Science of the Total Environment 544 (2016) 168–174

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Copper tolerant ecotypes of *Heliscus lugdunensis* differ in their ecological function and growth

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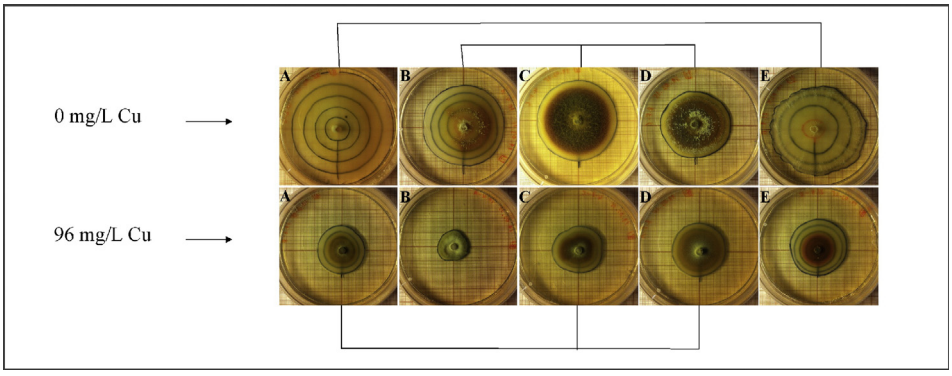
HIGHLIGHTS

- We studied intraspecific diversity in strains isolated from varying Cu gradients.
- 5 strains of *Heliscus lugdunensis* were grown on agar plates and in microcosms.
- Ecological function and growth were assessed under Cu exposure.
- Strains differed significantly in behaviour and morphology when exposed to Cu.
- Cu pollution may have led to evolved adaptations in species resulting in ecotypes.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 September 2015
Received in revised form 22 November 2015
Accepted 23 November 2015
Available online xxxx

Editor: D. Barcelo

Keywords:
Aquatic hyphomycetes
Intraspecific diversity
Metal tolerance
Mining
Fungal ecotypes
Litter decomposition

ABSTRACT

Metal tolerance in aquatic hyphomycetes varies with the level of pollution at the fungal isolation site. While the focus of previous research has been on the effects of metal exposure on interspecies diversity, intraspecies variation of aquatic hyphomycetes remains largely unexplored. In this study we investigate the effects of Cu on ecological function (litter decomposition) and growth of five strains of *Heliscus lugdunensis*, isolated from contaminated and un-contaminated streams, in order to examine whether strains are expressed as ecotypes with distinct growth and functional signatures in response to metal stress. When exposed to Cu, strains of *H. lugdunensis* differed significantly in their litter decomposition and reproductive activity (sporulation) as well as mycelial growth, corresponding to the Cu concentrations at their isolation site. Strains isolated from sites with high Cu concentrations induced the highest litter decomposition or invested most in growth. This study broadens our understanding of Cu pollution in streams, which may lead to evolved adaptations of Cu tolerant ecotypes of *H. lugdunensis* differing in their ecological function, behaviour and morphology when exposed to metals.

1. Introduction

Leaf litter from riparian zones is considered the main energy source for low order streams. Aquatic hyphomycetes decompose leaf litter and,

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http://dx.doi.org/10.1016/j.scitotenv.2015.11.119
0048-9697/

in this way, play an important ecological role in the energy transfer from riparian plant-litter to stream invertebrates and higher trophic levels as well as in nutrient cycling (Bärlocher, 1985; Graça et al., 2015; Krauss et al., 2011).

Metal pollution caused by industrial activities, particularly mining, poses risks to the environment and public health (De Miguel et al., 2014). At the individual scale, metal pollution in aquatic environments can have adverse effects on the ability of organisms to cope with oxidative stress, by inducing a misbalance between the production of antioxidant defences and reactive oxygen species within aquatic organisms (Mahboob, 2013; Valavanidis et al., 2006). The accumulation of metals in the aquatic food chain, through bio magnification, and their persistence in the environment are some of the characteristics thought to be the source of most pollution issues such as oxidative stress (Medeiros et al., 2010). At higher organizational levels, metal pollution is an evolutionary pressure, since metal sensitive genotypes are eliminated while only the resistant are able to reproduce. However, the differences in ecological performance between resistant and more sensitive organisms are yet to be fully understood in polluted aquatic systems. If one such genotype shift occurs in aquatic organisms which regulate major ecosystem functions, such as litter decomposition by aquatic hyphomycetes, shifts in the ecological balance can arise, revealing changes in ecological redundancy (Pascoal et al., 2005; Rosenfeld, 2002). In aquatic environments, one of the metals of concern is copper (Cu). In contrast to the metal concentration standards proposed by the European water framework directive, 0.001 mg/L (Defra, 2014), anthropogenic metal pollution can drastically increase Cu concentrations up to 240 mg/L in rivers affected by copper mining (Hudson-Edwards et al., 1999). In low concentrations, Cu is an essential metal that facilitates fungal lignin degradation by increasing transcription levels of laccase enzymes (Azevedo and Cássio, 2010; Baldrian, 2003). However, in high concentrations Cu inhibits fungal growth and reproduction in addition to affecting fungal capability to decompose litter (Azevedo and Cássio, 2010; Duarte et al., 2008; Miersch et al., 1997). The direct impact of metals on aquatic fungi can be assessed through investigations on physiological responses, such as growth and reproduction, and functional responses, such as litter decomposition.

Cu toxicity in aquatic hyphomycetes may cascade throughout the food chain causing changes in nutrient cycling and energy flow (Maltby, 2009). In response to high metal concentrations, some species of aquatic hyphomycetes have evolved tolerances by adapting biochemical responses such as antioxidant defences (Braha et al., 2007; Miersch et al., 1997) and adaptations in life history traits such as growth and sporulation (Azevedo and Cássio, 2010; Ferreira et al., 2010). Several studies reported that metal tolerance and conidial morphology of aquatic hyphomycete species vary according to the pollution levels at their isolation site, suggesting the possibility for genetic adaptation (Azevedo and Cássio, 2010; Braha et al., 2007; Ferreira et al., 2010). Whereas previous research has been mainly focusing on the effects of metal exposure on interspecies diversity of aquatic hyphomycetes, stress responses at intraspecies level remain largely unexplored. This raises the question of how in particular metal tolerant strains of aquatic fungal species differ in their ecological function in regard to litter decomposition and if they differentiate into ecotypes (Gadd, 1994; Sridhar et al., 2001). Here, the term "ecotype" is used to define fungal strains sampled from different areas or isolates from the same site, that exhibit differences in colony morphology and physiology when grown in standard media as well as challenging conditions (Haselwandter et al., 1994).

Heliscus lugdunensis is a ubiquitous aquatic hyphomycete commonly found in different aquatic environments, including metal polluted sites (Braha et al., 2007; Jaeckel et al., 2005; Miersch et al., 1997). We hypothesised that according to metal contamination at their site of origin, fungal strains would differ in their tolerance to Cu concentrations in terms of physiological and functional responses due to evolved intraspecific adaptations. The aim of this study was to explore whether strains of *H. lugdunensis* are expressed as ecotypes with distinct physiological and functional signatures in response to metal stress. Investigations were made on reproductive output, metabolic activity and decomposition activity as well as growth inhibition and growth rates of strains of *H. lugdunensis* isolated from reference streams, a moderately polluted stream and a highly polluted stream contaminated with Cu.

2. Materials and methods

2.1. Fungal activity under moderate Cu conditions

2.1.1. Experimental setup

2.1.1.1. Isolation of fungal strains. We used five fungal strains of *H. lugdunensis*, (A, B, C, D and E) isolated from different locations at Barroca River (A, B, D), Sinhel River (C) and Casinhas River (E) impacted respectively, by the Senhora da Guia, Escádia Grande and Panasqueira mines of Central Portugal in 2014. Fungal isolates were defined as strains based on their different sampling points, distances away from adjacent mines and/or based on morphology differences. Even though, strains A and B were isolated from the same site and same Cu concentration they were labelled as different strains since they showed clear differences in morphology prior to experiment begin as well as throughout the growth experiments. The isolation site of strains A and B was chosen as a reference site since the Cu concentration was closest to the Cu concentration standards proposed by the European water framework directive (Defra, 2014). The mean values of Cu concentration and other physicochemical parameters of the stream waters at the isolation sites in the year 2013–14, as well as distances of sampling points away from adjacent mines and mining activity are presented in Table 1. The Cu concentrations were measured by coupled graphite furnace Atomic Absorption Spectrometry (SOLAAR M Series equipment from Thermo–Unicam) and dissolved oxygen, electrical conductivity and pH were measured in situ with a multi-parameter instrument (Multi 340i, Geotech). NO_3, and PO_4 were determined by colorimetric method (Clesceri et al., 1998). The strains were maintained on 2% malt extract (SIGMA-ALDRICH) agar (SIGMA) on petri dishes at 15 °C and 12 h photoperiod.

2.1.1.2. Preparation of leaf litter discs. Alnus glutinosa (L.) Gaertn. (Alder) leaves were collected during Autumn from trees at Parque Verde do Mondego, Coimbra in 2014 and dried at room temperature. Alder leaves were chosen because it is a common riparian species in Europe and classified as good quality for consumers (Boyero et al., 2011). Leaves were leached for 16 h and 12 mm discs were punched out with a cork borer (Duarte et al., 2004). Sets of 20 discs each were autoclaved at 120 °C for 20 min (Uniclave 88, AJC), dried at 105 °C for 48 h (Thermo Scientific Heratherm) and weighed (AS 220/C/2, RADWAG) in sets to determine initial dry mass as well as incinerated at 550 °C for 6 h (Thermolyne F6000 Furnace) and weighted (UMX2, Mettler) in sets to determine ash free dry mass (AFDM).

2.1.2. Experimental design

To test for differences between strains ($n = 5$, replicates of 4) in decomposing leaf discs under increased Cu concentrations ($n = 3$), the sets of 20 weighted and dried leaf discs were introduced in Erlenmeyer flasks (150 ml capacity; $n = 60$). The flasks with leaf discs were randomly allocated for each strain and filled with 50 ml sterile distilled water for colonization of leaf discs with fungal strains. Fungal inoculates consisted of agar plugs (5 mm) taken from 1 month old overgrown fungal mycelium from each of the 5 strains and left for 6 days (Jaeckel et al., 2005). Microcosms were kept on an orbital shaker at 110 rpm and 15 °C (GFL 3017), 12 h photoperiod, and solutions were renewed by day three. At day six all microcosms were drained and allocated to one of the following treatments: (1) control 0 mg/L Cu;