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Contamination by uranium mine drainages affects fungal growth and interactions between fungal species and strains

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Abstract: The presence of aquatic hyphomycetes has been reported for several heavy metal-contaminated waters. Tolerance probably is one adaptation to coping with heavy metals. To help clarify this issue strains of two species of aquatic hyphomycetes (*Tricladium splendens* Ingold and *Varicosporium elodeae* Kegel) were isolated from a reference stream and a stream contaminated with heavy metals and grown on malt extract agar prepared with reference and contaminated water to characterize colony morphology, growth rate, growth inhibition and interaction among species and strains. In *V. elodeae* the morphology of colonies differed between strains. Colony diameter increased linearly over time with growth rates being lower for strains isolated from contaminated than from reference streams (mostly for *V. elodeae*). Strains from the contaminated stream grew faster in medium prepared with contaminated water than in medium prepared with reference water, while for strains from the reference stream there was no significant difference in growth rates on the two media. In interacting isolates radial growth toward the opposing colony was generally lower than toward the dish edge. Percentage growth inhibition was higher for isolates in intraspecific interactions (13–37%) than in interspecific interactions (3–27%). However differences in growth inhibition experienced by interacting isolates were observed only in three cases out of 16. The difference between the percentage inhibition caused and experienced by a given isolate was highest in interactions involving isolates with distinct growth rates. Our results suggest that strains from the reference stream tolerate heavy metals while strains from the contaminated stream seem to be adapted to contaminated waters. We hypothesize that in natural environments fungal species-specific limits of tolerance to metal contamination might determine an abrupt or gradual response of the original fungal community to mine pollution giving origin to a poorer fungal community dominated by adapted strains with distinct functional efficiency.

Key words: aquatic hyphomycetes, growth, interspecific interactions, intraspecific interactions, mine contamination, strains

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INTRODUCTION

Heavy metal contamination from mining and industrial activities is an important worldwide stressor of freshwaters with negative effects on aquatic communities even after activity cessation (Clements et al. 1992, Labrot et al. 1999, Niyogi et al. 2002). In central Portugal mining of radioactive ores lasted 90 y (1909–2001), and although all radium-uranium mines are closed tailings still exist in the open air and leaching introduces heavy metals to surrounding freshwaters (Carvalho et al. 2006, 2007; Antunes et al. 2007). Uranium mine drainage consists of a mixture of heavy metals (Antunes et al. 2007), many of which with known deleterious effects on aquatic hyphomycetes. Streams running through heavy metal-contaminated catchments usually have lower fungal species richness and diversity than their counterparts from noncontaminated areas (Bermingham et al. 1996, Raghu et al. 2001, Niyogi et al. 2002, Baudoin et al. 2008). Growth and sporulation of aquatic hyphomycetes usually are depressed by increasing concentrations of cadmium, copper and zinc (Abel and Bärlocher 1984, Miersch et al. 1997, Jaeckel et al. 2005) and community structure is altered by high concentrations of the last two (Duarte et al. 2004, 2008). However there seems to be a certain level of tolerance/adaptation of aquatic hyphomycetes to heavy metals because they have been found in highly contaminated waters (Sridhar et al. 2000, Krauss et al. 2001). Several studies reported higher tolerance of fungal strains isolated from contaminated streams to the contaminant metal than of conspecific strains isolated from reference streams (Chamier and Tipping 1997, Miersch et al. 1997, but see Baldrian and Gabriel 2002), which might suggest genetic adaptation. Furthermore strains of *Heliscus*

lugdunensis isolated from streams differing in heavy metal concentrations differed in conidia morphology and enzymatic activity (Braha et al. 2007). These differences might affect growth and interspecific interactions, with affects on community structure, leaf decomposition and nutrient cycling on lotic systems.

Several species of aquatic hyphomycetes have been found in watercourses contaminated with uranium (e.g. Ribeira da Pantanha, Urgeiriça mine; GenBank accession numbers 1219853, 1219862, 1219867); however little is known about the importance of changes in water quality on fungal species characteristics and ecological interactions. Considering the key role of fungi as leaf litter processors and their importance as modulators of invertebrate feeding activities (e.g. Canhoto and Graça 2008), it is essential to understand the functioning of ecosystems affected by anthropogenic activities such as mining.

Here we characterize colony morphology, growth rate, growth inhibition and interaction of strains of two species of aquatic hyphomycetes isolated from a reference stream and a stream contaminated by uranium drainages and grown in reference and contaminated media in the laboratory. We hypothesize that if the presence of these species in the contaminated stream is due to tolerance to increases in metal concentrations then both strains are expected to perform equally well in both media, while if the presence of these species is explained by genetic adaptation to the contaminants then each strain is expected to perform better in the medium prepared with water from the stream of origin.

MATERIALS AND METHODS

Aquatic hyphomycete isolates.—Strains of *Tricladium splendens* Ingold and *Varicosporium elodeae* Kegel were isolated from a reference stream (Ribeira de Alhões, 40°30′N, 7°52′W) and a stream contaminated with heavy metals (Ribeira da Pantanha, 40°59′N, 8°00′W), from single conidia associated with submerged leaves. Both streams are in central Portugal and have granitic substratum and vegetation composed mainly of *Alnus glutinosa* (L.) Gaertner, *Quercus robur* L. and *Castanea sativa* Miller. The contaminated stream receives drainage from an abandoned uranium mine, Urgeiriça, Nelas (Carvalho et al. 2006, 2007), resulting in increases of 8–371-fold in ionic concentrations, 15-fold in nitrogen concentration and up to 41-fold (U) in heavy metal concentrations relative to the reference stream. The reduction in ionic concentration in the contaminated stream in 2009 compared to 2008 was due to increased stream discharge after a rainy period in 2009 (TABLE I). Colonies were grown 15 d in malt extract agar medium (Difco; 10 g MEA L^{-1} distilled water) at 20 C before use.

TABLE I. Chemical composition of the reference and contaminated water used for the MEA media (2009) and of contaminated water sampled once in 2008

	Reference water	Contaminated water	
	2009	2008	2009
$N\text{-}NO_3$ (mg L^{-1})	0.2	0.5	3.4
SRP (μg L^{-1})	29.6	—	20.81
Ca (mg L^{-1})	0.3	218.3	126.2
Mg (mg L^{-1})	0.2	36.5	20.7
K (mg L^{-1})	0.1	4.1	2.1
Na (mg L^{-1})	1.6	50.4	25.0
Cl (mg L^{-1})	6.2	73.0	49.3
U (μg L^{-1})	0. 7	179.0	27.8
Fe (mg L^{-1})	0.04	0.09	0.24
As (μg L^{-1})	0.2	0.6	1.2
Zn (mg L^{-1})	0.01	0.11	0.02
Hg (μg L^{-1})	0.2	—	0.6
Cu (μg L^{-1})	15.7	15.8	12.2

Incubation media.—Malt extract agar media (10 g MEA L^{-1}) were prepared with reference water and contaminated water collected respectively from the reference and contaminated stream. Water was filtered (Millipore, APFF, 0.7 μm) and kept on ice until MEA media were prepared. Two subsamples of filtered water were poured into acid-washed plastic bottles and frozen at −18 C for later determination of nitrogen (ionic chromatography; Dionex DX-120, Sunnyvale, California) and soluble reactive phosphorus (SRP; ascorbic acid method; APHA 1995). A third subsample was acidified with HNO_3 (65%) to pH 2 for uranium (fluorometric analysis; Fluorat-02-2M analyzer, Lumex, Russia; Paulo et al. 2006) and other heavy metals determination (atomic absorption spectrophotometer; SOLAAR M Series, Thermo Electron Corp., Massachusetts). MEA media were autoclaved (121 C, 15 min) and used to fill 9 cm diam Petri dishes (20 mL) under aseptic conditions. Incubations took place at 20 C, under natural light/dark photoperiod Apr 2009.

Growth.—Growth rates of both species and strains were assessed in MEA medium prepared with reference or contaminated stream water in a fully factorial design with three replicates per treatment. Petri dishes were inoculated in the center with one agar plug (3 mm diam) cut from the leading edge of a growing colony (15 d old). Colony diameter was measured (± 0.5 mm) in two perpendicular directions at regular intervals for 17 (*V. elodeae*) and 24 (*T. plendens*) d, that is until the colony was ~ 1 cm from the dish edges (Rajashekhar and Kaveriappa 2000, Treton et al. 2004). Colonies diameter over time was compared among treatments by repeated measures ANOVA (species, strain and medium as categorical variables) followed by Tukey's test, with Statistica 6 software. Growth (mm d^{-1}) was determined from the slope of the linear regression between colony diameter and time (with free intercept).

Interactions.—Interactions between species and strains were assessed in Petri dishes filled with MEA medium prepared

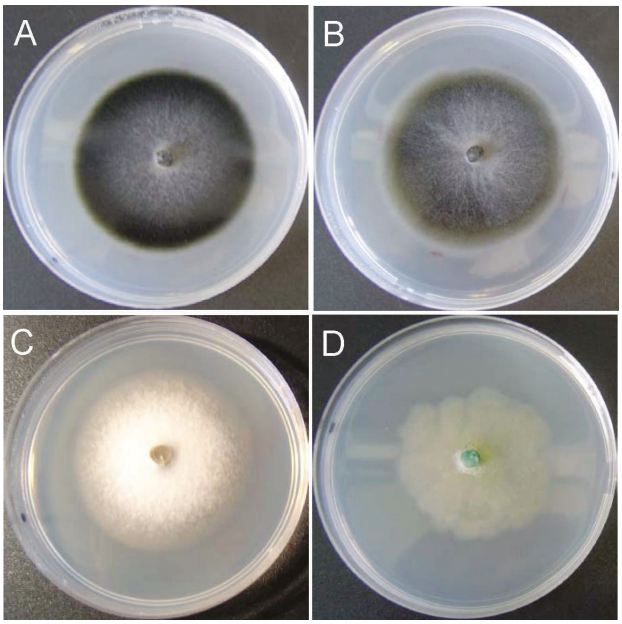

FIG. 1. *T. splendens* (A, B) and *V. elodeae* (C, D) strains isolated from a reference (A, C) and a contaminated (B, D) stream, growing in reference medium. *T. splendens* colonies are 24 d old and *V. elodeae* colonies are 17 d old.

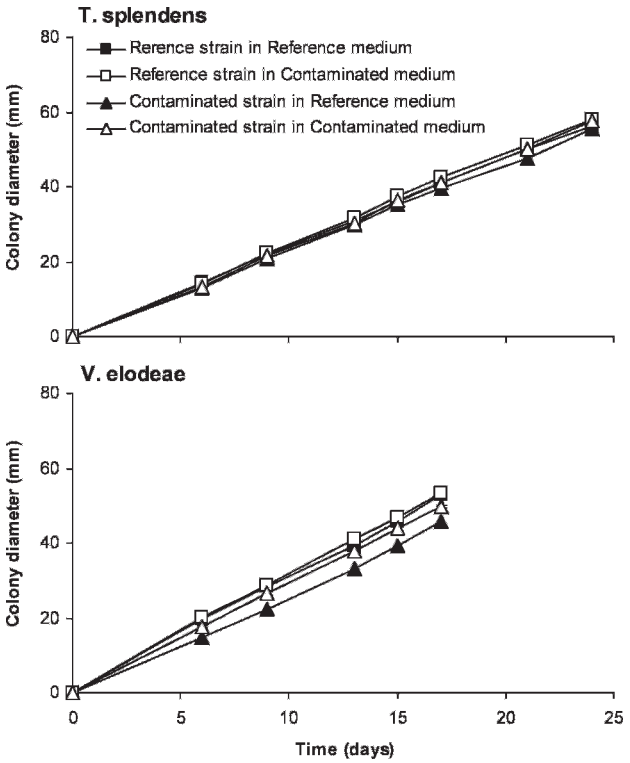

FIG. 2. Growth of *T. splendens* and *V. elodeae* strains isolated from a reference and a contaminated stream in MEA medium made with reference and contaminated stream water. SE bars are smaller than the symbols.

with reference or contaminated stream water in a fully factorial design with six replicates per treatment. There were six intraspecific interaction combinations (strain from reference stream times strain from reference stream, strain from contaminated stream times strain from contaminated stream, and strain from reference stream times strain from contaminated stream for each species) and two interspecific interaction combinations (*T. splendens* from reference times *V. elodeae* from reference and *T. splendens* from contaminated times *V. elodeae* from contaminated) for each medium type. Interactions were assessed by placing two agar plugs (3 mm diam), taken from the leading edge of growing colonies, at opposite sides of a Petri dish, 2.5 cm apart (Bärlocher 1991, Yuen et al. 1999). The interaction type between colonies was recorded, following Porter (1924) and Yuen et al. (1999), after 15 d or when the colonies met each other. The radial growth of each species, both toward the dish edge (R_1) and the opposing colony (R_2) (on a horizontal line across the center of both agar plugs), was measured (± 0.5 mm), and compared for each isolate by paired *t*-test. The percentage inhibition was calculated as $(R_1 - R_2)/R_1 \times 100$ (Shearer and Zare-Maivan 1988, Bärlocher 1991) and compared between opposing strains or species by paired *t*-test.

RESULTS

Growth.—Colony morphology and coloration did not differ between *T. splendens* reference and contaminated strains; colonies were black with a regular boundary resulting in a perfectly circular colony. On the other hand *V. elodeae* reference and contaminated strains were easily distinguishable; the reference strain was white with a regular boundary resulting in a perfectly circular colony, while the contaminated strain was green with an irregular boundary (FIG. 1).

Colony diameter increased linearly over time (FIG. 2) with growth 2.310–2.449 mm d^{-1} for *T. splendens* and 2.676–3.112 mm d^{-1} for *V. elodeae* (TABLE II). For *T. splendens* on contaminated medium no significant differences in growth rate between strains were observed (Tukey's test, $P = 0.096$), while in reference medium the reference strain grew faster than the strain isolated from the contaminated stream ($P = 0.041$); the reference strain did equally well in both media ($P = 0.115$), while the strain isolated from the contaminated stream grew faster in the contaminated medium ($P = 0.048$). For *V. elodeae* the reference strain grew faster than the strain isolated from the contaminated stream in both media (Tukey's test, $P < 0.008$); the reference strain had similar growth rates in both media ($P = 0.567$), while the strain isolated from the contaminated stream grew faster in the contaminated medium ($P = 0.001$) (TABLE II).

Interactions.—Radial growth toward the opposing colony was always lower than toward the dish edge (paired *t*-test, $P < 0.013$), except for *T. splendens* strain

TABLE II. Growth (mm d^{-1}) of *T. splendens* and *V. elodeae* strains isolated from a reference and a contaminated stream in MEA medium made with reference and contaminated stream water. Within the same species comparisons of colony diameter over time were made by repeated measures ANOVA followed by Tukey's test (different letters indicate significant differences at $P < 0.05$)

Species	Stream of origin	Medium	Growth rate	SE	R^2	P	Tukey's test
T. splendens	Reference	Reference	2.361	0.016	0.999	< 0.001	a
	Reference	Contaminated	2.449	0.025	0.998	< 0.001	a
	Contaminated	Reference	2.310	0.021	0.998	< 0.001	b
	Contaminated	Contaminated	2.417	0.018	0.999	< 0.001	a
V. elodeae	Reference	Reference	3.050	0.034	0.998	< 0.001	c
	Reference	Contaminated	3.112	0.036	0.998	< 0.001	c
	Contaminated	Reference	2.676	0.070	0.989	< 0.001	a
	Contaminated	Contaminated	2.914	0.028	0.999	< 0.001	b

from reference stream in the presence of *V. elodeae* strain from reference stream in contaminated medium where no inhibition occurred ($P = 0.275$). Nevertheless interactions between isolates were not extremely aggressive, with percentage growth inhibition 19–37% for intraspecific interactions within the same strain, 13–28% for intraspecific interactions between different strains and 3–27% for interspecific interactions. Yet only in three cases were there differences in the growth inhibition experienced by interacting isolates: (i) in reference medium *V. elodeae* strain from reference stream suffered a 27% inhibition in growth by, and caused a 12% inhibition in growth on, *T. splendens* strain of the same origin (paired *t*-test, $P = 0.010$); (ii) in contaminated medium *V. elodeae* strain from reference stream suffered a 24% inhibition in growth by, and caused a 3% inhibition in growth on, *T. splendens* strain of the same origin ($P = 0.005$); and (iii) in reference medium *V. elodeae* strain from reference stream suffered a 21% inhibition in growth by, and caused a 13% inhibition in growth on, *V. elodeae* strain from contaminated stream ($P = 0.031$) (TABLE III). Interaction among isolates were identified as being of three types: (i) mutual intermingling, when hyphae cross to the opposing colony in both directions (FIG. 3A, B); (ii) mutual inhibition at contact, when colonies of both species approach each other until the tips of the mycelium touch, and growth of both species ceases, which leads an area of low density mycelium between colonies (FIG. 3C, D); (iii) mutual inhibition at a distance, when colonies of both species approach each other until growth of both species ceases, which leads a space between colonies (FIG. 3E, F) (adapted from Porter [1924] and Yuen et al. [1999]) (TABLE III).

DISCUSSION

The presence of aquatic hyphomycetes in streams contaminated by uranium drainage confirms once again that at least some species of aquatic hyphomycetes may be tolerant while others may adapt to aquatic systems contaminated with heavy metals. In fact colony morphology of *V. elodeae*, but not of *T. splendens*, differed markedly between strains from reference and contaminated streams, irrespective of the growth medium, which might simply indicate intraspecific variability but also might suggest adaptation to the heavy metal concentrations in the stream water. Braha et al. (2007) also reported morphological differences on conidia produced by fungal strains isolated from streams differing in heavy metal concentrations. It therefore is plausible that distinct phenotypes might mirror resistance mutations (Bohannan and Lenski 2000).

Accordingly, and taking into account colony aspect, differences between strains were more pronounced for *V. elodeae* than for *T. splendens*. Growth of colonies was similar in pattern and rate to that observed for other species of aquatic hyphomycetes kept in similar conditions (Rajashekhar and Kaveriappa 2000). Growth of strains from the contaminated stream was generally lower than that of strains from the reference stream in both media. This might suggest that the strains from the reference stream, although tolerant to heavy metals, when historically submitted to the presence of heavy metal concentrations developed an adaptation as a result of a tradeoff between the energy channeled into growth and that required for resistance to high metal concentrations. Studies have explained the tolerance of some microorganisms to heavy metals by their ability to adsorb, bio-accumulate and/or transform metals (Bhainsa and D'Souza 1999, Singhal et al. 2004, Kalin et al. 2005, Fomina et al. 2007), for which energy might be required (Fowler 1987).

Strains from the reference stream had similar growth rates in both media, which indicates at least short term tolerance for heavy metals; it seems likely that the higher nitrogen concentration in the contaminated medium compensated to some extent for higher metal concentrations. Stimulation of fungal performance by increases in nutrient concen-

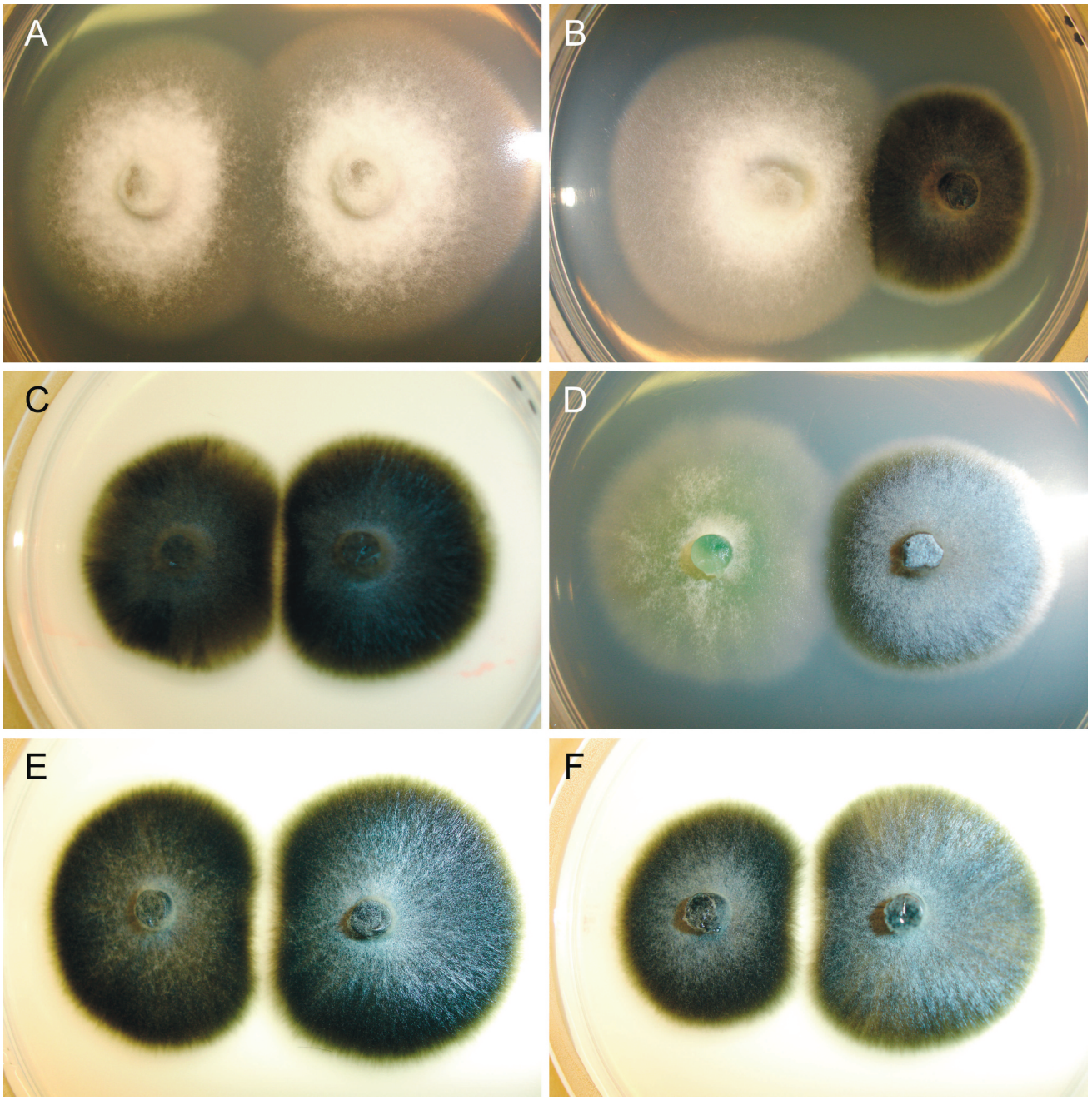

FIG. 3. Interaction types: mutual intermingling (A, *V. elodeae* from reference × *V. elodeae* from reference in reference medium; B, *V. elodeae* from reference × *T. splendens* from reference in contaminated medium), mutual inhibition at contact (C, *T. splendens* from reference × *T. splendens* from reference in contaminated medium; D, *V. elodeae* from contaminated × *T. splendens* from contaminated in contaminated medium), and mutual inhibition at a distance (E, *T. splendens* from reference × *T. splendens* from reference in reference medium; F, *T. splendens* from reference × *T. splendens* from contaminated in reference medium).

trations is well documented (Gulis and Suberkropp 2003, Ferreira et al. 2006). Whether this is a common situation in other metal-contaminated freshwaters is not clear. If so the presence of nitrogen might mitigate the potential deleterious effects of high metal concentration, contributing to resilience of a poorer fungal community in streams affected by mining wastewaters (Bermingham et al. 1996). On the other hand strains from the contaminated stream grew less in the reference than in the contaminated medium, which might indicate some degree of adaptation of these strains to high metal concentrations.

TABLE III. Inhibition of growth (average ± 1 SE) for each interacting species and interaction type (see text for definitions)

Medium	Interaction	Species	Stream of origin	Inhibition (%)	Species	Stream of origin	Inhibition (%)	Paired *t*-test (*P*)	Interaction type
Reference	Intraspecific	*T. splendens*	Reference	26 ± 3	*T. splendens*	Reference	24 ± 4	0.777	Mutual inhibition at a distance
	Intraspecific	*T. splendens*	Contaminated	26 ± 1	*T. splendens*	Reference	26 ± 1	0.936	Mutual inhibition at a distance
	Intraspecific	*T. splendens*	Contaminated	19 ± 3	*T. splendens*	Contaminated	20 ± 3	0.972	Mutual inhibition at a distance
	Intraspecific	*V. elodeae*	Reference	27 ± 2	*V. elodeae*	Reference	21 ± 1	0.108	Mutual intermingling
	Intraspecific	*V. elodeae*	Contaminated	13 ± 3	*V. elodeae*	Reference	21 ± 3	0.031	Mutual inhibition at contact
	Intraspecific	*V. elodeae*	Contaminated	21 ± 3	*V. elodeae*	Contaminated	29 ± 2	0.054	Mutual inhibition at contact
	Interspecific	*V. elodeae*	Reference	27 ± 3	*T. splendens*	Reference	12 ± 3	0.010	Mutual intermingling
	Interspecific	*V. elodeae*	Contaminated	22 ± 4	*T. splendens*	Contaminated	12 ± 3	0.179	Mutual intermingling
Contaminated	Intraspecific	*T. splendens*	Reference	24 ± 1	*T. splendens*	Reference	26 ± 5	0.726	Mutual inhibition at contact
	Intraspecific	*T. splendens*	Contaminated	28 ± 2	*T. splendens*	Reference	28 ± 2	0.946	Mutual inhibition at contact
	Intraspecific	*T. splendens*	Contaminated	21 ± 3	*T. splendens*	Contaminated	26 ± 2	0.201	Mutual inhibition at a distance
	Intraspecific	*V. elodeae*	Reference	24 ± 2	*V. elodeae*	Reference	29 ± 1	0.098	Mutual intermingling
	Intraspecific	*V. elodeae*	Contaminated	26 ± 2	*V. elodeae*	Reference	22 ± 1	0.230	Mutual intermingling
	Intraspecific	*V. elodeae*	Contaminated	37 ± 1	*V. elodeae*	Contaminated	32 ± 2	0.084	Mutual inhibition at contact
	Interspecific	*V. elodeae*	Reference	24 ± 2	*T. splendens*	Reference	3 ± 3	0.005	Mutual intermingling
	Interspecific	*V. elodeae*	Contaminated	17 ± 2	*T. splendens*	Contaminated	15 ± 3	0.746	Mutual intermingling

Studies have shown that strains from contaminated waters have higher tolerance to the contaminant metal than strains from reference streams (Chamier and Tipping 1997, Miersch et al. 1997).

For interacting isolates growth toward the opposing colony decreased compared to that toward the dish edge, which was a predicted outcome because isolates on the same dish compete for space and nutrients (Shearer and Zare-Maivan 1988, Bärlocher 1991, Yuen et al. 1999). However, contrary to expectations, inhibition of growth was higher in intraspecific interactions (within the same strain > between strains) than in interspecific interactions, although self inhibition was expected to be less pronounced than that between different strains or species (Bärlocher 1991). The difference between the percentage inhibition caused and experienced by a given isolate was highest in interactions involving isolates with distinct growth rates. Considering that a rich, solid media was used, this might be a result of competition for space as fast growing species rapidly extend their mycelium toward the opposing growing colony. This negative relationship between growth rate and percentage inhibition also was observed by Shearer and Zare-Maivan (1988) although not by Bärlocher (1991). Despite a nutrient-rich medium, we cannot rule out the possibility that there was nutrient depletion in the area between isolates or that there was production of metabolic products, which could have limited growth (staling; Robinson 1969, Arora and Upadhya 1978). Interactions between opposing isolates were classified as being of three types, with intermingling being the most common. This category includes the interactions where differences between the percentage inhibition caused and experienced by a given isolate were greatest. However in our study and in accordance with Bärlocher 1991 after both colonies had overgrown the Petri dish mutual intermingling was observed for all interaction pairs.

In conclusion differences in colony morphology and growth rates between strains (mostly for *V. elodeae*) and media (for strains from the contaminated stream) point to an adaptation of strains from the contaminated stream to heavy metal-contaminated waters. It seems possible that the adaptation evolved from a tolerance to metals presented by reference strains. These changes could justify the disappearance of the competitive advantage that the *T. splendens* strain from the reference stream had over *V. elodeae* from the same origin, not expressed when strain from the contaminated stream are opposed. We hypothesize that in natural environments fungal species-specific limits of tolerance to metal contamination might determine an abrupt or gradual response of the original fungal community to mine pollution, giving origin to a poorer fungal community dominated by tolerant strains with potentially distinct functional efficiency in relation to the community of origin. More research is needed at several levels of biological organization to understand the mechanisms that drive ecosystem processes (e.g. leaf decomposition) in metal-contaminated systems. At present it seems that metal-resistant strain communities might differ in their functional capabilities from original strains with potential consequences on stream nutrient cycling.

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