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Impacts of warming on aquatic decomposers along a gradient of cadmium stress

D. Batista, C. Pascoal, F. Cássio*

Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

ABSTRACT

We evaluated the effects of cadmium and temperature on plant-litter decomposition by examining diversity and activity of aquatic fungi and leaf consumption by *Limnephilus* sp., a typical invertebrate shredder of Iberian streams. Freshly fallen leaves were immersed in a stream to allow microbial colonization, and were exposed in microcosms to a gradient of cadmium (≤ 11 levels, ≤ 35 mg L^{-1}). Microcosms were kept at 15 °C, a temperature typically found in Iberian streams in autumn, and at 21 °C to simulate a warming scenario. The increase in temperature stimulated leaf decomposition by microbes, fungal reproduction and leaf consumption by the shredder. Conversely, increased cadmium concentrations inhibited fungal reproduction and diversity, and leaf consumption by the invertebrate. Cadmium concentration inhibiting 50% of fungal reproduction, microbial decomposition and leaf consumption by the shredder was higher at 15 °C than at 21 °C, suggesting that higher temperatures can lead to increased metal toxicity to aquatic decomposers.

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1. Introduction

The predicted increase in temperature, up to 6 °C till 2100 (IPCC, 2007), is expected to affect organisms and ecological processes in both terrestrial and aquatic ecosystems (Petchey et al., 1999; Parmesan and Yohe, 2003). Moreover, climate models predict an increase in the frequency and magnitude of extreme weather events, such as drought periods and intense rainfalls (Jentsch et al., 2007), changing the levels and bioavailability of contaminants in freshwaters as a result of runoff events from the surrounding soils. Metal contamination is an environmental problem in both developing and developed countries throughout the world. Metals can reach surface and ground waters by the leaching of ores and contaminated soils or through industrial activities. Pollution by metals is a matter of great concern because of its toxicity to living organisms and persistence in the environment. Cadmium (Cd) is considered one of the most toxic metals even at micro grams per litre level, and it can accumulate in aquatic organisms (Wang et al., 2009; Schaller et al., 2010).

In freshwaters, plant-litter decomposition is an important ecosystem process, which depends on the activity of microbial decomposers and invertebrate shredders (Graça, 2001; Pascoal et al., 2005). Fungi, particularly aquatic hyphomycetes, are known to play a key role in litter decomposition in streams (Baldy et al.,

2002; Pascoal and Cássio, 2004), and to enhance litter nutritional value for invertebrate shredders (Graça, 2001).

Higher stream water temperature can affect litter decomposition directly by promoting leaching of soluble compounds (Chergui and Pattee, 1990), and indirectly by enhancing microbial activity (Fernandes et al., 2009a) or stimulating leaf consumption by invertebrate shredders (Gonzalez and Graça, 2003; Azevedo-Pereira et al., 2006). Increased temperature can stimulate the productivity of fungal assemblages on leaves (Suberkropp and Weyers, 1996), and the growth and reproduction of some aquatic hyphomycete species (Rajashekar and Kaveriappa, 2000; Dang et al., 2009), resulting in faster litter decomposition (Fernandes et al., 2009a; Ferreira and Chauvet, 2011). The increase in temperature may also change the feeding behaviour of invertebrate shredders and the individual body elemental composition (Ferreira et al., 2010).

Several studies have demonstrated that metals, above a certain threshold concentration, can negatively affect the diversity and activity of aquatic hyphomycetes and depress plant-litter decomposition in streams (Sridhar et al., 2001; Duarte et al., 2004, 2008). Metals can also compromise survival, growth and reproduction of several invertebrate species (Barata and Baird, 2000; Vogt et al., 2007) and reduce the consumption rates of many invertebrates at environmentally realistic concentrations (Riddell et al., 2005; Felten et al., 2008).

In manipulative experiments, the effects of environmental stressors are usually tested individually and mostly targeting specific organisms. However, in natural ecosystems organisms are

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exposed to several stressors simultaneously and may not always respond as predicted from studies that used only single stressors (Duarte et al., 2008; Fernandes et al., 2009b). Moreover, studies based on organismal responses to stressors are clearly insufficient to predict the impact of stressors to the entire community and associated ecological processes. Therefore, studies at the community and multitrophic levels are warranted if we are to elucidate the combined effects of stressors on ecosystem functioning.

Higher temperatures can lead to increased toxicity of chemicals in conventional ecotoxicity experiments (Heugens et al., 2006), but the potential impacts on communities or processes in which organisms are involved have been seldom examined. We investigated the interactive effects of Cd and temperature on plant-litter decomposition by examining (1) leaf-associated fungal assemblages, and (2) the feeding behaviour of invertebrate shredders. Litter associated microbial assemblages were exposed to a gradient of Cd (11 levels, ≤ 35 mg L^{-1}) under two temperature regimes (ambient 15 °C and 21 °C to simulate a warming scenario), and fungal diversity and activity on decomposing leaves were monitored. In parallel, we conducted a feeding experiment where a common invertebrate shredder was allowed to feed on microbially-colonized leaves while exposed to metal and temperature treatments. Finally, we conducted a post-exposure feeding experiment to assess the invertebrate ability to recover after release from metal stress. We hypothesized that a 6 °C increase in temperature would stimulate fungal and invertebrate activity on decomposing leaves, while the opposite would occur under Cd stress. Because a rise in temperature may result in an increase in metal uptake by the organisms, we expected that warming would exacerbate the impacts of metals on detritus food-webs.

2. Materials and methods

2.1. Sampling site and microbial colonization of leaves

The sampling site was in the Algeriz Stream (Cávado River basin), a low-order stream in NW Portugal (41°350′N 8°220′W). The riparian vegetation is dominated by *Eucalyptus globulus* Labill. and *Pteridium aquilinum* Khun, and it also includes *Alnus glutinosa* (L.) Gaertn. and *Quercus robur* L. The stream bottom is composed by boulders, pebbles and sand.

Conductivity, pH and dissolved oxygen were measured in situ using a Multiline F/set 3 no. 400327 (WTW). Stream water had low conductivity (43 µS cm^{-1}), high dissolved oxygen concentration (100% saturation) and was slightly acidic (pH 6.8). Stream water samples were collected in glass bottles, transported in a cool box (4 °C), for quantification of inorganic nutrient concentrations with a HACH DR/2000 spectrophotometer (Hach, Loveland, CO) according to the manufacture protocols. Concentrations of nutrients were: 0.06 mg NO$_3^-$–N L^{-1}, 0.001 mg NO$_2^-$–N L^{-1}, <0.01 mg NH$_3$–N L^{-1}, and 0.03 mg PO$_4^{3-}$–P L^{-1}. Additional stream water samples were collected, filtered to retain suspended solids, and autoclaved (120 °C, 20 min) for the microcosm experiments.

In September 2009, leaves of *A. glutinosa* (alder) were collected immediately before abscission and dried at room temperature. The leaves were soaked in deionised water and cut into 12 mm diameter disks. Sets of leaf disks were placed into 0.5 mm mesh bags (16 × 20 cm) and were immersed in the stream to allow microbial colonization. After 7 days, leaf bags were retrieved and transported to the laboratory in a cool box for microcosm experiments.

2.2. Leaf decomposition by microbes

Fifty leaf disks from each bag were rinsed with deionised water and placed in 150 mL Erlenmeyer flasks. Three replicate microcosms were supplemented with Cd (0.015, 0.1, 0.5, 1, 1.5, 3.5, 4.5, 10, 20, 35 mg L^{-1}; added as chlorides, Sigma); Cd solutions were prepared in sterile stream water, and a final volume of 70 mL was used per microcosm. Three additional microcosms not supplemented with Cd were used as a negative control. One set of microcosms was incubated at 15 °C and another set at 21 °C (total of 66 microcosms). All microcosms were kept under shaking (120 rpm) for 20 days. Solutions were renewed every 4 days and conidial suspensions were preserved in formalin (2% final concentration) until fungal conidia were identified and counted. At the end of the experiment (after 20 days), leaf disks were harvested for estimation of leaf mass loss, fungal sporulation, biomass and diversity.

2.3. Fungal sporulation and biomass

Conidial suspensions were mixed with 200 µL of 0.5% Tween 80 and appropriate volumes were filtered (5 µm pore size, Millipore, Billerica, MA). Conidia on the filters were stained with 0.05% cotton blue in lactic acid. To determine the contribution of each aquatic hyphomycete species to the total conidial production, approximately 300 conidia per filter were identified and counted under a light microscope (400×, Leica Biomed, Heerbrug, Switzerland).

Six leaf disks from each replicate were used for quantification of litter ergosterol concentrations as a surrogate for fungal biomass (Gessner, 2005). Lipids were extracted from leaf disks by heating (80 °C, 30 min) in 0.8% of KOH/methanol, and the extract was partially purified by solid-phase extraction. Ergosterol was quantified by high-performance liquid chromatography (HPLC) using a LiChrospher RP18 column (250 mm × 4 mm, Merck), connected to a liquid chromatographic system (Beckmann Gold System, Brea, CA). The system was run isocratically with HPLC-grade methanol at 1.4 mL min^{-1} and 33 °C. Ergosterol was detected at $\lambda = 282$ nm and its concentration was estimated using standard series of ergosterol (Fluka) in isopropanol.

2.4. Fungal diversity from DNA fingerprints

DNA was extracted from three leaf disks using a soil DNA extraction kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer instructions. The ITS2 region of fungal rDNA was amplified with the primer pairs ITS3GC and ITS4 as described in Duarte et al. (2008). The forward primer had an additional 40-bp GC tail on the 5′end, which ensures separation during denaturing gradient gel electrophoresis (DGGE) gel (Duarte et al., 2008). For polymerase chain reaction (PCR) of fungal DNA, 12.5 µL of Go Taq, 0.5 µL of each primer, 1 µL of DNA and 10.5 µL of ultra pure water were used in a final volume of 25 µL. PCR reagents were from Promega except primers that were from Stabvida. DNA amplification was carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using the following program: initial denaturation at 95 °C for 2 min; followed by 36 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. Final extension was at 72 °C for 5 min (Duarte et al., 2008).

DGGE analysis was performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories). Samples of 20 µL from the amplification products of 380–400 bp were loaded on 8% (w/v) polyacrylamide gel in 1× Tris–acetate–EDTA (TAE) with a denaturing gradient from 30 to 70%. The gels were run at 55 V, 56 °C for 16 h and stained with 10 µL of Gel Star (Lonza) for 10 min. The gel images were captured under UV light in a transilluminator Eagle eye II (Stratagene, La Jolla, CA, USA).

2.5. Feeding experiment

Feeding experiments were performed with the invertebrate *Limnephilus* sp. (Trichoptera: Limnephilidae) because animals of this genus are common in Iberian streams and easy to maintain in the laboratory. The animals were collected at an unpolluted site of the Cávado River (NW Portugal) and acclimated in the laboratory under aerated stream water, at 15 °C, with a supply of alder leaves. The animals were kept under starvation for 24 h before exposure to stream water supplemented or not with Cd and microbially-colonized leaf disks. Microbial colonization was achieved as above, by immersing leaf disks (12 mm diameter) in the stream for 7 days. Leaf disks were then exposed or not to Cd treatments (0, 0.5 and 10 mg L^{-1}; 11 replicates) at 15 °C and 21 °C, for 20 days, before being offered to the animals. For each replicate, 1 animal (14.5 ± 1.8 mm length) and 12 leaf disks were placed in 250 mL Erlenmeyer flasks containing 150 mL of Cd solutions. All microcosms were aerated with air pumps for 6 days. Solutions were renewed every 3 days to remove excreted compounds and survivorship was registered twice a day during the experiment. At the end of the experiment, leaf disks and animals were frozen and lyophilized to a constant weight.

2.6. Post-exposure feeding experiment

For each replicate, 5 animals were placed in 500 mL Erlenmeyer flask containing 250 mL of different Cd concentrations (0, 0.015, 0.05, 0.1, 0.5, 1, 3.5, 10, 20, 35 mg L^{-1}; 3 replicates) and kept under aeration at 15 °C. The shredders were kept under starvation for 4 days while exposed to Cd. Then, animals were released from the metal, by transferring them to microcosms with stream water without added Cd, and were allowed to feed for 5 days on microbially-colonized leaf disks (20 disks; 12 mm diameter) non-contaminated with Cd. Leaf disks were colonized by microbial decomposers as above, by immersing the leaves in the stream for 7 days followed by 20 days in microcosms without added Cd. At the end of the experiment, leaf disks were frozen and lyophilized before weighed to estimate leaf consumption rates.

2.7. Leaf mass loss

Before and after animal feeding, leaf disks from each replicate were frozen and lyophilized to a constant weight. Sets of non-colonized leaf disks were used to estimate the initial dry mass of leaves.

2.8. Metal analysis

Lyophilized leaf disks, cases and animals from each treatment in the feeding experiment were ashed at 500 °C (>8 h) and digested with nitric acid (1 mL, 10%) and hydrochloric acid (1 mL, 10%) for quantification of Cd concentrations. Cadmium concentrations in the leaves, cases and larvae were analyzed using inductively coupled plasma–atomic emission spectrometry (ICP–AES) at the Scientific and Technological Research Assistance Centre (CACTI, University of Vigo, Spain).

2.9. Data analysis

The effects of Cd and temperature on leaf mass loss, fungal biomass and reproduction were tested by two-way analyses of variance (Two-way ANOVA) followed by Bonferroni post-tests (Zar, 1996). Data were homoscedastic and had a normal distribution, so no data transformation was done.

DGGE gels were aligned and the relative intensity of the bands in the gel was analyzed with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Each DGGE band was considered an operational taxonomic unit (OTU). Cluster analyses of fungal community structure, assessed as sporulating species or OTUs from DGGE fingerprints, were done by unweighted pair-group method average (UPGMA) using the Pearson correlation coefficient.

ANOVAs were done with Graph Pad Prism 5 (GraphPad software Inc., San Diego, CA) and Cluster analyses with CANOCO 4.5 (Microcomputer Power, NY, USA).

The length of the case of each animal was measured using a stereo microscope (Leica ZOOM 2000) before the experiment, and the animal dry mass was estimated by linear regression as $DM = 0.0029 \times CO - 0.0293$ ($r^2 = 0.73$, $p < 0.05$, $n = 36$), where DM is the animal dry mass (g) and CO is the length of the case (mm). Relative growth rates (RGR) of the animals (g animal dry mass g^{-1} animal dry mass day^{-1}) were calculated as $RGR = DM_g/(DM_f \times t)$, where DM_g is the dry mass gained during the elapsed time (t) given by the difference between final and initial dry mass (g) and DM_f is the final dry mass (g) (Ferreira et al., 2010). Relative consumption rates (RCR, g leaf dry mass g^{-1} animal dry mass day^{-1}) were calculated as $RCR = Le/(DM_f \times t)$, where Le is the litter dry mass eaten during the elapsed time (t) and DM_f is the final dry mass of animals (Ferreira et al., 2010). Leaf disks were weighed before and after being offered to the invertebrates.

The median effect concentration (EC_{50}), i.e. the concentration of Cd promoting 50% of effects, was estimated by probit analysis with SPSS software (Version 17.0 for windows, SPSS Inc, Chicago, USA). Data are presented as mean with 95% confidence limits.

3. Results

During the whole study, 19 sporulating fungal taxa were found on decomposing alder leaves. The dominant fungal species was *Articulospora tetracladia*, followed by *Tetrachaetum elegans* and *Infundibura* sp. (Table 1). *Alatospora acuminata*, *Anguillospora filiformis* and *Tetrachaetum elegans* were absent at high Cd concentrations (Cd $\geq$ 1.5 mg L^{-1}), whereas *Triscelosphorus* cf. *acuminatus* became dominant. *Tricladium attenuatum* was only present at 15 °C, while *Clavariopsis aquatica* was only present at 21 °C (Table 1).

In microcosms without Cd addition, estimates of fungal diversity were higher based on number of OTUs from DGGE fingerprints (19 and 17 OTUs at 15 °C and 21 °C, respectively) than from analysis of spores produced (15 fungal taxa at both temperatures) (Table 1). The exposure to increased Cd concentrations led to a decrease in fungal diversity, mainly when assessed as sporulating species (Table 1).

Cluster analysis of sporulating fungal taxa (Fig. 1a) and DGGE fingerprints (Fig. 1b) showed that fungal assemblages were mainly structured by Cd. In a general way, fungal assemblages exposed to the highest Cd concentration separated from the others. Moreover, assemblages exposed to low Cd concentrations grouped together and were separated from those exposed to intermediate Cd concentrations. At each metal concentration, temperature also discriminated fungal assemblages (Fig. 1a, b).

After 20 days in microcosms without Cd addition, fungal sporulation rates on leaves were 0.8×10^6 and 1.1×10^6 spores g^{-1} leaf dry mass day^{-1} at 15 °C and 21 °C, respectively (Fig. 2a). Fungal sporulation was stimulated by temperature but reduced by Cd, especially at concentrations higher than 0.5 mg L^{-1} (two-way ANOVA, $p < 0.0001$ and $p = 0.0004$, respectively; Bonferroni tests, $p < 0.05$). Temperature and Cd interactions were not significant ($p = 0.083$). The metal concentration that inhibited sporulation rate by 50% was higher at 15 °C ($EC_{50} = 1.889 \pm 0.006$ mg Cd L^{-1}) than at 21 °C ($EC_{50} = 1.685 \pm 0.001$ mg Cd L^{-1}).

Fungal biomass on alder leaves ranged from 185 to 226 µg ergosterol g^{-1} leaf dry mass in control microcosms (Fig. 2b). Fungal biomass was affected by Cd concentration, but not by temperature or interaction between factors (two-way ANOVA, $p < 0.0001$, $p = 0.427$ and $p = 0.117$, respectively). Consistently, the EC_{50} of Cd for fungal biomass was similar at both temperatures ($EC_{50} = 12.39 \pm 0.01$ and 12.29 ± 0.005 mg L^{-1} for 15 °C and 21 °C, respectively). Cadmium at concentrations $\geq$10 mg L^{-1} inhibited

Table 1
Percentage contribution of each fungal taxon to the total conidial production on alder leaves colonized in the Algeriz Stream and then exposed for 20 days in microcosms to different Cd concentrations at 15 °C and 21 °C.

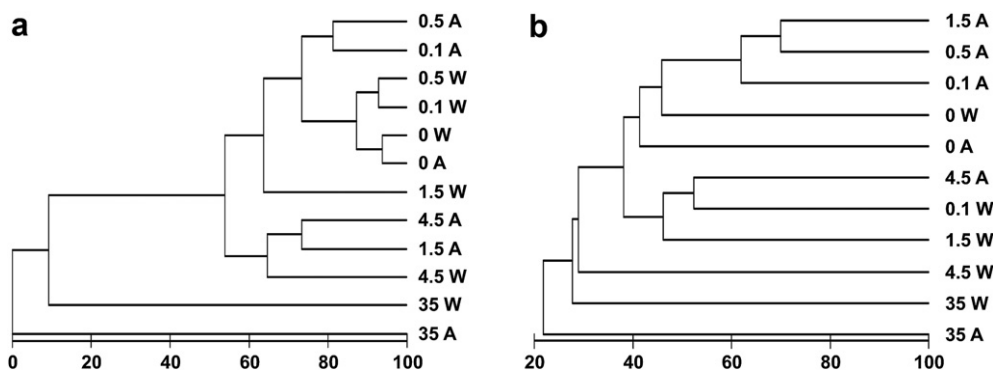| Cd concentration (mg L^{-1})											
Fungal taxa	15 °C/21 °C										
	0	0.015	0.1	0.5	1	1.5	3.5	4.5	10	20	35
Alatospora acuminata Ingold	0.2/0.1	0.4/0.1	0.1/0.1	0.1/0.3	—/0.1	—/—	—/—	—/—	—/—	—/—	—/—
Alatospora pulchella Marvanová	4.4/5.9	6.0/6.6	5.5/8.4	8.2/16.3	15.5/16.5	14.5/26.7	36.1/67.9	34.5/71.4	7.1/—	—/—	—/—
Anguillospora filiformis Greath.	4.0/1.9	1.2/1.1	3.0/2.3	0.7/0.4	0.4/0.7	1.3/0.1	—/—	—/—	—/—	—/—	—/—
Articulospora tetracladia Ingold	38.7/47.8	48.2/45.3	42.3/46.7	53.8/39.3	33.4/44.6	28.8/36.6	15.9/17.7	2.6/8.6	14.3/30.8	—/62.5	—/—
Clavariopsis aquatica De Wild.	0.2/0.3	—/0.3	—/0.1	—/0.3	—/0.1	—/0.2	—/—	—/—	—/—	—/—	—/—
Cylindrocarpon sp.	—/—	—/—	—/—	—/—	0.3/—	0.3/0.1	0.1/1.2	0.7/4.9	42.9/46.2	—/—	—/—
Flabellospora acuminata Descals	—/—	0.1/—	—/—	0.1/—	—/—	0.1/—	0.1/—	0.4/—	—/15.4	—/—	—/50.0
Fusarium sp.	—/—	0.1/—	—/—	0.1/—	—/—	—/0.1	0.1/0.3	1.3/—	14.3/7.7	16.7/25.0	—/—
Infundibura sp.	15.2/19.2	12.2/20.9	16.5/3.7	0.1/0.2	—/0.1	—/—	—/—	—/—	—/—	33.3/—	—/—
Lemonniera aquatica De Wild.	5.2/4.3	4.3/4.5	4.1/4.8	3.7/7.3	8.0/6.4	3.4/5.7	4.1/3.0	3.5/4.9	—/—	—/—	—/—
Lunulospora curvula Ingold	4.4/4.1	5.8/3.5	6.3/5.0	4.0/11.5	3.2/12.6	2.0/14.3	2.9/1.7	2.2/3.3	—/—	—/—	—/—
Tetrachaetum elegans Ingold	25.2/14.4	18.6/14.7	20.8/27.3	28.0/23.2	36.4/17.5	47.9/12.4	37.1/5.5	50.9/4.3	—/—	—/—	—/—
Tetracladium breve A. Roldán	0.1/0.4	0.5/0.3	0.2/—	0.3/0.1	0.4/—	0.1/—	0.3/0.1	0.2/—	—/—	—/—	—/—
Tricladium attenuatum S.H. Iqbal	—/—	0.8/—	0.1/—	0.1/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—
Tricladium chaetocladium Ingold	0.8/0.4	0.2/0.1	0.4/0.1	0.3/0.1	—/—	0.4/0.4	1.0/0.3	0.4/0.4	7.1/—	—/—	—/—
Tricladium splendens Ingold	0.4/0.1	0.8/0.5	0.6/0.6	0.3/0.3	1.8/0.5	1.0/0.3	1.8/1.2	2.4/1.0	—/—	—/—	—/—
Triscelosphorus cf. *acuminatus* Ingold	0.4/0.3	—/1.5	0.1/0.6	0.1/0.5	0.3/0.8	0.1/3.1	0.3/1.3	1.1/1.2	14.3/—	50.0/12.5	—/50.0
Unknown sigmoid	0.1/0.2	0.3/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—	—/—
Varicosporium elodeae W. Kegel	0.6/0.5	0.5/0.4	—/0.2	0.3/0.2	0.4/0.1	—/0.1	—/—	—/—	—/—	—/—	—/—
No. of species	15/15	16/14	13/13	16/14	11/12	12/13	12/11	12/9	6/4	3/3	0/2
No. of OTUs	19/17	n.d.	17/14	18/—	n.d.	16/14	n.d.	15/15	n.d.	n.d.	9/10

n.d.: not determined.

Fig. 1. Dendograms from fungal-sporulating species (a) and DGGE fingerprints of fungal DNA (b) on decomposing alder leaves colonized in the Algeriz Stream and exposed for 20 days in microcosms to different Cd concentrations (0, 0.1, 0.5, 1.5, 4.5 and 35 mg L^{-1}) at ambient temperature of 15 °C (A) and warming temperature of 21 °C (W). Clusters were constructed based on UPGMA analyses using the Pearson correlation coefficient.
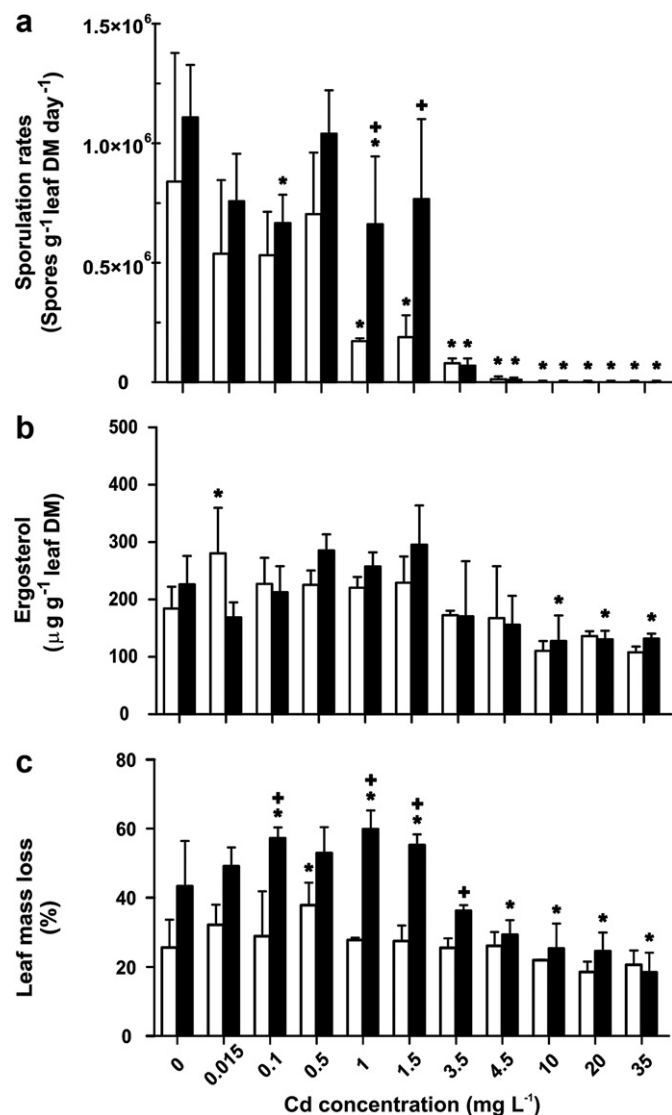

Fig. 2. Fungal sporulation rates (a), fungal biomass as ergosterol concentration (b) and mass loss of alder leaves (c) colonized in the Algeriz Stream and exposed for 20 days in microcosms to different Cd concentrations at ambient temperature of 15 °C (white bars) and warming temperature of 21 °C (black bars). *, significant effect of Cd comparing to control at each temperature; +, significant effect of temperature at each Cd level (two-way ANOVA, Bonferroni tests, $p < 0.05$). M + SEM.

fungal biomass at the highest temperature (Bonferroni tests, $p < 0.05$).

In control microcosms at 15 °C, alder leaves lost 26% of its mass in 20 days (Fig. 2c). Leaf mass loss was generally higher at 21 °C than at 15 °C and was also affected by Cd (two-way ANOVA, $p < 0.0001$ for both factors). The interaction between temperature and Cd was also significant ($p < 0.0001$). Leaf mass loss was stimulated at low Cd concentrations, but reduced at concentrations higher than 3.5 mg L^{-1} at the highest temperature (Bonferroni tests, $p < 0.05$). At 15 °C, no significant decrease in leaf mass loss was found. The EC$_{50}$ of Cd for leaf mass loss was about 4 times higher at 15 °C (EC$_{50} = 44.089 \pm 0.021$ mg L^{-1}) than at 21 °C (EC$_{50} = 9.513 \pm 0.016$ mg L^{-1}).

In microcosms without Cd addition, the relative consumption rates of alder leaves by the shredder *Limnephilus* sp. were 0.233 and 0.301 g leaf dry mass g^{-1} animal dry mass day^{-1} at 15 °C and 21 °C, respectively (Fig. 3a). The relative consumption rate was stimulated by temperature and inhibited by Cd, although the interaction between factors was significant (two-way ANOVA, $p = 0.005$, $p < 0.0001$ and $p = 0.028$, respectively). Inhibition by Cd was stronger when animals were exposed to the highest metal concentration (Bonferroni tests, $p < 0.05$). The concentration of Cd causing 50% reduction in the relative consumption rate of the shredder was higher at 15 °C (EC$_{50} = 3.643 \pm 0.015$ mg L^{-1}) than at 21 °C (EC$_{50} = 3.166 \pm 0.051$ mg L^{-1}). The relative growth rate of animals was only significantly affected by temperature, with greater values for animals exposed to 21 °C (two-way ANOVA, $p = 0.023$, Bonferroni tests, $p < 0.05$) (Fig. 3b). At the end of the feeding experiment, higher concentrations of Cd were found in leaves followed by animal case and body (Table 2). Moreover, the accumulation of Cd at 21 °C was consistently higher than at 15 °C (Table 2).

In the post-exposure feeding experiment, animals were exposed to Cd (4 days) and then were released from the stressor and allowed to feed on leaves for 5 days at 15 °C. In control microcosms, shredders consumed 54% of leaf mass in 5 days (Fig. 4). Leaf consumption by the shredders was strongly inhibited by pre-exposure to Cd concentrations $\geq$0.01 mg L^{-1} (one-way ANOVA, $p < 0.0001$; Bonferroni tests, $p > 0.05$). The Cd concentration inhibiting 50% of leaf consumption in the post-exposure feeding experiment was 0.161 ± 0.045 mg L^{-1}.

4. Discussion

Within the physiological range, biological processes, such as organismal growth and reproduction, are positively related to temperature (Fernandes et al., 2009a; Ferreira et al., 2010). This was
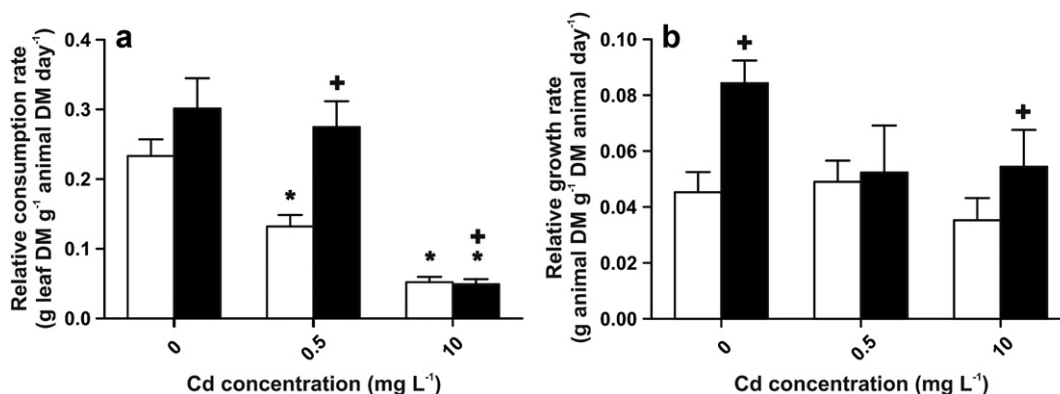
Fig. 3. Relative consumption rates of alder leaves (a) and relative growth rates (b) of the shredder *Limnephilus* sp. exposed to different Cd concentrations at ambient temperature of 15 °C (white bars) and warming temperature of 21 °C (black bars). *, significant effect of Cd comparing to control at each temperature; +, significant effect of temperature at each Cd level (two-way ANOVA, Bonferroni tests, $p < 0.05$). M + SEM.

confirmed in our study by the stimulation of both microbially-mediated litter decomposition and leaf consumption by the shredder *Limnephilus* sp. at the highest temperature (21 °C). The increase in the stream water temperature led to shifts in fungal species composition on decomposing leaves, probably because some species attained their upper thermal tolerance limit while others attained their optimum. These changes can affect litter decomposition because invertebrate shredders preferentially feed on leaves colonized by certain fungal species (Lecerf et al., 2005; Canhoto and Graça, 2008; Chung and Suberkropp, 2009).

The exposure to Cd led to a decrease in fungal reproductive activity and leaf decomposition, mainly at concentrations higher than 1.5 mg L^{-1}. Although we recorded a higher number of fungal species based on DNA fingerprinting than on morphology of released conidia, Cd exposure decreased fungal species richness assessed by both methodologies, particularly at the highest concentrations. Some species of aquatic hyphomycetes are able to tolerate high levels of Cd (Guimarães-Soares et al., 2007; Azevedo and Cássio, 2010), but both diversity and reproductive (spore) activity of fungal decomposers on leaves can decrease under metal exposure (Sridhar et al., 2001; Krauss et al., 2003; Duarte et al., 2008; Moreirinha et al., 2011). However, at low Cd concentrations we found a stimulation of leaf decomposition by microbes. In low doses, both essential and non-essential metals are reported to have a stimulatory effect on reproduction and growth of several aquatic organisms (Calabrese and Blain, 2005; Lefcort et al., 2008; Shen et al., 2009). According to Chapman (2002), under low stress, organisms are able to repair cellular damages and can even over-compensate and reduce background damage more effectively. Indeed, many studies have reported a stimulation of biological

responses (e.g. growth) at low doses of an inhibitor (Calabrese et al., 2007). This is known as hormesis and has been documented in microbes, plants and animals (Calabrese and Blain, 2005), but the implications of this phenomenon to ecological processes require further investigation.

Invertebrate survival and growth are commonly reported to be affected by Cd (Irving et al., 2003; Krauss et al., 2003; Wilding and Maltby, 2006; Brinkman and Johnston, 2008) and temperature (Turner and Williams, 2005; Ferreira et al., 2010). In our study, these two stressors affected the feeding behaviour of the invertebrate shredder *Limnephilus* sp. and effects were more pronounced at 21 °C. Also, for both fungal sporulation and leaf decomposition, the concentration of Cd at which 50% of effects were observed was higher at 15 °C than at 21 °C, suggesting that higher temperatures lead to increased toxicity of Cd. Elevated temperature and metal stress can, independently of each other, increase maintenance costs, reduce mitochondrial efficiency and impair the oxygen uptake and delivery to the tissues, leading to hypoxaemia and energy deficiency (Sokolova and Lannig, 2008). As a result, exposure to one of these stressors might sensitize organisms to the other stressor. Moreover, an increase in metabolic rates at elevated

Table 2
Cadmium concentration in leaves and in the case and larvae of the invertebrate shredder *Limnephilus* sp. in microcosms supplemented or not with Cd at 15 °C and 21 °C for 6 days. Data represent the pool of 11 replicates.

	Cd added (mg L^{-1})	Cd measured (mg g^{-1}) 15 °C/21 °C
Leaves	0	0.02/0.02
	0.5	0.25/0.33
	10	3.57/4.30
Case	0	0.01/0.01
	0.5	0.05/0.06
	10	0.65/0.89
Larvae	0	0.00/0.00
	0.5	0.02/0.02
	10	0.05/0.12
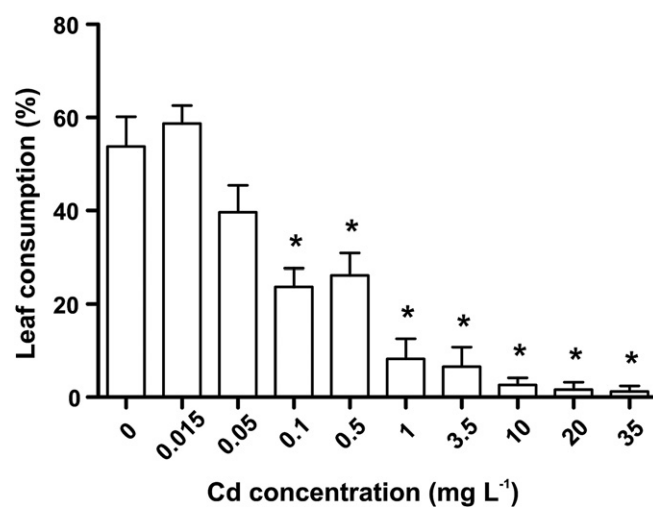
Fig. 4. Consumption of alder leaves during 5 days by the shredder *Limnephilus* sp. at 15 °C. The animals were previously exposed to different Cd concentrations for 4 days under starvation, before released from the stressor and supplied with non-contaminated leaves. *, significant effect of Cd comparing to control (one-way ANOVA, Bonferroni tests, $p < 0.05$). M + SEM.

temperatures is often found (Sokolova and Lannig, 2008) and may contribute to metal accumulation due to a higher energy demand. Indeed, we found a greater accumulation of Cd in microbially-colonized leaves and animals at the highest temperature probably contributing to a greater Cd toxicity. This may have implications in aquatic ecosystems, with possible consequences to water quality criteria for Cd in face of the ongoing global climate change.

In our study, fungal reproduction (EC_{50} = 1.69 mg Cd L^{-1}, 21 °C) was the most sensitive measure to Cd exposure, followed by leaf consumption by the shredder (EC_{50} = 3.17 mg Cd L^{-1}, 21 °C) and leaf decomposition by microbes (EC_{50} = 9.51 mg Cd L^{-1}). In natural freshwaters, Cd can occur at concentrations less than 0.1 μg L^{-1} (USEPA, 2001), but in metal-impacted streams higher concentrations have been reported (e.g., 67 μg L^{-1}, Bervoets et al., 2005; up to 0.84 mg L^{-1}, Lopes et al., 1999; 2.2 mg L^{-1}, Sridhar et al., 2005 in the stream water; 29 mg kg^{-1}, Krauss et al., 2005 in sediments), making the range of Cd concentrations tested here environmentally relevant.

Felten et al. (2008) found a $LC_{50\ 96\ h}$ = 82.1 μg Cd L^{-1} for the aquatic shredder Gammarus pulex. Although we used Cd concentrations up to 10 times higher, we did not observe mortality after 96 h of exposure. A possible explanation for this can be attributed to the protective role of the case in the Limnephilus sp. Indeed, more Cd was associated with the case than with the larvae. Moreover, Cd accumulation in invertebrates has been more strongly correlated with metal content in food than in stream water or sediment (Beltman et al., 1999; Tessier et al., 2000). We demonstrated that Cd accumulated four times more in leaves than in animal case or body, probably explaining the strong inhibition of feeding activity of the invertebrate shredder at both temperatures. Metal toxicity is generally assumed to occur through waterborne exposure and environmental regulations do not take into account the potential impact of food as a source of metal contamination to aquatic organisms (Brinkman and Johnston, 2008). When metal exposure occurs via food in addition to via water, water quality criteria and standards may not protect organisms in aquatic environments. Hence, future studies on the effects of dietary versus aqueous exposure to toxicants on aquatic invertebrates would be helpful to clarify metal toxicity to biota and protect ecosystems in more realistic scenarios.

In our work, leaf consumption rates by the shredder Limnephilus sp. decreased until 60% when animals exposed to Cd (≤35 mg L^{-1}) were released from metal stress and were allowed to feed on non-contaminated leaves. This agrees with other studies reporting a reduction in invertebrate feeding rates (17–90%) after pre-exposure to metals (Moreira et al., 2005; Soares et al., 2005). Alterations in the feeding behaviour are known to influence the organism physiological performance, interfering with specific life-history events such as development, growth and reproduction, and eventually causing changes at the population and community level (Maltby et al., 2002). Although in our study the exposure to Cd did not lead to animal death up to 96 h, invertebrate feeding activity was severely compromised. Therefore, the use of feeding rates as a sublethal endpoint might be a useful tool for assessing potential toxicity of contaminants in the environment.

Overall, our results showed that the increase in temperature stimulated microbial decomposition of leaf litter, fungal reproduction and leaf consumption by the shredder. Increased Cd concentrations inhibited reproduction and diversity of fungi, and leaf consumption by the invertebrate. The negative effects of Cd on fungal and shredder activity on leaves were more pronounced at the highest temperature. The combined effects of Cd and increased stream water temperature may compromise the diversity and activity of aquatic fungi and the feeding activity of sensitive shredders, with direct impacts to plant-litter decomposition and nutrient cycling in freshwaters. However, caution is needed when extrapolating results to natural ecosystems, because these are much more complex and variable than we can mimic in microcosm experiments. Although we found that higher temperature increased the toxicity of Cd to aquatic microbial decomposers and detritivores, the impacts of combined effects of chemicals and warming are still poorly understood. Because this type of information is relevant in the context of ongoing global change, further research is needed to better understand the current risks to freshwater biota and key ecosystem processes.

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