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Leaf litter decomposition of sweet chestnut is affected more by oomycte infection of trees than by water temperature

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ABSTRACT

Riparian forests are subjected to multiple disturbances, such as tree diseases caused by invasive pathogens, whose consequences on stream functioning are unknown. We assessed the impact of *Phytophthora cinnamomi* infection, and interactions with temperature, on microbial decomposition of *Castanea sativa* leaves. Leaves from healthy, symptomatic and highly symptomatic trees were incubated in the laboratory at 13 and 18 °C for 64 d. Infection significantly increased polyphenolic concentration and leaf toughness, reducing leaf decomposition and microbial respiration rates irrespective of temperature. Aquatic hyphomycete communities differed significantly in leaves from highly symptomatic trees. Fungal biomass was highest at 18 °C, irrespective of tree health status. None of the parameters were influenced by the tree health status × temperature interaction, suggesting that temperature rise may not synergistically increase the cross-ecosystem effects caused by *P. cinnamomi* in streams where litter decomposition is microbial-driven. Infection by *P. cinnamomi* alters the nutritional quality of leaves affecting the functioning of aquatic ecosystems.

1. Introduction

Forests represent about 30% of the Earth's surface (Bonan, 2008) and provide fundamental ecosystem services (Turner and Daily, 2008; Lewis et al., 2015; Trumbore et al., 2015). In addition to ensuring the maintenance and stability of ecological systems, forests are responsible for providing humans with several products and services such as food, timber, fresh water, climate control, nutrient cycling and primary production (MEA, 2005). Nowadays, forests are subjected to a wide range of disturbances worldwide (Trumbore et al., 2015; Prävälie, 2018), with invasive pathogenic species among those causing the strongest perturbations (Hubbart et al., 2016; Prävälie, 2018).

The soilborne oomycete *Phytophthora cinnamomi* is a generalist invasive pathogen that is widely distributed in all continents (Kamoun et al., 2015). Considered one of the most aggressive and devastating plant pathogens ever (Kamoun et al., 2015), *P. cinnamomi* is historically responsible for massive economic loss in agriculture, forestry and horticulture (Hansen et al., 2012; Hardham and Blackman, 2018). Its occurrence is linked to human activities such as soil transposition for construction activities (Dawson and Weste, 1985) and movement of infected nursery plants (Jung et al., 2016; Beaulieu et al., 2017), which allow dispersal over great distances. Several studies have evaluated the influence of abiotic factors on disease development caused by *P. cinnamomi* and some have indicated that temperature is an important factor for distribution and establishment of the pathogen. Hyphal growth is optimal at 20.0–32.5 °C (Zentmyer et al., 1976) and disease severity is highest at intermediate temperatures (Shearer et al., 1987; Martín-García et al., 2015). Models simulating potential range expansion of diseases under future climate change scenarios predict that rising temperatures, mainly in winter, will increase the area suitable for *P. cinnamomi* survival (Bergot et al., 2004; Thompson et al., 2014; Burgess et al., 2017). Temperature should therefore be considered when studying the impacts of *P. cinnamomi* infection.

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Unlike other *Phytophthora* species, *P. cinnamomi* has about 5000 host species (Jung et al., 2018) and has had disastrous impacts on natural ecosystems and biodiversity (Jung et al., 2018; Sena et al., 2018). Susceptible plant species of particular concern include sweet chestnut (*Castanea sativa*), holm oak (*Quercus ilex*) and cork oak (*Quercus suber*) in Europe (Camilo-Alves et al., 2013; Corcobado et al., 2013a; Dal Maso and Montecchio, 2015; Serrazina et al., 2015; Sena et al., 2018); jarrah (*Eucalyptus marginata*) in Australia (Hardham and Blackman, 2018; Sena et al., 2018); and American chestnut (*Castanea dentata*) in the USA (Hardham and Blackman, 2018; Sena et al., 2018). The impacts of *P. cinnamomi* on forests are multiple and are strongly associated with tree health status, which in turn depends on the duration of the infection (Camilo-Alves et al., 2013), soil water content (Camilo-Alves et al., 2013; Corcobado et al., 2013b) and the amount of inoculum in the soil (Solla et al., 2009). *P. cinnamomi* typically infects fine feeder roots, and infection is initiated by hyphae germinating from encysted zoospores (Hardham and Blackman, 2018). Hyphae grow intra- or intercellularly through the root cortex and into the central vascular bundle, causing cell degradation (Redondo et al., 2015). Blockage of the xylem through hyphal obstruction and deposition of material by the plant inhibits water movement from the roots to the shoots, resulting in water stress (Corcobado et al., 2013b; Ruiz-Gómez et al., 2015). In addition to altering water relations (Dawson and Weste, 1982; Corcobado et al., 2013b), *P. cinnamomi* reduces leaf mineral concentration and modifies plant performance after infection (Maurel et al., 2001; Corcobado et al., 2017; León et al., 2017). Fine root rot and collar girdling exacerbate the problem and lead to rapid leaf wilting and plant death (Robin et al., 2001; Osswald et al., 2014). It has been reported that the presence of *P. cinnamomi* in the rhizosphere of declining trees alters relations between ectomycorrhizal abundance and several soil properties (Corcobado et al., 2014). Therefore, even before the tree dies, physiological and structural changes caused by *P. cinnamomi* can affect ecosystem functioning, not only in plant and fungal communities (Ruiz Gómez et al., 2019), but also in detrital pathways dependent on plant litter.

Forest streams are especially vulnerable to changes in riparian vegetation, as they depend directly on the input of terrestrial plant material (Vannote et al., 1980). In streams, organic matter is decomposed mainly by aquatic hyphomycetes and macroinvertebrate shredders, ensuring the flow of nutrients and energy along the food web (Hieber and Gessner, 2002). Because infection by *P. cinnamomi* affects the physiology, fitness and ectomycorrhizal symbiosis of trees (Corcobado et al., 2013b, 2015; León et al., 2017; Hardham and Blackman, 2018; Sena et al., 2018), the pathogen could also be expected to affect leaf nutritional quality. Litter quality is a crucial factor that controls decomposer activity and decomposition rates. High quality litter (low in lignin and polyphenolics and high in nutrient concentration) is generally preferred and decomposed faster by microorganisms and invertebrates than low quality litter, which is more recalcitrant (Ferreira et al., 2015; Frainer et al., 2015; López-Rojo et al., 2018). Water temperature is an additional consideration, because it modulates litter decomposition by stimulating decomposer activity (Friberg et al., 2009; Ferreira and Chauvet, 2011a; Geraldes et al., 2012) and may display synergistic effects with dissolved nutrients (Ferreira and Chauvet, 2011b; Fernandes et al., 2014; but see Manning et al., 2018). It has also been suggested that decomposition of low quality litter responds more strongly than high quality litter to changes in water temperature (Conant et al., 2008; Gonçalves et al., 2013; Fernandes et al., 2014; Ferreira et al., 2015), but more research is needed to confirm this.

The cross-ecosystem impacts of *P. cinnamomi* infection of riparian trees on stream ecosystem functioning are unknown. We collected senescent leaf litter from *P. cinnamomi*-infected and non-infected *C. sativa* trees to test the following hypothesis: (1) the health status of *C. sativa* trees will influence the physico-chemical characteristics of leaf litter, with more recalcitrant leaves produced by infected than by healthy trees. We then assessed microbial-driven decomposition of leaf litter from *P. cinnamomi*-infected and non-infected *C. sativa* trees in laboratory microcosms at two temperatures, with the following hypotheses: (1) *C. sativa* leaf litter from infected trees will decompose slower than litter from healthy trees; (2) higher water temperature will stimulate microbial activity and consequently increase the rate of litter decomposition; and (3) interactions between the health status of *C. sativa* trees and temperature will influence leaf litter decomposition, with stronger effects of temperature being found for low quality litter, i.e. from infected trees.

2. Methods

2.1. Study area and leaf litter collection

To test whether *P. cinnamomi* infection influences leaf litter characteristics and decomposition rates, the highly susceptible *C. sativa* species was used. Sampling was performed in a *C. sativa* riparian forest in Hervás, Extremadura region, southwest Spain (40°15′N, 5°52′W; 805 m a.s.l.). The Hervás chestnut forest has a Mediterranean climate (mean air temperature $= 14.9 \pm 0.5$ °C; annual precipitation $= 1004.7 \pm 150.7$ mm) and is threatened by warming (e.g., mean air temperatures in 2016 and 2017 were 15.8 and 16.9 °C, respectively) and the occurrence of several *Phytophthora* species, thus providing an ideal condition for studying global change factors. In May 2017, isolations from the rhizosphere of several healthy and non-healthy trees enabled detection of *P. cinnamomi* in non-healthy symptomatic trees (Jung et al., 2018). Infected trees showed typical symptoms of ink disease (Jung et al., 2018), including branch dieback, reduced shoot growth and reduced leaf and nut size.

Five healthy (≤5% crown transparency; estimated visually), five symptomatic (21–40%) and five highly symptomatic (≥60%) *C. sativa* trees were selected. Trees were located close to a stream bank where soil and light conditions were favourable for growth. Trees were about 60–70 y old and had similar height and basal diameter (ca. 20 m and 80 cm, respectively). In November 2017, about 100 senescent leaves per tree were collected, air dried at room temperature, mailed to the University of Coimbra and stored in the dark until analysis.

2.2. Leaf litter characterisation

Subsamples of senescent leaves from each tree were ground to pass through a 0.5 mm screen (Retsch MM 400, Haan, Germany) and analysed for carbon (C) and nitrogen (N) (IRMS Thermo Delta V advantage with a Flash EA-1112 series; Thermo Fisher Scientific Inc., Waltham, MA, USA), phosphorus (P;APHA, 1995), polyphenolics (Graça et al., 2005) and lignin (Goering and Van Soest, 1970) concentrations. Three subsamples per tree were used. Concentrations were expressed as percentage of dry mass (% DM). Initial leaf toughness was determined with a penetrometer for nine leaf discs (12 mm diameter) per tree after discs had been soaked in distilled water for 1 h. Leaf toughness was expressed as the mass (g) required to force a blunt iron rod (1.55 mm diameter) through the leaf mesophyll (Graça et al., 2005). Specific leaf area (SLA) was determined for the same leaf discs after drying for 24 h at 105 °C and weighing, and results were expressed as $mm^2\ mg^{-1}$.

2.3. Leaf litter conditioning

After leaves were moistened, 12 mm diameter leaf discs were cut with a cork borer (avoiding primary and secondary veins), air dried for 72 h and weighed in sets of 20 discs. Each set of discs was enclosed in a 0.5-mm mesh bag, 5 cm × 7 cm, and incubated in a 15-L tank containing filtered stream water and ca. 2 L of a diverse litter mixture comprising leaves at different stages of degradation. Both water and litter were collected from a local oligotrophic stream (Ribeira da Sardeira, Lousã Mountain, central Portugal; 40°5′N, 8°12′W; 683 m a.s.l.) flowing through a broadleaf deciduous forest dominated by *C. sativa* and *Quercus robur* trees, in an area of very low human density. More information about the stream and the riparian vegetation can be found in previous studies (Gulis et al., 2006; Ferreira et al., 2016). To prevent inhibition of microbial colonisation of leaf discs by accumulation of polyphenols leached from the litter, the water in the tank was renewed every 2 d. The tank was kept for 10 d at 18 °C with aeration, under 12 h light and 12 h dark photoperiod, to ensure colonisation of leaf discs by microbial decomposers.

After conditioning, six sets of discs per tree were used in a microcosm experiment. Disc dry mass (DM) at the start of the experiment (day 0) was estimated by multiplying air-dry mass before conditioning by a conversion factor derived from an additional set of discs from each tree. The additional sets of discs were air dried and weighed (DM_1), conditioned as described above, dried for 24 h at 105 °C and weighed again after conditioning (DM_2). The conversion factor was estimated as DM_2/DM_1.

2.4. Experimental design

The effects of tree health status and temperature on decomposition rates of senescent *C. sativa* leaves were tested in microcosms in a controlled laboratory environment. Microcosms comprised 100-mL Erlenmeyer flasks filled with 40 mL filtered stream water renewed twice a week. All microcosms received one set of leaf discs and were placed on orbital shakers (100–120 rotations min^{-1}; GLF 3017, Burgwedel, Germany) under 12 h of light and 12 h of dark photoperiod (Fig. 1). In some areas of the world, including Portugal (Miranda et al., 2002), 5 °C increase has been predicted for stream water. Applying this extreme warming scenario, flasks were exposed to 13 and 18 °C to simulate present and future stream water temperatures in autumn.

Tree health status and temperature were crossed in a full factorial design (Fig. 1), allowing six treatments to be tested: (i) leaves of healthy trees at 13 °C, (ii) leaves of healthy trees at 18 °C, (iii) leaves of symptomatic trees at 13 °C, (iv) leaves of symptomatic trees at 18 °C, (v) leaves of highly symptomatic trees at 13 °C, and (vi) leaves of highly symptomatic trees at 18 °C. Leaf decomposition for each tree was destructively assessed after 15, 36 and 64 d of incubation. In total, there were 90 microcosms corresponding to 3 tree health statuses × 5 trees × 2 temperatures × 3 sampling dates (Fig. 1). In each microcosm, conidial production by aquatic hyphomycetes, microbial respiration rates, leaf toughness, fungal biomass and leaf mass were determined.

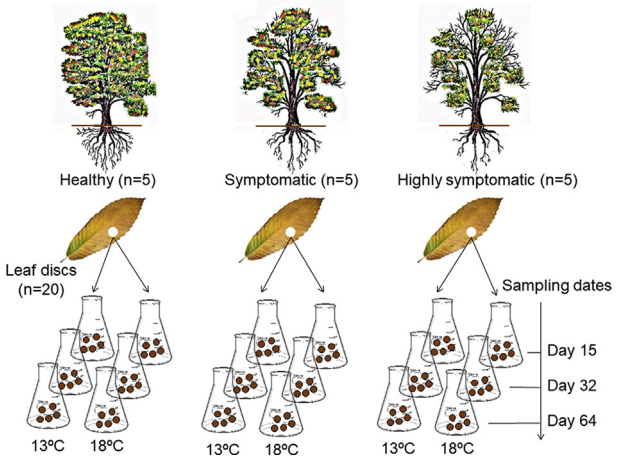

Fig. 1. Experimental design comprising five healthy and 10 *P. cinnamomi*-infected *Castanea sativa* trees, from which three sets of 20 discs of senescent leaves were incubated in water at 13 and 18 °C. After 15, 36 and 64 d incubation, microcosms were destructively assessed.

2.5. Conidial production

On each sampling date, the conidial suspensions of each microcosm were poured into 50-mL Falcon tubes, preserved with 2 mL 37% formalin and stored until processing. When preparing filters for conidia counting and identification, 150 μL 0.5% Triton X-100 was added to the suspension, which was stirred to ensure uniform distribution of conidia, and an aliquot of the suspension was filtered through cellulose nitrate filters (5 μm pore size; Fioroni, Ingré, France). Filters were stained with 0.05% cotton blue in 60% lactic acid and conidia were identified and counted under a microscope at 200× magnification (Leica, DM1000, Wetzlar, Germany) (Graça et al., 2005). The reproductive activity of the aquatic hyphomycetes was characterised by sporulation rate (expressed as the number of conidia mg^{-1} leaf DM d^{-1}) and species richness (expressed as the number of species $sample^{-1}$).

2.6. Microbial respiration

On each sampling date, five leaf discs from each microcosm were used to estimate microbial oxygen consumption rates as a surrogate for overall microbial metabolism. A closed six-channel dissolved oxygen measuring system (Strathkelvin 929 System, North Lanarkshire, Scotland) was used, with oxygen electrodes calibrated at 13 and 18 °C with a 0% O_2 solution (sodium sulphite in 0.01 M sodium borate) and 100% O_2 saturated stream water. Leaf discs were incubated at 13 or 18 °C in 3-mL chambers containing 100% O_2 saturated stream water. Additional chambers without leaf discs were used as controls. Oxygen consumption rates were determined as the difference between the O_2 concentration in the sample and the control over a 20 min interval, taken from the ca. 1 h of incubation, during which O_2 consumption was linear. Results were expressed as mg O_2 g^{-1} leaf DM h^{-1}.

2.7. Leaf toughness

After oxygen measurements, the same leaf discs were used to estimate leaf toughness as a surrogate for enzymatic maceration of leaf litter, applying the same penetrometer and method described above. Leaf toughness was expressed in g and the percentage of leaf toughness remaining relative to initial toughness (day 0) was estimated as (toughness remaining/initial toughness) × 100. Discs were dried for 24 h at 105 °C and weighed.

2.8. Fungal biomass

Five additional leaf discs from each microcosm were used to estimate ergosterol concentration as a surrogate for fungal biomass (Gessner and Chauvet, 1993; Graça et al., 2005). Leaf discs were promptly frozen at −20 °C, lyophilised overnight, weighed and used for extraction. Ergosterol was extracted in 10 mL KOH/

methanol (8 g L^{-1}) for 30 min at 80 °C. The extract was then purified by solid phase extraction (Waters Sep-Pak® Vac RC, 500 mg, Tc18 cartridges; Waters Corp, Milford, MA, USA) and quantified with high-performance liquid chromatography (HPLC; Dionex DX-120, Sunnyvale, CA, USA) by measuring absorbance at 282 nm. The HPLC system was equipped with the Thermo Scientific Syncronis C18 column (250 × 4 mm, 5 μm particle size; Thermo, Waltham, MA, USA). The Thermo Universal Uniguard holder 4/4.6 mm ID3 + Syncronis C18 (10 × 4 mm, 5 μm particle size) drop in guard pre-column (Thermo) was set at 33 °C. The mobile phase was 100% methanol and the flow rate was set at 1.4 mL min. Ergosterol concentration was converted into fungal biomass, assuming 5.5 μg ergosterol mg^{-1} fungal DM, and the results were expressed as mg fungal DM g^{-1} leaf DM.

2.9. Leaf mass

The remaining 10 leaf discs from each microcosm were dried for 24 h at 105 °C and weighed. Dry mass values of these discs were pooled with the values of the five discs used for microbial respiration determination and of the five discs used for fungal biomass determination to estimate total DM. Percentage of leaf mass remaining was calculated as (total DM remaining/initial DM) × 100.

2.10. Data analysis

To determine whether the physical and chemical characteristics of senescent *C. sativa* leaves were influenced by *P. cinnamomi* infection, a comparison was made of initial leaf toughness, SLA, polyphenolics, lignin, C, N and P concentrations, and C:N and C:P molar ratios among the three tree health statuses (healthy, symptomatic and highly symptomatic) by one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. The three subsamples were averaged and average values per tree (n = 5 per health status) were used in these analyses.

The fraction of DM (ln-transformed) over time was compared among treatments by analysis of covariance (ANCOVA), with 'tree health status' and 'temperature' as categorical factors and 'time' as covariate, followed by Fisher's test for multiple comparisons. Exponential decomposition rates on a per day basis (k, d^{-1}) were estimated for each treatment by linear regression of ln-transformed fraction of DM remaining over time, considering the intercept fixed at ln(1) = 0.

Leaf toughness, microbial respiration rates, fungal biomass and sporulation rates by aquatic hyphomycetes were compared among treatments by repeated-measures ANOVA (with 'tree health status' and 'temperature' as categorical factors), followed by Tukey's or Fisher's tests. Analysis of similarity (ANOSIM) was used to compare aquatic hyphomycete communities among treatments based on a Bray-Curtis dissimilarity matrix; specific sporulation rate data (log (x + 1)-transformed) were used.

Data were checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test) and transformed when necessary. Analyses were performed using Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA), except ANOSIM, which was performed using Primer 6 v6.1.11 (Primer-E Ltd, Plymouth, UK; Clarke and Gorley, 2001).

3. Results

3.1. Leaf litter characterisation

Leaves from highly symptomatic *C. sativa* trees had significantly higher polyphenolic concentration and toughness than leaves from symptomatic and healthy trees (Table 1). Carbon concentration and C:P ratio of leaves were marginally ($0.100 < p < 0.050$) higher in highly symptomatic trees, whereas P concentration and SLA were marginally higher in healthy trees (Table 1).

3.2. Leaf decomposition

Decomposition rates (k) of *C. sativa* leaves ranged from 0.0071 to 0.0112 d^{-1} (Table 2). The lowest value corresponded to leaves from the highly symptomatic trees decomposing at 13 °C, i.e., 66% mass remaining after 64 d of incubation (Fig. 2A; Table S1). Decomposition rates of leaves were influenced by tree health status (ANCOVA, $p < 0.001$), with significantly lower rates in the highly symptomatic than in the symptomatic and healthy trees (Fisher's test, $p < 0.001$) (Table 2; Table S2). Decomposition rates were not significantly influenced by the health status × temperature interaction (ANCOVA, $p = 0.288$) and by the temperature (ANCOVA, $p = 0.746$) (Table 2; Table S2).

Table 2
Exponential decomposition rates (k; mean ± SE) of leaves from healthy, symptomatic and highly symptomatic *Castanea sativa* trees ($n = 5$) in a riparian *Phytophthora cinnamomi*-infested forest incubated in microcosms at 13 and 18 °C. Regression determination coefficients are shown (R^2; $p < 0.001$ in all cases). Comparisons among tree health status categories and temperature were made using ANCOVA. Different letters indicate significant differences (Fisher's test, $p < 0.050$).

Tree health status	Temperature (°C)	k (d^{-1})	R^2
Healthy	13	0.0111 ± 0.0005 a	0.86
	18	0.0112 ± 0.0003 a	0.93
Symptomatic	13	0.0111 ± 0.0007 a	0.81
	18	0.0102 ± 0.0008 a	0.66
Highly symptomatic	13	0.0071 ± 0.0005 b	0.67
	18	0.0088 ± 0.0004 b	0.92

Table 1
Physical and chemical characteristics (mean ± SE) of senescent leaves from healthy, symptomatic and highly symptomatic *Castanea sativa* trees ($n = 5$) in a riparian *Phytophthora cinnamomi*-infested forest. Comparisons among tree health status categories were made using ANOVA; where significant p values were found (in bold), different letters indicate significant differences (Tukey's test, $p < 0.050$).

Leaf characteristic	Healthy	Symptomatic	Highly symptomatic	p
Toughness (g)	114 ± 9[a]	113 ± 10[a]	150 ± 6[b]	**0.014**
Specific leaf area (mm^2 mg^{-1})	29.64 ± 3.33	28.63 ± 2.30	21.52 ± 1.48	0.079
Polyphenolics (% DM)	9.81 ± 1.04[a]	10.00 ± 0.90[a]	15.41 ± 1.13[b]	**0.003**
Lignin (% DM)	25.05 ± 1.53	28.53 ± 0.60	29.25 ± 1.37	0.107
Carbon (% DM)	48.56 ± 0.51	49.25 ± 0.36	50.17 ± 0.34	0.052
Nitrogen (% DM)	0.61 ± 0.03	0.67 ± 0.09	0.90 ± 0.20	0.255
Phosphorus (% DM)	0.09 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.070
Carbon:nitrogen	94.81 ± 5.31	93.75 ± 12.17	87.44 ± 28.59	0.953
Carbon:phosphorus	1525 ± 250	2055 ± 320	2456 ± 228	0.087

DM, dry mass.

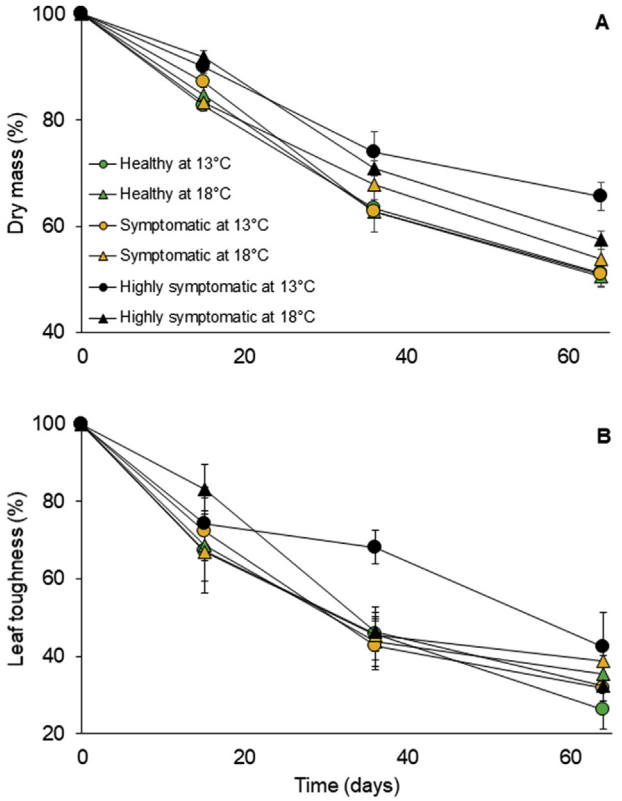

Fig. 2. (A) Dry mass and (B) leaf toughness of leaves from healthy, symptomatic and highly symptomatic *Castanea sativa* trees ($n = 5$) in a riparian *Phytophthora cinnamomi*-infested forest incubated in microcosms at 13 and 18 °C for 15, 36 and 64 d. Values are means ± SE ($n = 5$).

3.3. Leaf toughness

Leaf toughness decreased considerably during the first month (36 d; repeated-measures ANOVA, $p < 0.001$) then stabilised at 40% of initial values in most treatments (Fig. 2B; Table S1). Leaf toughness was not significantly influenced by tree health status (repeated-measures ANOVA, $p = 0.074$) and temperature (repeated-measures ANOVA, $p = 0.550$) (Table S3), although toughness loss was lower in leaves from highly symptomatic trees incubated at 13 °C (Fig. 2B).

3.4. Microbial respiration

Microbial respiration rates ranged from 0.14 to 0.55 mg O_2 g^{-1} DM h^{-1} (Fig. 3A; Table S1). Mean values were lowest in highly symptomatic trees during the first 2 weeks of incubation (15 d), irrespective of temperature, and increased in highly symptomatic trees until 36 d (Fig. 3A). Rates were relatively constant over time in the healthy and symptomatic categories (Fig. 3A). A significant tree health status × time interaction was observed (repeated-measures ANOVA, $p = 0.002$; Table S3). Microbial respiration rates were significantly influenced by tree health status (repeated-measures ANOVA, $p < 0.001$; Table S3), with lower values in leaves from highly symptomatic trees than in leaves from healthy and symptomatic trees (Tukey's test, $p < 0.01$). Microbial respiration rates were not significantly affected by temperature (repeated-measures ANOVA, $p = 0.124$) or the tree health status × temperature interaction (repeated-measures ANOVA, $p = 0.547$), although they were significantly influenced by the time × temperature interaction (repeated-measures ANOVA, $p = 0.017$) (Table S3).

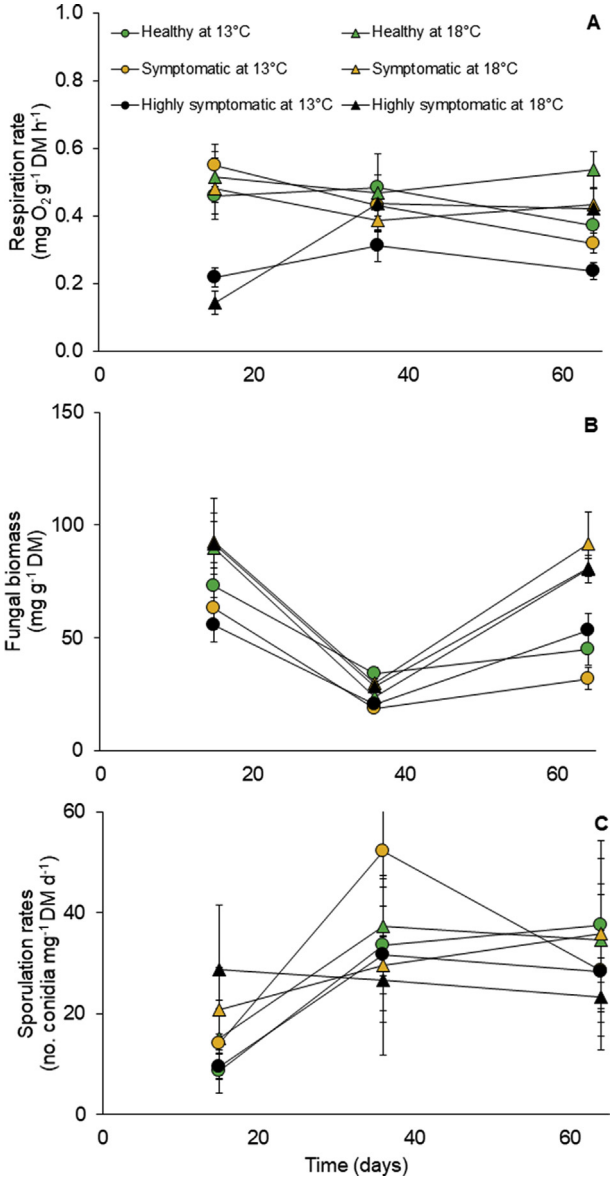

Fig. 3. (A) Microbial respiration rates, (B) fungal biomass and (C) sporulation rates by aquatic hyphomycetes on leaves from healthy, symptomatic and highly symptomatic *Castanea sativa* trees ($n = 5$) in a riparian *Phytophthora cinnamomi*-infested forest incubated in microcosms at 13 and 18 °C for 15, 36 and 64 d. Values are mean ± SE ($n = 5$).

3.5. Fungal biomass

Fungal biomass ranged from 18 to 93 mg g^{-1} DM, decreasing significantly during the 15–36 d period and increasingly significantly during the 36–64 d period (repeated-measures ANOVA, $p < 0.001$) (Fig. 3B; Table S1). Fungal biomass was not significantly influenced by tree health status (repeated-measures ANOVA, $p = 0.871$) but it was significantly influenced by temperature (repeated-measures ANOVA, $p < 0.001$) (Table S3) with higher values at 18 than at 13 °C (Fig. 3B).

3.6. Conidial production

Sporulation rates ranged from 9 to 52 conidia mg^{-1} DM d^{-1} and were significantly lower at 15 d than at 36 d and 64 d (repeated-measures ANOVA, $p = 0.002$; Tukey's test, $p = 0.002$) (Fig. 3C;

Table S1). Sporulation rates were not significantly influenced by tree health status or temperature, or their interaction (repeated-measures ANOVA, $p = 0.422$, $p = 0.664$ and $p = 0.942$, respectively; Table S3).

3.7. Aquatic hyphomycete assemblages

The cumulative number of aquatic hyphomycete species per treatment ranged from five to nine, with a total of 11 species identified across treatments (Table 3). Three species accounted for more than 80% of total conidial production: *Articulospora tetracladia* (42%), *Triscelophorus acuminatus* (25%) and *Tetrachaetum elegans* (15%) (Table 3; Fig. 4). Aquatic hyphomycete community structure was significantly influenced by tree health status (ANOSIM, global R = 0.37, $p = 0.002$). In particular, communities on leaves from healthy and symptomatic trees significantly differed from those on leaves from highly symptomatic trees (ANOSIM, global R = 0.72, $p = 0.001$ and global R = 0.39, $p = 0.001$, respectively; Fig. 4).

4. Discussion

Despite the extensive literature about the consequences of the invasive *P. cinnamomi* for terrestrial ecosystems (Cahill et al., 2008; Corcobado et al., 2014; Martín-Garcia et al., 2015; Sena et al., 2018; Domínguez-Begines et al., 2019), to the best of our knowledge this is the first study to report the effects of *P. cinnamomi* on aquatic ecosystem functioning. The results demonstrated that *P. cinnamomi* infection had a greater effect than water temperature on litter decomposition and aquatic hyphomycete community structure.

4.1. Phytophthora cinnamomi infection of sweet chestnut trees influences litter properties

The hypothesis that *P. cinnamomi* infection would change the physico-chemical characteristics of leaves was confirmed, due to higher concentrations of polyphenolic compounds and toughness in leaves from highly symptomatic trees, which may be associated with the enhanced concentration of metabolites related to plant defence (Mazid et al., 2011; Gallardo et al., 2019). In addition to

Table 3
Relative abundance (%) of aquatic hyphomycete species, based on conidial numbers, on leaves from healthy, symptomatic and highly symptomatic *Castanea sativa* trees (n = 5) in a riparian *Phytophthora cinnamomi*-infested forest incubated in microcosms at 13 and 18 °C for 15, 36 and 64 d. Species with highest relative abundance are highlighted in bold.

Aquatic hyphomycete species	Healthy 13 °C			Healthy 18 °C			Symptomatic 13 °C			Symptomatic 18 °C			Highly symptomatic 13 °C			Highly symptomatic 18 °C		
	15	36	64	15	36	64	15	36	64	15	36	64	15	36	64	15	36	64
Anguillospora crassa			0.3		0.1	0.6			0.3			0.3			0.3			0.1
Anguillospora filiformis	7.4	0.5		7.8	0.5		18.9	1.8	0.6	9.5	1.7	0.2	18.7	14.8	0.9	11.4	7.3	0.1
Articulospora tetracladia	**37.4**	**54.5**	**58.0**	**39.3**	**46.7**	**20.9**	**21.5**	**52.0**	**63.0**	**28.7**	**39.3**	**34.1**	**15.3**	**54.8**	**65.9**	**13.7**	**54.8**	**64.7**
Lemonniera cornuta		0.3		0.5	0.1		0.2			0.3			0.1			0.7		
Lemonniera terrestris	19.8	5.3	0.1	15.6	3.6	1.0	10.1	1.0		11.4	0.8		10.5	1.7	1.9	5.3	0.6	0.1
Lunulospora curvula	11.0	0.7	0.2	10.6	1.7	0.5	14.8	1.3	0.4	12.9	2.8		7.0	2.9	1.9	14.1	3.0	1.3
Tetrachaetum elegans	**24.4**	**3.2**	**0.5**	**24.7**	**0.5**	**0.6**	**34.4**	**1.6**	**0.2**	**37.2**	**2.5**	**1.0**	**46.5**	**25.7**	**6.9**	**54.9**	**12.6**	**1.4**
Tetracladium marchalianum									2.9			0.9						
Tricladium chaetocladium													0.2					
Triscelophorus acuminatus		**35.6**	**40.9**	**1.6**	**46.7**	**63.0**		**42.4**	**32.6**		**52.9**	**63.6**	**1.6**	**0.2**	**22.1**		**21.7**	**32.3**
Unidentified species						13.4							0.1					
Species richness (no. species treatment^{-1})	5	7	6	7	8	7	6	6	7	6	6	6	9	6	7	6	6	7

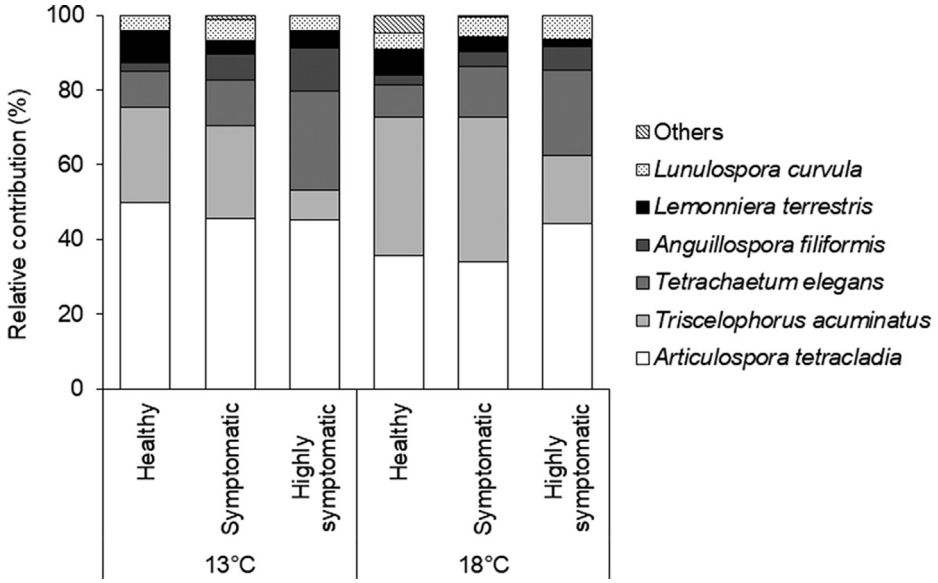

Fig. 4. Relative contribution (%) of aquatic hyphomycete species on leaves from healthy, symptomatic and highly symptomatic *Castanea sativa* trees ($n = 5$) in a riparian *Phytophthora cinnamomi*-infested forest incubated in microcosms at 13 and 18 °C. Others: species contributing less than 1% to total conidial production.

deterring herbivores (Solla et al., 2016), polyphenolic compounds are widely recognised for their antimicrobial activity (Constabel et al., 2014). Through the detection of specific proteins released by the pathogen, the infected tree increases the production of polyphenolic compounds that prevent spore germination, fungal infection and fungal colonisation of the vascular system of plants (Osswald et al., 2014). Lignin accumulation is a physical barrier that increases leaf toughness, preventing herbivory and the entry of pathogens into host cells (Canhoto and Graça, 1999; Moretti et al., 2007; Bieras and Sajo, 2009). The highest lignin concentration values were observed in leaves from highly symptomatic trees, and may be associated with the highest leaf toughness values observed in the same health status category (Gessner, 2005; Graça and Zimmer, 2005).

In agreement with previous literature (Weste and Chaudhri, 1982; Cahill et al., 1986; Maurel et al., 2001), P concentration was lower in leaves from highly symptomatic trees. Because *P. cinnamomi* induces root rot, collar girdling and vascular degradation in infected plants (O'Gara et al., 2015; Redondo et al., 2015; Ruiz-Gómez et al., 2015; Jung et al., 2018), it is expected that leaf nutritional composition will be affected. Moreover, P is a key element for root development and metabolic processes of plants (Richardson et al., 2011; Wu et al., 2013), and infected trees may have invested and consumed more P than healthy trees to counteract the effect of the pathogen, e.g., by producing new fine roots. The concentration of N in leaves has been reported to be higher (Fleischmann et al., 2010; Milanović et al., 2015) or lower (Maurel et al., 2001) in *Phytophthora*-infected than in healthy trees; the reason for this discrepancy is unknown. In our study, average values of N leaf concentration increased with disease incidence, but not significantly (Table 1).

Regardless of the causes of the different mineral concentration of infected plants, we infer that heavy infection of *C. sativa* trees by *P. cinnamomi* resulted in less nutritious litter due to the higher polyphenolic concentration and leaf toughness. This situation persisted throughout the experiment.

4.2. Tree health status has a greater effect than temperature on microbial activity and litter decomposition

Leaf litter decomposition was hampered in highly symptomatic trees, thus confirming the first hypothesis that *C. sativa* leaf litter from infected trees would decompose slower than litter from healthy trees. Slower leaf litter decomposition for highly symptomatic trees likely resulted from increased leaf litter recalcitrance as discussed above.

Leroy et al. (2011) and Grimmet et al. (2012) also showed that leaves from *Acer* species infected by a fungal endophyte (*Rhytisma punctatum* and *Rhytisma acerinum*, respectively) had significantly lower decomposition rates than uninfected leaves. However, in their case, N and P concentrations were higher in infected leaves, likely due to the accumulation of fungal endophyte biomass, and the authors attributed the lower litter decomposition rates to lower rates of decomposition of the fungal tissues themselves or to endophyte–hyphomycete interactions.

Increased temperature did not significantly stimulate microbial respiration and litter decomposition and, therefore, the second hypothesis was not confirmed. Some studies have shown higher metabolic activity of decomposers and litter decomposition at high rather than at low temperatures (Ferreira et al., 2015; Martins et al., 2017; Shah et al., 2017; Manning et al., 2018). Differences between the present results and previous literature may be explained by the different litter used. Temperature effects on decomposition processes are often modulated by the nutritional quality of litter (Fernandes et al., 2014; Ferreira et al., 2015).

The development of microbial decomposers is related to nutrient availability, e.g., N and P supply stimulate microbial growth and reproduction (Suberkropp, 1998; Griffths et al., 2012). In contrast, elevated concentrations of secondary or structural metabolites reduce litter palatability for consumers, either by toxicity effects of some compounds or by increased toughness, which in turn suppresses the development of microorganisms (Solla et al., 2016). Thus, it is presumed here that the temperature effect may have been hindered by the reduced nutritional quality of the litter used, as reported by several authors (Ferreira and Chauvet, 2011a; Gonçalves et al., 2013; Fernandes et al., 2014; Ferreira et al., 2015). Our results are in agreement with many studies reporting faster litter decomposition in high quality litter (Ferreira et al., 2012, 2015; Frainer et al., 2015; Lima-Fernandes et al., 2015).

Even though aquatic fungi generally represent the highest proportion of microbial decomposers in streams (Gessner et al., 2007; Krauss et al., 2011), microbial respiration rates reflect the biological activity of the entire microbial community (Graça et al., 2005), which includes bacteria (Graça and Abelho, 2005). Bacteria are more sensitive than fungi to the nutritional restrictions of litter, given their low enzymatic efficiency (Gulis and Suberkropp, 2003; Romaní et al., 2006), which may have contributed to the strong effect of health status on litter decomposition rates.

4.3. Tree infection by P. cinnamomi influenced aquatic hyphomycete communities on leaf litter

Fungal biomass was stimulated by temperature rise, as expected according to the literature (Dang et al., 2009; Ferreira and Chauvet, 2011a). However, sporulation rates were not higher at 18 than at 13 °C. This result is in agreement with other studies (Rajashekhar and Kaveriappa, 2000; Gonçalves et al., 2013) reporting that aquatic hyphomycete communities became acclimatised to the environmental conditions in which they were collected and thus did not respond to increased temperature during laboratory incubation. Again, the lack of influence of temperature on the reproductive output of fungi may be related to the effects of *P. cinnamomi* infection on the physico-chemical characteristics of leaf litter.

Throughout the experiment, and especially during the final stage, the lowest sporulation rates occurred on leaves from highly symptomatic trees. Considering that conidial production by aquatic hyphomycetes is limited by low nutrient concentration and high concentration of secondary compounds (Gulis and Suberkropp, 2003, 2004), we can assume that fungal reproductive activity is sensitive to changes in leaf nutritional quality in terms of P, polyphenolics and toughness. Phosphorus is a microbial growth-limiting nutrient (Rosemond et al., 2002) necessary for the nucleic acid synthesis involved in mycelium and conidial production (Ferreira and Chauvet, 2011a). In addition, polyphenols act as fungicides (Martín et al., 2008), and leaf toughness negatively affects hypha establishment on the leaf surface (Canhoto and Graça, 1999; Gessner, 2005). Thus, although mycelium accrual was observed, conidial production by aquatic hyphomycetes was probably limited by the lower nutritional quality of the leaves from highly symptomatic trees. These results are in agreement with previous studies indicating that litter quality is more important than temperature for the reproduction and activity of aquatic fungi (Fernandes et al., 2012; Gonçalves et al., 2013).

It was of particular interest that aquatic hyphomycete community structure was significantly influenced by *P. cinnamomi* infection. This may be due to the specific nutritional requirements of the different species involved (Bisht, 2013; Brosed et al., 2017), their different enzymatic performance (Bisht, 2013) and their ecological stoichiometry in the different substrates (Danger et al., 2016; Brosed et al., 2017). In the presence of *P. cinnamomi* tree infection,

the aquatic hyphomycete community structure was altered by a decrease in *T. acuminatus* and an increase in *T. elegans*. Several authors have suggested that fungi show specificity for certain substrates, which may help explain differences in community structure and decomposition rates among different litter substrates (Charcosset and Gardes, 1999; Gulis, 2001; Ferreira et al., 2006, 2017).

Changes in the composition or structure of aquatic hyphomycete communities may also indirectly affect decomposition rates and in turn alter the ecosystem functioning of headwater streams (Jabiol et al., 2013; Bärlocher and Sridhar, 2014). For instance, some invertebrates are able to differentiate among aquatic hyphomycete species and feed preferentially on certain species (Arsuffi and Suberkropp, 1984, 1985; Lecerf et al., 2005), suggesting that changes in the aquatic hyphomycete identity and dominance in litter may affect litter decomposition rates.

4.4. Ecological consequences of *P. cinnamomi* infection and temperature changes

The results show that microbial activity and leaf litter decomposition rates in microcosms are affected more by *P. cinnamomi* infection of trees than by temperature. This suggests that this invasive oomycete may change leaf litter characteristics and induce cross-ecosystem effects. Changes in leaf physico-chemical characteristics due to tree infection by *P. cinnamomi* may lead to input of recalcitrant litter into aquatic ecosystems. The reduction in decomposition rates evidenced by the results may affect nutrient cycling and increase the storage of low quality litter in the streambed, which in turn will presumably affect the occurrence, activity and survival of aquatic organisms.

The impact of tree infection by *P. cinnamomi* on other features of aquatic ecosystems such as water quality, food webs, nutrient cycling, dynamics and nutrient transfer between terrestrial and aquatic ecosystems deserves further study. Other effects may include changes in the amount and timing of litter inputs, as infected trees may produce less and smaller leaves, which are shed earlier (Pires et al., 1998). Infected trees may eventually die leaving an open space which may be occupied by other tree species, including opportunistic invaders (Bjelke et al., 2016). The consequences of all these changes occurring in aquatic systems should be addressed to better characterise the cross-ecosystem effects of tree infection.

Predicting the impact of temperature changes on species and communities is a central challenge in forestry and ecology. Increased air temperature could affect riparian tree species by giving native and invasive pathogens additional roles of unpredicted consequences. This study addresses, for the first time, interactions between an invasive pathogen and temperature with regard to parameters of leaf litter decomposition. The third hypothesis of this work is rejected, because none of the parameters assessed was influenced by the tree health status × temperature interaction. This suggests that temperature rise may not synergistically increase the cross-ecosystem effects of *P. cinnamomi* in streams where litter decomposition is microbial-driven.

To better characterise the impact of invasive pathogenic species on stream ecosystem functioning, additional tree-pathogen systems should be assessed. Further collaboration between forest pathologists and stream ecologists is, therefore, recommended. This work and ongoing research on the impact of other pandemics (e.g., *Phytophthora alni* and Dutch elm disease; unpublished results) on riparian ecosystem functioning are first steps in this direction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2019.07.005.

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