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Effects of Cadmium and Phenanthrene Mixtures on Aquatic Fungi and Microbially Mediated Leaf Litter Decomposition

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Abstract Urbanization and industrial activities have contributed to widespread contamination by metals and polycyclic aromatic hydrocarbons, but the combined effects of these toxics on aquatic biota and processes are poorly understood. We examined the effects of cadmium (Cd) and phenanthrene on the activity and diversity of fungi associated with decomposing leaf litter in streams. Leaves of *Alnus glutinosa* were immersed for 10 days in an unpolluted low-order stream in northwest Portugal to allow microbial colonization. Leaves were then exposed in microcosms for 14 days to Cd (0.06–4.5 mg L^{-1}) and phenanthrene (0.2 mg L^{-1}) either alone or in mixture. A total of 19 aquatic hyphomycete species were found sporulating on leaves during the whole study. The dominant species was *Articulospora tetracladia*, followed by *Alatospora pulchella, Clavatospora longibrachiata*, and *Tetrachaetum elegans*. Exposure to Cd and phenanthrene decreased the contribution of *A. tetracladia* to the total conidial production, whereas it increased that of *A. pulchella.* Fungal diversity, assessed as denaturing gradient gel electrophoresis fingerprinting or conidial morphology, was decreased by the exposure to Cd and/or phenanthrene. Moreover, increased Cd concentrations decreased leaf decomposition and fungal reproduction but did not inhibit fungal biomass production. Exposure to phenanthrene potentiated the negative effects of Cd on fungal diversity and activity, suggesting that the co-occurrence of these stressors may pose additional risk to aquatic biodiversity and stream ecosystem functioning.

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In streams with low autotrophic production, plant-litter decomposition provides carbon and energy for the functioning of aquatic food webs (Allan and Castillo 2007). This carbon is first processed through microbial activity, thus increasing the palatability of plant litter for aquatic invertebrates (Bärlocher 2005; Gessner et al. 2007). Among microorganisms, aquatic hyphomycetes play a key role in plant-litter decomposition in streams (Pascoal and Cássio 2004; Pascoal et al. 2005a) due to their ubiquity and their ability to produce a variety of extracellular enzymes that degrade the complex plant cell-wall polysaccharides (Bärlocher 2005; Gessner et al. 2007).

Human activities from agriculture, mining, and industry have contributed to the increase of contaminants entering streams that affect the activity and diversity of biotic communities (Niyogi et al. 2001; Pascoal et al. 2005a, b; Sridhar et al. 2001). Among pollutants, metals can have adverse effects on aquatic biota because of their toxicity and persistence in the environment (Rand et al. 1995). Although aquatic hyphomycetes have been found in metal-polluted streams, the diversity and activity of this group of fungi is constrained by the presence of metals (Bermingham et al. 1996; Niyogi et al. 2002; Pascoal et al. 2005b; Sridhar et al. 2005). The exposure of leaf-associated fungal communities to copper (Cu) and/or zinc (Zn) has been reported to alter the structure of aquatic hyphomycete communities and decrease fungal sporulation and leaf decomposition (Duarte et al. 2004, 2008a, 2009). Moreover, metals, including Cd, inhibit the growth and reproduction of several species of aquatic hyphomycetes, but reproduction appears to be more sensitive than growth

(Abel and Bärlocher 1984; Azevedo and Cássio 2010; Miersch et al. 1997).

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxicants containing two or more benzene rings that are known to have carcinogenic and mutagenic properties (Chaudry 1994). These compounds are components of coal, crude oil, and its derivatives and are ubiquitous in the environment because they are formed during forest fires, combustion of petroleum, and incineration of waste. Several studies have shown that microbes have the ability to metabolize and biodegrade PAHs and can potentially be used for bioremediation of contaminated environments (D'Annibale et al. 2006; Johnsen et al. 2005; Juhasz and Naidu 2000; Juhasz et al. 2000). For instance, the bacterium *Pseudomonas putida* degraded approximately 70% of the initial amount of phenanthrene (0.47 g L^{-1}) in 27 days (Cuny et al. 2004), and the aquatic hyphomycete *Heliscus lugdunensis* metabolized metabolites of complex PAHs, such as 1-naphthol (Augustin et al. 2006). PAHs do not dissolve easily in water, but they can bind strongly to particulate organic matter and accumulate in the sediments of rivers and lakes at high concentrations, affecting aquatic biota and humans through bioaccumulation (Gust and Fleeger 2006; Ribeiro et al. 2005; Scoggins et al. 2007).

In aquatic ecosystems, the simultaneous occurrence of PAHs and metals is quite common [e.g., streams in a mining district, central Germany (Krauss et al. 2005); urban streams in Scotland (Wilson et al. 2005); estuaries in Portugal (Guimarães et al. 2009)]. Although many studies have investigated the individual effects of these toxicants on organisms, relatively few have considered their effects in mixtures (but see Gust and Fleeger 2006), particularly at the community level. In this study, we assessed the effects of Cd and phenanthrene on the diversity and activity of aquatic fungal communities associated with decomposing leaves. Alder leaves were incubated in a stream to allow microbial colonization and then exposed to increasing concentrations of Cd ($\leq$4.5 mg L^{-1}) in the absence or presence of a fixed concentration of phenanthrene (0.2 mg L^{-1}). Although concentrations of Cd in the water column of metal polluted streams [e.g., 0.06 mg L^{-1} in northwest Portugal (Gonçalves 2001) and 2.2 mg L^{-1} in central Germany (Sridhar et al. 2005)] are generally lower than the maximum tested in our study, Cd can reach high concentrations in sediments [e.g., 29 mg kg^{-1} (Krauss et al. 2005)], making the range of Cd concentrations tested here environmentally relevant. In this study, the effects of Cd and phenanthrene on leaf-mass loss and fungal biomass, sporulation, and diversity were evaluated. We hypothesized that Cd and phenanthrene would restrict fungal diversity and activity and that this effect would be more severe in mixtures with increasing Cd concentrations. However, we also hypothesized that some species might be able to metabolize phenanthrene and thereby survive under the tested conditions.

Materials and Methods

Sampling Site

The sampling site is located at Algeriz, a low-order stream in the northwest Portugal (41°35′N 8°22′W). The riparian vegetation is dominated by *Eucalyptus globulus* Labill., *Quercus robur* L., *Alnus glutinosa* (L.) Gaertn., and *Rubus* sp.

Leaves of *A. glutinosa*, collected in October 2006, were air dried and kept at room temperature until used. The leaves were leached in deionised water for 48 h and cut into 12-mm diameter disks. Sets of 22 disks were placed into 42 fine-mesh bags (20 × 20 cm, 0.5-mm mesh size) to prevent invertebrate colonization. On March 20, 2007, leaf bags were immersed in a stream for 10 days to allow microbial colonization.

At the time of leaf immersion, stream water had a temperature of 11.8°C, a pH of 6.9, a redox potential of −8 mV, a concentration of dissolved oxygen of 10.8 mg L^{-1}, and a conductivity of 40 μS cm^{-1}, measured in situ with field probes (Multiline F/set 3 no. 400327, Wissenschaftlich-Technische Werkstätten GmbH & Co. KG WTW, Weilheim, Germany). Stream water was collected in dark glass bottles, transported on ice to the laboratory, and analyzed within 6 h for quantification of inorganic nutrients with a HACH DR/2000 photometer (Hach, Loveland, CO). Nutrient concentrations were as follows: orthophosphate 0.06 mg $P–PO_4^{3-}$ L^{-1} (HACH kit, program 490); nitrate 0.2 mg $N–NO_3^-$ L^{-1} (HACH kit, program 355); nitrite 0.005 mg $N–NO_2^-$ L^{-1} (HACH kit, program 371); and ammonium <0.01 mg $N–NH_3$ L^{-1} (HACH kit, program 385). Additional stream water was collected for microcosm experiments.

Microcosms

In the laboratory, sets of 22 leaf disks were placed into 150-mL Erlenmeyer flasks with 70 mL filtered (Macherey–Nagel MN-GF3, glass-fiber membranes) and sterilized (120°C, 20 min) stream water. The microcosms were supplemented with Cd chloride (Sigma) at final concentrations of 0.06, 0.6, 1.2, 3.6, and 4.5 mg L^{-1} Cd and phenanthrene (Fluka) at a final concentration of 0.2 mg L^{-1} added alone or in mixture (three replicates). Phenanthrene was solubilized in ethanol at a final concentration of 0.3%. Microcosms were incubated on a shaker (110 rpm, Certomat BS 3; Braun, Melsungen, Germany) at 15°C in the dark. The microcosm solutions were changed every 4 days, and the phenanthrene

concentration was checked daily by direct fluorescence spectrophotometry (LS 50 luminescence spectrometer; Perkin-Elmer, Foster City, CA) using the fixed wavelength technique (Watson et al. 2004; Yang et al. 2003). Phenanthrene concentration varied daily up to 50% and was maintained by adding appropriate amounts of phenanthrene ($\leq$23 µl). Microcosms without added toxicants and microcosms with ethanol, at the concentration used to solubilize phenanthrene, were used as controls (three replicates).

After 14 days of exposure, all microcosms were killed and the leaf disks were freeze-dried and weighed ($\pm$0.001 g) for determination of leaf dry mass remaining and then stored at −80°C for further assays. Sets of non-colonized leaf disks were used to estimate initial leaf dry mass.

Fungal Biomass

The concentration of ergosterol was measured to estimate fungal biomass associated with decomposing leaf disks. Sets of eight disks from each replicate microcosm were heated (80°C, 30 min) in 0.8% potassium hydroxide–methanol for lipid extraction, and the extract was purified by solid-phase extraction according to Gessner (2005). Ergosterol was quantified by high-performance liquid chromatography (HPLC; Beckmann Gold System; Brea, CA) using a LiChrospher RP18 column (Merck). The system was run isocratically with HPLC-grade methanol at 1.4 mL min^{-1} at 33°C. Peaks of ergosterol were detected at 282 nm, and standard series of ergosterol (Sigma) in isopropanol were used to estimate ergosterol concentration in the samples.

Fungal Diversity

After 10 and 14 days in microcosms, conidial suspensions were filtered (5-µm pore size; Millipore, Billerica, MA), and the spores were stained with cotton blue in lactic acid. Conidia were counted and identified under a microscope (400×; Leica Biomed, Heerbrugg, Switzerland).

DNA was extracted from sets of three leaf disks (one from each replicate) using an UltraClean Soil DNA kit (MoBio, Solana Beach, CA). The ITS2 region of fungal ribosomal DNA was amplified by polymerase chain reaction (PCR) with ITS3GC and ITS4 primers (White et al. 1990). The reaction mixture contained 4 µM each primer, 1 µL (approximately 50 ng) of DNA, 1.5 U Taq polymerase, 3 mM $MgCl_2$, 2 mM DNTPs, and 1 × Taq buffer ($KCl:(NH_4)_2SO_4$) in a final volume of 50 µL. Fungal DNA amplification was performed in an iCycler Thermal Cycler (BioRad, Hercules, CA), and PCR was started with denaturation of 2 min at 95°C, followed by 36 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 55°C, and extension for 1 min at 72°C. Final extension was at 72°C for 5 min (Nikolcheva and Bärlocher 2005; Duarte et al. 2008a).

PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) using a DCode Universal Mutation Detection System (BioRad). Samples of approximately 750 ng DNA were loaded on 8% polyacrilamide gel in 1× Tris–acetate-EDTA (TAE) with a denaturing gradient from 30 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). The gel was run at 55 V, 56°C for 16 h and was stained with 1 µg mL^{-1} ethidium bromide (BioRad). The images were captured under ultraviolet (UV) light in a transilluminator Eagle Eye II (Stratagene, La Jolla, CA).

Data Analysis

Leaf mass loss, fungal biomass, and sporulation were expressed as percentage of control. To achieve normal distribution and homocedasticity, data were arcsine square root-transformed (Zar 1996). Ethanol at the concentration used to solubilize phenanthrene did not significantly inhibit leaf mass loss, fungal biomass, and sporulation (Student t tests $p > 0.05$; Zar 1996). The effects of Cd and phenanthrene on leaf mass loss and ergosterol concentration were tested by two-way analysis of variance (ANOVA) (Zar 1996). Three-way ANOVA (Zar 1996) was used to test if Cd, phenanthrene, and exposure time affected sporulation rates and the number of leaf-associated aquatic hyphomycete taxa. Dunnett's post tests were performed to test which treatments significantly differed from control (Zar 1996). Statistical analyses were performed using Statistica 7.0 (Statsoft, Tulsa, OK).

The DGGE gel was aligned and normalized using Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium). Each DGGE band was treated as an individual operational taxonomic unit (OTU) and the number of OTUs was used as a measure of fungal diversity. A correspondence analysis (CA; Legendre and Legendre 1998) was used to ordinate treatments using data on fungal community structure based on sporulating species or OTUs (as relative intensity of each band in DGGE fingerprinting). The analyses were performed using CANOCO 4.5 (Microcomputer Power, Ithaca, NY).

Results

Effects of Cd and Phenanthrene on Fungal Activity

In the absence of Cd and phenanthrene, alder leaves lost 54 mg during 24 days (10 days colonization in the stream

plus 14 days in microcosms) corresponding to 56% of their initial dry mass. After leaf retrieval from the stream, fungal biomass on leaves was 78 μg ergosterol g^{-1} dry mass and attained 339 μg ergosterol g^{-1} dry mass at the end of the experiment. Fungal sporulation rate on decomposing leaves was 2.94×10^5 and 0.31×10^5 conidia g^{-1} dry mass day^{-1} after 10 and 14 days in microcosms, respectively.

Leaf-mass loss was significantly affected by Cd, phenanthrene, and the interaction between both toxicants (two-way ANOVA, $p < 0.01$ for all factors) (Fig. 1a). Significant inhibition effects were found after the exposure

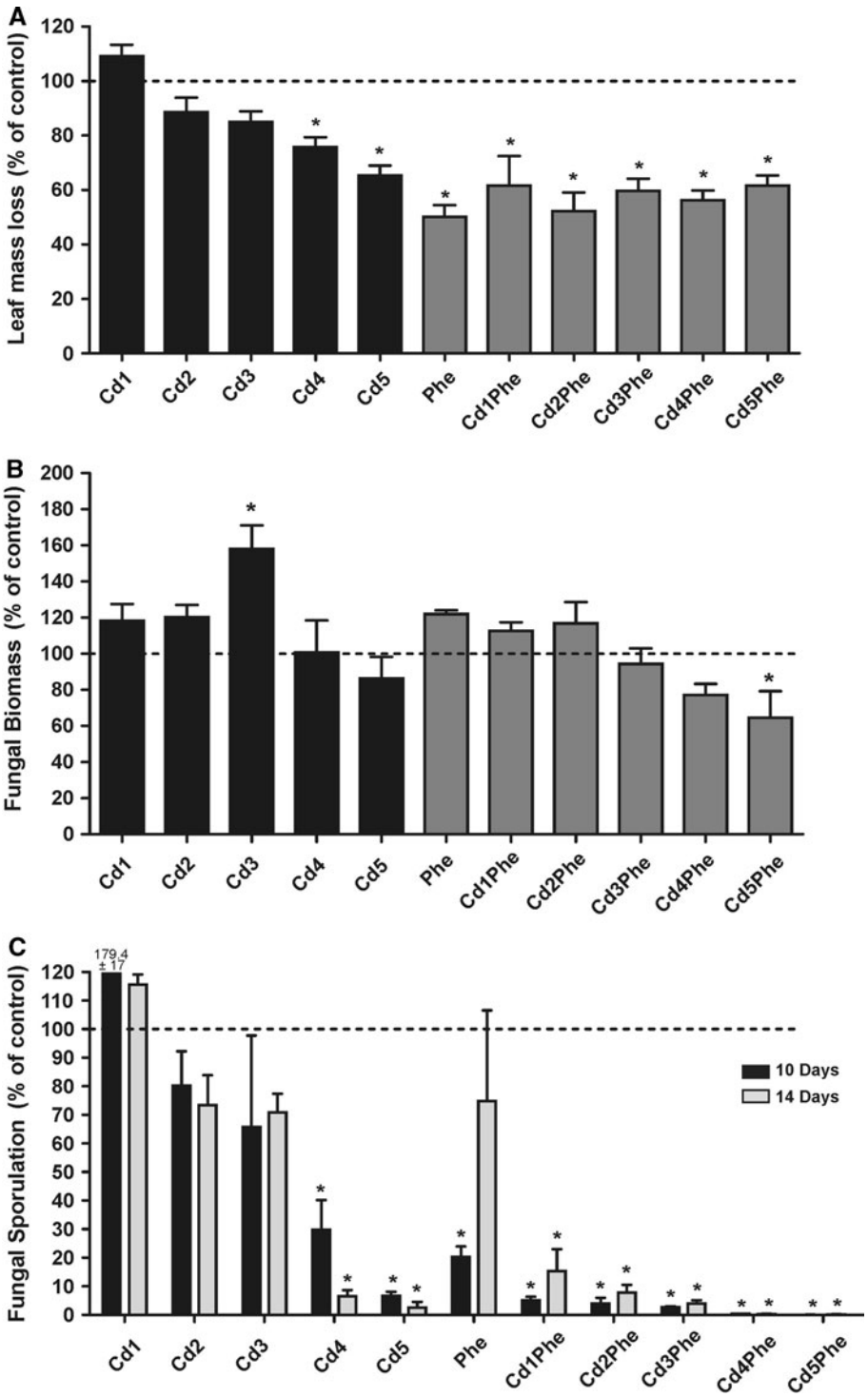

Fig. 1 Effects of Cd and/or phenanthrene on leaf-mass loss (**a**), fungal biomass (**b**), and fungal sporulation (**c**). Leaf-mass loss and fungal biomass were measured after 14 days of exposure, whereas fungal sporulation was measured after 10 and 14 days of exposure. $n = 3$, *error bars* indicate ± 1 SEM; * $p < 0.05$. Treatments were Cd1 = 0.06, Cd2 = 0.6 Cd3 = 1.2, Cd4 = L^{-1}, and Cd5 = 4.5 mg L^{-1} Cd. Phe (phenanthrene) = 0.2 mg L^{-1}

to Cd at high concentrations (≥3.6 mg L^{-1}; Dunnett's test, $p < 0.05$) and to phenanthrene alone or in mixture with Cd (Dunnett's test, $p < 0.01$).

Fungal biomass on decomposing leaves was significantly affected by Cd and phenanthrene (two-way ANOVA, $p = 0.001$ and $p = 0.003$, respectively) (Fig. 1b). A slight increase in biomass production was found at low Cd concentrations (with or without phenanthrene); this was significant in only one treatment (1.2 mg L^{-1}). Significant inhibition was found after exposure to mixtures of phenanthrene and Cd at the highest concentration (4.5 mg L^{-1}; Dunnett's test, $p < 0.05$).

Sporulation of aquatic hyphomycetes was significantly affected by the concentration of Cd and phenanthrene (three-way ANOVA, $p < 0.001$, for both comparisons) (Fig. 1c) but not by exposure time (three-way ANOVA, $p = 0.1$). Moreover, interactions between phenanthrene and exposure time and between the two toxicants were significant (three-way ANOVA, $p = 0.008$ and $p < 0.001$, respectively; Fig. 1c). At both exposure times, sporulation rate was inhibited by Cd concentrations ≥3.6 mg L^{-1} (Dunnett's test, $p < 0.05$) and by mixtures of Cd and phenanthrene at concentrations of Cd ≥ 0.06 mg L^{-1} (Dunnett's test, $p < 0.05$).

Effects of Cd and Phenanthrene on the Structure of the Fungal Community

During the study, 19 species of aquatic hyphomycetes were found sporulating on alder leaves (Table 1). Exposure to increasing Cd concentrations led to a decrease in the number of fungal species, particularly in microcosms supplemented with the highest Cd concentration (4.5 mg L^{-1}), in which only 8 fungal species were observed (Table 1). *Articulospora tectracladia* was the dominant species in control microcosms, contributing to 40% to the total conidial production. Exposure to the toxicants, especially in mixture, led to a decrease in the

Table 1 Fungal diversity on decomposing leaves as number and composition of sporulating species (after 10 and 14 days) and number of OTUs from DGGE fingerprints (after 14 days) after the exposure to Cd and phenanthrene alone or in mixture

Abb	Species	% of conidia											
		Ct	Cd1	Cd2	Cd3	Cd4	Cd5	Phe	Cd1Phe	Cd2Phe	Cd3Phe	Cd4Phe	Cd5Phe
AA	*A. acuminata* Ingold	1.8	1.7	3.6	2.7	10.4	1.0	13.0	–	0.1	0.5	–	–
AP	*A. pulchella* Marvanová	20.4	34.4	63.4	57.7	33.0	31.8	29.8	41.5	72.8	88.6	39.8	54.2
AF	*Anguillospora filiformis* Greath.	0.5	0.1	–	–	–	–	–	–	–	–	–	–
AT	*A. tetracladia* Ingold	40.0	19.8	14.7	1.4	4.1	17.7	7.7	15.1	2.1	0.7	–	6.3
CA	*Clavariopsis aquatica* De Wild.	2.0	0.1	<0.1	0.5	–	0.2	–	–	–	–	–	–
CL	*Clavatospora longibrachiata* (Ingold) Marvanová & Sv. Nilsson	15.9	36.6	2.8	0.1	0.6	–	–	–	–	–	–	–
DF	*Dimorphospora foliicola* Tubaki	–	–	–	–	–	–	0.8	0.1	–	–	–	–
LA	*Lemonniera aquatica* De Wild.	0.9	0.9	1.7	5.8	28.5	41.3	–	1.5	7.1	2.1	22.2	4.2
LC	*Lunulospora curvula* Ingold	0.2	0.3	<0.1	–	–	–	–	–	–	–	–	–
TE	*T. elegans* Ingold	6.6	4.1	12.2	27.5	12.8	–	33.7	38.5	16.3	5.4	2.3	4.2
TB	*Tetracladium breve* A. Roldán	0.1	0.1	0.4	0.1	–	–	0.6	–	–	–	–	–
TSt	*T. setigerum* (Grove) Ingold	–	–	–	–	–	0.2	0.8	–	–	–	–	–
TSp	*Tricladium splendens* Ingold	1.3	1.0	0.5	0.6	1.6	0.5	2.2	0.8	0.1	1.3	4.0	–
TA	*Triscelophorus cf. acuminatus* Nawawi	0.3	–	–	–	–	–	–	–	–	–	–	–
Un	Unknown branched species (50–30 μm)	0.4	0.2	0.7	–	–	7.5	0.1	2.4	1.1	1.5	31.8	31.3
S1	Sigmoid 1 (80–2 μm)	0.3	0.1	–	0.1	0.2	–	–	–	–	–	–	–
S2	Sigmoid 2 (50–1.5 μm)	0.2	0.1	–	–	–	–	–	–	–	–	–	–
S3	Sigmoid 3 (20–4 μm)	9.4	0.1	–	3.7	9.0	–	11.4	0.2	0.2	–	–	–
S4	Sigmoid 4 (110–1.5 μm)	<0.1	0.2	0.1	–	–	–	–	–	0.1	–	–	–
	Sum of conidial production	100	100	100	100	100	100	100	100	100	100	100	100
	No. of conidial morphotypes	17	16	12	11	9	8	10	8	9	7	5	5
	No. of DGGE OTUs	18	19	16	14	14	13	13	12	12	10	10	10

Treatments were Cd1 = 0.06, Cd2 = 0.6, Cd3 = 1.2, Cd4 = 3.6, and Cd5 = 4.5 mg L^{-1} Cd. Phe (phenanthrene) = 0.2 mg L^{-1} Ct = control; Abb = species abbreviation

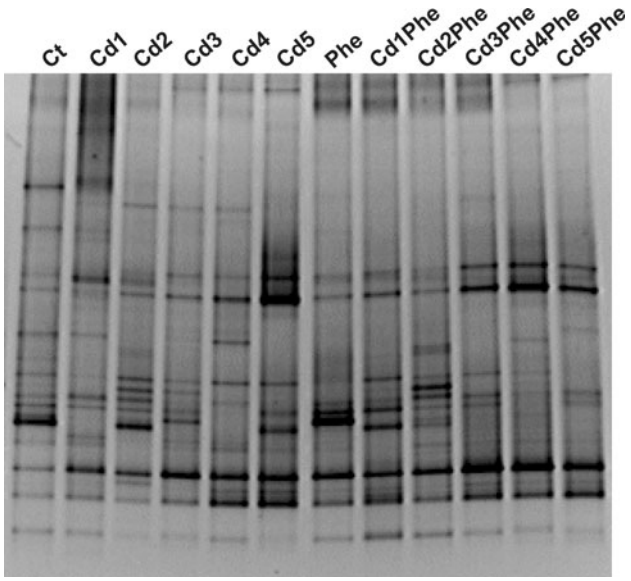

Fig. 2 DGGE fingerprints of the ITS2 region of rDNA of fungal communities on decomposing leaves. Treatments were Cd1 = 0.06, Cd2 = 0.6 Cd3 = 1.2, Cd4 = L^{-1}, and Cd5 = 4.5 mg L^{-1} Cd. Phe (phenanthrene) = 0.2 mg L^{-1}

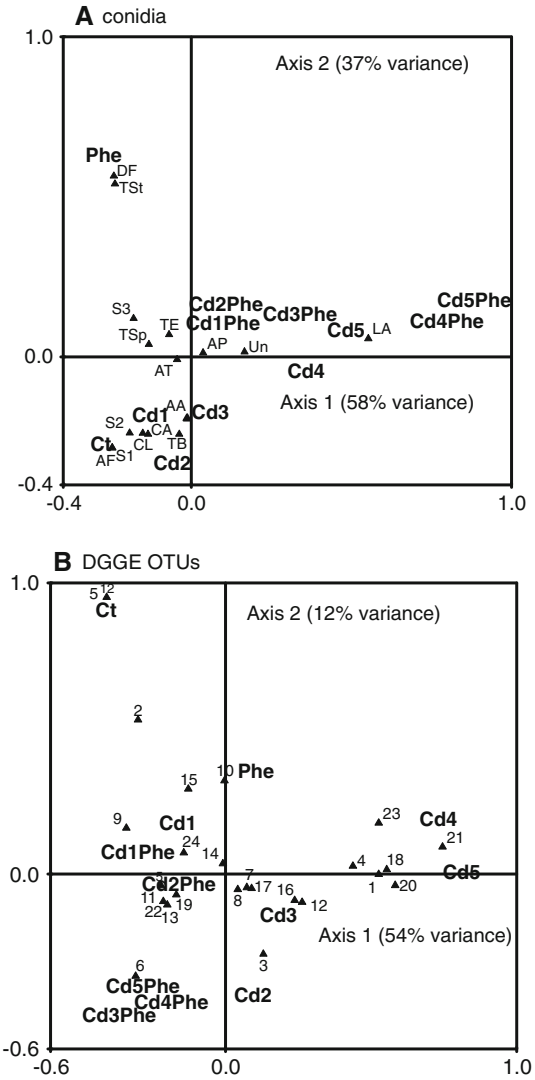

Fig. 3 CA diagrams for ordination of treatments using data of fungal community structure based on the 19 sporulating species (**a**) and on the 24 DGGE OTUs (**b**). Communities were exposed to Cd and/or phenanthrene for 14 days in microcosms. See Table 1 for species names and abbreviations in (**a**). Treatments were Cd1 = 0.06, Cd2 = 0.6 Cd3 = 1.2, Cd4 = L^{-1}, and Cd5 = 4.5 mg L^{-1} Cd. Phe (phenanthrene) = 0.2 mg L^{-1}

contribution of *A. tetracladia* (Table 1). In contrast, the exposure to Cd, alone or in mixture with phenanthrene, increased the contribution of the codominant species *Alatospora pulchella*. *Lemmoniera aquatica* seemed to be tolerant to Cd because its contribution increased with metal concentration, attaining 41.3% of the total conidial production at the highest Cd concentration (4.5 mg L^{-1}). Phenanthrene increased approximately 5 times the contribution of *Tetrachaetum elegans* to conidial production in the absence or presence of the lowest Cd concentration. An unidentified species became codominant in mixtures with high Cd concentrations.

DGGE of DNA of fungal communities on decomposing leaves showed 18 OTUs in control microcosms after 14 days (Table 1; Fig. 2). Exposure to increasing Cd concentrations led to a decrease in the number of OTUs, particularly in the presence of phenanthrene. Generally, higher fungal diversity was found from DGGE than from conidial identification.

The CA ordination of treatments using data of fungal communities based on conidial morphology and DGGE OTUs showed that exposure to Cd and phenanthrene altered the structure of the fungal community, with stronger effects for communities exposed to mixtures of both stressors (Fig. 3). Fungal communities based on conidial morphology were distributed along the gradient of Cd defined by the first axis (58% of the total variance) in the absence or presence of phenanthrene (Fig. 3a). The second CA axis (37% of the total variance) separated communities exposed to phenanthrene from all the others. Fungal communities based on DGGE OTUs were distributed along the gradient of Cd defined by the first axis (54% of the total variance), whereas the second axis (12% of the total variance) separated control communities from all the others and further separated communities exposed to mixtures of phenanthrene with increasing Cd concentrations (Fig. 3b).

Discussion

In the current study, the exposure of freshwater microbial decomposers to high Cd concentrations (≥3.6 mg L^{-1}) led

to a decrease in leaf decomposition. This might be explained by the reported negative effects of Cd on the activity of fungal extracellular enzymes that degrade plant cell-wall polymers (Baldrian et al. 1996). A poor use of resources was consistent with the observed decrease in fungal activity as sporulation rates in treatments with the highest Cd concentrations, particularly in the presence of phenanthrene. In previous studies, concentrations of Cd $\leq$ 0.1 mg L^{-1} inhibited growth and sporulation of pure cultures of aquatic hyphomycetes (Abel and Bärlocher 1984). However, in our study, low Cd concentrations did not inhibit, or tended to stimulate, fungal biomass as found in soil microbes (Shentu et al. 2008). It has been reported that fungi are able to grow at concentrations that inhibit fungal reproduction (Abel and Bärlocher 1984; Duarte et al. 2004, 2008a, 2009). Moreover, biomass of fungi on decomposing leaves is less sensitive to metals than that of bacteria (Duarte et al. 2008a, 2009). This agrees with the increased contribution of soil fungi to the total microbial biomass at low Cd concentrations (Shentu et al. 2008). Antagonistic relations between fungi and bacteria have been reported to occur during aquatic decomposition of plant litter (Mille-Lindblom and Tranvik 2003); therefore, if a decrease of bacterial biomass occurs under metal stress, this may weaken the antagonistic interactions and thus benefit fungi.

Although there are no studies in literature about the joint effects of metals and PAHs on aquatic microbially mediated processes, Cd and phenanthrene are reported to have variable effects on organisms depending on the concentration and magnitude of interactions between toxicants (Moreau et al. 1999). For instance, the exposure to a mixture of the two toxicants resulted in antagonistic effects on the feeding rate of the oligochaete *Ilyodrilus templetoni* (Gust and Fleeger 2006). In another study, Cd combined with phenanthrene decreased grazing rates of the copepod *Schizopera knabeni*, but no interactive effects on feeding were found (Silva et al. 2009). In soils, the addition of metals and PAHs, including Cd and phenanthrene, led to a greater biocidal effect on microbes when toxicants were amended together than alone (Maliszewska-Kordybach and Smreczak 2003; Shen et al. 2005). In our study, phenanthrene appeared to accentuate the negative effects of Cd because the highest inhibitions of fungal biomass and sporulation rates were in treatments with both toxicants.

The inhibitions of fungal biomass and sporulation by the toxicants were probably due to aquatic fungi channeling part of the energy available for growth and reproduction to the synthesis of compounds and/or enzymes involved in cellular detoxification processes. Indeed, aquatic hyphomycetes have been reported to be able to trigger defense mechanisms against metal exposure by increasing the activity of antioxidant enzymes (Azevedo et al. 2007, 2009; Braha et al. 2007) by producing thiol-containing compounds to sequester metal ions or scavenge reactive oxygen species (Guimarães-Soares et al. 2007; Jaeckel et al. 2005; Miersch et al. 1997). In contrast, some studies have reported that white-rot fungi are able to metabolize PAHs, including phenanthrene, by the phase I enzymes cytochrome P-450 monooxygenase and epoxide hydrolase (Bezalel et al. 1996, 1997; Capotorti et al. 2004). Other enzymes, such as manganese peroxidase, are also involved in the degradation of phenanthrene by white-rot fungi (Baborová et al. 2006). However, the addition of phenanthrene led to a more pronounced effect of Cd on the activity of several enzymes produced by soil microorganisms (Shen et al. 2005).

A decrease in aquatic hyphomycete diversity has been found in metal-polluted streams (Bermingham et al. 1996; Niyogi et al. 2002; Pascoal et al. 2005b; Sridhar et al. 2001). In addition, in our study, the exposure to increasing Cd concentrations caused a decrease in the number of fungal taxa, assessed as conidial morphotypes or DGGE OTUs, with the highest decreases in treatments with the highest Cd concentrations and when both toxicants were added together ($\sim$2–3 times lower than control microcosms). However, the effects did not appear to be so drastic when diversity was assessed based on DGGE. This is not surprising because analysis based on DNA offers the advantage of detecting species from nonsporulating mycelia, and sporulation has been consistently found to be sensitive to pollutants (Duarte et al. 2004, 2008a; Niyogi et al. 2002; Sridhar et al. 2001, 2005). Moreover, we cannot rule out the hypothesis that other fungal taxa than aquatic hyphomycetes could be present on decomposing leaves (see Seena et al. 2008).

Exposure to the toxicants also induced shifts in the structure of fungal community as shown by multivariate analyses based on both sporulating species or OTUs. In this and other studies, microbial communities exposed to high metal concentrations or mixtures of pollutants were most different from control communities (Duarte et al. 2004, 2008a, 2009). In our study, the dominance pattern of sporulating fungal species was affected by exposure to both toxicants; the dominant species in control, *A. tetracladia*, showed high sensitivity to toxicants similar to that found in field observations (Duarte et al. 2008b; Pascoal et al. 2005b), whereas *A. pulchella* was the most tolerant species to Cd, even in the presence of phenanthrene. Some species of aquatic hyphomycetes, such as *H. lugdunensis* and *Flagellospora curta*, have been reported to tolerate high levels of Cd (Azevedo and Cássio 2010; Braha et al. 2007; Guimarães-Soares et al. 2007) or to metabolize PAHs, such as naphtol (Augustin et al. 2006). Interestingly, in our study, some species were even stimulated by phenanthrene,

as in the case of the unknown species, which had increased spore production with exposure to phenanthrene.

Overall, results show that the presence of phenanthrene accentuated the deleterious effects of Cd on the diversity and activity of aquatic fungal decomposers, suggesting that the co-occurrence of these stressors may pose additional risk to aquatic biodiversity and stream ecosystem functioning. However, more experiments are needed to better understand the interactions between Cd, phenanthrene, and other stressors and to predict their impacts on aquatic detritus food webs.

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