biodiversity loss has important consequences for the structure and functioning of ecosystems (Boyero et al. 2021; Cardinale et al. 2006; Hooper et al. 2012). Importantly, the consequences of species loss are expected to vary depending on their role in the ecosystem and their position in the food web (Lourenço-Amorim et al. 2014). Thus, while the loss of herbivore species can induce algal blooms in fresh waters (Hillebrand 2009), the loss of predators can have cascading effects on lower trophic levels by inducing an increase in primary consumers due to predatory release, and hence a reduction in basal resources such as algae (Kurle and Cardinale 2011). Amphibian species losses are highly relevant in

this context for at least two reasons: firstly, amphibian larvae are key consumers in freshwater ecosystems (Hocking and Babbitt 2014; Whiles et al. 2006) and often include both herbivores (many anurans) and predators (urodeles); and secondly, amphibians suffer dramatic declines globally (Collins 2010; Luedtke et al. 2023; Wake and Vredenburg 2008), and there is virtually no information about the consequences that the reduction in amphibian diversity may have for freshwater ecosystem structure and functioning (Whiles et al. 2006).

Anuran larvae are important periphyton grazers that can control algal accrual and alter the structure of algal communities (Alonso et al. 2022; Connelly et al. 2008; Kupferberg 1997; Mallory and Richardson 2005; Ranvestel et al. 2004). Thus, they can have an influence on primary production, decomposition and nutrient cycling (Connelly et al. 2008; Rugenski et al. 2012; Schmidt et al. 2019). Anuran larvae compete with certain invertebrates, the former generally showing greater grazing efficiency (Alonso et al. 2022; Arribas et al. 2014; Barnum et al. 2022; Colón-Gaud et al. 2009; Connelly et al. 2008; Ranvestel et al. 2004; Rowland et al. 2017), although differences among tadpoles of different species are to be expected depending on larval size and their feeding and behavioural strategies (Kupferberg 1997; Schmidt et al. 2019). Moreover, while some anuran larvae can competitively exclude invertebrates (Kupferberg 1997), others may facilitate their access to underlying algal resources as a result of bioturbation or production of organic matter that can be used by more generalist invertebrates (Alonso et al. 2022; Ranvestel et al. 2004). On the other hand, urodele larvae are predators that can alter invertebrate communities by direct consumption (Arribas et al. 2014; Rowland et al. 2017; Urban 2013) and hence induce top-down effects on basal resources, as observed for the increases in periphyton accrual in the presence of salamander larvae (Blaustein et al. 1996; Holomuzki et al. 1994).

Emergent infectious diseases are among the main causes of amphibian declines worldwide, mostly due to fungi of the genus *Batrachochytrium* (Collins 2010; Daszak et al. 2003; Fisher and Garner 2020; Scheele et al. 2019). In particular, the species *Batrachochytrium dendrobatidis* LONGCORE, PESSIER & D.K. NICHOLS (hereafter "Bd") is a widely introduced generalist fungal pathogen which has caused amphibian declines and extinctions globally. This pathogen infects the keratinized skin of amphibian adults and larvae, leading to hyperkeratosis of the skin, which can cause deformation of mouthparts and reduction of growth in larvae, and high mortality after metamorphosis in sensitive

species (Fisher and Garner 2020; Fisher et al. 2009; Garner et al. 2009; Harjoe et al. 2022). In our study area, the montane wetlands of the Central System in Spain, the anuran *Alytes obstetricans* LAURENTI (common midwife toad) presents one of the largest larva and, at the same time, it is the species most affected by chytridiomycosis (Bosch et al. 2018; Bosch et al. 2001). The other described species of the *Batrachochytrium* genus, *B. salamandrivorans* A. MARTEL, BLOOI, BOSSUYT & PASMANS (hereafter "Bsal"), has been shown to affect western Palaeartic urodeles, such as *Salamandra salamandra* LINNAEUS in Central Europe or *Triturus marmoratus* LATREILLE (marbled newt) in the only affected locality in the Iberian Peninsula (Bosch et al. 2021). In susceptible urodeles, this pathogen infects the skin, leading to ulceration and causing mortality rates of almost 100% in adults (Fisher and Garner 2020; Martel et al. 2014; Martel et al. 2020; Stegen et al. 2017).

In this study, we used a model freshwater ecosystem representing a simplified amphibian community of montane ponds in central Spain (Bosch et al. 2018), to assess how the loss of amphibian species affected by chytrid infections (Bd and Bsal) may affect the structure of periphyton communities and associated ecosystem processes. Our amphibian community was composed of three anuran species (*A. obstetricans*, *Bufo spinosus* DAUDIN and *Pelophylax perezi* LÓPEZ-SEOANE) and one urodele species (*T. marmoratus*) and was intended to represent a common amphibian community from montane areas of the Iberian peninsula (García-París et al. 2004). Using mesocosms, we compared the original scenario that consisted of the whole, four-species community (control) with four scenarios simulating the reduction or loss of the most threatened species: the anuran *A. obstetricans* and the urodele *T. marmoratus*. We kept total larval abundance constant in all scenarios, with increases in the abundance of the remaining species following the loss of a given species. Our response variables were: periphyton biomass accrual; chlorophyll *a*, *b* and *c* concentrations, as indicators of the biomass of total algae, green algae and diatoms, respectively; inorganic mass, as indicator of sediment accumulation; the autotrophic index, the ratio between periphyton biomass and chlorophyll concentration to assess the proportion of autotrophic and heterotrophic organisms in periphyton (Steinman 2006); and algal community structure. We hypothesized that:

(i) The reduction or loss of the most threatened anuran species, *A. obstetricans*, which larvae are periphyton grazers, would result in: higher periphyton biomass accrual, chlorophyll concentration and inorganic mass accumulation; and changes in algal community structure. Grazer diversity is known to reduce periphyton accrual

through complementarity effects related to different grazing efficiencies in invertebrates (Brönmark et al. 1991; Hertonsson et al. 2008; Hillebrand et al. 2009), and we expected this to occur also in tadpoles of different species (Kupferberg 1997).

(ii) The loss of the urodele species, *T. marmoratus*, which larvae are predators, would result in lower periphyton biomass accrual and lower chlorophyll concentration through a trophic cascade involving zooplankton. Zooplankton feeds on periphyton and significantly reduces its biomass in a density-dependent manner (Kazanjian et al. 2018; Masclaux et al. 2012). Thus, we expected that, in the control scenario, *T. marmoratus* would control the density of zooplankton, their preferred prey (Sánchez-Hernández 2020), but with the loss of *T. marmoratus* and its predatory pressure, zooplankton density would increase and their feeding on periphyton (Blaustein et al. 1996) would lead to lower algal accrual.

(iii) The simultaneous loss of *A. obstetricans* and *T. marmoratus* would likely lead to a lesser increase in algal biomass accrual, chlorophyll concentration and inorganic mass than the loss of only *A. obstetricans*, due to the opposite effects of the loss of both species (as explained in the previous hypotheses), but still resulting in a net positive effect on periphyton variables due to the stronger grazing pressure of tadpoles compared to invertebrates (Alonso et al. 2022).

MATERIALS AND METHODS

Amphibian species

The common midwife toad (*A. obstetricans*, Family Alytidea, Order Anura) is distributed through high pluviosity regions in Western Europe. Larvae can live in stream pools and temporary or permanent ponds where they graze on periphyton, thus controlling algal accrual (Alonso et al. 2022), but they can also feed on detritus. Larval stage duration varies from 3 months in lowlands to several years in montane habitats, what determines their size, ranging from 7 cm in lowlands to 11 cm in montane habitats (García-París et al. 2004).

The Iberian green frog (*P. perezi*, Family Ranidae, Order Anura) widely distributed species in freshwater habitats across the Iberian Peninsula. The larval stage lasts between 8 and 12 weeks, but at high altitudes metamorphosis may be prevented during the first year, forcing larvae to remain in the water. Tadpoles live mainly at the bottom of ponds and

streams with abundant vegetation, and feed by aspiring detritus and grazing periphyton. Larval length usually reaches 6 cm (García-París et al. 2004).

The spiny toad (*B. spinosus*, Family Bufonidae, Order Anura) is widely distributed through the western Mediterranean region (Iberian Peninsula, west of France, Morocco, Algeria and Tunisia). Larvae live at the bottom of lentic waters or low flow areas of streams, feeding by grazing periphyton and collecting organic material deposited at the bottom of ponds. The larval stage is variable depending on environmental conditions, ranging from 2 to 4 months, and usually reaching lengths of 3-4 cm (García-París et al. 2004).

The marbled newt (*T. marmoratus*, Family Salamandridae, Order Caudata) is a common newt in western France and the northern Iberian Peninsula, in temporal and permanent ponds with abundant vegetation. The larval stage lasts 3 months and tadpoles can reach 4-7 cm. Larvae feed on aquatic invertebrates, and adults feed on amphibian larvae (García-París et al. 2004).

Experimental set-up

A mesocosm experiment was carried out in June-July 2022 at the 'Centro de Investigación, Seguimiento y Evaluación' facilities (Guadarrama Mountains National Park, Spain). Mesocosms were thirty-five 80-L tanks filled with 20 L of filtered stream water, with a light-dark regime of 12:12 h. Water physicochemical parameters during the experiment were (mean ± SE): temperature, 17.53 ± 0.23 ºC; pH, 7.78 ± 0.00; conductivity, 132.83 ± 3.10; O_2 concentration, 8.50 ± 0.07 mg l^{-1}; O_2 saturation, 103.97 ± 1.15 %; dissolved inorganic nitrogen, 189.11 ± 89.35 µg N L^{-1}; and soluble reactive phosphorus, 548.03 ± 213.44 µg P L^{-1}. Mesocosms contained natural sediment and aquatic mosses of the genus *Fontinalis* collected from a pond in Guadarrama Mountains National Park to simulate their natural habitat.

Periphyton

Two hundred and ten marble tiles of 33.6-cm^2 were colonized by periphyton through their incubation in an outdoor artificial pond located at the facilities, for one month before the beginning of the experiment (Pérez-Calpe et al. 2021). At the beginning of the experiment,

each mesocosm received 6 tiles containing (mean ± SE): 5.60 ± 1.15 g m^{-2} of biomass, 11.84 ± 3.42 g m^{-2} of inorganic mass, 966.22 ± 462.14 µg m^{-2} of Chl-*a*, 233.21 ± 78.94 µg m^{-2} of Chl-*b*, an undetermined quantity below our detection limit of Chl-*c* (obtained from 30 extra tiles incubated in the same pond at the same time), and then a combination of amphibian larvae, as explained below.

Amphibian larvae

Alytes obstetricans larvae were raised in captivity at the facilities, while larvae of the other species were collected from several ponds in the Guadarrama Mountains National Park. Just before the start of the experiment, we measured (0.05 mm precision) larval total length (TL) and snout-vent length (SVL) using ImageJ software (v. 1.46r). Tadpoles of *A. obstetricans* and *P. perezi* were at Gosner stage 26, except for some *P. perezi* that were at Gosner stages 30 and 31. Tadpoles of *B. spinosus* ranged from Gosner stages 26 to 37, and larvae of *T. marmoratus* presented a development stage ranging from 47 to 56 following Liozner and Dettlaff (1991). Invertebrates, mainly Ostracoda with a lesser proportion of larvae of Culicidae (Diptera) and *Habroleptoides* (Ephemeroptera), were added to all mesocosms as food for *T. marmoratus*, including the mesocosms without this species in order to maintain the same conditions in all of them as they could affect periphyton accrual (Kazanjian et al. 2018; Tachet et al. 2010).

Experimental design

The experimental design included five scenarios simulating a simplified amphibian community in our study area, and the changes occurring following the *A. obstetricans* population collapse due to chytridiomycosis (Bosch et al. 2018; Bosch et al. 2001; Bosch and Rincón 2008; Martínez-Solano et al. 2003) and the potential emergence of Bsal, which has occurred in the north-eastern Iberian Peninsula (Martel et al. 2020). There were seven replicates per scenario, therefore 35 mesocosms in total. All mesocosms contained the same number of individuals (16), but the presence and proportion of different species varied among scenarios. Control mesocosms contained eight larvae of *A. obstetricans*, two of *B. spinosus*, four of *P. perezi* and two of *T. marmoratus*, representing a typical situation which falls within the variability observed in the study area before 1997, with *A. obstetricans* as

the dominant species and lesser presence of other amphibians (Bosch et al. 2018; Martínez-Solano et al. 2003).

The first scenario of species loss (S1) represented the first years (2002-2005) after the emergence of Bd and hence the reduction of *A. obstetricans* abundance, with a concomitant increase in the other anuran species. Mesocosms contained two larvae of *A. obstetricans*, four of *B. spinosus*, eight of *P. perezi* and two of *T. marmoratus,* similar to the proportion of these species at that time; *A. obstetricans* 6.2 ± 0.4 %, *B. spinosus* 19.0 ± 1.0 %, *P. perezi* 64.9 ± 1.2 %, and *T. marmoratus* 9.9 ± 0.5 % (Bosch et al. 2018; Bosch and Rincón 2008; Martínez-Solano et al. 2003). The reduction of *A. obstetricans* benefited the other species, which have the same habitat preferences than *A. obstetricans*, but are less affected by Bd. This was particularly true for *P. perezi,* a highly competitive species and abundant in any suitable habitat, which showed a population growth after the *A. obstetricans* decline. *Bufo spinosus* also benefited, as it is usually outcompeted by *A. obstetricans* and thus displaced from the best foraging sites due to the greater larval size of the latter (Bosch and Rincón 2008; Richter-Boix et al. 2007a; Richter-Boix et al. 2004). *Triturus marmoratus* maintained its density because this species does not compete with *A. obstetricans*, as they feed on different resources, and *A. obstetricans* tadpoles are too large to be a suitable prey for *T. marmoratus* larvae.

The second scenario (S2) represented the effects of the prevalence of Bd after several years, with the total disappearance of *A. obstetricans* from the area and thus a further increase in the abundance of the other anurans (five *B. spinosus*, nine *P. perezi*) and no changes in *T. marmoratus* (two). This would result in an amphibian community similar to the observed in the study area 20 years after the emergence of Bd (2013-2016, A. obstetricans 3.3 ± 0.7 %, B. spinosus 15.4 ± 0.8 %, P. perezi 72.1 ± 0.8 %, T. marmoratus 9.2 ± 1.5 %; Bosch et al. 2018). The third scenario (S3) simulated the emergence of Bsal and hence the loss of *T. marmoratus*, as observed in the Montnegre i el Corredor Natural Park (northeastern Iberian peninsula; Martel et al. 2020), with a concomitant increase in the other species (nine *A. obstetricans*, two *B. spinosus*, five *P. perezi*). We did not experimentally test for the reduction (rather than loss) of *T. marmoratus* because of its high mortality following the appearance of Bsal (Martel et al. 2020). The fourth scenario (S4) simulated the extinction of both *A. obstetricans* and *T. marmoratus*, due to the presence of both Bd and Bsal, resulting in a community composed solely of *B. spinosus* (five) and *P. perezi* (11; Figure S1).

Sample collection and processing

At day 14, tiles and larvae were collected. Tile surfaces were scrubbed into 100 mL of distilled water and then divided in three subsamples. One of them was filtered in pre-incinerated (5 h, 500 ºC) and pre-weighed filters (GF/F, 0.7 µm), dried (72 h, 70 ºC), weighed (0.01 mg precision) to quantify periphyton dry mass (DM) and, afterwards, incinerated (5 h, 500 ºC) and reweighed to estimate ash-free dry mass (AFDM; g m^{-2}) and inorganic mass (ash mass, g m^{-2}). The second subsample was also filtered (GF/F, 0.7 µm), and then chlorophyll was extracted from the filters by submerging them in 90% acetone in darkness (24 h, 4 ºC). To ensure the complete separation of materials, samples were sonicated (60 Hz) and centrifuged (2500 rpm). Concentrations (µg Chl m^{-2}) of chlorophyll *a* (Chl-*a*), chlorophyll *b* (Chl-*b*) and chlorophyll *c* (Chl-*c*) were estimated spectrophotometrically by measuring absorbance at 750, 665, 647 and 630 nm (Steinman 2006). The three types of Chl were assessed since they are indicative of the biomass of total algae, green algae, and diatoms and related taxa, respectively. The biofilm autotrophic index (AI) was calculated as the ratio between AFDM and Chl-*a* concentration (Steinman 2006). The last subsample was preserved in acidic Lugol solution (0.4 %) and used to characterize the periphytic algal community. Taxonomic identification to the lowest level possible (genus) and cell counting were performed using an optical microscope and a Neubauer chamber at ×200 magnification following Bellinger and Sigee (2015). For each sample, density (cell m^{-2}) and taxon richness (number of taxa per enclosure) were calculated.

Amphibian larvae were measured using ImageJ at the end of the experiment to calculate mean growth for each species (% length) in each mesocosm as the difference between final and initial length divided by initial length, for both TL and SVL, as indicator of interspecific competition. Most periphyton related variables (biomass, inorganic mass, Chl concentration and cell density) were divided by anuran fresh mass (FM) in each mesocosm to avoid variability due to different larval sizes. Anuran FM was estimated using a TL-FM or SVL-FM relationship (*B. spinosus,* $FM = 0.001 \times TL^{1.8441}$, $r^2 = 0.4642$, n = 9, Gosner stages 26-34; *P. perezi*, $FM = 0.0177e^{0.2129 \times SVL}$; $r^2 = 0.9433$, n = 11, Gosner stages 26; *A. obstetricans*, $FM = 0.0314e^{0.0791 \times TL}$, $r^2 = 0.8838$, n = 10, Gosner stages 26) obtained from 9-11 extra larvae for each species, whose length was measured as the experimental ones and which were then weighed (0.1 mg precision).

Data analysis

We quantified differences in our response variables (periphyton biomass, Chl *a*, *b* and *c* concentrations, biofilm AI, inorganic mass, algal density and richness, and larval growth) among scenarios (i.e., the control and scenarios S1-S4) with linear models (gls function, "nlme" R package, Pinheiro et al. 2007), following the statistical requirements of the models and log transforming the variables to achieve normality when necessary, with scenario as fixed factor. When a variable was significantly affected by scenario ($\alpha = 0.05$), differences among scenarios were explored with Tukey tests (ghlt function of the "multcomp" R package; Hothorn et al. 2008; Zar 1999).

Differences in algal community structure among scenarios were analysed with non-metric dimensional scaling (NMDS) based on the Bray Curtis similarity index, using cell density (cell m^{-2}) data (metaMDS function of the "vegan" R package, Oksanen et al. 2007) and permutational multivariate analysis of variance (*adonis* function of the "vegan" R package). We used an indicator value index to identify the most representative taxa of each community (multipatt function of the "indicspecies" R package, De Cáceres 2013). All statistical analyses were performed with R version 4.1.2.

RESULTS

Despite the absence of significant differences in periphyton biomass, this variable presented a strong trend to be higher in the control (C, mean ± SE: 1.10 ± 0.23 g m^{-2} g^{-1}) than in scenarios of *A. obstetricans* loss (S2, 0.34 ± 0.10) and *A. obstetricans* and *T. marmoratus* loss (S4, 0.31 ± 0.09; Table S1, Fig. 1A). Inorganic mass did not present significant differences either, but it showed the same trend as biomass (C, 4.44 ± 1.09 g m^{-2} g^{-1}; S2, 1.40 ± 0.41; S4, 1.28 ± 0.35; Table S1, Fig. 1B).

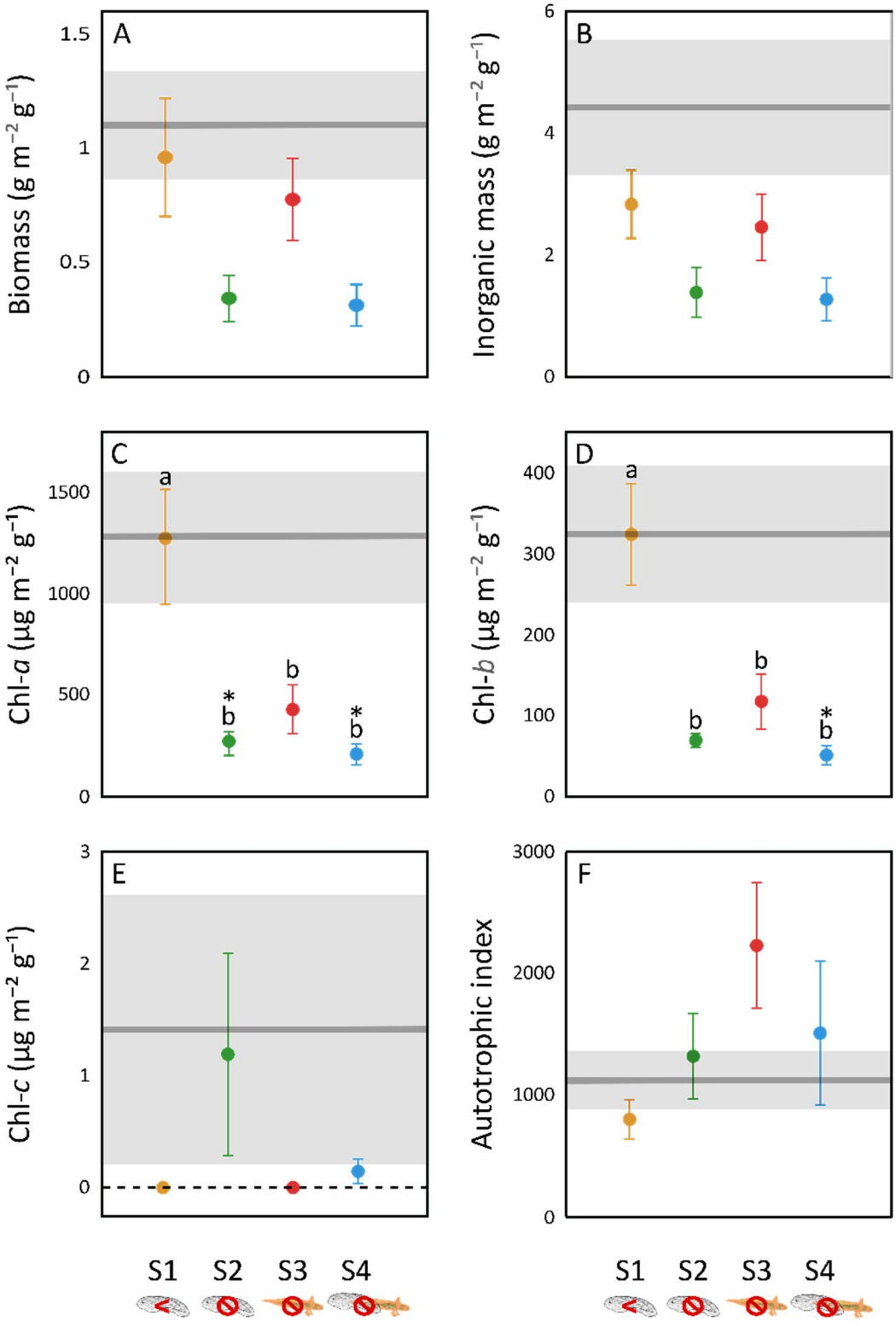

Fig. 1 Effects of amphibian species reduction and loss in a simplified amphibian community composed of *A. obstetricans, Bufo spinosus, Pelophylax perezi* and *T. marmoratus* on periphyton biomass (a), inorganic mass (b), Chl-*a* (c), Chl-*b* (d) and Chl-*c* (e) concentrations and the autotrophic index (f). The mean value for the control is represented by a grey bar; mean values of scenarios by circles [yellow: *Alytes obstetricans* reduction scenario (S1); green: *A. obstetricans* loss (S2); red: *Triturus marmoratus* loss (S3); blue: *A. obstetricans* and *T. marmoratus* loss (S4)]; and standard error by grey shadowing (control) and whiskers (scenarios S1–S4); asterisks indicate significant differences with the control and different letters indicate significant differences among scenarios.

Chlorophylls showed similar patterns, but differences among scenarios were significant: Chl-*a* concentration was higher in the control (C) and the scenario with *A. obstetricans* reduction (S1) than in the other scenarios (S2-S4), with no significant differences between the control and the scenario with *T. marmoratus* loss (C, 1284.10 ± 314.83 µg m^{-2} g^{-1}; S1, 1274.12 ± 241.48; S2, 267.36 ± 47.55 ; S3, 423.33 ± 122.22 ; S4, 204.34 ± 51.25; Table S1, Fig. 1C); Chl-*b* concentration was higher in the scenario with *A. obstetricans* reduction (S1) than in other scenarios (S2-S4), with significant differences also between the control and the scenario with *A. obstetricans* and *T. marmoratus* loss (C, 325.05 ± 83.29 µg m^{-2} g^{-1}; S1, 324.51 ± 61.92; S2, 69.82 ± 8.79; S3, 117.50 ± 33.40; S4, 51.50 ± 11.64; Table S1, Fig. 1D); and Chl-*c* could not be analysed, since most values were below our detection limits (Fig. 1E). The biofilm AI did not vary among scenarios (Table S1, Fig. 1F). We did not observe any effect on interspecific competition, since larval length was not significantly affected by the scenario for any species, neither in TL nor in SVL (Table S1).

Algal density was highest in the control (C, 324818.1 ± 51791.7 cell m^{-2} g^{-1}) and lowest in the scenarios with *A. obstetricans* loss (S2, 35622.5 ± 9160.5) and *A. obstetricans* and *T. marmoratus* loss (S4, 36555.8 ± 6731.3; Table S1, Fig. 2A). Algal taxon richness did not show significant differences (Table S1, Fig. 2B). Algal assemblage structure varied depending on the scenario, being significantly different in the control and the scenario with *A. obstetricans* reduction compared to scenarios with *A. obstetricans* loss and loss of both species (C vs S2, C vs S4, S1 vs S2, S1 vs S4), and also differing between the scenario with *A. obstetricans* loss and the one with *T. marmoratus* loss (S2 vs S3; Table S2, Fig. 3). The indicator value index showed that the control and the scenario with *A. obstetricans* reduction (S1) were characterized by *Microthamnion* NÄGELI, and the control also presented *Oscillatoria* VAUCHER EX GOMONT as a characteristic taxon. The assemblages in all scenarios were mainly composed by green algae and, in general, dominated by *Navicula* BORY, *Microthamnion* and *Chlorella* BEIJERINCK (Table S3).

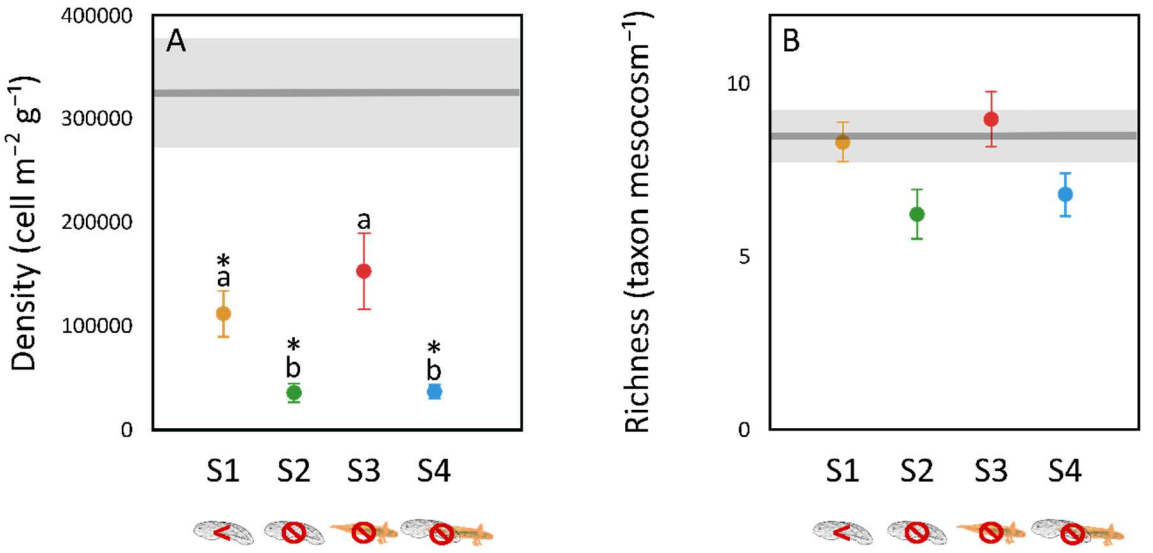

Fig. 2. Effects of amphibian species reduction and loss in a simplified amphibian community composed of *A. obstetricans, Bufo spinosus, Pelophylax perezi* and *T. marmoratus* on cell density (a) and taxon richness (b). The mean value for the control is represented by a grey bar; mean values of scenarios by circles [yellow: *Alytes obstetricans* reduction scenario (S1); green: *A. obstetricans* loss (S2); red: *Triturus marmoratus* loss (S3); blue: *A. obstetricans* and *T. marmoratus* loss (S4)]; and standard error by grey shadowing (control) and whiskers (scenarios S1–S4); asterisks indicate significant differences with the control and different letters indicate significant differences among scenarios.

DISCUSSION

Our experiment showed that changes in the composition of an amphibian community, representative of montane ponds in our study area, can alter ecosystem structure through changes in primary producer assemblages, which occurred even at the short term of our experiment. The loss of the anuran *A. obstetricans*, which larvae are herbivores, promoted significant changes in algal communities, reducing periphyton biomass, chlorophyll *a* and *b* concentrations, and algal cell density. These effects, which are counterintuitive and contrary to our first hypothesis, could be explained if other periphyton grazers in the community (i.e., *P. perezi* and *B. spinosus*), which replaced *A. obstetricans*, had higher grazing activity (in relation to a faster development, which is known for *B. spinosus*; Richardson 2001; Richter-Boix et al. 2007b) or higher grazing efficiency than *A. obstetricans* (such as differences in resource exploitation demonstrated for tadpoles of other species; Diaz-Paniagua 1985; Kupferberg 1997). However, our experiment did not allow to identify the specific mechanism involved here, because treatments with each species isolated would have been necessary.

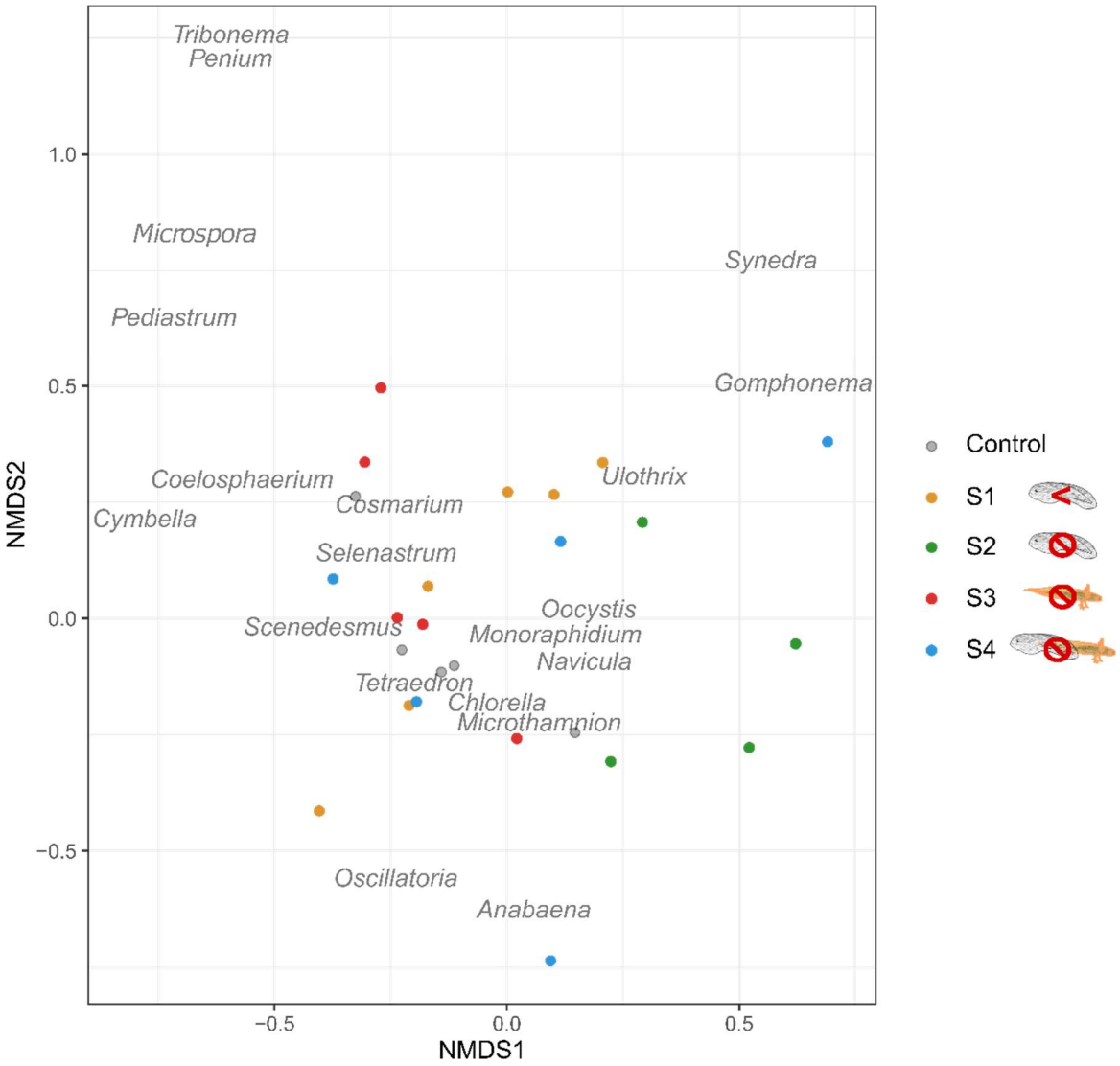

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination of periphyton algae in the different scenarios of amphibian species reduction and loss in a simplified amphibian community composed of *Alytes obstetricans, Bufo spinosus, Pelophylax perezi* and *Triturus marmoratus* [grey: control; yellow: *Alytes obstetricans* reduction scenario (S1); green: *A. obstetricans* loss (S2); red: *Triturus marmoratus* loss (S3); blue: *A. obstetricans* and *T. marmoratus* loss (S4)]. Stress = 0.1343

The similar results of the control and the scenario with reduction of *A. obstetricans* (S1) in all variables, except for algal cell density, may be due to the fact that large *A. obstetricans* tadpoles outcompete the other smaller, more active species, such as *B. spinosus* (Richter-Boix et al. 2007a). This competition can reduce the grazing activity of *B. spinosus* tadpoles, maybe displacing them from foraging areas and forcing them to feed on less efficient resources such as suspended particles (Richter-Boix et al. 2004), and resulting in similar periphyton accrual despite the lower density of *A. obstetricans* in scenario S1. However, we did not find a similar effect in the scenario with loss of *A. obstetricans* (S2),

despite the increase in *P. perezi* density, a species also observed to outcompete *B. spinosus* (Richter-Boix et al. 2007a). These differences between *A. obstetricans* and *P. perezi* may be due to differences in their ecology (Richter-Boix et al. 2007a; Richter-Boix et al. 2006), for example in their activity and feeding strategies, as observed for tadpoles of other species, even taxonomically close species (Richardson 2001).

Algal cell density and assemblage structure were also affected by the loss of *A. obstetricans*. Cell density decreased when this species disappeared, and the filamentous green algae (*Microthamnion*) and cyanophytes (*Oscillatoria*) that were characteristic taxa of the control community were considerably reduced. This may have been the result of the higher activity of other tadpoles in the absence of *A. obstetricans*. Despite the fact that filamentous green algae such as *Microthamnion* are usually avoided in favour of other algae (Kupferberg 1997), they are more easily removed from the substrate due to their greater size (Guo et al. 2022), and the higher grazing activity in the absence of *A. obstetricans* is likely to increase bioturbation. *Oscillatoria* density was reduced in all scenarios compared to the control, maybe by removal due to bioturbation or consumption by tadpoles, as it has been observed to be an important part of their diet in tropical streams (dos Santos Protázio et al. 2020; Santos et al. 2016) and a higher grazing pressure could cause a greater effect in the preferred resource.

In the scenario with *T. marmoratus* loss (S3), we found a reduction in Chl *a* and *b* concentrations similar to that observed in the absence of *A. obstetricans*. This supports our hypothesis of a top-down control of *T. marmoratus* larvae on periphyton due to the consumption of planktonic crustaceans by newt larvae. Larvae of another urodele common in our study area, the fire salamander (*Salamandra salamandra*), have been observed to enhance periphyton accrual through top-down control on macroinvertebrates and zooplankton (Blaustein et al. 1996), and larvae of other urodeles such as the tiger salamander (*Ambystoma trigrinum* GREEN), marbled salamander (*Ambystoma opacum* GRAVENHORST) or spotted salamander (*Ambystoma maculatum* SHAW) are known to control zooplankton abundance (Holomuzki et al. 1994; Urban 2013). The same effects could be expected for *T. marmoratus*, since their main prey are planktonic crustaceans (Cladocera and Copepoda; Braña et al. 1986; Santos et al. 1986), and therefore they could reduce zooplankton communities, leading to lower grazing pressure on periphyton (Hann 1991; Kazanjian et al. 2018; Masclaux et al. 2012) and, consequently, higher periphyton accrual. Another possibility is that *T. marmoratus* may stimulate periphyton growth

through nutrient release by excretion, as observed with other freshwater predators such as crayfish (Arribas et al. 2014) or dragonfly larvae (Costa and Vonesh 2013). Therefore, with the loss of *T. marmoratus*, the reduction in nutrient cycling could inhibit periphyton growth.

The scenario with loss of both species (S4) showed a trend towards lower periphyton accrual, but it was similar to the scenario with loss of *A. obstetricans* (S2), suggesting that anuran tadpole grazing had a stronger effect on algal communities than the top-down effect induced by small urodeles such as *T. marmoratus*. This was to be expected, as tadpoles are more efficient grazers than invertebrates in streams and they cause stronger reductions in periphyton accrual (Alonso et al. 2022). Finally, in contrast with other studies where interspecific competition reduced tadpole growth rates (Richter-Boix et al. 2007a; Richter-Boix et al. 2004), in our study tadpoles did not show significant differences among scenarios in their growth regardless of the species, which could be expected due to the short duration of the experiment. In particular, *A. obstetricans* and *P. perezi* can remain as tadpoles for more than one year (García-París et al. 2004; Garriga et al. 2017; Scheidt and Uthleb 2005). This suggests that short-term experiments are not the most appropriate to observe effects of competition between tadpoles on their growth, even if the effects of the interactions of the different amphibian species can be already observed in periphyton.

CONCLUSIONS

The total loss of amphibians from freshwater ecosystems is known to impact ecosystem structure and functioning through changes in periphyton assemblages (Alonso et al. 2022; Barnum et al. 2022; Colón-Gaud et al. 2010; Connelly et al. 2008; Mallory and Richardson 2005; Ranvestel et al. 2004) but, to date, little was known about the effect of the disappearance of particular species. In this study, we show how montane pond ecosystem structure and functioning can be affected by the loss of an anuran and a urodele that play different ecological roles in the ecosystem, even when total amphibian larval density is maintained. The loss of an anuran species, in this case *A. obstetricans*, a species suffering a dramatic decline in some regions of its distribution area (Bosch et al. 2018), resulted in a reduction of periphytic algae and a change in the algal community, even in the short term. Similar but smaller effects were observed for the loss of the urodele *T. marmoratus*, a species which has been depleted from ponds infected by Bsal (Martel et al. 2020). Understanding the ecological effects of the loss of different amphibian species, particularly

those more vulnerable to extinction, is important if we are to predict the consequences that such extinctions entail for freshwater ecosystems.

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CONFLICT OF INTEREST

The authors have no competing interests to declare that are relevant to the content of this article.

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SUPPLEMENTARY MATERIAL

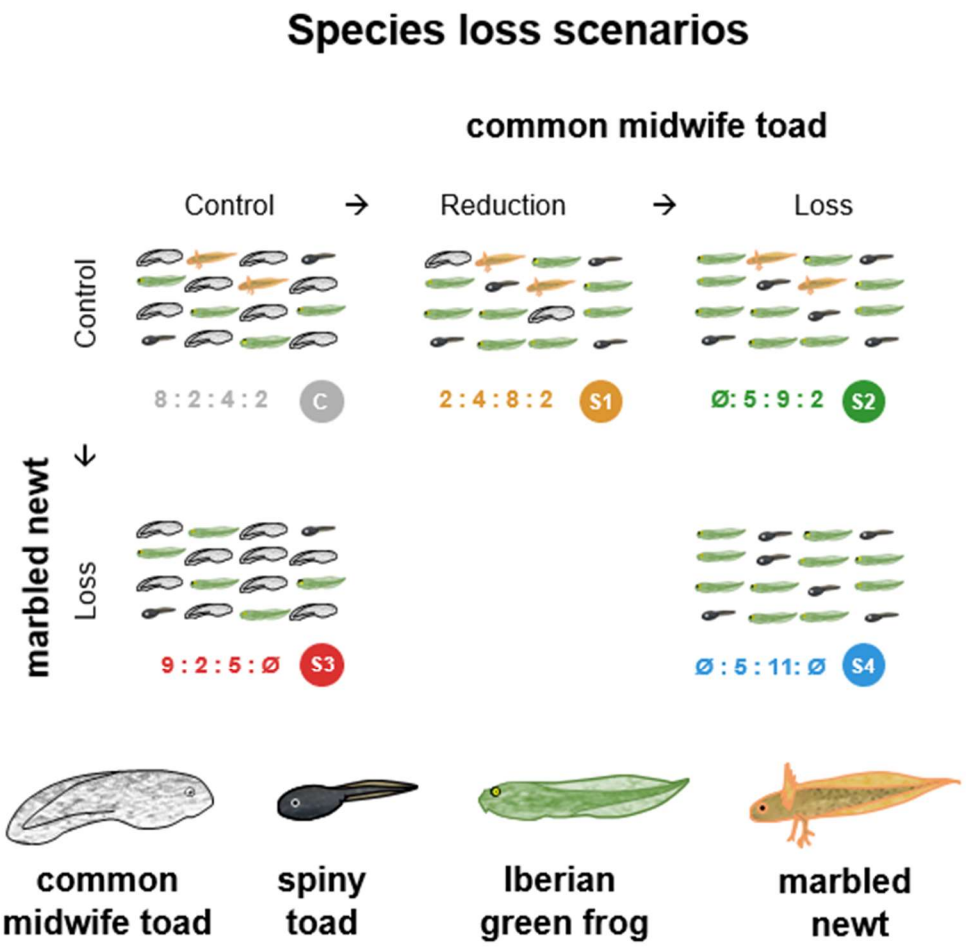

Figure S1. Schematic diagram of the experimental design showing the proportion of each species (common midwife toad, spiny toad, Iberian green frog and marbled newt) in each scenario (control and species loss scenarios S1-S4).

Table S1. Results of linear models exploring the effects of scenarios of amphibian species reduction and loss (control, S1-S4) on periphyton biomass (g m^{-2} anuran g^{-1}); inorganic mass (g m^{-2} anuran g^{-1}); chlorophyll *a* and *b* concentrations (µg m^{-2} anuran g^{-1}); autotrophic index; common midwife toad, spiny toad, Iberian green frog and marbled newt growth (prop.) in total length (TL) and snout-vent length (SVL); algal density (cell m^{-2} anuran g^{-1}) and taxon richness (taxa per mesocosm); df = degrees of freedom; F = F-statistic; p = p-value.

Variable	df	F	p
Biomass	4, 21	2.39	0.083
Inorganic mass	4, 21	2.70	0.058
Chlorophyll *a*	4, 20	6.01	0.002
Chlorophyll *b*	4, 20	6.06	0.002
Autotrophic index	4, 20	1.74	0.181
Midwife toad TL growth	2, 14	1.20	0.331
Midwife toad SVL growth	2, 14	1.14	0.348
Spiny toad TL growth	4, 19	1.74	0.184
Spiny toad SVL growth	4, 19	1.03	0.419
Iberian green frog TL growth	4, 21	0.93	0.468
Iberian green frog SVL growth	4, 21	0.29	0.880
Marbled newt TL growth	2, 12	3.37	0.069
Marbled newt SVL growth	2, 12	2.94	0.091
Algal density	4, 20	8.35	<0.001
Algal richness	4, 20	2.03	0.129

Table S2. Results of PERMANOVAs exploring the effects of scenarios of amphibian species reduction and loss (control, S1-S4), as well as significant paired comparisons, on algal assemblage structure; df = degrees of freedom; F = F-statistic; R^2 = adjusted R^2; p = p-value. Stress = 0.134264.

Factor	df	F	R^2	p	Sign. paired comparisons
Scenario	4	2.16	0.3014	0.004	Control vs S2; S1 vs S2; S2 vs S3;

Table S3. Relative contribution (%) of each algal taxon to algal abundance in each scenario of amphibian species reduction and loss [control, reduction of common midwife toad (S1), loss of common midwife toad (S2), loss of marbled newt (S3) and loss of common midwife toad and marbled newt (S4)]. Mean density and accumulated taxon richness are given at the bottom of the table. Bold type represents species with a contribution to total density higher than 10%.

Treatment		**Control**	**S1**	**S2**	**S3**	**S4**
Phylum	**Genus**					
Cyanobacteria	*Anabaena* BORY EX BORNET & FLAHAULT				3.66	
Chlorophyta	*Chlorella* BEIJERINCK	**10.06**	**19.11**	**16.36**	**17.42**	**13.84**
Cyanobacteria	*Coelosphaerium* NÄGELI				7.49	1.87
Charophyta	*Cosmarium* CORDA EX RALFS		0.20		0. 41	
Bacillariophyta	*Cymbella* C.AGARDH					0.26
Bacillariophyta	*Gomphonema* EHRENBERG		0.29	1.48	0.18	0.81
Chlorophyta	*Microspora* THURET				2.96	
Chlorophyta	*Microthamnion* NÄGELI	**39.67**	**34.37**	**34.70**	**18.39**	**14.48**
Chlorophyta	*Monoraphidium* KOMÁRKOVÁ-LEGNEROVÁ	2.66	5.42	5.92	4.56	9.00
Bacillariophyta	*Navicula* BORY	**22.86**	**11.45**	**32.44**	**21.98**	**34.63**
Chlorophyta	*Oocystis* NÄGELI EX A.BRAUN	0.56	3.09	4.75	0.56	0.61
Cyanobacteria	*Oscillatoria* VAUCHER EX GOMONT	9.67			5.14	6.52
Chlorophyta	*Pediastrum* MEYEN	1.67				
Charophyta	*Penium* BRÉBISSON EX RALFS				0.10	
Chlorophyta	*Scenedesmus* MEYEN	6.61	**10.10**		7.42	7.97
Chlorophyta	*Selenastrum* REINSCH	0.95	2.06	0.40	2.23	2.76
Bacillariophyta	*Synedra* EHRENBERG	0.33	0.52			0.85
Chlorophyta	*Tetraedron* KÜTZING	3.05	7.24	1.18	6.67	6.37
Ochrophyta	*Tribonema* DERBÈS & SOLIER				0.83	
Chlorophyta	*Ulothrix* KÜTZING	1.90	6.16	2.78		
Density (cell m^{-2} anuran g^{-1})		324818.05 ± 51791.65	111866.88 ± 22314.00	35622.46 ± 9160.54	152970.18 ± 36827.00	36555.84 ± 6731.26
Accumulated taxon richness		12	12	9	16	13

Chapter 6

SALAMANDER LOSS ALTERS MONTANE STREAM ECOSYSTEM FUNCTIONING AND STRUCTURE THROUGH TOP-DOWN EFFECTS

Alonso, A., Bosch, J., Pérez, J., Rojo, D., & Boyero, L. Salamander loss alters montane stream ecosystem functioning and structure through top-down effects. *Biodiversity and Conservation*, *under review.*

ABSTRACT

Amphibians are among the most endangered taxa worldwide, but little is known about how their disappearance can alter the functioning and structure of freshwater ecosystems, where they live as larval stages. This is particularly true for urodeles, which often are key predators in these ecosystems. The fire salamander (*Salamandra salamandra*) is a common predator in European fresh waters, but the species is declining due to habitat loss and the infection by fungal pathogens. We studied the consequences of fire salamander loss from three montane streams, by comparing two key ecosystem processes (periphyton accrual and leaf litter decomposition) and the structure of three communities (periphyton algae, aquatic hyphomycetes and invertebrates) using instream enclosures with and without salamander larvae. Salamander loss did not cause changes in invertebrate abundance or community structure, except for one stream where abundance increased in the absence of salamander larvae. However, salamander loss led to lower periphyton accrual, changes in algal community structure and slower leaf litter decomposition, with no associated changes in fungal communities or microbial decomposition. The changes observed may have been caused by release of salamander larvae predatory pressure on invertebrates, which could have promoted their grazing on periphyton, in contrast to their preference for leaf shredding in the presence of salamander. Our study demonstrates an important role of salamander larvae in montane streams through top-down control of lower trophic levels and thus in regulating key stream ecosystem processes.

INTRODUCTION

Biodiversity is currently declining at rates that are comparable to those reported for past mass extinctions and unprecedented to human history (Barnosky et al. 2011; Ceballos et al. 2017). Beyond the ethical implications of such an event, due to the intrinsic value of biodiversity (Ghilarov 2000; IPCC 2014), there is now ample evidence that biodiversity loss can have profound effects on ecosystems through changes in the structure of biological communities and shifts in the rates of fundamental ecosystem processes (Boyero et al. 2021; Cardinale et al. 2011; Cardinale et al. 2006; Hooper et al. 2012). Freshwater ecosystems suffer extinction rates considerably higher than those of marine or terrestrial

ecosystems (Reid et al. 2019; Sala et al. 2000) and amphibians, which larval stages usually inhabit fresh waters, are particularly vulnerable to extinction (Collins 2010; Luedtke et al. 2023; Wake and Vredenburg 2008). Among the multiple causes of such extinction are emergent infectious diseases (Fisher and Garner 2020; Scheele et al. 2019), invasive species (Falaschi et al. 2020), climate change (Blaustein et al. 2010), habitat loss and fragmentation (Becker et al. 2007; Gallant et al. 2007) and pollution (Blaustein et al. 2003). However, oddly enough, the effects of amphibian species losses on freshwater ecosystem functioning and structure are still poorly known (Whiles et al. 2006).

Salamanders are important predators in both freshwater and terrestrial ecosystems, representing a significant biomass in temperate forests (Semlitsch et al. 2014), and are key drivers of nutrient and energy flows between these environments (Davic and Welsh Jr 2004; Hocking and Babbitt 2014). However, salamanders are declining in Europe due to the infection of the fungal pathogens *Batrachochytrium dendrobatidis* LONGCORE, PESSIER & D.K. NICHOLS and *B. salamandrivorans* A. MARTEL, BLOOI, BOSSUYT & PASMANS (Bosch et al. 2018; Bosch and Martínez-Solano 2006; Martel et al. 2014; Stegen et al. 2017). These pathogens could lead to salamander extinctions, especially if *B. salamandrivorans* increased its current distribution area, which may drive ecosystem changes that are mostly unknown. In terrestrial ecosystems, salamander adults are known to reduce leaf litter decomposition through changes in invertebrate and microbial communities (Laking et al. 2021), but the potential effects of salamander larvae on freshwater ecosystems have received less attention (Hocking and Babbitt 2014; Rowland et al. 2017). Available data on other urodeles suggest that they could potentially control the abundance of invertebrates (Arribas et al. 2014; Rowland et al. 2017; Urban 2013) and ultimately influence periphyton accrual via consumptive effects (Blaustein et al. 1996; Holomuzki et al. 1994). Other possible effects on periphyton, based on evidence from freshwater predators other than urodeles, include non-consumptive effects through reduction of prey foraging activity to avoid predation (Davenport et al. 2020; Peckarsky et al. 1993; Preisser et al. 2005), or by nutrient release though excretion (Arribas et al. 2014; Costa and Vonesh 2013; Iwai and Kagaya 2007; Small et al. 2011).

In this study, we experimentally assessed how the loss of salamander larvae from montane streams affected several key ecosystem processes (i.e., periphyton biomass accrual; chlorophyll *a*, *b* and *c* concentrations; inorganic mass accrual; and total and microbially-mediated leaf litter decomposition) and associated organisms (i.e., algal,

aquatic hyphomycete and invertebrate community structure). We hypothesized that salamander loss would release invertebrates from predatory pressure, leading to: (i) higher invertebrate grazing activity and hence lower periphyton accrual and chlorophyll concentration, with concomitant changes in algal community structure; (ii) higher leaf litter decomposition mediated by invertebrates, again because of the increase in invertebrate activity as a result of predatory release; (iii) lower microbial activity (i.e., primary production, decomposition and aquatic hyphomycete sporulation) due to the absence of nutrients released by salamander excretion, with concomitant changes in aquatic hyphomycete community structure; and (iv) higher invertebrate abundance in the absence of consumption by salamander, with associated changes in invertebrate community structure.

MATERIALS AND METHODS

Study site and species

The study was conducted in three low-order streams located in the Peñalara Massif (Guadarrama Mountains National Park, Central Spain; Table 1, Fig. 1). This is a montane habitat (1800–2430 m), with low mean annual precipitation (694 mm) and marked seasonality (temperature annual range: 28°C). Annual mean temperature is 6.6°C, with snowy and cold winters (mean temperature 0.1°C) and warm and dry summers (mean temperature 15°C and precipitation of 109 mm) [WorldClim database (Fick and Hijmans 2017)]. The substrate is mostly granitic and covered by heathlands dominated by *Cytisus oromediterraneus* RIV. MART. and *Juniperus communis nana* SYME, with isolated *Pinus sylvestris* L. and *Salix atrocinerea* BROT. around the streams. As usually observed in temperate running waters, few amphibian species live in streams of the study area, mainly the fire salamander (*Salamandra salamandra* L.) and the Iberian stream frog (*Rana iberica* BOULENGER), and, in the most stagnant areas, the Iberian green frog (*Pelophylax perezi* LÓPEZ-SEOANE) and the common midwife toad (*Alytes obstetricans* LAURENTI). The study sites were close to the treeline and had well oxygenated water, pH ca. 6.5, low conductivity, low nutrient concentration and temperatures ca. 10°C at the time of the study (Table 1). The streams were chosen as they are the two largest streams in the area and the main tributary of one of them, thus being less likely to dry during the summer than other nearby streams.

Table 1. Physicochemical characteristics of the study streams during the experimental period (mean ± SE; n=3-5). DIN = dissolved inorganic nitrogen, SRP = soluble reactive phosphorus.

Variable	Stream 1	Stream 2	Stream 3
Latitude (ºN)	40.84398	40.83668	40.83563
Longitude (ºW)	3.94376	3.95362	3.95333
Altitude (m a. s. l.)	1895	1942	1949
Temperature (ºC)	9.09 ± 0.70	12.16 ± 0.31	9.33 ± 0.24
Conductivity (µS cm^{-1})	3.33 ± 3.33	2.00 ± 1.53	5.33 ± 0.66
pH	6.95 ± 0.07	6.63 ± 0.13	6.44 ± 0.04
Dissolved O_2 (mg l^{-1})	9.08 ± 0.06	8.38 ± 0.10	8.59 ± 0.10
% Saturation O_2	97.87 ± 1.20	99.00 ± 1.66	94.57 ± 1.05
Flow (l s^{-1})	18.35 ± 15.97	13.16 ± 11.51	6.05 ± 2.62
DIN (µg l^{-1})	139.25 ± 26.65	91.51 ± 12.96	41.75 ± 18.12
SRP (µg l^{-1})	4.06 ± 0.61	2.89 ± 0.48	9.49 ± 1.88

The fire salamander (*S. salamandra*, Family Salamandridae, Order Caudata) is a common urodele in southern and central Europe (from the Iberian Peninsula to the Balkans), occupying a great range of altitudes (from the sea level to 2000 m a.s.l.). It is abundant in the study area, despite the decrease it has suffered in the last decades (Bosch et al. 2018). Larvae live in a great variety of freshwater ecosystems, both temporal and permanent ponds and streams, while adults are mainly terrestrial. Larval development lasts 3-4 months, but they can maintain their larval stage for more than a year in cold habitats such as the study area, and they can reach a size of 7 cm before metamorphosis. Larvae feed mainly on aquatic invertebrates, but their diet also includes smaller amphibian larvae. Fire salamanders are not considered threatened but, despite their abundance, populations have decreased in some areas due to habitat loss, the spread of invasive species and infection by the fungal pathogens *B. dendrobatidis* and *B. salamandrivorans* (Bosch et al. 2018; Bosch et al. 2006; García-París et al. 2004; Martel et al. 2014; Stegen et al. 2017).

The leaf litter used in the experiment (described below) belonged to the two tree species present in the study area: willow and pine. The willow (*Salix atrocinerea*, Family

Salicaceae) is a riparian tree distributed along Western Europe and North Africa, and especially abundant in the Iberian Peninsula. In the study area, the willow is the most common riparian tree despite the low tree cover. Willow leaves are rich in nutrients, but they are tough and present high concentration of tannins, leading to intermediate decomposition rates (Alonso et al. 2021; Webster and Benfield 1986). The pine (*Pinus sylvestris*, Family Pinaceae) is a common tree species along Eurasia, and the dominant tree in the study area. Pine needles are tough, poor in nutrients and they present high concentration of toxic compounds, therefore presenting very low decomposition rates (Casas et al. 2013; Martínez et al. 2013a; Martínez et al. 2013b; Webster and Benfield 1986).

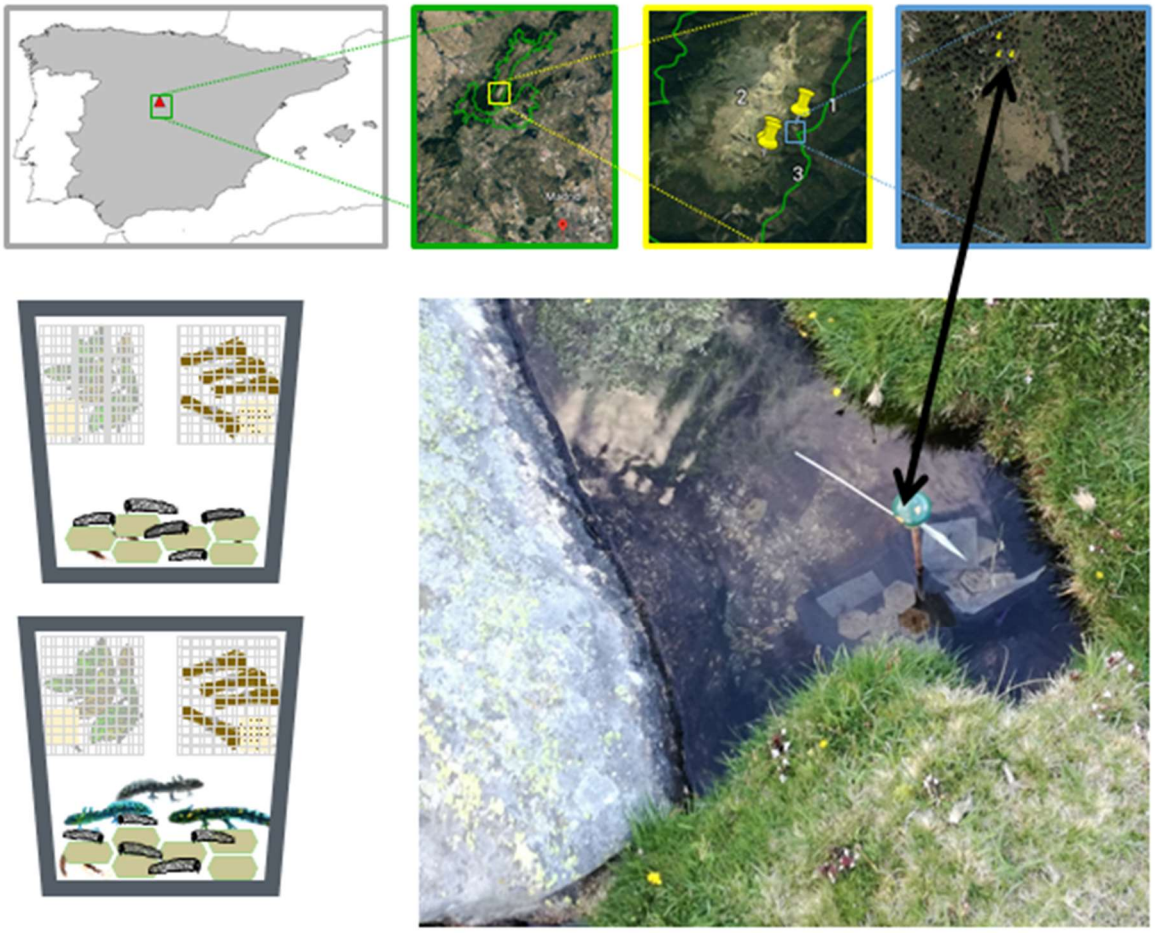

Fig. 1. Location of the Guadarrama Mountains National Park (green) in Central Spain and the three studied streams (yellow), with an example of pool location within one of the streams (blue) and a diagram of the experimental design of the different treatments (i.e., control and salamander loss).

Experimental procedure

We conducted a 35-day, in-situ experiment in May-June 2023. We used three streams, with six enclosures per stream. There were two enclosures in each pool, all of them containing ceramic tiles allowing periphyton growth, leaf litter enclosed within mesh bags, and macroinvertebrates (see below for details); additionally, one enclosure in each pool contained three salamander larvae, while the other contained no salamander. Enclosures consisted of plastic baskets (17 × 17 × 13 cm) closed by a 2-mm glass fibre mesh that allowed water and small invertebrate access, but precluded salamander larvae from moving in or out. They had the bottom covered by sediment from the corresponding stream, on top of which we placed: six marble tiles (33.6 cm^2 surface area per tile), used as standardized substrata for periphyton growth; two coarse-mesh bags (5-mm, 10 × 10 cm) containing 2.0 ± 0.1 g of leaf litter, one containing willow and the other pine (with pine needles grouped into four tied packs per bag to prevent accidental losses), which were used to quantify total decomposition; and within each coarse-mesh bag, one fine mesh bag (500-µm, 5 × 5 cm) containing 0.20 ± 0.01 g of leaf litter from the corresponding species, which was used to quantify microbial decomposition (Fig. 1). At the beginning of the experiment, each enclosure received invertebrates collected through a Surber sample at the same pool, in order to facilitate invertebrate colonization of the enclosures.

The 138 tiles used in the experiment had been previously incubated in a pond located nearby (40.88694º N, 3.88613º W) for 19 days. At the beginning of the experiment, 30 extra tiles were used to calculate initial periphyton biomass, chlorophyll concentrations and algal assemblage structure (mean ± SE; biomass: 0.26 ± 0.02 g m^{-2}; sediment mass: 0.51 ± 0.11 g m^{-2}; Chl-*a*: 4254.05 ± 1152.50 µg m^{-2}; Chl-*b*: 786.20 ± 128.96 µg m^{-2}; Chl-*c*: below detection limit). The willow leaves had been collected immediately after natural abscission from the forest floor next to the University of the Basque Country (43.330º N, 2.972º W) in autumn 2022. The pine needles had been collected from dry branches at the Guadarrama Mountains National Park (40.826º N, 3.958º W) in spring 2023. All leaves had been air-dried at room temperature in the laboratory and stored in the dark until needed. An extra set of five fine mesh bags of each species had been incubated for 48 h to estimate mass losses due to leaching [i.e., the difference between ash-free dry mass (AFDM) of non-incubated and incubated litter divided by AFDM of non-incubated litter) and initial carbon (C), nitrogen (N) and phosphorus (P) concentrations (Table 2)]. The fire salamander larvae used in the experiment were overwintering larvae collected at the study area (Guadarrama

Mountains National Park), which total length was measured (TL ± SE: 6.98 ± 0.14; 0.001 mm precision) using ImageJ software (v. 1.46r) just before the start of the experiment.

Table 2. Initial traits of the different leaf litter types used in the study (mean ± s.e.; n=5): carbon, nitrogen and phosphorus concentrations, and elemental molar ratios.

Variable	*Salix atrocinerea*	*Pinus sylvestris*
Carbon (C; % DM)	51.90 ± 23.21	51.41 ± 23.00
Nitrogen (N; % DM)	1.51 ± 0.68	1.46 ± 0.65
Phosphorus (P; % DM)	0.009 ± 0.004	0.017 ± 0.008

The enclosures were sampled at day 35. The surface of the six tiles of each enclosure was scrubbed into 100 mL of distilled water and frozen (-20 ºC) until subsequent analysis in the laboratory. The leaf litter bags were enclosed individually in zip-lock bags and transported in a refrigerated cooler to the laboratory. The sediment was rinsed with stream water through a 500-µm sieve to collect the invertebrates, which were preserved in 70% ethanol until subsequent identification. Tile solutions were divided in three subsamples. One of them was filtered in pre-incinerated and pre-weighted filters (GF/F, 0.7 µm), dried (72 h, 70 ºC), weighed to quantify periphyton dry mass (DM) and, afterwards, incinerated (5 h, 500 ºC) and weighed to estimate biomass (AFDM) and inorganic mass. The second was filtered (GF/F, 0.7 µm) for the quantification of chlorophyll, which was extracted from filters by submerging them in acetone 90% in darkness (12 h, 4 ºC). To ensure the complete separation of materials, samples were sonicated (60 Hz) and centrifuged (2000 rpm). Then, chlorophyll *a* (Chl-*a*), chlorophyll *b* (Chl-*b*) and chlorophyll *c* (Chl-*c*) concentrations (mg Chl m^{-2}) were measured spectrophotometrically by measuring absorbance at 750, 665, 647 and 630 nm. The biofilm autotrophic index (AI) was calculated as the ratio between AFDM and Chl-*a* concentration, to assess the ratio between autotrophic and heterotrophic organisms in periphyton (Steinman 2006). The last subsample was preserved with acidic Lugol's solution (0.4 %) and used to characterize periphyton algal community. Taxonomic identification to the lowest level possible (genus) and cell counting was performed using an optical microscope and a Neubauer chamber at ×200 magnification following Bellinger

and Sigee (2015). For each sample, abundance (cell m^{-2}) and taxon richness (number of taxa per enclosure) were calculated.

The leaf litter material from each bag was rinsed with filtered (100 µm) stream water on a 500-µm sieve to remove sediment and associated invertebrates. Leaf litter was then oven-dried (70º C, 72 h), weighed to determine final DM, incinerated (500º C, 4 h) and weighed to determine final AFDM. Invertebrates were stored with those collected from the sediment of the same enclosure. Decomposition (%) was calculated as the difference between initial and final AFDM divided by initial AFDM, with initial AFDM corrected by leaching losses. Total decomposition was obtained from the coarse mesh bags, and microbial decomposition from the fine mesh bags. Invertebrates retrieved from each enclosure (coarse mesh bags and sediment) were identified to the lowest taxonomic level possible (typically genus) using Tachet et al. (2010). For each enclosure, abundance (ind. m^{-2}) and taxon richness (number of taxa per enclosure).

To stimulate fungal sporulation, a fraction of leaf litter from coarse mesh bags (0.25 ± 0.02 g) was placed in Erlenmeyer flasks filled with 25 mL of filtered stream water (glass fibre filters, Whatman GF/F; pore size: 0.7 µm). Flasks were incubated for 48 ± 2 h on a shaker at 100 rpm and 5 ºC. Conidial suspensions were poured into 50-mL Falcon tubes, pre-stained with 2 drops of 0.05 % trypan blue in 60% lactic acid, preserved with 2 mL of 35% formalin and adjusted to 40 mL with distilled water. Each conidial suspension received 100 µL of 0.5 % Triton X-100 and was homogenized with a magnetic stirrer. Then, 10 mL were filtered (glass fibre filters, 25-mm diameter, pore size 5 µm, Millipore SMWP, Millipore Corporation) and filters were stained with 0.05% cotton blue in 60% lactic acid. Conidia were identified and counted with a microscope at ×200 magnification. For each sample, abundance (conidia g^{-1} d^{-1}) and taxon richness (number of taxa per mesh bag) were calculated.

Data analyses

Differences in periphyton biomass, chlorophyll concentrations, the autotrophic index, inorganic mass, and algal and invertebrate abundance and richness among treatments were examined with linear models (*lme* function of the "nlme" R package, Pinheiro et al. 2007), with salamander loss and stream as fixed factors and pool as random factor. Differences in total and microbial decomposition and aquatic hyphomycete sporulation and richness were

also examined with linear models, with salamander loss, leaf species and stream as fixed factors and pool as random factor. When there were significant differences among stream levels ($\alpha = 0.05$), these were explored with Tukey tests (*ghlt* function of the "multcomp" R package; Hothorn et al. 2008; Zar 1999).

The structure of invertebrate, algal and aquatic hyphomycete communities was analysed with non-metric dimensional scaling (NMDS) based on the Bray Curtis similarity index using abundance data (*metaMDS* function of the "vegan" R package, Oksanen et al. 2007). Permutational multivariate analysis of variance (*adonis* function of the "vegan" R package) was used to test whether communities varied depending on salamander loss, stream and leaf species (the last one only in the case of aquatic hyphomycetes). An indicator value index (*multipatt* function of the "indicspecies" R package, De Cáceres 2013) was used to identify the most representative taxa for each community. All statistical analyses were performed with R statistical software, version 4.1.2.

RESULTS

Periphyton biomass tended to be higher in the absence of salamander, but the pattern was not significant, neither there were significant differences among streams or there was interaction between both factors (Table S1, Fig. 2A). Chlorophyll *a*, *b* and *c* concentrations showed the same trend as periphyton biomass, but these variables were significantly lower in the absence of salamander (Table S1, Fig. 2B, Fig. 2C, Fig. 2D). In addition, Chl-*c* concentration was also affected by the interaction between stream and salamander presence: Chl-*c* concentration decreased in all cases due to salamander loss, but the magnitude of the decrease varied among streams, with greater reduction in stream 3 (Table S1, Fig. 2D). The autotrophic index was significantly higher in absence of salamander (Table S1, Fig. 2F) and inorganic biomass did not show any significant variation (Table S1, Fig. 2E).

Algal abundance was significantly reduced in the absence of salamander (Table S1, Fig. 3A), but algal taxon richness was similar across treatments (Table S1, Fig. 3B). Algal community structure was significantly affected by salamander loss (Table 3): communities in the presence of salamander were characterized by diatoms of the genera *Achnanthes* BORY, *Gomphonema* EHRENBERG and *Amphora* EHRENBERG EX KÜTZING, whereas

communities in the absence of salamander did not present any characteristic genera (Table S2).

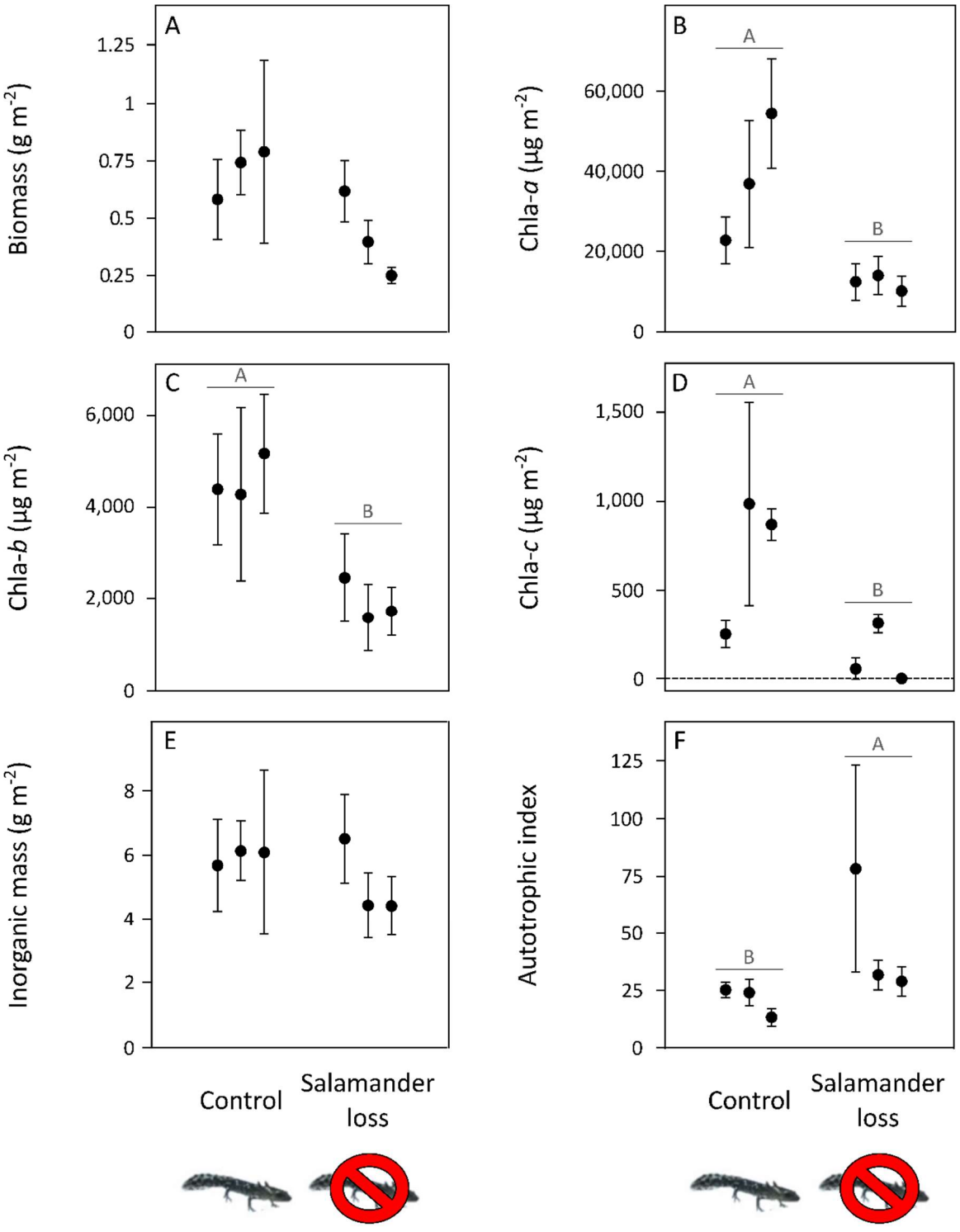

Fig. 2. Periphyton biomass (g m^{-2}; a), Chl-*a* (b), Chl-*b* (c) and Chl-*c* (d) concentrations (µg m^{-2}), inorganic mass (g m^{-2}; e) and autotrophic index (f) in the control and salamander loss treatment in the three streams (stream 1, stream 2 and stream 3, in that order). Circles represent means and whiskers standard errors. Different letters indicate significant differences among treatments.

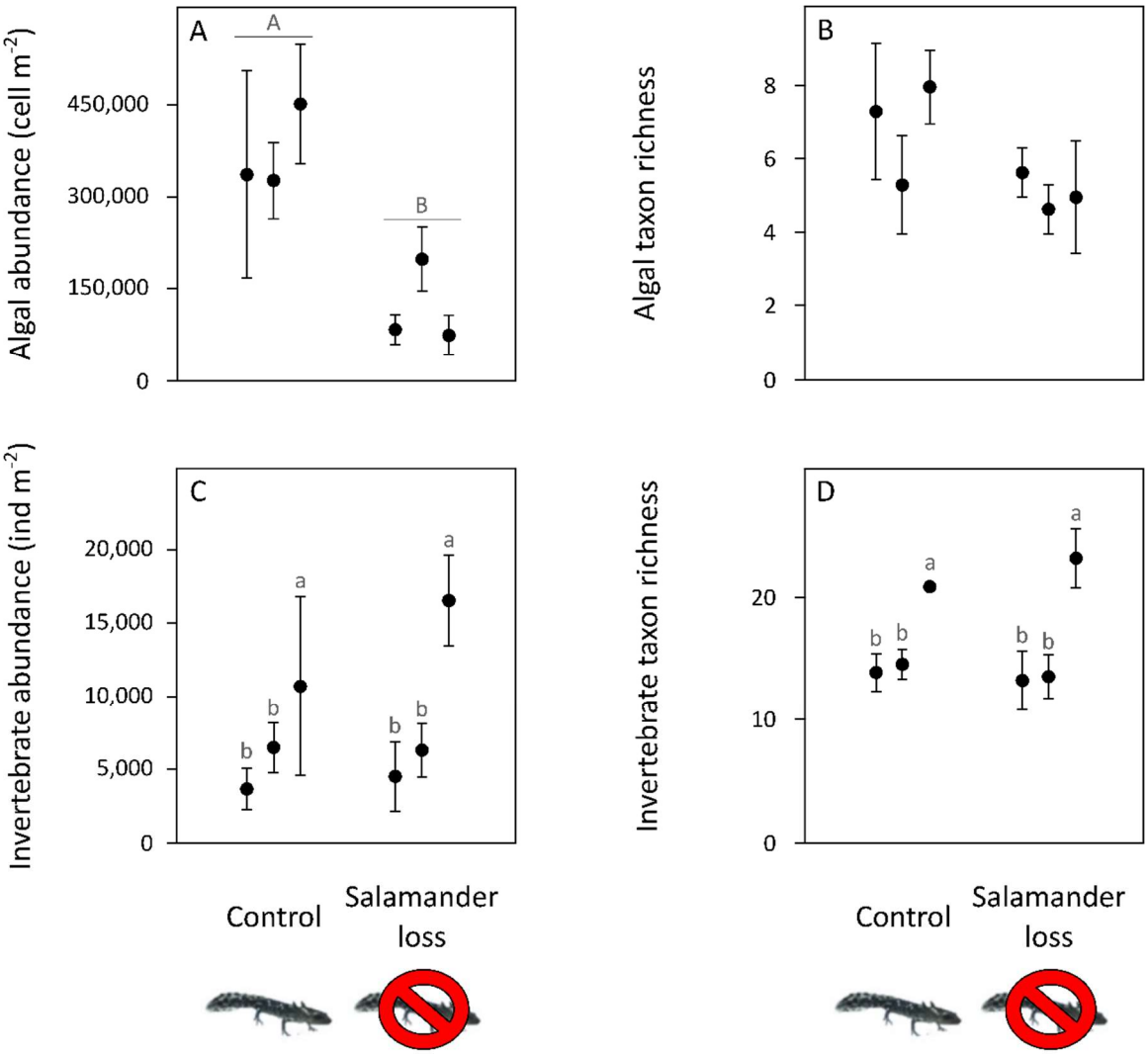

Fig. 3. Algal abundance (cell m^{-2}; a), algal taxon richness (b), invertebrate abundance (individual m^{-2}; c) and invertebrate taxon richness (d) in the control and salamander loss treatment in the three streams (stream 1, stream 2 and stream 3, in that order). Circles represent means and whiskers standard errors. Different letters indicate significant differences among treatments or streams.

Total leaf litter decomposition was lower in the absence of salamander, and higher for pine than willow. There were also differences among streams, and the interaction between stream and leaf type was significant, being highest in stream 3 and lowest in stream 2, and with greater differences among streams for pine needles than for willow leaves (Table S3, Fig. 4A). On the other hand, microbial decomposition was mainly driven by leaf type, being in this case higher for willow than pine; the interactions between leaf type and the other factors were significant, but there was no identifiable pattern (Table S3, Fig. 4B).

Table 3. Results of PERMANOVA exploring the effects of salamander loss, stream (streams 1, 2 and 3) and their interaction on algal (stress = 0.09899264) and invertebrate community structure (stress = 0.0765613); and of salamander loss, stream, leaf type (*Salix atrocinerea* and *Pinus sylvestris*) and their interactions on aquatic hyphomycete community structure (stress = 0.1075228); df = degrees of freedom, F = F-statistic, R^2 = adjusted R^2, p = p-value.

Community	Factor	df	F	R^2	p
Algae	Salamander loss	1	2.91	0.159	0.017
	Stream	1	1.24	0.068	0.263
	Salamander loss×Stream	1	1.19	0.065	0.283
Macroinvertebrate	Salamander loss	1	0.79	0.045	0.619
	Stream	1	3.39	0.193	0.002
	Salamander loss×Stream	1	0.41	0.023	0.928
Aquatic hyphomycetes	Salamander loss	1	1.25	0.029	0.250
	Stream	1	2.63	0.061	0.015
	Species	1	10.53	0.246	<0.001
	Salamander loss×Stream	1	0.55	0.013	0.822
	Salamander loss×Species	1	1.06	0.025	0.366
	Stream×Species	1	1.39	0.032	0.201
	Salamander loss×Stream×Species	1	0.46	0.011	0.887

Aquatic hyphomycete sporulation rate and species richness were higher in willow than pine and varied among streams, with no effect of salamander loss (Table S3, Fig. 4C, Fig. 4D). Aquatic hyphomycete community structure also varied with leaf type and stream (Table 3), with distinctive species in each case. Communities in willow leaves were characterized by *Anguillospora filiformis* GREATH., *Articulospora tetracladia* INGOLD, *Neonectria lugdunensis* (SACC. & THERRY) L. LOMBARD & CROUS, *Flagellospora curvula* INGOLD, *Tetrachaetum elegans* INGOLD, *Alatospora pulchella* MARVANOVÁ, *Tricladium splendens* INGOLD, *Lemonniera aquatica* DE WILD., *Lemonniera terrestris* TUBAKI and *Clavariopsis aquatica* DE WILD., whereas there were no characteristic species of the communities in pine needles. Stream 1 was characterized by *A. filiformis*, stream 2 by *F. curvula* and stream 3 by *T. splendens, L. aquatica, L. terrestris, Alatospora acuminata* Ingold and *C. aquatica* (Table S4).

Invertebrate abundance varied among streams and there was a significant interaction between salamander loss and stream: abundance was higher in stream 3, being greater in the absence of salamander, but in the other two streams there were no differences depending on salamander presence (Table S1, Fig. 2C). Taxon richness varied only among streams, being higher in stream 3 (Table S1, Fig. 2D). Invertebrate community structure also varied among streams (Table 3): stream 1 was characterized by *Chloroperla*

(Plecoptera); stream 2 by *Hydroporus* (Coleoptera), Simuliidae (Diptera), *Nemoura* (Plecoptera) and Ceratopogoninae (Diptera, shared with stream 3); and stream 3 by *Ecdyonurus* (Ephemeroptera), Tanypodinae (Diptera), *Thremma* (Trichoptera), *Oulimnius* (Coleptera), *Goera* (Trichoptera), *Holocentropus* (Trichoptera), *Limnius* (Coleptera), *Micrasema* (Trichoptera), Hirudinea and *Pisidium* (Sphaeriida; Table S5).

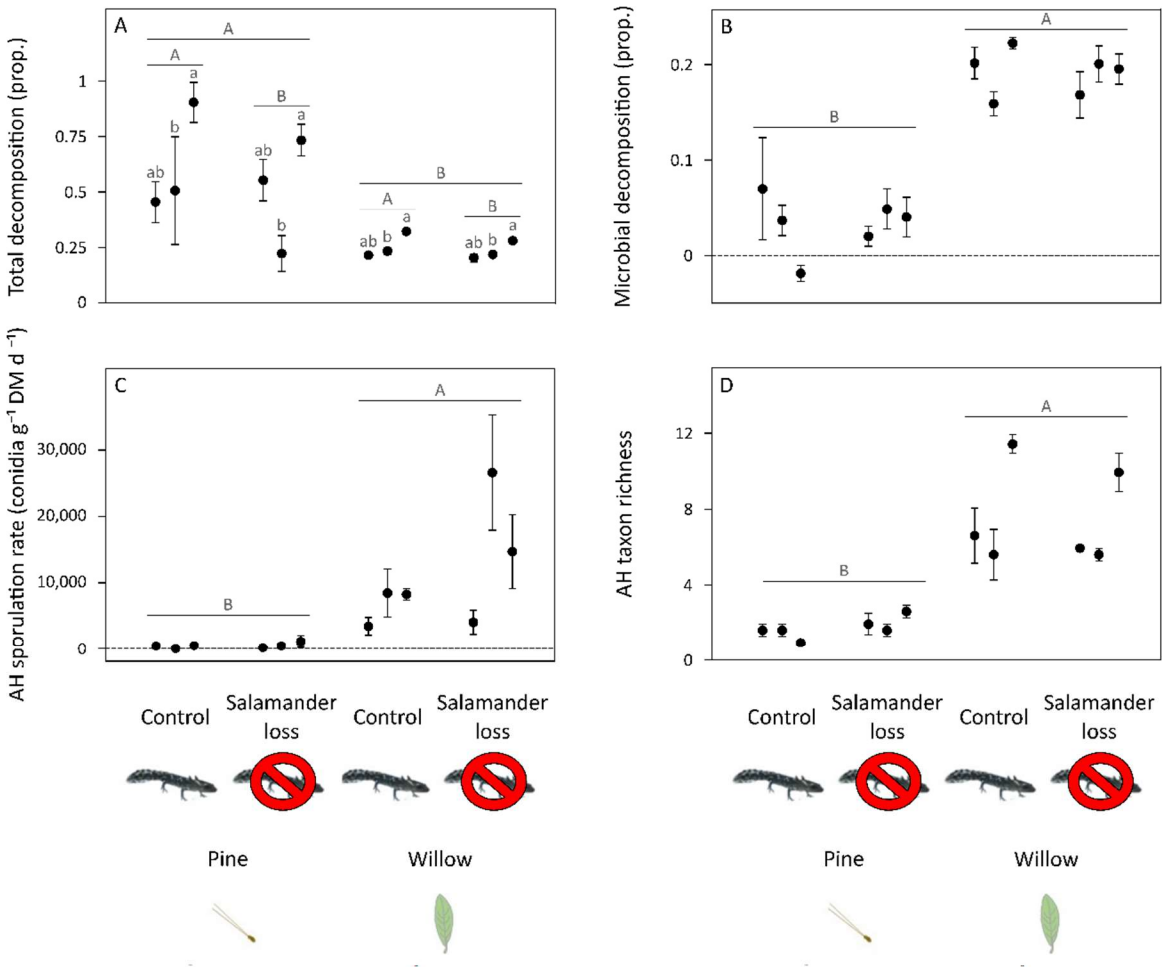

Fig. 4. Total (a) and microbial (b) decomposition (prop.), aquatic hyphomycete sporulation rate (conidia d^{-1} g^{-1}; c) and aquatic hyphomycete taxon richness (d) in the control and salamander loss treatment, in willow leaves and pines needles and in the three streams (stream 1, stream 2 and stream 3, in that order). Circles represent means and whiskers standard errors. Different letters indicate significant differences among treatments, leaf species or streams.

DISCUSSION

Our experiment demonstrates that salamander loss can alter the functioning of montane stream ecosystems, mainly through top-down effects that induce changes in both the green

and brown food web pathways (López-Rojo et al. 2022). Some of these effects have similarities with those reported for predatory invertebrates such as stonefly or dragonfly larvae (Peckarsky et al. 1993; Werner and Peacor 2006). Such effects could be consumptive –as salamander larvae prey on invertebrates and thus reduce their abundance, thus driving cascading effects on basal resources (Blaustein et al. 1996; Holomuzki et al. 1994; Urban 2013)– and/or non-consumptive –as they can induce antipredator strategies in invertebrates and thus reduce their foraging activity or increase the preference for safer habitats. Both of them might induce significant effects on basal resources, as observed with other freshwater predators (Bernot and Turner 2001; Davenport et al. 2020; Peckarsky et al. 1993; Preisser et al. 2005; Simon et al. 2004; Wudkevich et al. 1997).

Effects of salamander loss on the green pathway were reflected in several variables in our experiment. In enclosures with no salamander, algal abundance and chlorophyll concentration were reduced compared to enclosures with salamander, indicating a decrease in periphyton accrual as a result of higher invertebrate grazing (Kurle and Cardinale 2011). Moreover, the increase in the autotrophic index in the absence of salamander, together with the smaller change in periphyton biomass compared to chlorophyll concentration, indicated that salamander larvae promoted the growth of the autotrophic components of periphyton, maybe due to the release of nutrients by salamander activity. These nutrients could benefit primary producers in these oligotrophic streams, as observed for some anuran tadpoles (Kupferberg 1997), fish (Small et al. 2011), crayfish (Arribas et al. 2014) and dragonfly larvae (Costa and Vonesh 2013). The algal community shifted after salamander loss, with several genera –*Achnanthes*, *Gomphonema* and *Amphora*– particularly affected; this had been previously observed with dragonfly larvae, whose absence induced a reduction in the same genera (Werner and Peacor 2006). The greater effect on these diatom genera could be related to their large size (Alonso et al. 2022b; Ranvestel et al. 2004; Werner and Peacor 2006), which could facilitate their assimilation in invertebrate digestive tubes and make them less likely to pass through the gut and recolonize the substrate compared to green algae, cyanobacteria or smaller diatoms (Blaustein et al. 1996; Connelly et al. 2008; Peterson and Boulton 1999; Peterson and Jones 2003). Another possible explanation is that they may be more affected by salamander bioturbation (Barnum et al. 2022; Ranvestel et al. 2004), but this is unlikely given the absence of differences in sediment accrual between enclosures with and without salamander.

The brown food web pathway was also altered by the loss of salamander, but the effect on leaf litter decomposition was opposite to our expectation. Total decomposition decreased, thus not reflecting the increase in invertebrate shredding activity as a result of release from predation that we expected (Atwood et al. 2013; Rezende et al. 2015). It is possible that antipredator strategies, and thus the release from predation, are more obvious for grazers than for shredders, given that stone (and tile) surfaces are much more exposed areas compared to leaf packs, where invertebrates can hide more easily, as observed with fish predators (Bernot and Turner 2001; Wudkevich et al. 1997). Moreover, pine and willow are more or less recalcitrant leaves and hence a more appropriate shelter (safer habitat) than other, more palatable, leaf types that disappear more rapidly (Jabiol et al. 2014). In the presence of salamander, invertebrates may thus preferentially feed on leaf litter instead of periphyton and thus avoid the risk of being seen and preyed upon by salamanders.

In contrast to total decomposition, microbial decomposition was not affected by salamander loss, neither were aquatic hyphomycete sporulation rate nor community structure. This suggests that microbial decomposers are not affected by any trophic cascade effects of salamander mediated by invertebrates, which has been previously observed in terrestrial ecosystems where fire salamander adults control invertebrate communities but not the mycobiome (Laking et al. 2021). The lack of effect on aquatic hyphomycetes can explain the absence of effect on microbial decomposition, since aquatic hyphomycetes are the main microbial decomposers in streams (Suberkropp 1998). Our results contrast with studies on anurans, where tadpole excretion stimulated microbial decomposition due to nutrient release (Connelly et al. 2011; Ramamonjisoa and Natuhara 2018; Rugenski et al. 2012). In our study, the high contents of tannins and other inhibitory compounds in willow leaves and pine needles (Alonso et al. 2021; Casas et al. 2013; Martínez et al. 2013b) may have been more limiting for fungal decomposers than a moderate increase in nutrient availability due to salamander excretion (Ramamonjisoa and Natuhara 2018). Also, water renewal due to stream flow may have precluded any effects of salamander loss on microbial decomposers related to nutrient availability, which could be expected to be greater in lentic freshwaters (Rubio-Ríos et al. 2021), although for other freshwater predators, such as fish and crayfish, their nutrient excretion has been observed to be relevant also in running waters (Arribas et al. 2014; Small et al. 2011).

Both total and microbial decomposition were affected by leaf type, but in opposite ways. Microbial decomposition was higher in willow than pine, and so were aquatic hyphomycete sporulation rate and taxon richness, as could be expected (Webster and Benfield 1986). The low specific leaf area, low nutrient concentration and high toxic concentration of pine needles often lead to low microbial colonization and decomposition (Martínez et al. 2013b), whereas willow leaves, despite their low nutrient concentration, have been found to support aquatic hyphomycete communities similarly to those of leaves with higher nutrient concentration (Alonso et al. 2022a; Alonso et al. 2021). Total decomposition was higher in pine than willow, contrasting with previous studies where pine needles decomposed very slowly (Martínez et al. 2013a; Martínez et al. 2013b). The high decomposition rate of pine needles in our study probably represented not only consumption, but also losses related to other invertebrate activities. For example, pine needles can be used by caddisflies to build their cases, as these larvae usually prefer recalcitrant materials for their higher protective value (Casas et al. 2013; Whiles and Wallace 1997). Also, needle fragments could be lost due to stream flow even though they were initially tied, because invertebrate shredders may cut them, and their narrow shape makes it easier for big fragments to be removed and flushed out of the bags, compared to willow leaves.

Invertebrate communities were not altered by salamander loss, and abundance was increased in only one of the three streams, suggesting low consumptive effects of salamander on invertebrates. This contrasts with a study conducted in temporary ponds, where salamander presence significantly reduced invertebrate abundance (Blaustein et al. 1996). The higher effect in temporary habitats could be due to an earlier arrival of predators compared to prey, which contrasts with permanent habitats where overwintering salamanders and invertebrate prey always coexist (Blaustein et al. 1996). While invertebrate abundance could also be affected by predator closeness through their chemical cues (Boyero 2011; Boyero et al. 2006; Boyero et al. 2011; Correa-Araneda et al. 2017; Wesner et al. 2012), this is unlikely in our study because enclosures with and without salamander were adjacent and because salamander larvae were present in the stream, so chemical cues could be present regardless of the treatment. The only stream where invertebrate abundance increased as a result of salamander loss was the stream with higher invertebrate abundance and taxon richness, suggesting that a higher consumptive effect could be expected in streams with less limiting environmental conditions (i.e., not as highly

oligotrophic as montane streams). In streams with higher invertebrate density, the number of encounters and therefore captures would increase (Griffith et al. 2023; Kratz 1996) and may result in more evident effects of salamander loss on invertebrates.

CONCLUSIONS

We have shown that fire salamander larvae can control periphyton community structure and leaf litter decomposition through trophic cascades induced by consumptive and non-consumptive effects on invertebrates in montane stream ecosystems. Our results thus suggest that salamanders are key predators that regulate ecosystem structure and functioning through top-down control, and their loss may lead to ecosystem changes that could be critical in these ecosystems, where there are few amphibian species and thus species replacement is unlikely. This is even more relevant considering that salamanders are suffering severe declines (Bosch et al. 2018; Martel et al. 2014; Stegen et al. 2017), as are other amphibians worldwide (Collins 2010; Luedtke et al. 2023). Our study highlights the importance of studying the functional and structural consequences of amphibian loss for ecosystems, particularly for understudied taxa such as urodeles (Hocking and Babbitt 2014), and the need for improving protection measures for amphibians that prevent the loss of biodiversity and hence the alteration of ecosystems.

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COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIAL

Table S1. Results of ANOVA exploring the effects of salamander loss, stream (streams 1, 2 and 3) and their interaction, on periphyton biomass (g m^{-2}), chlorophyll *a*, *b* and *c* concentration (µg m^{-2}), inorganic mass (g m^{-2}), autotrophic index, algal abundance (cell m^{-2}) and species richness (number of species $sample^{-1}$), and invertebrate abundance (cell m^{-2}) and taxon richness (number of taxa $sample^{-1}$). df = degrees of freedom, F = F-statistic, p = p-value.

Variable	Factor	df	F	p
Periphyton biomass	Salamander loss	1, 5	4.64	0.084
	Stream	2, 6	2.91	0.131
	Salamander loss×Stream	2, 5	1.89	0.245
Chl-*a* concentration	Salamander loss	1, 5	21.54	0.006
	Stream	2, 6	0.87	0.467
	Salamander loss×Stream	2, 5	1.66	0.280
Chl-*b* concentration	Salamander loss	1, 5	11.69	0.019
	Stream	2, 6	0.22	0.810
	Salamander loss×Stream	2, 5	0.18	0.839
Chl-*c* concentration	Salamander loss	1, 5	39.38	0.002
	Stream	2, 6	2.93	0.130
	Salamander loss×Stream	2, 5	8.33	0.026
Inorganic mass	Salamander loss	1, 5	1.52	0.273
	Stream	2, 6	0.22	0.807
	Salamander loss×Stream	2, 5	1.94	0.237
Autotrophic index	Salamander loss	1, 5	7.48	0.041
	Stream	2, 6	0.73	0.519
	Salamander loss×Stream	2, 5	0.91	0.460
Algal abundance	Salamander loss	1, 5	10.40	0.023
	Stream	2, 6	2.76	0.141
	Salamander loss×Stream	2, 5	0.85	0.480
Algal richness	Salamander loss	1, 5	2.38	0.184
	Stream	2, 6	0.84	0.475
	Salamander loss×Stream	2, 5	0.38	0.703
Invertebrate abundance	Salamander loss	1, 5	1.23	0.318
	Stream	2, 6	5.24	0.048
	Salamander loss×Stream	2, 5	21.57	0.004
Invertebrate richness	Salamander loss	1, 5	0.04	0.852
	Stream	2, 6	8.14	0.020
	Salamander loss×Stream	2, 5	0.28	0.768

Table S2. Algal genus found in each treatment and stream (cell m^{-2}; mean ± SE).

		Stream 1		Stream 2		Stream 3	
Genus	Phylum	Control	Salamander loss	Control	Salamander loss	Control	Salamander loss
Achnanthes	Bacillariphyta	59294.2 ± 40675.0	22587.8 ± 8395.1	158656.7 ± 33745.3	65136.3 ± 20822.9	81937.1 ± 42831.4	12795.8 ± 6987.0
Amphora	Bacillariphyta	1782.0± 891.2	790.0 ± 790.0	900.2 ± 900.2		16993.7 ± 10150.3	891.0 ± 891.0
Anabaena	Cyanobacteria						5731.9 ± 5731.9
Chlorella	Chlorophyta			2884.3 ± 1720.8		936.9 ± 936.9	
Cladophora	Chlorophyta	10839.2 ± 6236.9					5731.9 ± 5731.9
Coelosphaerium	Cyanobacteria	74055.7 ± 38141.2	18169.5 ± 18169.5				
Eunotia	Bacillariphyta	900.2 ± 900.2					
Gomphonema	Bacillariphyta	40536.8 ± 21003.5	7642.6 ± 1347.9	16075.1 ± 5468.3	15560.7 ± 3381.7	88532.5 ± 32106.9	14412.5 ± 7942.0
Melosira	Bacillariphyta		5474.7 ± 4344.0	7330.2 ± 6030.5	4133.6 ± 4133.6	7164.9 ± 2291.7	17893.9 ± 12921.8
Monoraphidium	Chlorophyta	7201.6 ± 7201.6	1873.9 ± 1873.9			1818.8 ± 910.6	
Navicula	Bacillariphyta	41207.4 ± 19754.4	23028.7 ± 5245.9	129133.6 ± 24363.3	104267.7 ± 20242.4	85501.2 ± 15410.3	16846.7 ± 5136.4
Oscillatoria	Cyanobacteria	54912.6 ± 54912.6		13503.1 ± 13503.1	7440.5 ± 7440.5		
Pinnularia	Bacillariphyta					900.2 ± 900.2	
Scenedesmus	Chlorophyta		3343.6 ± 3343.6			3527.3 ± 3527.3	
Selenastrum	Chlorophyta		790.0 ± 790.0				
Stigeoclonium	Chlorophyta	44091.7 ± 44091.7				18904.3 ± 18904.3	
Surirella	Bacillariphyta	900.2 ± 900.2					
Synedra	Bacillariphyta				1653.4 ± 1653.4	881.8 ± 881.8	1846.3 ± 924.8
Tabellaria	Bacillariphyta	3610.0 ± 2380.0	1671.8 ± 1671.8	900.2 ± 900.2	2590.4 ± 1782.8		
Ulothrix	Chlorophyta					33509.7 ± 33509.7	

Table S3. Results of ANOVA exploring the effects of salamander presence, stream(streams 1, 2 and 3), leaf species (*Salix atrocinerea* and *Pinus sylvestris*) and their interactions, on total and microbial decomposition (%), and aquatic hyphomycete sporulation rate (conidia d^{-1} g^{-1}) and species richness (number of species $sample^{-1}$). df = degrees of freedom, F = F-statistic, p = p-value.

Variable	Factor	df	F	p
Total decomposition	Salamander loss	1, 16	8.16	0.011
	Stream	2, 6	18.82	0.003
	Species	1, 16	33.89	<0.001
	Salamander loss×Stream	2, 16	2.32	0.130
	Salamander loss×Species	1, 16	0.43	0.519
	Stream×Species	2, 16	4.21	0.034
	Salamander loss×Stream×Species	2, 16	1.19	0.329
Microbial decomposition	Salamander loss	1, 16	2.89	0.108
	Stream	2, 6	0.37	0.708
	Species	1, 16	3428.42	<0.001
	Salamander loss×Stream	2, 16	1.92	0.179
	Salamander loss×Species	1, 16	29.83	<0.001
	Stream×Species	2, 16	18.76	<0.001
	Salamander loss×Stream×Species	2, 16	4.40	0.030
Aquatic hyphomycete sporulation rate	Salamander loss	1, 15	2.06	0.172
	Stream	2, 6	9.30	0.015
	Species	1, 15	82.57	<0.001
	Salamander loss×Stream	2, 15	1.24	0.318
	Salamander loss×Species	1, 15	0.51	0.488
	Stream×Species	2, 15	2.52	0.114
	Salamander loss×Stream×Species	2, 15	0.02	0.984
Aquatic hyphomycete richness	Salamander loss	1, 15	0.24	0.634
	Stream	2, 6	9.28	0.015
	Species	1, 15	119.24	<0.001
	Salamander loss×Stream	2, 15	0.02	0.976
	Salamander loss×Species	1, 15	0.76	0.397
	Stream×Species	2, 15	1.21	0.327
	Salamander loss×Stream×Species	2, 15	1.56	0.242

Table S4. Aquatic hyphomycete species found in each treatment, leaf litter species and stream (conidia d^{-1} g^{-1}; mean ± SE).

	Pine leaves					
	Stream 1		Stream 2		Stream 3	
Species	Control	Salamander loss	Control	Salamander loss	Control	Salamander loss
Alatospora acuminata	161.4 ± 161.4		36.0 ± 36.0	68.2 ± 68.2	311.7 ± 311.7	927.4 ± 869.5
Alatospora pulchella						
Anguillospora filiformis	215.6 ± 154.3	57.0 ± 57.0	39.6 ± 39. 6			21.0 ± 21.0
Articulospora tetracladia	142.9 ± 142.9	206.5 ± 70.1	83.3 ± 42.8	135.8 ± 93.2	154.5 ± 154. 5	260.0 ± 76.2
Clavariopsis aquatica						
Culicidospora aquatica						
Flagellospora curvula		36.1 ± 36.1	39.6 ± 39.6	393.9 ± 295.4		52.7 ± 27.9
Heliscella stellata		28.5 ± 28.5				
Hydrocina chaetocladia						
Lemonniera aquatica						
Lemonniera terrestris						
Neonectria lugdunensis	49.7 ± 49.7					
Taeniospora gracilis						
Tetrachaetum elegans						
Tricladium angulatum						
Tricladium curvisporum						
Tricladium splendens						
Tripospermum myrti						
Triscelophorus monosporus						

Table S4. Cont.

	Willow leaves					
	Stream 1		Stream 2		Stream 3	
Species	Control	Salamander loss	Control	Salamander loss	Control	Salamander loss
Alatospora acuminata	80.0 ± 17.8	32.1 ± 17.1	225.9 ± 113.4	426.5 ± 78.4	602.3 ± 228.3	667.7 ± 317.9
Alatospora pulchella			50.6 ± 25.4	775.8 ± 610.4	239.4 ± 123.9	473.1 ± 333.2
Anguillospora filiformis	1404.8 ± 344.2	1804.6 ± 848.1	377.4 ± 170.5	506.9 ± 220.5	404.9 ± 151.3	569.1 ± 277.8
Articulospora tetracladia	1661.1 ± 950.4	1600.2 ± 497.2	4308.2 ± 2954.5	8431.9 ± 5193.1	2918.8 ± 738.8	5324.3 ± 1526.3
Clavariopsis aquatica	15.1 ± 15.1	12.6 ± 12.6		23.0 ± 23.0	697.4 ± 446.1	1595.5 ± 869.3
Culicidospora aquatica		19.5 ± 19.5				
Flagellospora curvula	135.6 ± 94.0	534.6 ± 409.1	3591.8 ± 1322.3	16447.6 ± 4728.0	4712.6 ± 2247.8	2738.4 ± 1133.8
Heliscella stellata			23.7 ± 23.7			
Hydrocina chaetocladia	15.1 ± 15.1	24.5 ± 24.5				
Lemonniera aquatica					904.3 ± 406.6	2577.8 ± 1066.3
Lemonniera terrestris			23.7 ± 23.7		82.3 ± 40.4	231.3 ± 118.2
Neonectria lugdunensis	212.1 ± 92.3	153.7 ± 103.7	26.9 ± 26.9	77.2 ± 77.2	484.7 ± 225.5	188.3 ± 109.4
Taeniospora gracilis					12.7 ± 12.7	
Tetrachaetum elegans	30.2 ± 15.1	12.2 ± 12.2		92.0 ± 92.0		137.8 ± 69.9
Tricladium angulatum	30.1 ± 30.1					
Tricladium curvisporum					40.7 ± 22.1	
Tricladium splendens					260.3 ± 111.3	380.6 ± 164.0
Tripospermum myrti			13.4 ± 13.4			
Triscelophorus monosporus	30.1 ± 30.1	12.6 ± 12.6				

Table S5. Macroinvertebrate taxa found in each treatment and stream (individual m^{-2}; mean ± SE).

		Stream 1		Stream 2		Stream 3	
Taxa	Order	Control	Salamander loss	Control	Salamander loss	Control	Salamander loss
sC. Acari						11.5 ± 11.5	23.1 ± 23.1
Acentrella	O. Ephemeroptera		11.5 ± 11.5		11.5 ± 11.5		
Agabus	O. Coleoptera		11.5 ± 11.5				
Amphinemura	O. Plecoptera	11.5 ± 11.5	11.5 ± 11.5				
Ancylus	O. Basommatophora			57.7 ± 57.7		23.1 ± 23.1	
Baetis	O. Ephemeroptera	276.8 ± 69.2	392.2 ± 197.1	726.6 ± 477.4	795.9 ± 490.6	830.5 ± 522.5	1164.9 ± 364.2
sF. Ceratopogoninae	O. Diptera			57.7 ± 11.5	69.2 ± 52.9	46.2 ± 30.5	138.4 ± 52.9
sF. Chironominae	O. Diptera	669.0 ± 476.7	922.7 ± 732.5	2825.8 ± 1606.0	2641.3 ± 1151.2	253.8 ± 120.4	1903.1 ± 283.2
Chloroperla	O. Plecoptera	115.3 ± 50.3	126.9 ± 57.7			23.1 ± 23.1	57.7 ± 11.5
Cordulegaster	O. Odonata			11.5 ± 11.5	11.5 ± 11.5	11.5 ± 11.5	11.5 ± 11.5
Dupophilus	O. Coleoptera						11.5 ± 11.5
Ecdyonurus	O. Ephemeroptera	34.6 ± 20.0	11.5 ± 11.5			149.9 ± 30.5	207.6 ± 52.9
Elmis	O. Coleoptera	23.1 ± 11.5					
Elodes	O. Coleoptera		11.5 ± 11.5				
Ephemerella	O. Ephemeroptera		11.5 ± 11.5				
Goera	O. Trichoptera					565.2 ± 340.4	461.4 ± 231.5
Habroleptoides	O. Ephemeroptera	11.5 ± 11.5	57.7 ± 41.6			34.6 ± 0.0	69.2 ± 69.2
Habrophlebia	O. Ephemeroptera	530.6 ± 351.9	380.6 ± 202.8	991.9 ± 180.2	899.7 ± 196.8	1280.3 ± 889.2	1038.1 ± 566.5
Helophorus	O. Coleoptera			11.5 ± 11.5			
C. Hirudinea		69.2 ± 20.0	161.5 ± 30.5		11.5 ± 11.5	484.4 ± 222.5	1084.2 ± 502.4
Holocentropus	O. Trichoptera	23.1 ± 11.5	34.6 ± 34.6	138.4 ± 138.4	173.0 ± 173.0	426.8 ± 102.5	542.1 ± 140.3
Hydraena	O. Coleoptera			11.5 ± 11.5			
Hydroporus	O. Coleoptera			69.2 ± 20.0	23.1 ± 11.5	11.5 ± 11.5	11.5 ± 11.5
Isoperla	O. Plecoptera		92.3 ± 46.1		11.5 ± 11.5	80.7 ± 30.5	69.2 ± 0.0
Leuctra	O. Plecoptera	484.4 ± 363.5	1095.7 ± 695.6	46.1 ± 30.5	11.5 ± 11.5	530.6 ± 94.4	1349.5 ± 488.1
Limnephilinae	O. Trichoptera	853.5 ± 272.7	484.4 ± 255.1	265.3 ± 50.3	138.4 ± 40.0	519.0 ± 91.6	415.2 ± 144.1
Limnius	O. Coleoptera				11.5 ± 11.5	57.7 ± 30.5	80.7 ± 41.6
F. Limoniidae	O. Diptera						11.5 ± 11.5

Table S5. Cont.

		Stream 1		Stream 2		Stream 3	
Taxa	Order	Control	Salamander loss	Control	Salamander loss	Control	Salamander loss
Micrasema	O. Trichoptera					2745.1 ± 1684.3	2249.1 ± 1541.3
Micronecta	O. Hemiptera			138.4 ± 121.5	57.7 ± 57.7		
Nemoura	O. Plecoptera			299.9 ± 219.2	149.9 ± 75.6	23.1 ± 23.1	34.6 ± 20.0
C. Oligochaeta		34.6 ± 0.0	34.6 ± 20.0	392.2 ± 189.9	761.3 ± 642.1	576.7 ± 426.8	1511.0 ± 1256.3
sF. Orthocladiinae	O. Diptera	242.2 ± 59.9	311.4 ± 156.0	173.0 ± 52.9	103.8 ± 20.0	126.9 ± 23.1	496.0 ± 50.3
Oulimnius	O. Coleoptera			149.9 ± 80.7	265.3 ± 80.7	945.8 ± 440.1	899.7 ± 390.0
Pisidium	O. Sphaeriida	23.1 ± 11.5		11.5 ± 11.5	34.6 ± 34.6	126.9 ± 46.1	553.6 ± 364.6
Protonemura	O. Plecoptera	11.5 ± 11.5		23.1 ± 23.1		34.6 ± 34.6	23.1 ± 23.1
F. Psychodidae	O. Diptera			11.5 ± 11.5		11.5 ± 11.5	11.5 ± 11.5
Scarodytes	O. Coleoptera	11.5 ± 11.5					
Sericostoma	O. Trichoptera		23.1 ± 23.1				46.1 ± 30.5
Sialis	O. Megaloptera		11.5 ± 11.5				
F. Simuliidae	O. Diptera	11.5 ± 11.5		23.1 ± 11.5	23.1 ± 11.5		
sF. Tanypodinae	O. Diptera	334.5 ± 98.6	438.3 ± 75.6	173.0 ± 121.5	207.6 ± 124.8	657.4 ± 207.6	2099.2 ± 475.4
Thremma	O. Trichoptera					34.6 ± 20.0	69.2 ± 40.0
F. Tipulidae	O. Diptera						11.5 ± 11.5
Velia	O. Hemiptera					11.5 ± 11.5	

General Discussion

This thesis addressed the impacts of non-random species loss caused by emergent diseases in two taxonomic groups –trees and amphibians– on the functioning and structure of freshwater ecosystems. Most biodiversity-ecosystem functioning research to date has focused on random species loss (Wardle, 2016), despite the fact that vulnerability to extinction varies greatly among species (Lepš, 2004), depending on their conservation status or their susceptibility to different stressors (Kominoski et al., 2013). Thus, the studies contained in this thesis are among the few that focus on how loss of particular species vulnerable to extinction can alter ecosystems, hence their potential relevance for future research as well as for species conservation strategies and ecosystem management. In this general discussion, we first summarize the results contained in each of the thesis chapters (Table 1) and then provide an extended review of some key results (Fig. 1) that we consider are worth of further attention.

OVERVIEW OF MAIN RESULTS

The first three chapters of the thesis focused on how the loss of vulnerable riparian tree species affects headwater stream ecosystems, which rely on leaf litter as the main basal resource of a predominantly brown food web. We found that losing different species altered leaf litter decomposition and associated communities but in different directions, depending on leaf litter traits of the species lost. Thus, a field experiment (Chapter 1) demonstrated that loss of alder (*Alnus glutinosa*), with highly palatable leaf litter, reduced decomposition via complementarity effects among leaf litter types in mixtures –which were promoted by alder and disappeared in its absence–, effect that was accompanied by changes in aquatic hyphomycete and invertebrate communities. A microcosm experiment (Chapter 2) found similar –but weaker– effects as a result of alder loss, whereas effects of losing oak (*Quercus robur*), with low leaf litter palatability, were opposite. Another microcosm experiment (Chapter 3) demonstrated that infection of alder (*A. lusitanica*) by *Phytophthora alni* tended to enhance its leaf litter palatability and decomposition but, following the disappearance of diseased trees and their replacement by the invasive black locust (*Robinia pseudoacacia*), the trend was just opposite, with strong reduction of decomposition and important changes in aquatic hyphomycete communities.

Table 1. Main results obtained in this thesis, consisting of effects of reduction and loss of different tree and amphibian species as a result of emergent diseases. Arrows indicate the direction of the response (non-significant when they are between parenthesis); "*" indicates significant, non directional differences; and "≈" indicates no effect. Community refers to species composition; AH = aquatic hyphomycete; N = nitrogen; P = phosphorus; Chl = chlorophyll.

Chapter #	Chapter 1	Chapter 2					Chapter 3		Chapter 4	Chapter 5				Chapter 6
Type of environment	Headwater streams	Stream microcosms					Stream microcosms		Montane streams	Pond mesocosms				Montane streams
Treatment or scenario	Alder loss	Alder reduction	Alder loss	Oak reduction	Oak loss	Alder and oak loss	Alder infection	Alder replacement by black locust	Midwife toad loss	Midwife toad reduction	Midwife toad loss	Marbled newt loss	Midwife toad and marbled newt loss	Fire salamander loss
Total decomposition	↓	(↓)	(↓)	≈	(↑)	≈								↓
Microbial decomposition		≈	≈	≈	(↑)	≈	(↑)	(↓)						≈
AH sporulation	↓	≈	(↓)	(↑)	(↑)	(↑)	≈	↓						≈
AH richness	↓	≈	≈	≈	≈	≈	(↑)	≈						≈
AH community	*	≈	≈	*	*	*	≈	*						≈
Fungal biomass							(↓)	(↓)						
Invertebrate abundance	≈													≈
Invertebrate richness	≈													≈
Invertebrate community	*													≈
Invertebrate growth		≈	≈	≈	≈	≈			↓					
Invertebrate N concentration		≈	↑	≈	≈	↑								
Invertebrate P concentration		≈	↑	↑	↑	↑								
Periphyton biomass									↑	≈	(↓)	(↓)	(↓)	(↓)
Chl-*a* concentration									↑	≈	↓	(↓)	↓	↓
Chl-*b* concentration									≈	≈	(↓)	(↓)	↓	↓
Chl-*c* concentration									(↑)	≈	≈	≈	≈	↓
Autotrophic index									≈	≈	≈	(↑)	≈	↑
Sediment mass									≈	(↓)	(↓)	(↓)	(↓)	≈
Algal abundance									(↑)	↓	↓	(↓)	↓	↓
Algal richness									(↑)	≈	(↓)	≈	(↓)	≈
Algal community									*	≈	*	≈	*	*

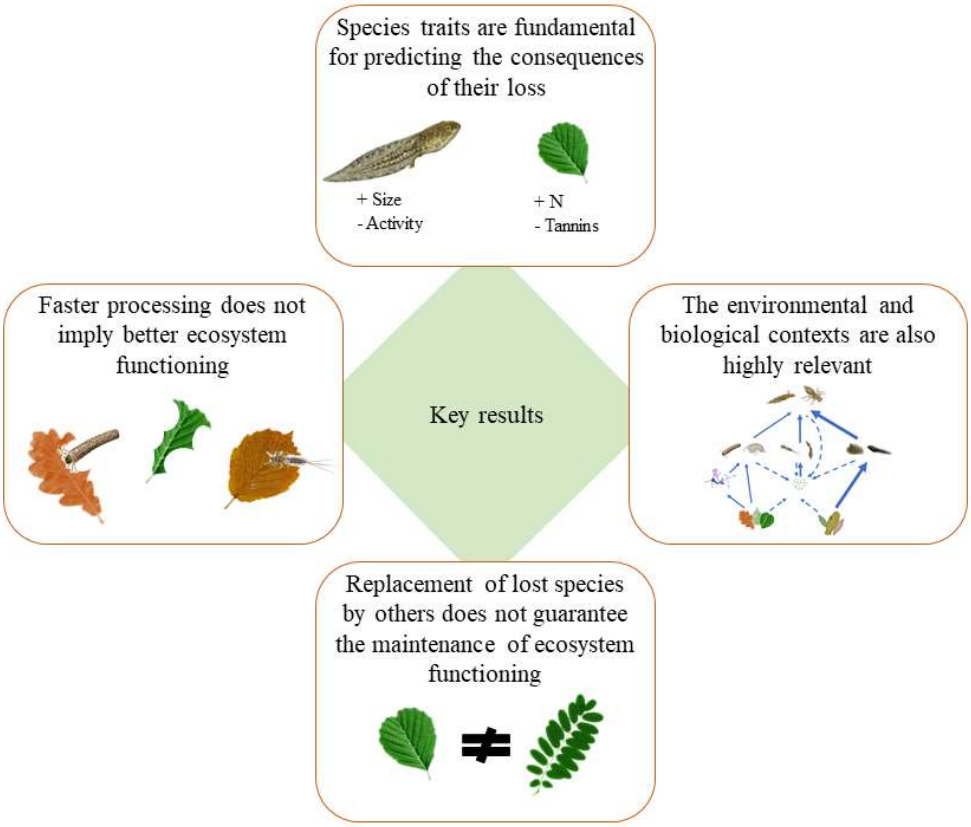

Fig. 1. Key results of this thesis about the consequences of species loss on freshwater ecosystems.

The other three chapters focused on amphibian loss due to *Batracochytrium* fungi and the consequences for montane streams and ponds, where periphyton is the main basal resource and a green food web prevails, being amphibian larvae major periphyton grazers. We showed that loss of anurans and urodeles induced changes in periphyton accrual and algal communities, which depended on competitive and trophic interactions with other species in the amphibian community. Thus, a field experiment conducted in montane streams (Chapter 4) showed that loss of common midwife toad (*Alytes obstetricans*), in the absence of other amphibian grazers, promoted periphyton biomass accrual but inhibited invertebrate growth, possibly because tadpoles facilitated invertebrate feeding. In contrast, a mesocosm experiment simulating ponds (Chapter 5), where common midwife toad loss was compensated by gain of other amphibian species, found reduced periphyton biomass accrual due to competitive release of more active species, and a similar –but weaker– effect of marbled newt (*Triturus marmoratus*) loss through its effects on zooplankton. Finally, another field experiment (Chapter 6) demonstrated that loss of salamander (*Salamandra salamandra*) released invertebrates of predatory pressure so they shifted their foraging

preferences from leaf litter –a safer resource in the presence of predators– towards periphyton, resulting in reduced periphyton biomass accrual.

KEY RESULT 1: SPECIES TRAITS ARE FUNDAMENTAL FOR PREDICTING THE CONSEQUENCES OF THEIR LOSS

We evidenced that loss of different vulnerable species can have contrasting effects on ecosystems and communities, depending on their traits that modulate interactions among species, such as leaf litter palatability or amphibian feeding mode and larval size. For example, loss of two tree species with contrasting leaf litter traits, alder and oak –of high and low palatability, respectively–, resulted in opposite effects on leaf litter decomposition. These effects will translate into a deceleration and acceleration of biomass and energy transfer among trophic levels within the brown food web, respectively for alder and oak loss, with likely further effects in the longer term due to changes in invertebrate and aquatic hyphomycete communities (Gessner et al., 2010). In montane ponds, the loss of common midwife toad and marbled newt reduced periphyton accrual in both cases, but through different mechanisms: the former is a periphyton grazer, its loss causing competitive release of more active grazers, whereas the latter is a predator and its loss releases zooplankton from predatory pressure. Therefore, our results show that understanding the trophic role of each species vulnerable to extinction within the food web, as well as its specific traits compared to other species in the community, can improve the prediction of changes in competitive and trophic interactions that can lead to fundamental changes in ecosystems.

KEY RESULT 2: THE ENVIRONMENTAL AND BIOLOGICAL CONTEXTS ARE ALSO HIGHLY RELEVANT

We observed that some outcomes of species loss depended on the experimental conditions. Thus, alder loss resulted in reduced decomposition and fungal sporulation in all cases, but effects were weaker in microcosms than in the field. This could be explained by the simplified conditions of microcosms, with the presence of only one detritivore species that precluded interactions among consumer species which often influence decomposition (Tonin et al., 2018). In microcosms, the presence of alder in a mixture may enhance decomposition of other species due to nutrient transfer through fungal hyphae (Handa et al., 2014) or nutrient leaching (Gessner et al., 2010; López-Rojo et al., 2019) but, in the

field, alder may also attract detritivores to the mixture and thus indirectly favour consumption of other species (Ferreira et al., 2012). Moreover, when oak was lost at the same time as alder in microcosms, the effect of alder loss disappeared because it was compensated by an opposite effect of oak loss (López-Rojo et al., 2020), indicating that losing more than one species simultaneously renders different effects than losing single species. For amphibians, loss of common midwife toad reduced periphyton accrual in ponds containing other anuran species, which were more active but competitively inferior (Richter-Boix et al., 2007), but had the opposite effect in montane streams where there were no similarly efficient grazers. Also, while the algal taxa more affected by amphibian loss in montane streams were large diatoms, we did not observe this trend in microcosms, where diatoms were scarce because conditions possibly favoured green algae or because the high tadpole density in mesocosms may have limited the growth of large diatoms. In streams, neither common midwife toad nor salamander loss induced changes in sediments, since water flow overshadowed any effect of bioturbation, whereas in mesocosms with no flow different species combinations showed different degrees of sediment accumulation explained by tadpole activity of different species (Ranvestel et al., 2004). Our results highlight the relevance of studying real-case scenarios, where single or multiple species are likely to disappear and others will remain, for more accurate predictions of ecosystem changes. It may be advisable to prioritize field studies, although some experiments require more controlled conditions; e.g., simulating loss of a given tree species can be hard because excluding its leaf litter from experimental units does not guarantee its absence in the stream, especially for more recalcitrant leaves that remain in the water for long periods of time.

KEY RESULT 3: REPLACEMENT OF LOST SPECIES BY OTHERS DOES NOT GUARANTEE THE MAINTENANCE OF ECOSYSTEM FUNCTIONING

Although some species share traits that lead to their inclusion in a given functional group, these species are likely to have differences in other traits that can lead to different effects on ecosystems (Rosenfeld, 2002). We showed this for alder and black locust, both N-fixing trees, which nevertheless differed in leaf litter palatability and hence in their effects on decomposition. The black locust is an invasive species susceptible to replace alder after its loss, given its high success in disturbed riparian habitats of southern Europe (Vítková et al., 2020), hence the importance of comparing the effects of both species on ecosystems.

Black locust leaf litter has lower nutrient and higher phenolic concentrations than alder leaf litter, traits that inhibit microbial activity and reduce fungal biomass and sporulation and leaf litter decomposition (McArthur et al., 1994; Medina-Villar et al., 2015). In consequence, key ecosystem process rates were reduced when black locust replaced alder. Also important to consider is the fact that alder infection comes before this species is lost and replaced, with leaf litter of infected alder being even more labile than that of healthy alder, so the shift from infected alder to black locust is even larger than could be expected from the direct replacement of healthy alder (Compson et al., 2018). A good assessment of the health status of vulnerable species is thus recommended to improve predictions about how species replacement will alter ecosystems.

KEY RESULT 4: FASTER PROCESSING DOES NOT IMPLY BETTER ECOSYSTEM FUNCTIONING

While faster ecosystem processes may seem equal to better performance, this is not always the case. For example, the more rapid disappearance of leaf litter from streams can be detrimental for some consumers in the absence of more persistent resources (Marks, 2019). We observed that, being alder a faster decomposer and containing more nutrients, leaf litter of other species induced a more beneficial response in the detritivore *Sericostoma pyrenaicum*, which assimilated more nutrients when fed leaf litter mixtures containing oak, willow (*Salix atrocinerea*) and hazel (*Corylus avellana*). Such mismatch between leaf litter nutrient concentrations and detritivore nutrient assimilation has been observed before (Siders et al., 2021) and could be caused by slower nutrient leaching from more recalcitrant litter that allows higher nutrient assimilation by detritivores along a more extended period of time, especially when recalcitrance is not coupled with high concentrations of inhibitory substances (Compson et al., 2015; Compson et al., 2018; Siders et al., 2021). More recalcitrant leaf litter can also offer a more stable substrate for invertebrates (Sanpera-Calbet et al., 2009; Compson et al., 2013), as we observed for hazel, which can be beneficial for communities and in the longer term for the ecosystem. Therefore, it seems important to keep in mind that faster is not better and that the existence of varied species traits in mixtures can be beneficial for other species or for the ecosystem for different reasons.

FINAL CONSIDERATIONS

There are several further facts that emerged from this thesis, which may be useful to consider when designing future studies about impacts of biodiversity loss on ecosystems (Fig. 2). Firstly, there are many species other than those studied here, which are vulnerable to extinction due to emergent diseases and deserve attention. For example, many tree species such as elms, ashes, myrtles, chestnuts or oaks are strongly affected by pathogens (Loo, 2009; Landolt et al., 2016; Berthon et al., 2018; Jung et al., 2018), but the potential effects of their loss are virtually unknown. Amphibians are suffering dramatic declines worldwide, but knowledge about impacts of amphibian loss on freshwater ecosystems is very scarce, with the existence of only a few studies, most of them conducted in tropical streams and focused on total disappearance of amphibians rather than the loss on particular species (Whiles et al., 2006; Hocking & Babbitt, 2014). Urodeles in particular have received very little attention (Hocking & Babbitt, 2014), so they should be especially considered in future research.

Secondly, this thesis revealed that different types of freshwater ecosystems can be differently impacted by species loss, with headwater forest streams being more likely affected through brown food webs, whereas green pathways are more strongly altered in montane streams and ponds. Thus, extending our research to other types of freshwater ecosystems, such as ponds, lakes or lower stream reaches, would be advisable. Thirdly, this thesis and previous studies have revealed how important it is to consider multiple ecosystem processes and characteristics of communities when predicting the consequences of stressors on ecosystems, as shown here for species loss and elsewhere for environmental stressors such as climate change (Pérez et al., 2021). Overall, investigations about how biodiversity loss impacts ecosystem functioning should continue implementing realistic scenarios and widening its scope to include less studied ecosystems and taxonomic groups, in order to reach a comprehensive understanding of the consequences of plausible extinctions and to improve conservation and management measures to mitigate those effects.

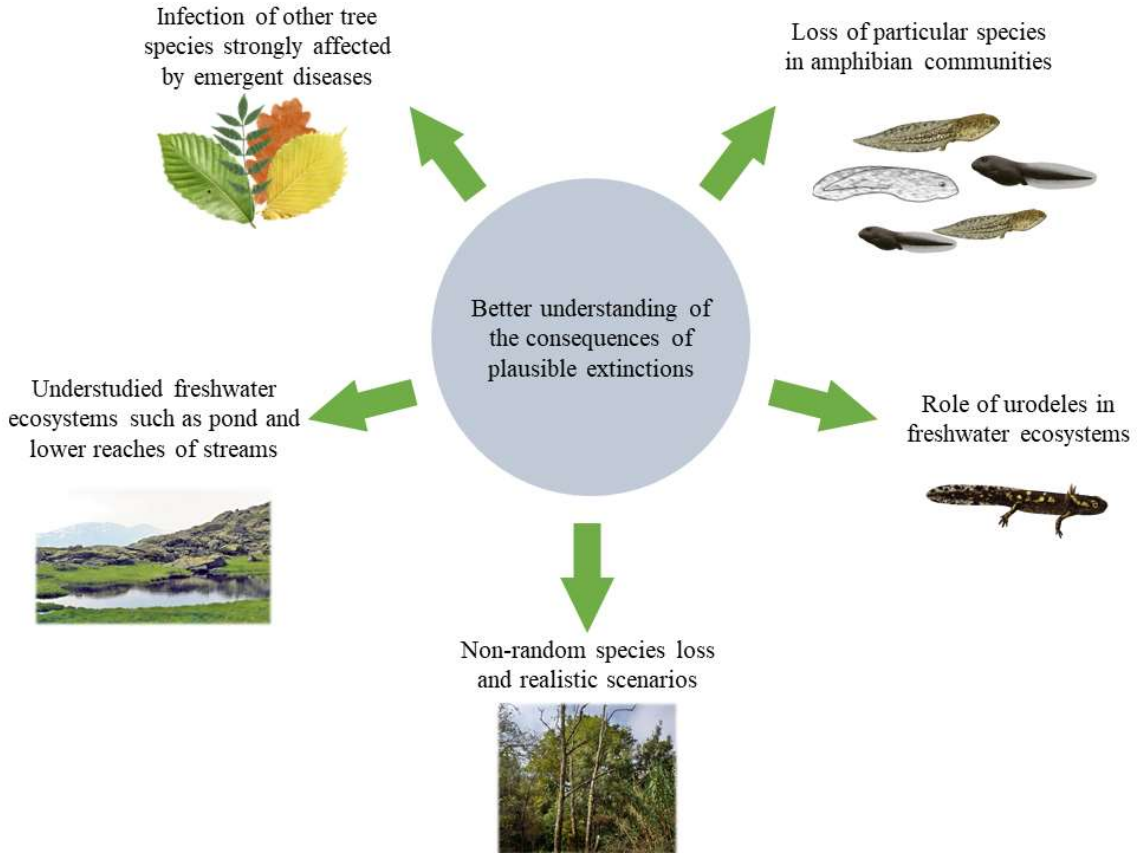

Fig. 2. Suggestions for future studies on effects of biodiversity loss on freshwater ecosystem functioning and structure.

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General Conclusions

1. Species loss can strongly alter freshwater ecosystem functioning and structure, but the direction and magnitude of effects depend on the identity of the lost species and their traits. *Loss of a tree species with high leaf litter palatability reduced leaf litter decomposition, whereas loss of a tree species with low palatability had the opposite effect.*

2. Effects of species loss on ecosystems depend on the biological context. *Loss of a grazing anuran species enhanced periphyton accrual in the absence of other anurans but had the opposite effect when other, more active grazing species replaced the lost species.*

3. Effects of species loss on ecosystems depend on the environmental context. *The presence of flow in streams precluded any effects of species loss on sediment accumulation, which were observed in pond mesocosms with no flow.*

4. Disease caused by fungal infections can affect ecosystems before the species disappears. *Leaf litter of infected trees was more labile and decomposed faster than that of healthy trees.*

5. Replacement of lost species by others of the same functional group does not guarantee the maintenance of ecosystem functioning. *Replacement of an N-fixing native species by an N-fixing invader resulted in decreased leaf litter decomposition.*

6. Loss of top predators alters ecosystem processes related to basal resources through trophic cascades. *Loss of urodele species altered periphyton accrual due to release of predation on zooplankton, and leaf litter decomposition through shifts in invertebrate feeding preferences.*

7. Research about impacts of biodiversity loss on freshwater ecosystems should focus on realistic scenarios of non-random species loss. *Different species traits, species combinations, ecosystem types and stressor dynamics rendered different outcomes.*