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# Interactive effects of aluminium and phosphorus on microbial leaf litter processing in acidified streams: A microcosm approach

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ABSTRACT

Decline in pH, elevated aluminium (Al) concentrations, and base cations depletion often covary in acidified headwater streams. These parameters are considered as the main factors reducing microbial activities involved in detritus processing, but their individual and interactive effects are still unclear. In addition to its direct toxicity, Al can also reduce the bioavailability of phosphorus (P) in ecosystems through the formation of stable chemical complexes. A three week microcosm experiment was carried out in acid conditions to assess the interactive effects of Al (three levels: 0, 200, and 1000 μg $L^{-1}$) and P (25, 100, and 1000 μg $L^{-1}$) on alder leaf litter processing by an aquatic hyphomycete *consortium*. Our results showed that Al alone reduced fungal growth and altered fungal decomposer activities. High P levels, probably through an alleviation of Al-induced P limitation and a reduction of Al toxic forms, suppressed the negative effects of Al on detritus decomposition.

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## 1. Introduction

Leaf litter breakdown is a key process in woodland streams, where primary production is notably limited by the riparian canopy and the oligotrophic status of these streams (Vannote et al., 1980). Indeed, in such heterotrophic ecosystems, allochthonous litter inputs represent the major source of energy and nutrients for all the detritus-based food web (Fisher and Likens, 1972; Webster and Meyer, 1997). Various studies reported that this ecosystem process is severely impaired in headwater streams subjected to anthropogenic acidification (Chamier, 1987; Mulholland et al., 1987; Meegan et al., 1996; Dangles et al., 2004), mainly through its negative impacts on acid-sensitive key shredders (Dangles and Guérold, 1998, 2001; Simon et al., 2009) and on aquatic hyphomycetes involved in leaf litter processing (Chamier, 1987; Baudoin et al., 2008). In particular, the latter are considered as the main mediators of microbial leaf litter conditioning (Gessner and Chauvet, 1994), which is an essential step before leaf litter consumption by stream detritivores (Bärlocher and Kendrick, 1981; Arsuffi and Suberkropp, 1988).

For instance, several factors have been identified as potentially responsible for reduced activities of microbial decomposers in acidified streams. Acidic pH and low calcium (Ca) concentrations can notably reduce the efficiency of pectin lyase activity, which is involved in leaf litter maceration (Chamier and Dixon, 1982; Jenkins and Suberkropp, 1995). Other studies reported that aluminium (Al) mobilized from soils to surface waters as a result of acid depositions can affect microbial communities. Indeed, under acidic conditions, monomeric forms of Al are known to be toxic for most aquatic organisms (Gensemer and Playle, 1999) including microorganisms (Piña and Cervantes, 1996). Increasing Al concentrations can decrease microbial respiration on decaying leaves (Dangles et al., 2004) and alter growth and conidial production of aquatic hyphomycetes (Chamier and Tipping, 1997). In addition, recent studies on microbial enzyme activities suggested that acidification and, in particular, elevated Al concentrations in streams could induce a phosphorus (P) limitation for microbial decomposers that could, in turn, constrain leaf litter processing (Simon et al., 2009; Clivot et al., 2013b). Indeed, Al could interfere with the P cycle in acidified waters through abiotic interactions (Kopácek et al., 2000) and inhibition of extracellular phosphatases (Bittl et al., 2001), thus

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decreasing P availability and potentially limiting microbial decomposer activities (Grattan and Suberkropp, 2001).

While Al appears to be an important factor that could potentially alter microbial leaf litter decomposition (Clivot et al., 2013b), high Al concentrations and base cation depletion are generally associated with pH decline in impacted streams. Disentangling the influence of each factor in field-experiments is not facilitated by these covarying parameters, which could lead to confound their mutual effects. By isolating one or a few of these parameters, laboratory microcosm experiments could allow to accurately evaluate their respective effects on microbial decomposers.

The main objective of the current study was to assess, *in vitro* and in acidic conditions, the role of Al toxicity and the indirect effects of this metal on phosphorus availability for microbial decomposers. In a microcosm experiment, leaf litter disks pre-inoculated with an assemblage of 7 aquatic hyphomycete species were exposed in artificial acidic water to 9 different combinations of Al and P levels. After 3 weeks, we assessed leaf litter mass loss, fungal biomass and community structure, Al and nutrient content of leaves and acid phosphatase activities. We expected that i) under acid stress and low P concentrations, high Al concentrations would reduce leaf litter processing by impacting microorganisms and notably by increasing their P limitation and that, ii) a high level of P could alleviate the negative effects of Al on microbial leaf litter processing.

## 2. Material and methods

### 2.1. Leaf litter collection and conditioning

Senescent leaves of alder (*Alnus glutinosa* L.) were collected at abscission in October 2010, air-dried and stored at room temperature until used. Leaf litter disks (16-mm diameter) were cut avoiding the central vein of the leaf and sterilized in an autoclave (120 °C, 15 min). To remove soluble substances, the initial step of leaf litter leaching (Petersen and Cummins, 1974; Bärlocher, 2005) was performed during five days at 12 °C in flasks on a rotary shaker (70–90 rpm) with daily change of sterilized ultrapure Milli-Q water (Millipore, Molsheim, France).

### 2.2. Mineral medium

The mineral medium of our experiment was adapted from those used by Kilham et al. (1998) and Gessner and Chauvet (1993), but it was more diluted to more closely correspond to conditions of low mineralization that prevail in acidified headwater streams (see Guérold et al., 2000). This medium consisted of 7.4 mg of $CaCl_2$, 7.4 mg of $MgSO_4-7H_2O$, 8.5 mg of $NaNO_3$, 10.1 mg of $KNO_3$, 0.2 mg of $MnSO_4-H_2O$, 0.2 mg of $ZnSO_4-H_2O$, 0.02 mg of $Na_2MoO_4-2H_2O$, 0.02 mg of KI, 0.008 mg of $CoCl_2-6H_2O$ and 0.005 mg of $NiCl_2-6H_2O$ in 1 L of ultrapure Milli-Q water.

### 2.3. Experimental conditions

The mineral medium was supplemented with or without $AlCl_3$ to obtain 3 different levels of Al (0, 200 and 1000 μg Al $L^{-1}$, called $Al_0$, $Al_{200}$ and $Al_{1000}$, respectively), 200 and 1000 μg $L^{-1}$ corresponding to typical Al concentrations that can be found in moderately and highly impacted streams, respectively (Guérold et al., 2000; Baudoin et al., 2008).

Three levels of P (25, 100 and 1000 μg P $L^{-1}$, called $P_{25}$, $P_{100}$ and $P_{1000}$, respectively) were obtained by adding $NaH_2PO_4$ to the mineral medium. The concentration of 25 μg P $L^{-1}$ corresponds to the highest P concentrations found in oligotrophic aquatic systems (Dodds et al., 1998). Concentrations of 100 and 1000 μg P $L^{-1}$ were used to determine if a higher P supply can alleviate the potential Al-induced P limitation and/or mitigate the Al toxicity.

The pH of solutions was adjusted to 4.5 with HCl (0.1 M) in order to establish acidic conditions close to those found in acidified streams and at which bioavailable and soluble forms of Al predominate (Piña and Cervantes, 1996). Real P concentrations were (mean ± SD) 25.2 ± 5.9 μg $L^{-1}$, 98.8 ± 1.7 μg $L^{-1}$ and 983.9 ± 29.4 μg $L^{-1}$ in $P_{25}$, $P_{100}$, and $P_{1000}$, respectively, whereas real Al concentrations reached 207 ± 13 and 975 ± 57 μg $L^{-1}$ in $Al_{200}$ and $Al_{1000}$, respectively.

### 2.4. Fungal inoculum

Seven common aquatic hyphomycete species constituted the inoculum of leaf litter (*Alatospora acuminata* (ALAC), Ingold; *Anguillospora crassa* (ANCR), Ingold; *Clavariopsis aquatica* (CLAQ), De Wild.; *Clavatospora longibrachiata* (CLLO), Ingold; *Heliscus lugdunensis* (HELU), Sacc. and Therry; *Tetrachaetum elegans* (THEL), Ingold; and *Tricladium chaetocladium* (TRCH), Ingold). These species were selected as non-comigrating in PCR-DGGE analyses (for conditions, see PCR-DGGE part below) in order to follow distinctly each species contribution to fungal assemblage on leaf litter. Single conidial isolates of these aquatic hyphomycetes were previously obtained from streams and species were grown and maintained on 2% Malt Agar plates. For this study, subcultures of each species were grown in $P_{25}$ solution supplemented with 5 g $L^{-1}$ of glucose as carbon source. An equivalent biomass of each hyphomycete mycelium (~5 mg of mycelial dry mass) was rinsed twice with $P_{25}$ solution to eliminate carbon traces. The seven strains were then ground together in 30 mL of $P_{25}$ solution and the homogenate was used to inoculate leaf litter.

### 2.5. Microcosms

Four sets of 525 leaf disks were each inoculated with 1 mL of fungal mycelium homogenate in 500 mL Erlenmeyer flasks containing 250 mL of $P_{25}$ solution. Flasks were then incubated at 12 °C on a rotary shaker for five days to allow fungal colonization of leaf litter. After this inoculation step, disks were placed in microcosms or were used to determine initial leaf parameters.

Seventy-five mL glass aeration chambers simulating stream turbulence conditions (Suberkropp, 1991; Dang et al., 2005) were used to perform the microcosm experiment. Sets of 40 leaf disks were randomly distributed in 36 microcosms containing 50 mL of solution from one of the 9 different combinations of Al and P (4 replicates each). Microcosms were incubated in the dark at 12 °C for 3 weeks. To limit changes in water physico-chemical properties, mineral media were renewed 3 times a week.

### 2.6. Water analysis and Al concentrations and speciation

Water analysis were performed on initial solutions and on solutions collected at the end of the experiment, *i.e.* on the last date of the experiment after 3 days of leaf litter incubation (all solutions being renewed every 2 or 3 days). Ion concentrations in the solutions were determined by inductively coupled plasma atomic emission spectroscopy using a JY38-Plus ICP-AES apparatus (K, Ca, Mg, Na, Al, Si), and by ion chromatography using a Dionex DX 300 model (F, Cl, $SO_4$, $PO_4$, $NO_3$). Al speciation was obtained using the method developed by Boudot et al. (1994, 2000) and described in detail in Maitat et al. (2000). In absence of Al-Fluoride species, a flash extraction with a pH 5 buffered 8-OH-quinoline reagent allowed the determination of all inorganic monomeric Al species (Alim). The Al not extracted represent organic Al and non-monomeric Al. The different species of Al in Alim were estimated at room temperature by equilibrium calculations with WHAM v. 6.09. The equilibrium constants used for Al reactions are given in Supplementary information (Table S1).

### 2.7. Leaf litter mass loss

Initial leaf disk mass was estimated from 4 sets of 40 disks randomly sampled at the start of the microcosm experiment. Then, 25 disks from each microcosm were used to assess the remaining mass of leaf litter. All leaf disks were dried at 60 °C for 48 h to constant mass and weighed to the nearest 0.1 mg (dry mass, DM). Leaf litter decomposition was evaluated by subtracting the final leaf disks mass without fungal biomass to the initial mass of control disks. Results were expressed as percentage of remaining DM.

### 2.8. Leaf litter Al and nutrient contents

Al on leaf disks was determined using 50 mg of dry leaf litter. After mineralization of leaf litter with 200 μL of $HNO_3$ (69%) and 200 μL of ultrapure Milli-Q water for 48 h at 70 °C, 5 mL of ultrapure Milli-Q water were added and the extract was centrifuged for 15 min at 1100 rpm. Al concentration on the supernatant was determined by atomic absorption spectrophotometry (AAnalyst 100; Perkin Elmer and Varian SpectrA A-300).

To determine N and P contents of leaf litter, ten dry leaf disks were finely ground. The N content was then assessed using a CHN elemental analyzer and organic P content was determined after oxidation by sodium persulphate in alkaline conditions using the AFNOR method (1990). Al and P contents were expressed as μg $g^{-1}$ of DM and N content was expressed as mg $g^{-1}$ of DM.

### 2.9. Fungal biomass

Five leaf disks were used to determine leaf-associated fungal biomass. Solid-phase extraction and reversed phase high performance liquid chromatography were performed to assess the ergosterol concentration on leaf litter (Gessner and Schmitt, 1996). A conversion factor of 5.5 mg ergosterol per g of mycelial dry mass was applied to determine the fungal biomass (Gessner and Chauvet, 1993). The fungal biomass was expressed as mg $g^{-1}$ of DM.

### 2.10. PCR-DGGE

A two-step PCR-DGGE protocol (Oros-Sichler et al., 2006) was performed to compare fungal assemblages between treatments. For each microcosm, five leaf disks were finely ground and total DNA from leaf litter was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Fungal 18S rRNA gene fragments were amplified using NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and EF3 (5′-TCCTCTAAATGACCAAGTTTG-3′) primers. Diluted amplicons (1:500) obtained were used as the template for the second amplification with primers NS1 and

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