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1	Structural and functional responses of leaf-associated fungal communities to chemical
2	pollution in streams
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24	Keywords: chemical stress, translocation, ligninolytic enzymes, aquatic fungi.

1 SUMMARY

1. In forested lotic ecosystems, organic matter decomposition processes, involving aquatic 2 fungi and their ligninolytic activities play a key role. In this study, the relationship 3 4 between realistic water chemical pollution (including nutrients, metals and pesticides) and 5 the biomass, structure and enzyme activities of leaf-associated fungal communities was 6 evaluated. 7 2. The experiment was performed through a microcosm approach by comparing fungal communities from a less-polluted upstream site to a more-polluted downstream site in the 8 9 agricultural basin of the Auzon stream (Puy-de-Dôme region, Centre France). The 10 resistance as well as the ability of these fungal communities to recover from pollution was also assessed through a translocation experiment. 11 3. Results showed a lower laccase activity and higher band richness in fungal communities 12 from the downstream site comparing to the upstream site, that can partially be explained 13 by the greater pesticide toxicity (r = -0.50) and nutrient concentration (r = -0.45, dissolved 14 15 organic carbon) of downstream waters. 4. Specifically, a negative relationship between laccase activity and nitrate concentration was 16 observed, irrespectively of the studied site, whereas the relationship between nitrate and 17 18 phenol oxidase/peroxidase appeared much weaker. 5. The translocation experiment evidenced i) a fast decrease in laccase activity concomitant 19 with a shift in fungal structure after stress exposure, and ii) a recovery ability in terms of 20 21 laccase activity and fungal community structure two weeks after stress removal. 6. The present study underlines the sensitivity of leaf-associated fungal communities in 22 23 terms of laccase activity and community structure to variations in chemical stress, as well as their ability to recover once the stress is removed. This study highlights the potential 24 25 use of laccase as indicators of stream water chemical pollution.

2 INTRODUCTION

3 Low-order forested streams, representing more than 70% of the total stream network in most of European and US basins (Benda et al. 2004; Kristensen & Globevnik 2014), are seasonally 4 5 fueled with allochthonous organic matter coming from the riparian forest (Mninshall 1967). These organic matter inputs, mostly in the form of leaf litter, can represent up to 90% of the 6 7 total organic matter in streams (Mninshall 1967; Triska et al 1970; Cummins 1974; Wallace & Webster 1996). Consequently, the decomposition of leaf litter becomes a central process in 8 9 these ecosystems since it i) intervenes in various biogeochemical cycles (C, P, N and S; Webster & Meyer 1997), ii) provides mineral nutrients to the entire stream food web (Gessner 10 & Chauvet 1994), and iii) returns CO₂ to the atmosphere (Gholz et al. 2000). Leaf litter 11 12 decomposition process in streams is performed by the action of both physical (abrasion, 13 leaching and/or fragmentation) and biological factors; the latter including the action of 14 microorganisms and macroinvertebrates (Mann 1975; Gessner, Chauvet & Dobson 1999; 15 Gaudes et al. 2009). While macroinvertebrates shredder taxa account for the major portion of the leaf mass loss in streams (Suberkropp & Klug 1976), it has been estimated that 16 microorganisms (including fungi and bacteria) can contribute up to 25-45% of the leaf mass 17 18 loss, even if these estimations vary depending on leaf species and sites studied (Hieber & 19 Gessner 2002; Baldy et al. 2007). Although the contribution of each microbial group on leaf 20 litter decomposition remains hard to evaluate, especially because of the difficulty to separate 21 them from the leaf matrix, it is widely accepted that microbial decomposition is mostly attributed to the action of aquatic fungi (Krauss et al. 2011; Canhoto, Gonçalves & Bärlocher 22 23 2016). Indeed, aquatic fungi (mostly the group of aquatic hyphomycetes) represent the major microbial biomass colonizing the leaf substratum (> 95% of the total microbial biomass; 24 25 Hieber & Gessner 2002), and possess a large panel of extracellular hydrolytic and oxidative

1 enzymes such as cellulases, hemicellulases and/or laccases (Abdullah *et al.* 1989;

2 Chandrashekar & Kaveriappa 1991; Abdel-Raheem & Ali 2004) capable to breakdown leaf
3 structural compounds (e.g. cellulose, pectin, lignin).

4 In the actual scenario of streams pollution, mostly resulting from the anthropogenic 5 activity occurring in the watersheds (i. e. agriculture, industry, urbanization), it has been 6 proved that chemical pollution (by heavy metals, nutrients and xenobiotics) can threaten 7 aquatic fungal communities and their decomposition abilities (Artigas et al. 2012), and by extension the entire ecosystem functioning (Duarte et al. 2008; Maltby, Brock & van den 8 9 Brink 2009). Amongst those pollutions, the effects of nutrients on aquatic fungal communities 10 is certainly one of the most studied (Gessner et al. 1999). Although nutrient enrichment (by phosphorus and nitrogen) is commonly associated with increases in fungal biomass, diversity, 11 and activity (Artigas, Romaní & Sabater 2004; Hagen, Webster & Benfield 2006; Magbanua 12 et al. 2010), some studies also observed a negative effect of the nutrients, depending on the 13 concentration and nature of the amendments (Baldy et al. 2002; Duarte et al. 2009b). For 14 15 instance, high nitrate and ammonium concentrations in eutrophic streams have been shown to decrease fungal diversity and biomass, as well as their potential for decomposing leaf litter 16 17 (Baldy et al. 2002; Duarte et al. 2009b). Effects of metals (cadmium, copper, and zinc) are 18 commonly associated with reductions in fungal species richness, biomass, sporulation rates, and leaf decay rates (Krauss et al. 2001; Duarte, Pascoal & Cassio 2009a; Zubrod et al. 2015). 19 Recently, field experiments have attempted to assess the effects of pesticides on microbial 20 litter decomposition in agricultural streams (Krauss et al. 2003; Rasmussen et al. 2012; 21 22 Fernández et al. 2015). In most of the cases, the exposure to pesticides lead to declines in 23 fungal species richness, concomitant with decreases in litter decay rates (Krauss et al. 2003; Rasmussen et al. 2012). More precisely, the type of the pesticide molecule as well as its mode 24 25 of action would determine its potential impact on target and non-target leaf microorganisms

1	(Maltby et al. 2009). This is the case of fungicides, being more toxic to aquatic fungi than
2	other pesticides, due to their direct action on fungal physiology (Artigas et al. 2012;
3	Fernández et al. 2015; Zubrod et al. 2015) but also indirectly affecting bacteria co-existing
4	with fungi (Artigas et al. 2014). Up to now, however, the effects of multi-stressors, including
5	pesticides and environmental stressors (e.g. temperature, droughts, nutrients) in leaf-
6	associated fungal communities are weakly studied in the literature (Fernández et al. 2015).
7	Besides, Piscart et al. (2011) did not find any correlation between pesticide pollution and
8	microbial decay rates of leaves in a stream draining vineyard area in France. The absence of
9	correlations was partially explained by the low temperatures recorded in their experiment
10	which may have masked pesticides impact on microbial litter decomposition.
11	Translocation experiments are suitable approaches to assess such multi-stress impacts
12	on stream microbial communities (including aquatic fungal communities). Indeed, this
13	approach, which consist on transferring biological communities from polluted sites to pristine
14	sites and vice versa (i. e. Ivorra et al. 1999; Proia et al. 2013), permits to identify
15	resistance/resilience mechanisms set up by microbial communities to cope stress (Sridhar et
16	al. 2005; Duarte et al. 2009a). At present, various studies have performed translocation
17	experiments on periphytic biofilms (mostly targeting diatom communities dynamics; Ivorra et
18	al. 1999; Morin et al. 2012). To our knowledge, two studies have performed translocation
19	experiments to assess metal pollution on leaf-associated fungal communities (Sridhar et al.
20	2005; Duarte et al. 2009a) but none regarding the overall influence of nutrients, metals and
21	pesticides. Studies on metals showed that copper, zinc, and manganese exposure stopped litter
22	decomposition and declined sporulation rates. However, these studies also showed that fungal
23	communities were able to recover when stress by metals was removed after translocation from
24	a polluted site to a cleaner one.

The present study investigates the sensitivity of leaf-associated fungal communities to 1 2 stream water pollution (including nutrients, metals, and pesticides) by comparing communities from upstream (less polluted) and downstream (more polluted) sites of an 3 agricultural stream (the Auzon, Puy-de-Dôme region, Centre France). Our main objective is to 4 assess the resistance and recovery abilities of leaf-associated fungal communities to stream 5 6 water pollution using a translocation experiment in laboratory microcosms. As base 7 hypothesis we presume that stream water pollution observed downstream (higher nutrients, metals, and pesticides concentrations) would promote shifts in the structure and activity of 8 leaf-associated fungal communities comparing to communities from the upstream site (less 9 10 polluted). As second hypothesis, we expected that chemical stress exposure due to 11 translocation from upstream to downstream would lead to fungal communities similar to that of downstream in terms of both structure and activity. In the other hand, the proved recovery 12 13 potential of leaf-associated fungal communities from single stressors suggest enough abilities in such communities to recover after stress removal during the translocation from downstream 14 to upstream. 15

16

17 MATERIALS AND METHODS

18 Study sites and preparation of fungal inoculums

Leaf-associated fungal communities were obtained from the Auzon, a third-order forested
stream draining a basin surface area of 6074 ha in the Puy-de-Dôme region (Centre France).
Two study sites were selected for this work: a less-polluted upstream site (first order section)
close to the river source (45°41'30.0"N 3°04'58.5"E; Channonat) and a more-polluted
downstream site (fourth order section) before the confluence of the Auzon with the Allier
River (45°43'30.3"N 3°12'27.4"E; Cournon d'Auvergne). The riparian forest, mainly

composed of Alnus glutinosa (L.) Gaertn and Corylus Avellana (L.) species, was well 1 2 preserved at both upstream and downstream sites. However, streambeds were composed by gravel and cobbles in the upstream section and by sand in the downstream section. Land use 3 variations throughout the watershed lead to an upstream section mostly occupied by forests 4 and prairies and a downstream section devoted to agriculture (38% of the total watershed 5 6 surface area, mainly cereal crops) and some dispersed urbanizations (10%). According to the 7 report on phytosanitary molecules detection in surface waters from the Puy-de-Dôme region, the Auzon is one of the most herbicide-polluted streams, especially in its downstream section 8 reaching concentration peaks of aminomethylphosphonic acid (AMPA), glyphosate, and S-9 metolachlor above 1 μ g L⁻¹ (Phyt'eauvergne 2014). 10

Fungal communities were obtained from the upstream and downstream sites of the 11 Auzon in October 2014 using Alnus glutinosa (Alnus) leaf species as substratum. Freshly 12 fallen Alnus leaves were harvested from the upstream site, dried 72h at room temperature, and 13 placed in a total of six bags (1 mm of mesh size), each containing 10 g of the pre-dried leaves. 14 15 Three of these litter bags were placed upstream and the three others downstream in order to obtain fungal communities representative of the two sites. After three weeks of colonization 16 17 in-stream, litter bags were retrieved and transported to the laboratory where leaves were 18 cleaned with filtered (0.2 µm) stream water, cut into small circles (2 cm in diameter) and placed in sterile 250 mL flasks (1 flask per bag). Each flask contained 10 leaf disks filled with 19 100 mL of filtered (0.2 µm) stream water from each respective site (Gessner & Chauvet 20 1993). Fungal mycelia growth and sporulation was achieved by incubations at 19 °C under 21 22 agitation at 180 rpm (Artigas et al, 2008). After 48 h, water suspension and leaf disks that 23 were crushed and well mixed, were transferred into sterilized flasks and used as inoculum for the subsequent microcosm experiment. 24

25

1 Microcosm experiment and sampling

In order to assess only the effect of stream water chemistry on microbial communities 2 colonizing leaf litter, the experiment was performed in microcosm under controlled condition 3 4 of temperature (room temperature, 19 ± 0.1 °C), light (dark/light 13 h : 11 h, 846 ± 50 and 0 umol photons cm⁻² s⁻¹, respectively) and flow (0.1 L s^{-1}) . In total, six glass indoor stream 5 6 channels $(1 \times w \times d = 63 \text{ cm} \times 11 \text{ cm} \times 4 \text{ cm})$, representing the upstream (3 replicate 7 channels) and downstream (3 replicate channels) sites of the Auzon were set up in a thermoregulated room. Each channel was independent and connected to a separate 50 L tank through 8 9 an aquarium pump (Newjet 1200, Newa, Italy) allowing water recirculation. Each tank was 10 filled with river water taken directly from the corresponding study sites (upstream and downstream) of the Auzon. After one-day stabilization of microcosm devices with stream 11 water, seven packs of pre-dried *Alnus* leaves (5 g dry weight per pack) were deployed in each 12 channel, supplemented with 100 mL of the corresponding fungal inoculum described above. 13 The first sampling was performed shortly after having released the fungal inoculum into the 14 15 channel (week 0). The other samplings were performed weekly for a total of four weeks (weeks 1, 2, 3 and 4). The river water was replaced each seven days to avoid nutrient 16 limitation and samplings were performed just before water replacement. 17

18 Translocation was performed in the middle of the experiment (after the sampling at week 2, the same day), 14 days after the beginning of the experiment) by transferring two 19 randomly chosen leaf packs from i) upstream to downstream channels (Ups \rightarrow Dws) and ii) 20 downstream to upstream channels (Dws \rightarrow Ups). One week after (7 days, week 3) the first 21 translocated samples were sampled. Translocated samples were used to assess the impact 22 23 (resistance) and the recovery (resilience) of leaf-associated fungal communities to stream water pollution. Fungal communities having remained permanently in upstream and 24 25 downstream channels were monitored in parallel during translocation.

At each sampling time (weeks 0, 1, 2, 3, and 4), one randomly selected leaf pack was 1 2 sacrificed from each channel and leaf disks (1 cm in diameter) were obtained for further analyses on the fungal community structure (DGGE), ergosterol concentration, extracellular 3 enzyme activities (laccase, peroxidase and phenol oxidase), leaf dry weight, and lignin 4 content. While extracellular enzyme activity measurements were performed the same 5 6 sampling date, samples for fungal community structure, ergosterol concentration and lignin 7 content were stored frozen at -20 °C until analyses. Additionally, water samples from each channel were taken at each sampling time to determine nutrients (nitrate, phosphate, and 8 dissolved carbon), multi-residues (including pesticides) and metals (cadmium, copper, 9 10 manganese, zinc) concentrations.

11

12 Water physical and chemical parameters

In each stream channel, water temperature, dissolved oxygen, conductivity and pH were 13 measured at each sampling time using portable probes (ProODO YSI, Cond340i WTW, 14 pHMeter CG818, respectively). Soluble reactive phosphorus (Murphy & Riley 1962) and 15 nitrate concentrations (Nitrat-Test kit, Merck, Darmstadt, Germany) in water were measured 16 spectrophotometrically. Total dissolved carbon (TDC), dissolved organic carbon (DOC) and 17 inorganic carbon (DIC) as well as nitrogen concentrations were measured using a total 18 organic carbon analyzer (TOC-VCPN, Shimadzu, Kyoto, Japan). In parallel, water samples 19 20 taken directly from the river were sent to Laboratoire de Touraine (COFRAC agreement number 1-0677) for pesticide multi-residues and heavy metals analyses. Predicted toxicity of 21 pesticides in water samples was estimated according to the toxic units (TU) concept (Sprague 22 23 1971). TU for each pesticide and metabolite compound was calculated according to the following equation: 24

$$TU = \frac{Ci}{EC50i}$$

Where C_i is the actual concentration of the pesticide in the water sample and EC_{50i} is the 1 median acute effect of this pesticide inhibiting 50% of the growth rate of standard aquatic 2 3 microbial species. Predicted toxicity of the pesticide cocktail, noted as $\Sigma T U$ s, was also 4 calculated at both upstream and downstream sites for each week as the sum of all TU for the pesticides detected in a single water sample. Since toxicity data (EC_{50}) for pesticides on 5 aquatic fungi are not available in the literature (Maltby 2009; Maltby et al. 2009), EC₅₀ values 6 7 of microalgae (Scenedemus subspicatus, Scenedesmus abundans, Pseudokirchneriella subcapitata and Chlamydomonas angulosa) were used as surrogates (see Rasmussen et al. 8 2012). 9

10

11 Biomass and structure of fungal communities

Ergosterol concentration determination was used as proxy for fungal biomass in leaves 12 (Gessner & Schmitt 1996). Lipids were extracted from leaves (fifteen leaf disks per sample) 13 after incubation in 0.14 M KOH methanol at 80 °C during 30 min. Extracts were purified and 14 concentrated using solid-phase extraction (tC18 cartridges, Sep-Pak Vac RC, 500 mg, Waters) 15 and then analyzed by a high pressure liquid chromatography system (Lachrom L-7400, 16 17 Merck-Hitachi, Tokyo, Japan) equipped with an ODS-2 Hypersil column (250 × 4.6 mm, 5 um particle diameter; Thermo Scientific, San Jose, CA, U.S.A.) and using methanol as the 18 mobile phase at a flow rate of 1.4 mL min⁻¹. Ergosterol was detected at 282 nm and quantified 19 according to an ergosterol standard (ergosterol purity \geq 95%, Sigma-Aldrich, St. Louis, 20 Missouri, USA) ranging from 0 to 200 μ g mL⁻¹. Results were expressed in μ g ergosterol per g 21 of leaf dry mass (DM). 22

1	Total DNA extraction was performed from 5 leaf disks per sample using the FastDNA
2	SPIN Kit for soil (MP Biomedicals, Santa Ana, California, USA) following the
3	manufacturer's instructions with some minor modifications. Briefly, two homogenization
4	steps (FastPrep, speed 6.0 for 40 seconds) were applied instead of one in order to improve
5	fungal mycelium extraction from the leaf matrix. Extracted DNA was quantified using a
6	Nanodrop (Nanodrop TM 2000, Wilmington, Delaware, USA), amplified by polymerase chain
7	reaction (PCR) targeting the Internal Transcribed Spacer (ITS) region 2 of the fungal rRNA
8	and then analyzed using denaturing gradient gel electrophoresis (DGGE) method. PCR mix
9	was composed of 2.5 μ l of MgCl (50 mM), 0.5 μ l of BSA (50 mg mL ⁻¹), 1 μ L dNTPs (10
10	mM), 0.3 μ L of Taq polymerase (5 U μ L ⁻¹), 2 μ L of each primers (10 pmol μ L ⁻¹), 5 μ L of 10X
11	buffer and 4 μ L of DNA (10 ng L ⁻¹), completed with H ₂ O to a final volume of 50 μ L. Primers,
12	purchased from MWG Biotech, were the forward ITS 3, with a GC tail attached in 5' (5'-
13	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC CCG CCC CGC ATC GAT
14	GAA GAA - 3') and the reverse ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC 3') (White et
15	al. 1990). PCR conditions were set at 95 °C for 5 minutes, followed by 35 steps (30 s at 95
16	°C, 45 s at 55 °C and 1 minute at 72 °C) and a final elongation phase of 7 minutes at 72 °C.
17	PCR products were then checked on 1% agarose gel (size expected ranging 200-450 bp) and
18	quantified using the DNA Quantification Kit, Fluorescence Assay (Sigma-Aldrich, Saint-
19	Louis, USA) before loading into DGGE. DGGE was run with 500 ng of PCR product per
20	sample, using the TIGEK 2401-220 device (CBS Scientific, San Diego, North Carolina, USA)
21	in 1X TAE buffer for 16 h, with a temperature of 59 °C and a voltage of 90 V. Gels contained
22	7.5% (w/v) of polyacrylamide and a linear urea-formamide denaturing gradient ranging of
23	25%-48% (100% = 7M urea and 40% (v/v) formamide). At the end of the electrophoresis,
24	gels were stained in 1X TAE buffer containing 1/20000 dilution of Gel star (Lonza, Rockland,
25	USA) and digitized using a BioSpectrumAC Imaging System (UVP, Upland, California,

USA). DGGE bands profiles were then analyzed using the Gelcompare2 software (Applied
 Maths, Belgium) in order to obtain the fungal band matrix, based on presence/absence of
 bands from each sample. Using this matrix, a cluster analysis was performed to assess
 homologies among experimental conditions tested.

5

6 Mass loss and lignin determination in leaves

Decomposition rates of *Alnus* leaves (*k*) were evaluated from leaf mass loss. Leaf mass loss was surveyed between weeks 0 to 4 in the different experimental conditions. The percent loss in dry mass (DM, oven-weight after 48 h at 60 °C) from ten leaf disks (pooled in one replicate sample) permitted to calculate decay rates using an exponential decay model: $M_t = M_0 e^{-kt}$, where M_0 is the initial DM (g), M_t is the final DM (g) at time *t*, and *k* is the breakdown coefficient (Petersen & Cummins 1974).

Lignin content in *Alnus* leaves was measured using the acetyl bromide procedure described by Iiyama & Wallis (1988). Five leaf disks were digested with 2.5 mL of acetyl bromide solution in acetic acid (25%, w/w) at 70 °C for 30 minutes. Extracts were then transferred into 50 mL flasks and rinsed with a 15 mL acetic acid up to a final volume of 50 mL. Results were obtained spectrophotometrically and expressed as lignin percentage per leaf DM, using the following equation:

%Lignin =
$$\frac{A}{\varepsilon Lignin \times l \times C} \times 100$$

19 Where A is the absorbance of the sample at 280 nm, ε is the absorption coefficient for lignin 20 ($\varepsilon_{\text{lignin}} = 20 \text{ g}^{-1} \text{ L cm}^{-1}$), 1 is the path length of the beam in cm and C is the concentration of the 21 sample in g of DM L⁻¹.

1 Extracellular enzyme activities

Phenol oxidase (EC 1.14.18.1), peroxidase (EC 1.11.1.7), and laccase (EC 1.10.3.2) activities 2 were measured in leaf samples (three leaf disks per sample) using 3,4-Dihydroxy-L-3 4 phenylalanine (L-DOPA) and 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) commercial substrates analogues (Sigma-Aldrich, St. Louis, Missouri, USA). All enzyme 5 6 activities were conducted under saturating conditions of substrate (previously determined 7 from substrate saturating curves) and according to the protocols of Sinsabaugh, Osgood & Findlay (1994a) and Johannes & Majcherczyk (2000). Phenol oxidase and peroxidase 8 activities assays consisted in dark incubations of 3 leaf disks per replicate at 19 °C under 9 10 agitation in glass test tubes containing 2 mL of water from the corresponding channel with 2 mL of 1.5 mM L-DOPA (final concentration) in acetate buffer (pH 4.5). Peroxidase activity 11 assay received an addition of 0.2 mL of 0.3% H₂O₂. After 1 hour incubation, 12 spectrophotometric readings at 460 nm were performed for phenol oxidase and peroxidase 13 using the Ultrospec 2000 (Pharmacia Biotech, Trowbridge, UK). Net peroxidase activity was 14 15 obtained after subtraction of phenol oxidase activity. Laccase activity was determined from 3 leaf disks per replicate, following the oxidation of 2 mL ABTS (3 mM, final concentration) in 16 0.1 M of citrate-phosphate buffer (pH 4.5) with 2 mL of water from the corresponding 17 channel at 19 °C, in the dark and under agitation (150 rpm) for 1 h. Absorbance was then 18 determined at 420 nm. All enzyme activities were corrected by control, performed with water 19 from the corresponding site (no leaf disks), and expressed in μ mol substrate h⁻¹ g leaf DM⁻¹. 20

21

22 Data analyses

Differences between upstream and downstream sites for water physical and chemical
parameters (oxygen, conductivity, pH, nutrients, metals, and pesticides) were evaluated by a

two-way ANOVA assessing the sources of variation for the factors time (weeks 0 to 4) and 1 2 site (upstream, downstream) as well as their interactions. Differences between fungal community descriptors (ergosterol concentration, fungal band richness and extracellular 3 enzyme activities) and litter characteristics (lignin content) were assessed using a first two-4 way ANOVA on upstream and downstream samples for the whole experiment (factors time : 5 6 weeks 0 to 4; factors condition : upstream and downstream) and a second two-way ANOVA 7 on all samples at week 3 and 4 corresponding to the translocation experiment (factors time: 8 week 3 and 4; factors condition: upstream, downstream, translocation upstream to downstream, and translocation downstream to upstream). All data were transformed using the 9 10 Box Cox function prior to the ANOVA tests to meet assumptions of normality and homoscedasticity of data residuals (Box and Cox, 1964), and were complemented with post 11 hoc multiple comparison tests (Tukey's test, P < 0.05). 12

From the fungal fingerprints obtained after DGGE analyses, the fungal band 13 presence/absence matrix was transformed to Euclidean distance, and fungal communities 14 15 were classified by a joining tree analysis using the Ward's hierarchical clustering method (Ward 2 criterion). Moreover, two redundancy analyses (RDA) were performed to determine 16 17 relationships between water chemical and fungal community parameters. As described for 18 ANOVA tests, a first RDA (RDA_{UpDw}) was performed on upstream and downstream samples 19 for the whole experiment, whereas the second RDA (RDA_{Transloc}) was performed on 20 translocated and non-translocated samples at weeks 3 and 4 only. Briefly, fungal community descriptors data matrices were composed of 5 parameters: ergosterol concentration, fungal 21 22 band richness and extracellular enzyme activities (phenol oxidase, peroxidase, and laccase). 23 Water physico-chemical data matrices included 15 parameters: dissolved oxygen, pH, conductivity, and concentrations of metals (Cu, Cd, Mn, Zn), phosphate, nitrate, dissolved 24 25 carbon and nitrogen concentrations and ΣTU_s . Lignin percentage in leaves was also included

in the above data matrix. All data from the matrices were transformed prior to analysis using the Hellinger transformation according to Legendre & Gallagher (2001), and a forward selection was performed to select water chemical parameters significantly correlated with fungal community descriptors using a Monte Carlo permutation test (anova.cca function, 999 unrestricted permutations, P = 0.05).

- All statistical analyses were computed using the R software, and RDA as well as related
 methods were performed using the Vegan package (http://cran.r-project.org/).
- 8

9 **RESULTS**

10 Physical and chemical characteristics of stream water

11 A total of 17 pesticide compounds (14 herbicides, 2 insecticides, and 1 fungicide) were detected in water samples from the Auzon stream (Table 1). Overall, pesticides displayed 12 relatively low concentrations (below 0.05 μ g L⁻¹) except for the herbicide glyphosate and its 13 metabolite the aminomethylphosphonic acid (AMPA) (ranging 0.10-1.40 μ g L⁻¹, 14 irrespectively of the studied site), the aminotriazole and the diuron. While only three 15 molecules were detected at the upstream site (glyphosate, AMPA, and lindane), the whole 17 16 pesticide compounds were detected downstream. Potential pesticide toxicity calculations, 17 represented as the ΣTU s, were on average (weeks 1 to 4) thirty times higher downstream 18 $(35.96 \times 10^{-3} \pm 4.78 \times 10^{-3})$ compared to upstream $(1.14 \times 10^{-3} \pm 0.39 \times 10^{-3})$ (ANOVA, Site 19 effect, P < 0.0001). This higher pesticide toxicity observed downstream can be attributed to 20 both the number of pesticide compounds and the presence of diuron ($TU = 2.06 \times 10^{-2}$), 21 oxadiazon ($TU = 1.81 \times 10^{-3}$) and terbutryne ($TU = 3.13 \times 10^{-3}$), contributing to the highest 22 TU observed downstream (Table 1). In addition, heavy metals (Cu, Zn, Mn, Cd) displayed 23 very low concentrations at both sites (Table 1) though their concentrations were significantly 24

1	higher downstream compared to upstream (ANOVA site effect, $P < 0.05$). Conductivity, pH,
2	dissolved carbon (total and organic), and phosphate concentrations were also greater at the
3	downstream site (ANOVA, site effect, $P < 0.001$ for each parameter; Table 1 and Figure 1.1
4	and 1.2). Conversely, no differences were observed for total nitrogen and nitrate
5	concentrations between sites (ANOVA, site effect, $P = 0.1210$ and $P = 0.1197$ respectively).
6	More precisely, average nitrate concentrations were 0.51 \pm 0.04 mg L ⁻¹ and 0.53 \pm 0.04 mg L ⁻¹
7	¹ in upstream and downstream channels, respectively (Figure 1.2). At week 2, N-NO ₃ ⁻
8	concentration peaked up to 0.70 \pm 0.10 mg L^{1} upstream and 0.78 \pm 0.09 mg L^{1} downstream
9	(ANOVA time effect, $P < 0.0001$).

11 Litter decomposition rates and lignin content

12 Decay rates of Alnus leaves calculated between weeks 0 and 4 were similar between downstream (0.0162 day⁻¹) and upstream (0.0101 day⁻¹) sites, as well as after translocation 13 experiments, although no statistical analysis were performed. Besides, lignin content in Alnus 14 leaves (data not shown) decreased from 10.45 ± 1.39 %DW (week 0) to 4.30 ± 0.52 %DW 15 (week 4) upstream, representing a loss of 58%. Downstream, lignin loss from leaves appeared 16 lower than upstream (29%) with values ranging from 8.20 ± 0.79 %DW (week 0) to $5.82 \pm$ 17 1.63 %DW (week 4). Specifically, the major decrease in lignin content from leaves was 18 observed at week 2 in upstream and downstream samples (ANOVA, time effect, P < 0.0001). 19 20 However, no effects of translocation were observed in lignin loss from leaves.

21 Biomass and structure of fungal communities

- Ergosterol concentration in *Alnus* leaves showed values ranging from $80.25 \pm 11.45 \ \mu g \ gDM^{-1}$
- 23 to $417.37 \pm 24.56 \ \mu g \ g D M^{-1}$ upstream and $61.84 \pm 2.79 \ \mu g \ g D M^{-1}$ to $321.87 \pm 84.24 \ \mu g$
- 24 gDM⁻¹ downstream (Figure 2.1). The major shifts in ergosterol concentrations were between

1 week 0 and week 1 (Tukey's test, P < 0.0001), evidencing the colonization phase of *Alnus* 2 leaves by fungi. No significant differences were observed in ergosterol concentration between 3 upstream and downstream sites during the four weeks of study (Tukey's test, P > 0.05), but 4 concentrations tended to be slightly higher upstream. Translocation did not have any effects 5 on ergosterol concentration (Figure 2.2).

Fungal band richness tended to increase from weeks 0 to week 4 (ANOVA, P < 0.05) in
both upstream (from 21 ± 1 to 44 ± 1 band) and downstream (from 36 ± 1 to 49 ± 1 band)
samples, evidencing a fungal species succession on leaves. However, fungal band richness
was on average (weeks 0 to 4) higher in downstream samples (S = 41 ± 1) compared to
upstream samples (S = 34 ± 3, Tukey test, Site effect, P < 0.05). Moreover, fungal band
richness responded to both translocations (Dws → Ups and Ups → Dws), displaying samples
with intermediary values (Tukey's test, P > 0.05; Table 2).

Cluster analysis, performed on fungal community presence/absence matrix, showed a 13 14 sample ordination, first by time and second by site (Figure 3). Effects of translocation on 15 fungal community structure were perceptible at week 3, and characterized by the splitting of translocated samples from their origin, despite still being in the same cluster. However, such 16 effects became more evident at week 4. After stress removal (Dws \rightarrow Ups translocation, week 17 4) translocated samples tended to form a separate cluster with those of upstream (less polluted 18 site) and to separate from those of downstream (site of origin). Conversely, after stress 19 exposure (Ups \rightarrow Dws translocation, week 4) translocated samples tended to form a separate 20 cluster with those of downstream (more polluted site) and to separate from those of upstream 21 (site of origin, Figure 3). 22

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24 Enzymatic activities

Peroxidase and phenol oxidase activities measured in *Alnus* leaves were not different between
upstream and downstream samples (Tukey test, *P* > 0.05) (Figure 4A1, B1), though a clear
time effect was observed in both activities (ANOVA, time effect, *P* < 0.0001). Time effects
were characterized by peaks of activity at week 2, multiplying by two-to-four the basal
activity values recorded at the other sampling times in both upstream and downstream
samples (Figure 4A1, B1). Translocation of leaves had no effect on the above mentioned
enzyme activities (figure 4A2, B2).

In contrast, laccase activity showed differences between sites (Tukey's test, site effect, P 8 < 0.0001), with higher average values (weeks 0 to 4) in upstream fungal communities (1.84 \pm 9 0.29 U gDW⁻¹ h⁻¹) comparing to those from downstream (0.39 \pm 0.12 U gDW⁻¹ h⁻¹, Figure 10 4C1). Laccase activity also showed significant differences over time, especially at week 2, 11 where a 3 to 5 times drop of activity in upstream samples was observed (ANOVA, time 12 effect, P < 0.0001). Concerning translocation experiments, after stress exposure (Ups \rightarrow Dws 13 translocation, week 4) samples displayed an intermediate state, characterized by a decrease in 14 15 laccase activity compared to upstream samples (samples of origin) and approached to the activity recorded in downstream samples at the same date (Tukey's test, P < 0.05, Figure 16 4C2). Similarly, after stress removal (Dws \rightarrow Ups translocation, week 4) translocated samples 17 18 displayed an increase in laccase activity, reaching values similar to those from the upstream samples (Tukey's test, P < 0.05). 19

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21 Relationship between water chemistry and fungal community parameters

RDA_{UpDw}, performed with upstream and downstream samples between weeks 0 to 4 (Figure
5.1), displayed two axes representing 85.97 % of the total variance explained by biological
and environmental descriptors. The first Axis (RDA1 explaining 70.38% of the variance) was

mainly correlated with nitrate concentration (r = 0.76) characteristic of samples at week 2 1 2 located in the right up corner of the RDA_{UDDw} (peak of nitrate concentration), and coincided with high phenol oxidase and peroxidase activities, but low laccase activity. The second axis 3 (RDA 2 explaining 15.59% of the variance) appeared mostly linked with heavy metals 4 concentration (Zn, r = -0.45) and pesticides toxicity (TU, r = -0.46), characteristic of samples 5 from the downstream site showing higher values of fungal band richness and lower ergosterol 6 7 concentrations. In contrast to the RDA_{UpDw}, the RDA_{Transloc} displayed two axes representing a much lower percent of the total variance (46.46%). Here, only nitrate concentration (NO3) 8 was retained by the permutation test as explicative of the variance in biological descriptors 9 10 (Figure 5.2). Again, the first axis (RDA1 explaining 38.88% of the variance) was mainly correlated with nitrate concentration (r = -0.59), but here coinciding with low rates of the 11 three ligninolytic activities (laccase, phenol oxidase and peroxidase). Specifically, samples 12 13 translocated from the downstream site to the upstream site at week 4 appeared in the right up corner of the RDA, and displayed low nitrate concentrations but high ligningolytic activities. 14 In addition, fungal band richness appeared also negatively related to the nitrate concentration 15 in water. 16

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18 DISCUSSION

Despite confinement effects due to our microcosm approach performed under controlled
conditions (temperature, light and flow), the obtained decay rates of *Alnus glutinosa* leaves
appeared similar between sites (0.0101-0.0162 day⁻¹) and were in the same range of that
found in field experiments ((k=0.0054 day⁻¹) Chauvet 1987; (k=0.0161 day⁻¹) Canhoto &
Graça 1996; (k=0.035 day⁻¹) Hieber & Gessner 2002). Only water, supplied to upstream and
downstream leaf-associated fungal communities, was different, coming from the two different
sites of the Auzon stream. Therefore, differences observed in terms of ligninolytic activity and

structure of fungal communities responsible of *Alnus* leaves decomposition can be essentially
 attributed to variations in stream water chemistry. Among all chemical parameters measured
 in the stream water, nitrate concentration was tightly related to ligninolytic activities
 expression in fungal communities.

5 As expected, the Auzon appeared as a contaminated stream (Phyt'eauvergne 2014, Water 6 agency, 2003). While DOC concentrations categorized its upstream and downstream sites in 7 two different class of quality according to the water agency classification system (respectively 8 poor and bad), nutrients (NO₃, PO₄) and pesticides categorized the two sites into the same 9 "good" class of quality (Table 1). Despite such small differences in terms of water quality 10 between sites, upstream water led to microbial communities with higher laccase activity and a lower fungal band richness comparing to those grown in downstream water. Besides, no 11 differences in fungal biomass (ergosterol concentration) between communities were observed. 12 The influence of stream water chemistry on the microbial community descriptors was also 13 supported by the translocation of leaves from upstream to downstream waters (stress 14 15 exposure) which induced a sharp decrease in laccase activity concomitant with changes in the 16 structure of the fungal community, but again without influence on fungal biomass. These results seems to support the "stress gradient" hypothesis proposed by Niyogi et al (2002), 17 18 stating that biodiversity has a low threshold of response to a stress, whereas biomass and function remains stable but decrease only under high stress. However, laccase activity 19 20 appeared to be very sensitive to water chemical stress.

Ligninolytic activities from fungal communities colonizing *Alnus* leaves were the most
sensitive parameters to nitrate variations in our study. Indeed, nitrogen effect (including
nitrate), have already been observed in other studies working on microbial communities from
forest soils (Gallo *et al.* 2004; Baldrian 2006; Sinsabaugh 2010) and streams (Artigas *et al.*2004). Despite the fact that ligninolytic activity regulation is still unclear, several studies

reported negative correlations between nitrogen and both laccase, phenol oxidase and 1 2 peroxidase activities, as observed in the second RDA (RDA_{Transloc}) of our study. However, our study showed also that after a two-fold increase of nitrate concentrations probably resulting 3 from the rainfall event occurring at week 2, laccase activity tended to be inhibited (Pearson, r 4 = -0.497, P < 0.05), whereas peroxidase and phenol oxidase activities tended to be stimulated 5 (Pearson, r = 0.807 and r = 0.549 respectively, P < 0.05, RDA_{UnsDws}, Figure 5.2). The fact 6 7 that ligninolytic activities responded differently to the nitrate increase is surprising. Usually, they evolve in the same manner, since laccases represent one of the largest class of phenol 8 oxidases (Baldrian 2006; Sinsabaugh 2010). Generally, phenol oxidases and peroxidase 9 10 activities are assayed using L-DOPA as substrate, the latter activity being calculated as the incremental response relative to phenol oxidases (Sinsabaugh, Osgood & Findlay 1994b; 11 Sinsabaugh 2010), whereas laccase activities are assayed using a more specific substrate: the 12 13 ABTS ((Baldrian 2006; Junghanns et al. 2009)). Consequently, the differential relationships obtained between ligninolytic activities and nitrate concentration can be due to the choice of 14 15 two different substrates for activity assays (Bach et al. 2013). Besides, the antagonistic results 16 between laccase and the other two ligninolytic activities can also reflect the effect of translocation in microbial communities. Indeed, RDA_{UpsDws} was performed on upstream and 17 18 downstream samples over the four weeks of the experiment, whereas RDA_{Transloc} was performed on week 3 and 4 only. Consequently, the greater ligninolytic activities of 19 translocated samples at week 4 (right up corner of the RDA_{Transloc}) may reflect a transient 20 21 response of communities to the translocation from the more polluted to less polluted site. Altogether, our results suggest that laccase activity is more sensitive to nitrate than phenol 22 23 oxidase and peroxidase activities.

Site effects were also observed on laccase activity, with higher values upstream compared
to downstream. Here, differences were neither explained by nitrate concentrations nor by

differences in fungal biomass (ergosterol concentration) since no differences were observed 1 2 between sites. These observations suggest that other factors may be involved in laccase activity regulation. We observed a negative correlation between dissolved organic carbon 3 (DOC) and laccase activity (Pearson, r = -0.45, P < 0.05) as well as between Toxic Units 4 (TU) and laccase activity (Pearson, r = -0.50, P < 0.05). As suggested in others studies, we 5 6 hypothesized that both DOC quantity and quality (Elisashvili et al. 2006; Mikiashvili et al. 7 2006; Romaní, Artigas & Ylla 2012) or the presence of some pesticide compounds could be important in laccase activity regulation (Zapp et al. 2011; Oliveira et al. 2013). Alternatively, 8 shifts in fungal communities structure, highlighted by changes in fungal band richness, can 9 10 partially explain the differences in laccase activity observed between sites and after translocation (Treton, Chauvet & Charcosset 2004; Frey et al. 2004; Osono 2007). When 11 looking at the fungal band absence/presence matrix, fungal species specific of the upstream 12 13 fungal communities were not found in the downstream communities. Selection and/or shifts in dominance between species can be due to changes in water chemistry. As reported by Abdel-14 15 Raheem & Shearer (2002) and Abdel-Raheem & Ali (2004) in culture experiments, not all aquatic fungal species have shown the ability to produce all types of ligninolytic enzymes. 16 Besides, since laccases have an indirect role in carbon uptake by fungi from leaf litter 17 18 (Baldrian 2006), the greater DOC concentrations (and perhaps different DOC quality) in water downstream may have led to the establishment of fungal species low-laccase producers, 19 more competitive than some high-laccase procuders upstream. Our study reveals that stress by 20 21 chemical pollution can induce shifts in the structure of fungal communities accompanied by decreases in laccase activity. However, no effect on the overall leaf mass loss was observed, 22 suggesting a certain functional redundancy in these communities (Pascoal, Cássio & 23 Marvanová 2005) probably explained by compensation mechanisms in the organic matter 24 degradation by extracellular enzymes. 25

When stress by pollution was removed through the translocation from the polluted site 1 2 (downstream) to the less polluted site (upstream), leaf-associated fungal communities displayed the ability to recover as stated in our base hypothesis. Both laccase activity and 3 fungal community structure tended to get closer to that of fungal communities from upstream 4 within 2 weeks. Such observations are consistent with other translocation studies, which 5 6 showed that aquatic hyphomycetes communities were able to recover in terms of structure, 7 decomposing activity (decay rates), and sporulation rates when translocated from highly metal-exposed sites to cleaner sites in about two to three weeks (Sridhar et al. 2005 in the 8 field; Duarte et al. 2009a in microcosms). Regarding at the fungal band presence/absence 9 10 matrix, this fast recovery in the structure of leaf fungal communities can actually mostly be explained by the disappearance of species in the translocated samples. Since fungal 11 12 communities are very sensitive to nutrient variations in streams (Suberkropp & Chauvet 1995; 13 Bärlocher & Corkum 2003), the combined effects of low nutrient concentrations in the upstream channels and the osmotic stress resulting from a sudden change of water chemistry 14 15 (conductivity values were 2.7 times higher downstream than upstream) may have affected the 16 dominance of certain fungal species from downstream communities.

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Overall our study indicates that variations in stream water chemistry, resulting from 18 19 anthropogenic activities in the watershed, induce changes in the structure and enzymatic pathway of leaf-associated fungal communities. However, recovery assessment allowed us to 20 21 evidence an almost full recovery in these communities which encourages river restoration programs. But linking fungal community responses to stream water chemistry remains 22 23 difficult considering the amount of stress compounds as well as their potential interactions (antagonistic, synergistic) and few EC_{50} data for pesticides on aquatic fungi are available in 24 the literature, which decrease the reliability of pesticide risk assessment in these communities 25

1	(Maltby et al. 2009). Thus, future researches should take into account aquatic fungi as species
2	sensitive to the chemical quality of stream waters.
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- 1 <u>Table 1:</u> Physical and chemical characteristics and pesticide compounds detected (Herbicides
- 2 (H), Insecticides (I) and Fungicides (F)) in the upstream and downstream waters of the Auzon
- 3 stream during the laboratory experiment. Values are means and (\pm) standard errors of the five
- 4 sampling times for nutrients (n = 15) and four sampling times for pesticides and heavy metals
- 5 (n = 12). Significant differences between sites (ANOVA, P < 0.05) are shown by an asterisk.
- 6 TU calculation were based on EC_{50} of Scenedemus subspicatus^a, Pseudokirchneriella
- 7 subcapitata^b, Scenedesmus quadricauda^c, Scenedesmus abundans^d and Chlamydomonas
- 8 angulosa^e.

		Upstream		Downstream	
		Concentration	mean TU	Concentration	mean TU
	Dissolved organic Carbon (mg L ⁻¹)	$14.32~\pm~9.57$		$19.71 \pm 8.45 *$	
	Total Carbon (mg L ⁻¹)	$31.87~\pm~9.25$	$63.59 \pm 7.28 *$		
	Phosphate ($\mu g L^{-1}$)	$41.56~\pm~2.34$		$64.82 \pm 4.13 *$	
Nutrients	Conductivity (μ S cm ⁻¹)	204.54 ± 2.77		$552.93 \pm 13.35 *$	
	pH	$7.18~\pm~0.17$		$7.94 \pm 0.15 *$	
	Total Nitrogen (mg L ⁻¹)	1.64 ± 0.09		1.92 ± 0.53	
	Nitrate (mg L ⁻¹)	2.44 ± 0.19		2.67 ± 0.21	
	Cadmium (μ g L ⁻¹)	$0.024~\pm~0.0$		$0.03 \pm 0.002 *$	
Heavy	Copper ($\mu g L^{-1}$)	2.9 ± 0.4		$4.3 \pm 0.2 *$	
metals	Manganese ($\mu g L^{-1}$)	15.75 ± 3.75		$24.5 \ 3 \pm \ 1.68 \ *$	
	Zinc (μ g L ⁻¹)	54.3 ± 3.5		77.17 ± 3.51 *	
	Aminotriazole ($\mu g L^{-1}$) (H) ^a	< 0.005		0.065 ± 0.0427	2.83 x 10 ⁻⁵
	Biphenyl ($\mu g L^{-1}$) (F) ^e	< 0.005		$0.01 \pm 0.01 *$	7.69 x 10 ⁻⁶
	AMPA ($\mu g L^{-1}$) (H) ^a	0.705 ± 0.241	1. 10 x 10 ⁻³	$0.476 \pm 0.157 *$	7.45 x 10 ⁻⁴
	Glyphosate ($\mu g L^{-1}$) (H) ^c	$0.18~\pm~0.068$	4.09 x 10 ⁻⁵	$0.332 \pm 0.101 *$	7.55 x 10 ⁻⁵
	Oxadiazon ($\mu g L^{-1}$) (H) ^a	< 0.005		0.007 ± 0.001	1.81 x 10 ⁻³
	Dinoterb ($\mu g L^{-1}$) (H) ^a	< 0.030		$0.02~\pm~0.02$	2.70 x 10 ⁻⁶
	Dichloprop ($\mu g L^{-1}$) (H) ^b	< 0.020		$0.022 ~\pm~ 0.008$	3.25 x 10 ⁻⁷
	MCPA ($\mu g L^{-1}$) (H) ^b	< 0.005	0.033 ± 0.014		4.10 x 10 ⁻⁷
Xenobiotics	Mecoprop (μ g L ⁻¹) (H) ^b	< 0.005		0.033 ± 0.012	1.39 x 10 ⁻⁷
	Prosulfocarb ($\mu g L^{-1}$) (H) ^b	< 0.005		0.002 ± 0.002	4.08 x 10 ⁻⁵
	Lindane ($\mu g L^{-1}$) (I) ^d	$0.0017 ~\pm~ 0.0008$	7.00 10-7	0.004 ± 0.0003	1.45 x 10 ⁻⁶
	Tributyl phosphate ($\mu g L^{-1}$) (H) ^a	< 0.005		$0.01~\pm~0.007$	7.69 x 10 ⁻³
	Imidacloprid ($\mu g L^{-1}$) (I) ^a	< 0.005		$0.027 ~\pm~ 0.004$	2.65 x 10 ⁻⁶
	Terbumeton ($\mu g L^{-1}$) (H) ^a	< 0.005		$0.008 ~\pm~ 0.008$	8.33 x 10 ⁻⁴
	Terbuthylazine ($\mu g L^{-1}$) (H) ^b	< 0.005		0.013 ± 0.013	1.04 x 10 ⁻³
	Terbutryne ($\mu g L^{-1}$) (H) ^b	< 0.005		0.008 ± 0.002	3.13 x 10 ⁻³
	Diuron ($\mu g L^{-1}$) (H) ^c	< 0.005		0.056 ± 0.004	2.06 x 10 ⁻²
	Σ	0.887	1.14 x 10 ⁻³	1.124	3.60 x 10 ⁻² *

<u>Table 2:</u> Fungal band richness in leaf-associated fungal communities obtained during the
 translocation experiment (translocated samples are highlighted in *italics*). Values are means
 (weeks 3 and 4) ± standard errors for each experimental condition. Significant differences
 between conditions for each week are shown (a ≠ b, Tukey's test, P < 0.05).

Experimental conditions	Time (weeks)			
	3		4	
Upstream	20.67 ± 0.33	a	37.67 ± 2.19 a	
Ups ->Dws	25.33 ± 0.67	a	<i>41</i> ± 0.58 ab	
Downstream	$36.33~\pm~1.20$	b	49.33 ± 1.20 b	
Dws -> Ups	34.33 ± 3.48	b	42.33 ± 2.03 ab	

1 FIGURE LEGENDS

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Figure 1: Dissolved inorganic (DIC) and organic (DOC) concentrations (1) as well as nitrate
(NO3) and phosphate (PO4) concentrations (2) measured in the upstream (Ups) and
downstream (Dws) site of the Auzon stream. Values are means and standard error (n = 3), and
significant differences are show by brackets with asterisks for conditions and asterisks only
for time (n.s. when not significant, Tukey's test, *P* < 0.05).

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9 Figure 2: Ergosterol concentration on Alnus leaves in upstream and downstream samples for 10 the whole experiment (1) and in all samples after translocation (2). Values are means (n = 3)and standard errors for the four experimental conditions [upstream samples (Ups), 11 12 downstream samples (Dws), samples translocated from control to polluted site (Ups->Dws) 13 and samples translocated from polluted to control site (Dws->Ups)]. Significant differences between conditions for each week are shown by brackets with asterisks (n.s. when not 14 significant, Tukey's test, P < 0.05). 15 16 Figure 3: Clustering analysis on the structure of the Alnus-associated fungal communities, 17 representing the upstream samples (Ups), downstream samples (Dws), samples translocated 18 from control to polluted site (Ups->Dws) and samples translocated from polluted to control 19 20 site (Dws->Ups) during the four weeks of experiment. Clustering was obtained using the Ward method (Ward dissimilarity index) on fungal DGGE absence/presence matrix. 21

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23 Figure 4: Values of the peroxidase (A), phenol oxidase (B) and laccase (C) activities

24 measured in *Alnus* leaves in upstream and downstream samples for the whole experiment (1)

and in all samples after translocation (2). Enzymatic activities are expressed as the amount of 2,3-dihydroindole-5,6-quinone-2-carboxylate (DIQC for peroxidase and phenol oxidase) and 3 oxidized 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS for laccase) released 4 per unit of leaf DM and time (h). Values are means and standard errors (n =3), and significant 5 differences between conditions for each week are shown by brackets with asterisks and 6 superscript (n.s. when not significant, a \neq b, Tukey's test, *P* < 0.05).

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8 Figure 5: Redundancy analysis (RDA) performed on upstream and downstream samples for the whole experiment (RDA_{UpDw}, 1) and in all samples after translocation (RDA_{Transloc}, 2). 9 10 Both RDA displays biological variables (bold and solid lines) constrained by environmental ones (dashed lines). Only variables that significantly explained the response of leaf-associated 11 12 fungal communities to physical and chemical parameters are shown (Permutation test, P <0.05). Biological variables are composed of phenol oxidase (PhOx), peroxidase (Perox) and 13 14 laccase (Lacc) activities, ergosterol concentration (Ergo) and band richness (Band). 15 Environmental variables are composed of conductivity (Cond), pH, lignin percentage (Lignin), pesticide toxicity (TU), nutrient (Nitrate (NO3), Phosphate (PO4), Inorganic carbon 16 (DIC)) and heavy metals concentrations (zinc (Zn)). 17





Figure 3. Rossi et al.



Cluster Dendrogram



Figure 5.1. Rossi et al.



Figure 5.2. Rossi et al.

