Effects of zinc on leaf decomposition by fungi in streams: studies in microcosms

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Abstract

The effect of zinc on leaf decomposition by aquatic fungi was studied in microcosms. Alder leaf disks were precolonized for 15 days at the source of the Este River, and exposed to different zinc concentrations during 25 days. Leaf mass loss, fungal biomass (based on ergosterol concentration), fungal production (rates of [1-^{14}C]acetate incorporation into ergosterol), sporulation rates and species richness of aquatic hyphomycetes were determined. At the source of the Este River decomposition of alder leaves was fast and 50% of the initial mass was lost in 25 days. A total of 18 aquatic hyphomycete species were recorded during 42 days of leaf immersion. *Articulospora tetracladia* was the dominant species, followed by *Lunulospora curvula* and two unidentified species with sigmoid conidia. Cluster analysis suggested that zinc concentration and exposure time affected the structure of aquatic hyphomycete assemblages, even though richness had not been severely affected. Both zinc concentration and exposure time significantly affected leaf mass loss, fungal production and sporulation, but not fungal biomass. Zinc exposure reduced leaf mass loss, inhibited fungal production and affected fungal reproduction by either stimulating or inhibiting sporulation rates. The results of this work suggested zinc pollution might depress leaf decomposition in streams due to changes in the structure and activity of aquatic fungi.

Introduction

Leaf decomposition is essential to the functioning of stream ecosystems [31, 32] and includes leaching, microbial degradation and physical and biotic fragmentation [19, 50]. The predominant microorganisms associated with leaf decomposition are fungi, particularly aquatic hyphomycetes [45] which are an ecological group of fungi adapted to clean and well-aerated flowing waters [5]. Aquatic hyphomycetes degrade leaf litter and transform it into a more suitable resource for invertebrate detritivores [45].

Contamination by heavy metals in aquatic environments arises from industrial processes (e.g. mining, smelting, finishing and plating of metals and dye manufacture) and from pipes and tanks in domestic systems [38]. Some heavy metals, such as zinc, are involved in growth, metabolism and differentiation of organisms, but others have no apparent biological function. Most heavy metals are toxic above a concentration which varies with the organisms, the physico-chemical properties of the metal and environmental factors [15].

Heavy metals affect fungal communities by reducing species diversity and selecting for resistant/tolerant species [15]. The ubiquitous and sometimes dominant presence of aquatic fungi in metal polluted streams [41] have increased interest on the effect of heavy metals on fungi during decomposition of organic matter. If the fungal community colonizing leaves is perturbed then the rate of decomposition and utilization by detritivores may be affected, leading to alterations in nutrient cycling, energy flow, and hence to changes in food web structure [28].

Miersch *et al.* [29, 30] found that cadmium, copper and zinc inhibit growth of aquatic hyphomycetes and others reported that cadmium [1] and aluminium [11] inhibit both growth and reproduction of these fungi. In addition, conidium production by aquatic hyphomycetes was found to be more sensitive to heavy metals than growth [1, 7]. In heavy metal polluted streams, a decrease in the richness of aquatic hyphomycete species [8, 34] and a reduction in conidium production and fungal biomass [41] were found. However, fungal biomass and microbial respiration associated with decomposing leaves was reported to be high at sites affected by mine drainage [34]. Leaf decomposition rates usually are low in waters affected by mine drainage [8, 33].

Microcosm systems have played an important role in furthering the knowledge of ecological processes in stream ecosystems and can be particularly useful when studying the impact of harmful compounds, such as heavy metals, since *in situ* additions are rarely

possible due to their toxicity and accumulation in the biota. A critical aspect of a microcosm is its ability to accurately portray fate and effects of contaminants [13].

In this work, microcosm experiments were used to assess the effects of zinc on leaf decomposition by aquatic fungi. In parallel, leaf decomposition was followed in the stream to validate microcosm results. Stream experiments were carried out at the source of the Este River (Northwest Portugal), where alder leaf disks were immersed for 42 days. The Este River flows through the city of Braga and its Industrial Park where heavy metals, including zinc, become a problem in both stream water [21] and sediments [39]. For microcosm experiments, leaves colonized for 15 days in the stream were collected and subsequently exposed for 25 days to zinc concentrations of 0.5, 15 and 150 µM, which are within the range occurring in either pristine or impacted streams [e.g. 33, 34]. To evaluate zinc effects on the natural fungal assemblages, leaf mass loss, fungal biomass and production, sporulation rates and diversity of aquatic hyphomycetes were determined.

Methods

Study site and stream water

The Este River is located in the Northwest of Portugal in an agricultural area with high demographic density, and flows through the city of Braga and its Industrial Park, where food, textile and metalworking industries are located. Previous analytical studies in the stream water [21] and sediments [39] reported trace levels of heavy metals at the source of the Este River and pollution by zinc and other heavy metals downstream, particularly at the Industrial Park of Braga. The stream experiments were conducted at the source of the Este River, where the stream is about 30 cm deep and 50 cm wide, the bottom consists of granite rock, pebbles and gravel. The riparian vegetation consists of *Eucalyptus globulus* Labill., *Pinus pinaster* Aiton, *Pteridium aquilinum* Khun and *Juncus* sp.

Temperature, pH and conductivity of the stream water were measured *in situ* with field probes (Multiline F/set 3 nº 400327, WTW). On 9 April 2002, stream water was collected into sterile glass bottles, transported in a cold box (4 °C) and used within 24 h for chemical and microbial analyses. Chemical oxygen demand, concentration of nitrate and orthophosphate, and microbial population densities in the stream water were determined according to standard methods [2]. Magnesium and calcium concentrations were determined using a kit (4824 DR-LT, LaMotte). Data from physical, chemical and microbiological parameters (Table 1) indicated moderately acidic softwater with elevated nutrient concentrations and low densities of culturable microorganisms in the flowing water. On 9 April 2002, additional stream water was collected, filtered (5 µm pore size) and sterilized (120 ºC, 20 min) for further assays.

Stream experiment

The leaves of *Alnus glutinosa* (L.) Gaertner were collected from trees in September 2001 immediately before abscission and dried at room temperature. The leaves were pre-leached in deionized water for 48 hours, cut into 12 mm-diameter disks and sets of 80 disks were placed into 0.5 mm mesh bags (16 x 20 cm). On 11 April 2002, 60 leaf bags were immersed at the source of the Este River and after 30 minutes of leaf immersion 4 leaf bags were collected to determine the initial mass of leaf disks. On each sampling date (12, 15, 28, 34, 40 and 42 days) 4 randomly chosen bags were retrieved, except after 15 days of leaf immersion when additional 32 leaf bags were collected for microcosm experiments. In the laboratory, the disks from each replicate were rinsed with deionized

water to remove sediments and any adhering invertebrates. A subset of 15 leaf disks was placed into 100 ml Erlenmeyer flasks with 40 ml of sterile stream water under aeration (2 days, 18 ºC) to induce sporulation. A second subset of 8 leaf disks was stored in 5 ml of 0.8% KOH-methanol (-20 ºC) for subsequent ergosterol analysis. A third subset of 57 leaf disks was used to determine leaf mass loss.

Zinc experiments in microcosms

The content of each of 32 bags retrieved after 15 days of stream immersion were placed in 250 ml Erlenmeyer flasks with 100 ml of sterile stream water. A total of 24 flasks were supplemented with $ZnCl_2$ at pH 6.0 ± 0.2 to give final concentrations of 0.5, 15 and 150 µM. The remaining 8 flasks served as controls. All flasks were continuously aerated with aquarium pumps during the experiment at 18 ºC. Every 2 days (but consecutively on days 4 and 5), all leaf disks of each replicate were transferred to another Erlenmeyer flask containing fresh solution with the same zinc concentration. After 2, 13, 19 and 25 days of zinc exposure, conidium suspensions from 4 replicates of each treatment were filtered to estimate sporulation rates. Four replicates of each treatment were sacrificed after 13 and 25 days of zinc exposure and leaf disks from each replicate were used as follows: 8 leaf disks were stored for ergosterol measurements as indicated above, 12 leaf disks were used to estimate fungal production and 60 leaf disks were used to estimate leaf mass loss.

Sporulation rates

Suspensions of conidia released from leaf disks during 2 days were mixed with 50 µl of 0.5% Tween 80 and filtered (5 µm pore size, Millipore); the retained conidia were stained with 0.1% cotton blue in lactic acid. At least 300 conidia were identified and counted to determine the relative proportion of each species. Sporulation rates were expressed as conidia mg^{-1} detrital dry mass d^{-1}.

Fungal biomass

Concentration of ergosterol was measured to estimate fungal biomass associated with decomposing leaf disks. Lipids were extracted from leaf disks by heating in 0.8 % KOH-methanol (30 min, 80 ºC) and the extract was purified by solid-phase extraction, according to Gessner and Schmitt [20]. Ergosterol was quantified by high performance liquid chromatography (HPLC) using a Lichrospher RP18 column (250 x 4 mm, Merck)

connected to a Beckmann Gold liquid chromatographic system. The system was run isocratically with HPLC-grade methanol at 1.4 ml min^{-1} and 33 ºC. The peaks of ergosterol were detected at 282 nm and standard series of ergosterol (Sigma) in isopropanol were used to estimate the ergosterol concentration in the samples. Ergosterol concentration was converted to fungal biomass assuming an ergosterol concentration of 5.5 µg mg^{-1} mycelial dry mass [16].

Fungal production

Fungal production on decomposing leaf disks was estimated by measuring rates of [1-^{14}C]acetate incorporation into ergosterol [18, 48], using an empirical conversion factor of 19.3 µg fungal biomass $nmol^{-1}$ of acetate incorporated [48]. Sets of 6 leaf disks were placed in 25 ml Erlenmeyer flasks containing 4 ml of sterile stream water. In one set of leaf disks per replicate microcosms, 200 µl of formaldehyde (2% final concentration) was added 30 min before the addition of radiolabeled acetate to determine background level of radioactivity. The reaction was started by the addition of sodium [1-^{14}C]acetate (Amersham) to a final concentration of 5 mM (specific activity, 48 MBq $mmol^{-1}$). After 2 hours of incubation at 18 ºC on a shaker (100 rpm, 25 mm path, Certomat HK, B. Braun Biotech International), the uptake of radiolabeled acetate was stopped by adding formaldehyde (2% final concentration). Leaf disks were filtered (glass microfibre filters, GF/C Whatman), washed twice with 4 ml of cold deionized water, placed in 5 ml of 0.8 % KOH-methanol and stored at -20 ºC until ergosterol extraction as indicated above. The eluting ergosterol fractions from HPLC (2 injections per replicate) were collected into a vial containing 10 ml of scintillation fluid (Optiphase Hisafe 2, Perkin-Elmer), stored overnight and the radioactivity was counted in a Packard Tri-Carb 2200 CA.

Under these experimental conditions the isotope dilution was negligible, incorporation of acetate was linear for at least 4 h and saturation of acetate had been achieved (data not shown).

Leaf mass loss

Leaf disks of each replicate collected on each sampling date were dried at 50 ºC to a constant mass (72 h ± 24 h) and weighed to the nearest 0.001 g.

Statistical analyses

Rate of leaf decomposition (k) was estimated according to the exponential model [37] after ln transformation as follows: $\ln (M_t/M_0) = -kt + b$, where M_t is the leaf detrital dry mass remaining at time t, M_0 is the initial dry mass, t is the time in days and b is the Y intercept.

To achieve normality for analysis of variance (ANOVA) arcsine square root transformation was applied to data in percentage and ln transformation was applied to all other data. Two-way analysis of variance (Two-way ANOVA) with zinc concentration and exposure time as factors was used to test for differences in leaf mass loss, fungal biomass, richness and sporulation rates [51]. Two-way ANOVA was also used to test for differences between stream and microcosm controls during the time of experiment. To determine where differences occurred the multiple comparison Tukey's test was used [51]. All the analyses were performed with $P<0.05$ as the criterion of significance. For univariate statistical analysis the program Prism 4.0 for Macintosh (GraphPad software Inc., San Diego) was used. Nontransformed data were used for graphic presentation of the means and standard errors of the mean (SEM).

Similarities in aquatic hyphomycete assemblages among treatments were quantified by Bray Curtis index [9] after ln (x+1) transformation. The resulting symmetric matrices were subjected to cluster analysis by Unweighed Pairgroup Method Average (UPGMA) [25], using the statistical package ADE-4 for Macintosh [49].

Results

Leaf mass loss, fungal biomass and sporulation in the stream

The decomposition rate of alder leaf disks at the source of the Este River was 0.074 ± 0.005 d^{-1} (k ± standard error; intercept=121%, r^2=0.89). Leaf mass loss was highest between 15 and 34 days of leaf immersion (Fig.1), this corresponds to the time where maximum values for fungal biomass (ranging from 124 to 141 mg g^{-1} leaf detrital dry mass) were reached. Sporulation rates of aquatic hyphomycetes reached a peak (2900 conidia mg^{-1} leaf detrital dry mass d^{-1}) after 15 days of leaf immersion and declined thereafter (Fig.1). A total of 18 aquatic hyphomycete species were found over the entire stream study (Table 2), with a maximum of 16 species after 28 days of leaf immersion. *Articulospora tetracladia* was the dominant species making up more than 50% of the total number of conidia released till 34 days of leaf decomposition. Other major species were *Lunulospora curvula* and two unidentified species with sigmoid conidia (Sigmoid 1 and Sigmoid 2). *Tricladium chaetocladium* and *Tricladium splendens* increased their relative contribution to conidium production in the later stages of leaf decomposition.

Effects of zinc on leaf decomposition by aquatic fungi in microcosms

Alder leaf disks colonized for 15 days at the source of the Este River had lost about 25% of the initial detrital dry mass (Fig. 1) when they were exposed for 25 days to different zinc concentrations. Leaf mass loss was significantly affected by both concentration of zinc and exposure time (Two-way ANOVA, P=0.0002 and P<0.0001, respectively; Fig. 2A). After 13 days in microcosms, leaf mass loss was significantly lower than in the controls only at the highest zinc concentration (Tukey, P<0.05), whereas at 25 days leaf mass loss was significantly lower at all tested concentrations (Tukey, P<0.01 for all comparisons). Similar leaf mass loss was observed in both control microcosms and stream experiment during the study period (Two-way ANOVA, P=0.58).

Fungal biomass associated with alder leaf disks, estimated from ergosterol concentration, was 124 mg g^{-1} leaf detrital dry mass after 15 days of stream immersion but before zinc exposure (Fig. 1). Fungal biomass was not significantly affected by subsequent exposure to zinc for up to 25 days (Two-way ANOVA, P=0.69 and P=0.26, respectively; Fig. 2B). In control microcosms fungal biomass on leaves was similar (13 days) or higher (25 days) than that in the stream at the corresponding time of the experiment (Two-way ANOVA, Tukey, P>0.05 and P<0.05, respectively).

Rates of fungal production, assessed as rates of radiolabeled acetate incorporation into ergosterol, were significantly affected by both zinc concentration and exposure time (Two-way ANOVA, $P=0.02$ and $P<0.0001$; Fig. 2C). Lower fungal production was found after 13 days of fungal exposure at the highest zinc concentration in comparison with either the control or the other treatments, but these differences were not significant (Tukey, $P\geq0.5$). However, significantly lower fungal production was found after 25 days in all zinc treatments (Tukey, $P< 0.01$ for all comparisons).

Sporulation rates of aquatic hyphomycetes from alder leaf disks were significantly affected by zinc concentration, exposure time to zinc and time-zinc concentration interaction (Two-way ANOVA, $P<0.0001$ for all factors; Fig. 2D). After 2 days of zinc exposure no significant differences in sporulation rates of aquatic hyphomycetes were observed between the control and all the treatments (Tukey, $P>0.5$). At other sampling dates, 150 µM of zinc significantly decreased the sporulation rates in comparison with those from either the control or lower zinc concentrations (Tukey, $P<0.001$ for all comparisons). After 25 days of zinc exposure, the sporulation rate of aquatic hyphomycetes exposed to 15 µM was significantly greater than those from either the control (Tukey, $P=0.02$) or all the other treatments (Tukey, $P<0.01$ for all comparisons).

A total of 17 aquatic hyphomycete species were observed during the microcosm experiment (Table 2). The comparison of the aquatic hyphomycete assemblage on leaves in the stream with that developing in the microcosm control revealed that the relative contribution of *A. tetracladia* to the total released conidia decreased (Two-way ANOVA, Tukey $P<0.001$ for all times), while that of Sigmoid 1 increased (Two-way ANOVA, Tukey, $P<0.01$ for all times). The presence of zinc depressed the relative contribution to total conidium production by *L. curvula* for either the highest concentration at the earliest time or all concentrations at later times (Two-way ANOVA, Tukey, $P<0.05$). The highest zinc concentration (150 µM) led to a substantial decrease in the relative abundance of Sigmoid 1 (Two-way ANOVA, Tukey, $P<0.01$ for all times) and a strong increase in the relative importance of Sigmoid 2 (Two-way ANOVA, Tukey, $P<0.01$ for all times), which accounted for more than 70% of the total conidium output. The richness in aquatic hyphomycetes was not significantly affected by zinc, except after 13 days of exposure to 150 µM (Two-way ANOVA, Tukey, $P<0.05$).

The similarity in the structure of aquatic hyphomycete assemblages among treatments, classified by UPGMA cluster analysis, showed two major hierarchical

divisions (Fig. 3). Treatments with 150 µM of zinc at later exposure times (days 13 and 25) had similar aquatic hyphomycete assemblages, that differed from those of the other treatments. Among the latter, controls and the lowest zinc concentration at earlier exposure times (days 2 and 13) diverge from the remaining treatments, namely the lowest zinc concentration at the latest time, intermediate zinc concentration at all times and the highest zinc concentration at the earliest time. Overall, the dendogram suggested that both zinc concentration and exposure time affected aquatic hyphomycete assemblages on decomposing alder leaves.

Discussion

Decomposition and fungal colonization of alder leaves in the Este River

Alnus glutinosa is reported as having fast decomposing leaves [37], with decomposition rates for this species ranging from 0.011 d^{-1} [36] to 0.064 d^{-1} [4]. At the source of the Este River the rate of alder leaf decomposition was very high ($k=0.074\ d^{-1}$) and 50% of the initial mass of the leaves was lost in 25 days. Several factors could have contributed to the rapid decomposition of alder leaves in the current study. Firstly, elevated concentrations of nutrients, such as nitrate and phosphate, could have stimulated leaf decomposition as observed in other studies [23, 35, 46] by increasing microbial activity [23, 40, 46]. Secondly, the temperature in the stream water may also have been partly responsible for the high fungal activity and the rapid leaf mass loss since this study was conducted in Spring, when temperatures between 17 and 20 ºC are commonly found in streams of this region (C. Pascoal, personal observation). The stream water temperature reported in this work was not particularly high (Table 1) probably because it was measured at the beginning of the study. Thirdly, the use of pre-leached leaf disks might have accelerated the removal of soluble compounds, such as phenolics that inhibit fungal colonization [6]. And finally, the low frequency of sampling at earlier times of the stream experiment may have also contributed to overestimate leaf decomposition rate, as suggested by the high Y intercept value of the linear regression.

Fungal biomass (based on ergosterol concentration) and sporulation rates of aquatic hyphomycetes associated with alder leaves immersed in the Este River increased rapidly to maximum values and then declined. In contrast to conidium production, fungal biomass levelled off during the period of greatest leaf mass loss. The pattern of changes in fungal sporulation and biomass during leaf decomposition was similar to that found by other authors [17, 27, 43]. In the present work, maximum values for both conidium production (2900 conidia mg^{-1} leaf detrital dry mass d^{-1}) and fungal biomass (141 mg g^{-1} leaf detrital dry mass) were within the upper range of values reported for decomposing alder leaves in other streams (conidia [6, 12, 17, 41] and biomass [4, 14, 22]).

During alder leaf immersion in the Este River, a total of 18 aquatic hyphomycetes species were recorded and the species richness was comparable with that found in both Portuguese [6, 36] and Spanish [12] streams. Changes in the aquatic hyphomycete assemblage were mainly due to shifts in the relative frequencies of a few species that appeared early and persisted during leaf decomposition, which agrees with an earlier

suggestion by Bärlocher [5]. *A. tetracladia* was the dominant species during the whole study, followed by *L. curvula* and two unidentified species with sigmoid conidia. *T. chaetocladium* and *T. splendens* were present at all sampling dates, but their relative contributions to conidium production were higher at later times of leaf decomposition.

Effects of zinc on leaf decomposition by aquatic fungi in microcosms

In control microcosms, fungal biomass (from ergosterol concentration) associated with decomposing leaves equalled or exceeded that in the stream, but was similar to the maximum reported in the literature [47]. Differences in temperature and oxygen supply regimes might have contributed to the differences found between fungal biomass in the stream and microcosms, since these abiotic factors are known to affect the growth of fungi [26]. Fungal biomass was not significantly affected by zinc, even at the highest concentration (Fig. 4). The elevated fungal biomass associated with leaves before zinc exposure could have contributed to this finding. Because ergosterol is considered to be a good indicator of fungal biomass [47], it is unlikely that zinc affected mycelium viability. Some aquatic hyphomycete species are relatively tolerant to heavy metals [1, 24, 29] and concentrations of zinc up to 400 µM did not affect the radial growth in 9 out of 10 aquatic hyphomycete isolates [29]. In addition, fungal biomass on decomposing leaves was found to be high at sites with elevated concentration of zinc [34], but was reported to be low in streams polluted by heavy metals [41].

The estimates of fungal production based on rates of radiolabeled acetate incorporation into ergosterol can be used as a measure of fungal activity in leaf decomposition studies [48]. In this work, the maximum fungal production on alder leaves was similar to or higher than reported from studies in both streams [3, 44] and microcosms [18]. The presence of zinc at all tested concentrations significantly inhibited fungal production after 25 days of exposure. This fact could account for the decreased leaf mass loss caused by zinc, particularly at later exposure time. The significant inhibition of fungal production by zinc was not accompanied by an equal effect on fungal biomass. Fungal biomass on leaves is the net result of several processes of gains and losses, thus it is conceivable that, even when fungal production is diminished, a considerable portion of fungal biomass might remain on leaves if mycelium fragmentation and/or sporulation are reduced.

Conidium production is considered an indirect measure of aquatic hyphomycete decomposing activity [27], since strong correlations between leaf decomposition rates and peaks of ergosterol and sporulation have been found [17]. It has been recognised that fungal reproduction is more sensitive than mycelial growth to heavy metals [1, 7] and conidium production was severely restricted in heavy metal polluted streams [41]. In the present study, the exposure of fungi colonising leaves to the highest zinc concentration led to a significant decrease in conidium production, while the lowest zinc concentration did not show any significant effect. The synthesis of sulphur-rich compounds by aquatic hyphomycetes [29, 30] is a common fungal response to heavy metal exposure [15] and may constrain the energy available for reproduction, which could explain the decreased conidium production at high zinc concentration. Moreover, since zinc is an essential metal ion, it is possible that zinc may also stimulate conidium production. In this work, 15 µM of zinc led to an increased conidium production, which was not accompanied by an increase in fungal biomass and production. On the contrary, in that treatment fungal production and leaf mass loss were significantly reduced at the end of the experiment.

Several studies have shown that the diversity of aquatic hyphomycetes colonizing leaves is restricted in streams receiving mine effluents [8, 34]. However, a total of 17 and 24 species were recorded associated with exposed leaves, natural plant detritus and foams in two extremely heavy metal polluted streams [42]. In the current study, both zinc concentration and exposure time affected the structure of aquatic hyphomycete assemblages, as shown by cluster analysis. Changes in the relative abundance of dominant species were noticed (Table 2). The relative abundance of *L. curvula* decreased in either the highest zinc concentration at earlier exposure times or lower concentrations at later times; and the relative abundance of Sigmoid 1 substantially decreased, while Sigmoid 2 became dominant at the highest zinc concentration. Nevertheless, the overall species richness of aquatic hyphomycetes was not severely affected by zinc, since effects were only found after13 days of exposure to the highest concentration.

It has been demonstrated that downstream of a mine effluent discharge the rate of leaf decomposition was reduced [8]. This was accompanied by decreased microbial metabolic activity and a decline in the richness of aquatic hyphomycete species colonizing leaves [8]. In the present work, leaf mass loss was affected by both zinc and exposure time. The highest zinc concentration led to a significant decrease in leaf mass loss during the whole treatment, whereas lower zinc concentrations only had a significant effect at later

exposure time. The reduction in leaf mass loss was probably due to the negative effects of zinc on the structure and activity of aquatic fungal assemblages associated with decomposing leaves. Microcosms seemed appropriate to study the impact of zinc on leaf decomposition by aquatic fungi, since leaf mass loss was similar in both the microcosm control and the stream. However, microcosms are simplified representations of real ecosystems and extrapolating these observations to the broader environment should be done with caution.

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References

1. Abel TH, Bärlocher F (1984) Effects of cadmium on aquatic hyphomycetes. Appl Environ Microbiol 48:245-251
2. APHA (1998) Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, DC
3. Baldy V, Chauvet E, Charcosset J-Y, Gessner MO (2002) Microbial dynamics associated with leaves decomposing in the mainstem and floodplain pond of a large river. Aquat Microb Ecol 28:25-36
4. Baldy V, Gessner MO (1997) Towards a budget of leaf litter decomposition in a first-order woodland stream. C R Acad Sci Paris Sér III 320:747-758
5. Bärlocher F (1992) The Ecology of Aquatic Hyphomycetes. Springer-Verlag, Berlin
6. Bärlocher F, Canhoto C, Graça MAS (1995) Fungal colonization of alder and eucalypt leaves in two streams in central Portugal. Arch Hydrobiol 133:457-470
7. Bermingham S, Maltby L, Cooke RC (1996) Effects of a coal mine effluent on aquatic hyphomycetes. II. Laboratory toxicity experiments. J Appl Ecol 33:1322-1328
8. Bermingham S, Maltby L, Cooke RC (1996) Effects of a coal mine effluent on aquatic hyphomycetes. I. Field Study. J Appl Ecol 33:1311-1321
9. Bray RJ, Curtis JT (1957) An ordination of the upland forest communities of southern Wiscosin. Ecol Monogr 27:325-349
10. Canhoto C, Graça MAS (1996) Decomposition of *Eucalyptus globulus* leaves and three native leaf species (*Alnus glutinosa*, *Castanea sativa* and *Quercus faginea*) in a Portuguese low order stream. Hydrobiologia 333:79-85
11. Chamier A-C, Tipping E (1997) Effects of aluminium in acid streams on growth and sporulation of aquatic hyphomycetes. Environ Pollut 96:289-298
12. Chauvet E, Fabre E, Elósegui A, Pozo J (1997) The impact of eucalypt on the leaf associated aquatic hyphomycetes in Spanish streams. Can J Bot 75:880-887
13. Clark JR, Cripe CR (1993) Marine and estuarine multi-species test systems. In: Calow P. (ed) Handbook of Ecotoxicology, vol 1. Blackwell Science Ltd, London, England, pp 227-247
14. Fabre E, Chauvet E (1998) Leaf breakdown along an altitudinal stream gradient. Arch Hydrobiol 141:167-179
15. Gadd GM (1993) Transley review Nº47. Interactions of fungi with toxic metals. New Phytol 124:25-60

16. Gessner MO, Chauvet E (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Appl Environ Microbiol 59:502-507

17. Gessner MO, Chauvet E (1994) Importance of stream microfungi in controlling breakdown rates of leaf litter. Ecology 75:1807-1817

18. Gessner MO, Chauvet E (1997) Growth and production of aquatic hyphomycetes in decomposing leaf litter. Limnol Oceanogr 42:496-505

19. Gessner MO, Chauvet E, Dobson M (1999) A perspective on leaf litter breakdown in streams. Oikos 85:377-384

20. Gessner MO, Schmitt AL (1996) Use of solid-phase extraction to determine ergosterol concentrations in plant tissue colonized by fungi. Appl Environ Microbiol 62:415-419

21. Gonçalves MAP (2001) Determinação de metais pesados em águas superficiais recolhidas no Rio Este. MSc Dissertation, University of Minho, Braga, Portugal.

22. Graça MAS, Ferreira RCF, Coimbra CN (2001) Litter processing along a stream gradient: the role of invertebrates and decomposers. J N Am Benthol Soc 20:408-420

23. Gulis V, Suberkropp K (2003) Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. Freshw Biol 48:123-134

24. Krauss G, Bärlocher F, Schreck P, Wennrich R, Glässer W, Krauss G-J (2001) Aquatic hyphomycetes occur in hyperpolluted waters in central Germany. Nova Hedwigia 72:419-428

25. Legendre P, Legendre L (1998) Numerical Ecology. Developments in Environmental Modelling, 20, 2nd ed. Elsevier Science BV, Amsterdam

26. Madigan MT, Martinko JM, Parker J (2000) Biology of Microorganisms, 9th ed. Prentice-Hall International, Inc. New Jersey, pp 147-162

27. Maharning AR, Bärlocher F (1996) Growth and reproduction in aquatic hyphomycetes. Mycologia 88:80-88

28. Maltby L (1992) Detritus processing. In: P Calow, GE Petts (eds) The Rivers Handbook: Hydrological and Ecological Principles, vol 1. Blackwell Scientific Publications Ltd, Oxford, pp 331-353

29. Miersch J, Bärlocher F, Bruns I, Krauss G-J (1997) Effects of cadmium, copper, and zinc on growth and thiol content of aquatic hyphomycets. Hydrobiologia 346:77-84

30. Miersch J, Tschimedbalshir M, Bärlocher F, Grams Y, Pierau B, Schierhorn A, Krauss G-J (2001) Heavy metals and thiol compounds in *Mucor racemosus* and *Articulospora tetracladia*. Mycol Res 105:883-889

31. Minshall GW (1967) Role of allochthonous detritus in the trophic structure of a woodland springbrook community. Ecology 48:139-149

32. Minshall GW, Petersen RC, Cummins KW, Butt TL, Sedell JR, Cushing CE, Vannote RL (1983) Interbiome comparison of stream ecosystem dynamics. Ecol Monogr 53:1-25

33. Niyogi DK, Lewis Jr WM, McKnight DM (2001) Litter breakdown in mountain streams affected by mine drainage: biotic mediation of abiotic controls. Ecol Appl 11:506-516

34. Niyogi DK, McKnight DM, Lewis Jr WM (2002) Fungal communities and biomass in mountain streams affected by mine drainage. Arch Hydrobiol 155:255-271

35. Pascoal C, Cássio F, Gomes P (2001) Leaf breakdown rates: a measure of water quality? Int Rev Hydrobiol 86:407-416

36. Pascoal C, Pinho M, Cássio F, Gomes P (2003) Assessing structural and functional ecosystem condition using leaf breakdown: studies on a polluted river. Freshw Biol (in press)

37. Petersen RC, Cummins KW (1974) Leaf processing in a woodland stream. Freshw Biol 4:343-368

38. Rand GM, Wells PG, McCarty LS (1995) Introduction to aquatic toxicology: effects, environmental fate, and risk assessment. In: GM Rand (eds) Fundamentals of Aquatic Toxicology, 2nd ed. Taylor and Francis, London, pp 3-66

39. Soares HMVM, Boaventura RAR, Machado AASC, Esteves da Silva JCG (1999) Sediments as monitors of heavy metal contamination in the Ave river basin (Portugal): multivariate analysis of data. Environ Pollut 105:311-323

40. Sridhar KR, Bärlocher F (2000) Initial colonization, nutrient supply, and fungal activity on leaves decaying in streams. Appl Environ Microbiol 66:1114-1119

41. Sridhar KR, Krauss G, Bärlocher F, Raviraja NS, Wennrich R, Baumbach R, Krauss G-J (2001) Decomposition of alder leaves in two heavy metal-polluted streams in central Germany. Aquat Microb Ecol 26:73-80

42. Sridhar KR, Krauss G, Bärlocher F, Wennrich R, Krauss G-J (2000) Fungal diversity in heavy metal polluted waters in central Germany. Fungal Diversity 5:119-129

43. Suberkropp K (1991) Relationships between growth and sporulation of aquatic hyphomycetes on decomposing leaf litter. Mycol Res 95:843-850

44. Suberkropp K (1995) The influence of nutrients on fungal growth, productivity, and sporulation during leaf breakdown in streams. Can J Bot 73:1361-1369

45. Suberkropp K (1998) Microorganisms and organic matter decomposition. In: RJ Naiman, RE Bilby (eds) River Ecology and Management: Lessons from the Pacific Coastal Ecoregion, Springer, New York, pp 120-143

46. Suberkropp K, Chauvet E (1995) Regulation of leaf breakdown by fungi in streams: influences of water chemistry. Ecology 76:1433-1445

47. Suberkropp K, Gessner MO, Chauvet E (1993) Comparison of ATP and ergosterol as indicators of fungal biomass associated with decomposing leaves in streams. Appl Environ Microbiol 59:3367-3372

48. Suberkropp K, Weyers HS (1996) Application of fungal and bacterial production methodologies to decomposing leaves in streams. Appl Environ Microbiol 62:1610-1615

49. Thioulouse J, Chessel D, Dolédec S, Olivier J-M (1997) ADE-4: a multivariate analysis and graphical display software. Statistics and Computing 7:75-83

50. Webster JR, Benfield EF (1986) Vascular plant breakdown in freshwater ecosystems. Annu Rev Ecol Syst 17:567-594

51. Zar JH (1996) Biostatistical Analysis, 3rd ed. Prentice-Hall, Englewood Cliffs, New Jersey

Table 1 - Physical, chemical and microbiological characteristics of the stream water at the source of the Este River on 9 April 2002.

Parameter	Value
pH	5.8*
Conductivity ($\mu s\ cm^{-1}$)	42*
Temperature (ºC)	14.1*
Nitrate (mg $NO_3^-\ l^{-1}$)	4
Orthophosphate (mg $PO_4^{-3}\ l^{-1}$)	0.17
Magnesium (ppm)	2
Calcium (ppm)	10
Chemical oxygen demand (mg $O_2\ l^{-1}$)	28
Total heterotrophs (CFU ml^{-1})	80
Total coliforms (CFU ml^{-1})	<1
Fecal coliforms (CFU ml^{-1})	<1

CFU, colony-forming units;
* measurements were done *in situ* at 9 a.m.

Table 2 - Percentage contribution of individual aquatic hyphomycete species to the total conidium production during alder leaf disks decomposition at the source of the Este River (ST) and in microcosms (M) exposed to different zinc concentrations. Microcosm treatments: M0, control; M0.5, 0.5 µM zinc; M15, 15 µM zinc; and M150, 150 µM zinc. Numbers in parentheses indicate exposure time to zinc. Values represent mean of 4 replicates.

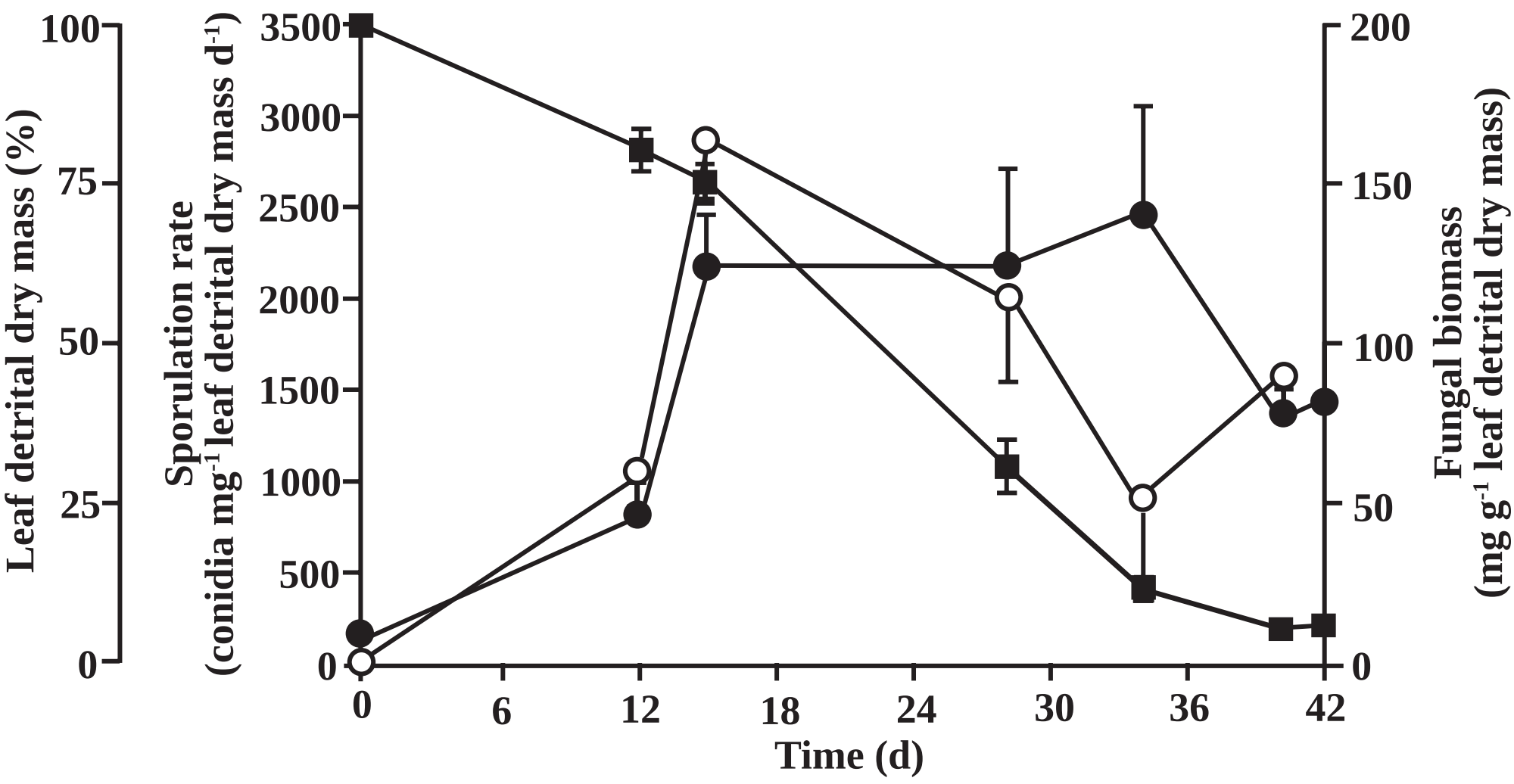
Species	Time of experiment (days)																
	12	15	28 (13)					34 (19)					40 (25)				
	ST	ST	ST	M0	M0.5	M15	M150	ST	M0	M0.5	M15	M150	ST	M0	M0.5	M15	M150
Alatospora acuminata Ingold	0.5	0.8	1.4	0.6	0.5	0.1	-	1.6	0.2	0.7	0.1	-	2.1	0.2	1.9	<0.1	-
Anguillospora filiformis Greathead	0.1	0.9	0.1	0.3	0.3	0.4	-	0.1	0.4	0.3	0.3	0.1	<0.1	3.2	<0.1	0.2	<0.1
Articulospora tetracladia Ingold	81.4	58.8	65.5	10.9	6.0	21.0	2.5	51.0	3.3	3.2	8.1	3.1	31.7	6.9	5.3	14.9	17.3
Cylindrocarpon sp.	-	-	-	-	<0.1	0.1	-	-	0.1	-	0.6	<0.1	-	-	-	-	-
Clavariopsis aquatica De Wildeman	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-
Clavatospora longibrachiata (Ingold) Marvanová and S. Nilsson	-	0.2	0.1	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-
Dimorphospora foliicola Tubaki	0.3	0.2	1.0	<0.1	0.1	0.1	0.1	0.4	-	<0.1	-	0.6	<0.1	-	<0.1	0.1	0.3
Heliscus lugdunensis Saccardo and Thérry	-	-	<0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heliscella stellata (Ingold and Cox) Marvanová and S. Nilsson	-	0.1	0.5	-	-	-	-	2.3	-	-	-	-	-	-	-	-	-
Lemonniera aquatica De Wildeman	1.0	2.3	0.5	0.9	0.2	0.3	5.2	1.1	1.3	0.2	0.3	1.2	2.3	4.7	0.3	0.3	0.7
Lemonniera terrestris Tubaki	-	-	-	-	-	-	-	-	0.2	<0.1	0.1	0.8	-	-	-	-	-
Lunulospora curvula Ingold	3.5	15.0	2.7	7.5	8.0	7.9	1.0	8.0	19.0	2.7	2.9	0.7	23.1	20.7	2.1	1.6	0.8
Mycocentrospora sp.	-	-	-	-	<0.1	-	-	-	-	-	-	-	-	-	-	-	-
Sigmoid 1 (40-60 x 1.0-1.5 µm)	9.0	16.4	13.8	74.8	68.9	55.1	10.3	15.5	46.8	67.2	76.1	11.6	18.5	54.4	83.5	77.1	9.2
Sigmoid 2 (20-30 x 1.0-1.5 µm)	2.4	3.3	11.2	4.1	15.4	14.5	80.2	8.1	28.1	25.1	11.2	80.8	9.0	9.4	5.7	5.1	70.3
Sigmoid 3 (40-60 x 3.5-4.0 µm)	0.3	0.5	0.6	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.1	0.3	-	-	0.3	0.3	1.0
Tetracladium breve Roldán	0.2	-	-	<0.1	-	-	-	-	-	<0.1	-	-	-	-	-	-	0.1
Tricladium chaetocladium Ingold	0.7	1.2	0.2	0.3	0.1	<0.1	0.3	5.5	0.3	0.2	0.1	0.5	5.2	0.4	0.6	0.4	0.1
Tricladium splendens Ingold	0.5	<0.1	0.2	<0.1	<0.1	0.1	0.1	4.0	-	<0.1	<0.1	0.1	7.8	-	0.1	-	0.2
Triscelophorus sp.	-	0.1	1.8	-	<0.1	-	-	-	-	-	-	-	-	-	-	-	-
Varicosporium elodeae Kegel	0.1	0.2	0.4	0.3	0.1	0.2	0.1	1.9	-	0.1	<0.1	0.1	0.2	0.1	0.2	-	-
Total number of species	13	15	16	13	15	13	10	15	11	14	13	13	11	9	12	10	11

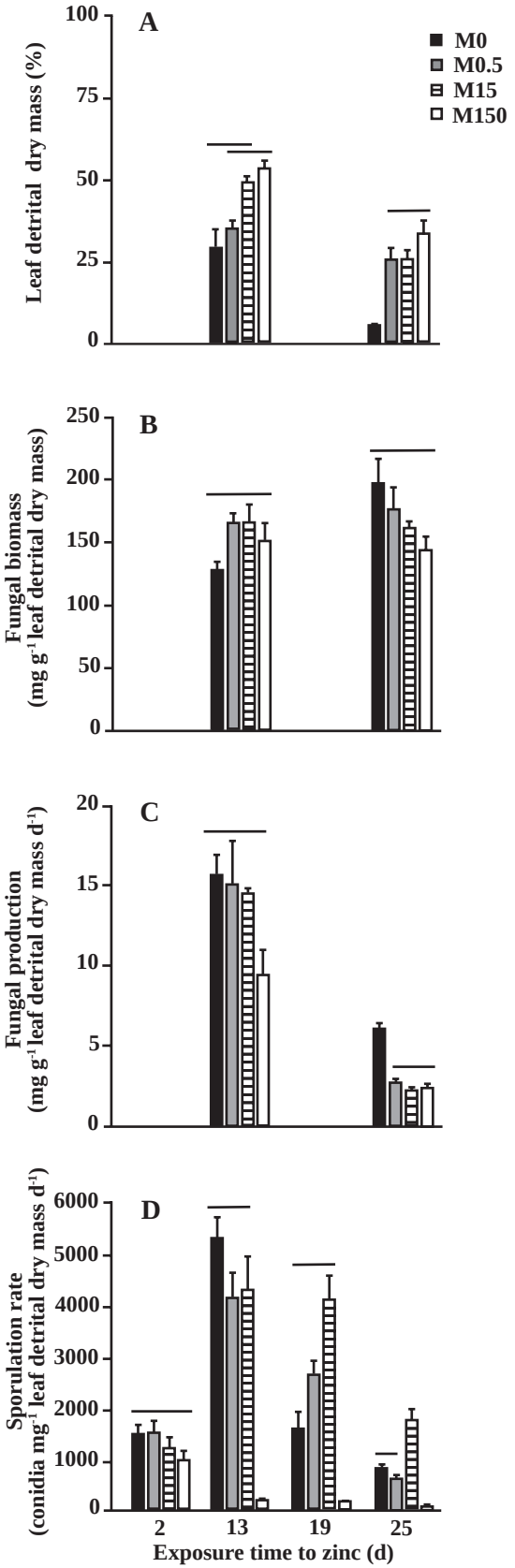
Legends

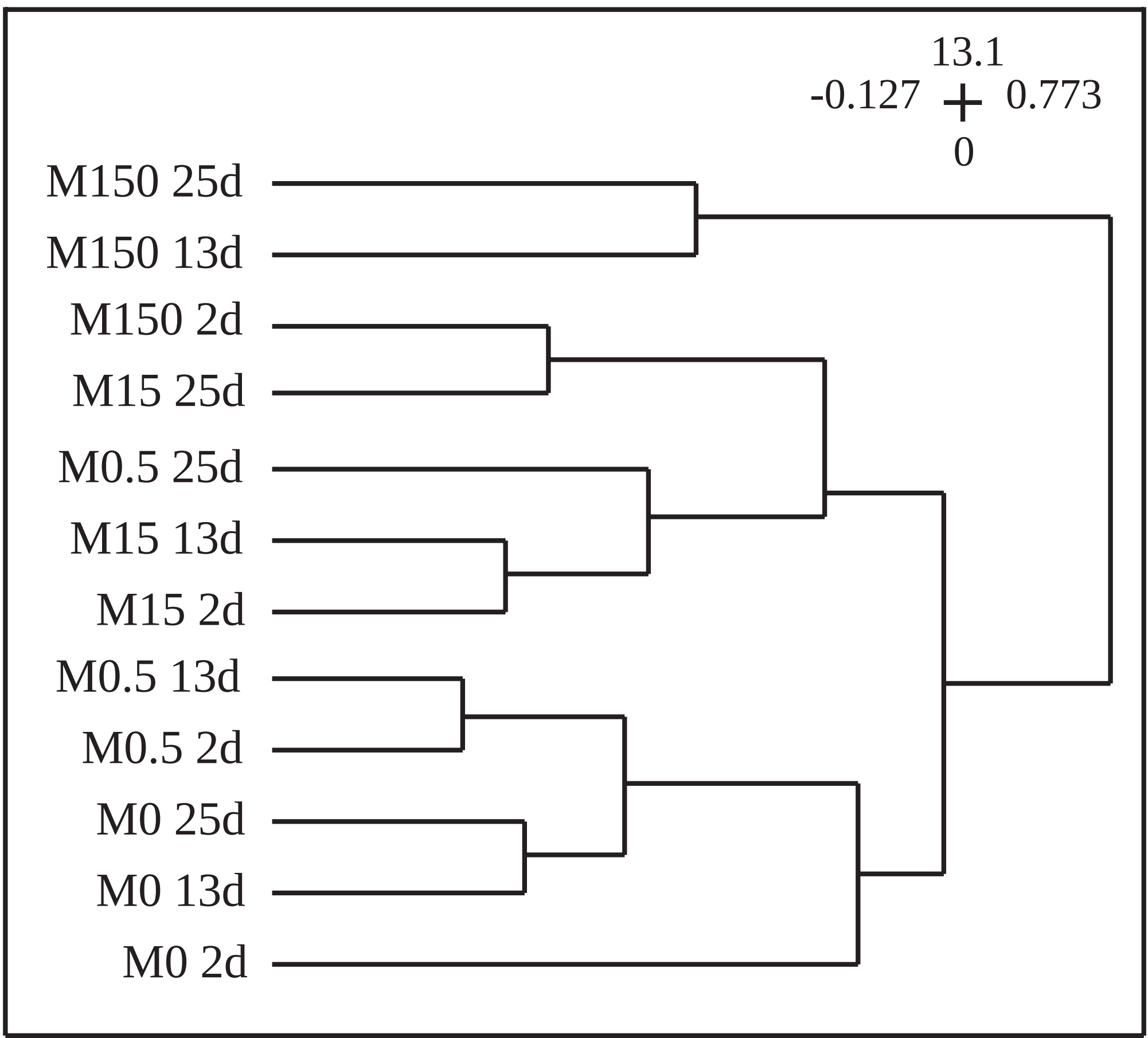
Fig. 1. Decomposition of alder leaf disks at the source of the Este River. Leaf mass loss (■), sporulation rates of aquatic hyphomycetes (○) and fungal biomass (●). N=4, error bars indicate ± 1 SEM.

Fig. 2. Leaf decomposition by fungal assemblages on leaf disks immersed at the source of the Este River for 15 days and subsequently exposed to zinc in microcosms. (**A)** Mass loss of alder leaf disks, (**B)** fungal biomass, (**C**) fungal production and (**D**) sporulation rates of aquatic hyphomycetes. Treatments: M0, control; M0.5, 0.5 µM zinc; M15, 15 µM zinc; and M150, 150 µM zinc. Horizontal bars above histograms indicate a lack of significant differences between treatments (Tukey's multiple comparison, $P>0.05$). N=4, error bars indicate ± 1 SEM.

Fig. 3. Dendogram from UPGMA cluster analysis based on Bray-Curtis similarity matrix constructed from aquatic hyphomycete assemblages on leaf disks immersed at the source of the Este River for 15 days and subsequently exposed to zinc in microcosms. Treatments are indicated in Fig. 2.


Leaf detrital dry mass (%)
100
75
50
25
0
Sporulation rate
(conidia mg-1 leaf detrital dry mass d-1)
3500
3000
2500
2000
1500
1000
500
0
Fungal biomass
(mg g-1 leaf detrital dry mass)
200
150
100
50
0
0
6
12
18
24
30
36
42
Time (d)


A
M0
M0.5
M15
M150
Leaf detrital dry mass (%)
100
75
50
25
0
B
Fungal biomass
(mg g-1 leaf detrital dry mass)
250
200
150
100
50
0
C
Fungal production
(mg g-1 leaf detrital dry mass d-1)
20
15
10
5
0
D
Sporulation rate
(conidia mg-1 leaf detrital dry mass d-1)
6000
5000
4000
3000
2000
1000
0
2
13
19
25
Exposure time to zinc (d)


13.1
-0.127
0.773
0
M150 25d
M150 13d
M150 2d
M15 25d
M0.5 25d
M15 13d
M15 2d
M0.5 13d
M0.5 2d
M0 25d
M0 13d
M0 2d