Leaf-associated fungal diversity in acidified streams: insights from combining traditional and molecular approaches

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Summary

We combined microscopic and molecular methods to investigate fungal assemblages on alder leaf litter exposed in the benthic and hyporheic zones of five streams across a gradient of increasing acidification for 4 weeks. The results showed that acidification and elevated Al concentrations strongly depressed sporulating aquatic hyphomycetes diversity in both zones of streams, while fungal diversity assessed by denaturing gradient gel electrophoresis (DGGE) appeared unaffected. Clone library analyses revealed that fungal communities on leaves were dominated by members of Ascomycetes and to a lesser extent by Basidiomycetes and Chytridiomycetes. An important contribution of terrestrial fungi was observed in both zones of the most acidified stream and in the hyporheic zone of the reference circumneutral stream. The highest leaf breakdown rate was observed in the circumneutral stream and occurred in the presence of both the highest diversity of sporulating aquatic hyphomycetes and the highest contribution to clone libraries of sequences affiliated with aquatic hyphomycetes. Both methods underline the major role played by aquatic hyphomycetes in leaf decomposition process. Our findings also bring out new highlights on the identity of leaf-associated fungal communities and their responses to anthropogenic alteration of running water ecosystems.

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Introduction

Fungi play a crucial role in detritus-based lotic ecosystems as main microbial mediators of allochthonous leaf litter decomposition (Findlay and Arsuffi, 1989; Baldy *et al*., 1995; Gulis and Suberkropp, 2003). Aquatic fungi, through their leaf-degrading enzyme capabilities, contribute to microbial leaf processing and improve the nutritional quality of leaf litter for further consumption by invertebrate detritivores (Bärlocher and Kendrick, 1981; Arsuffi and Suberkropp, 1988). Aquatic hyphomycetes, a polyphyletic group of fungi, are thought to dominate microbial communities growing on decaying leaves (Bärlocher, 1992; Suberkropp, 1992; Gessner *et al*., 2007).

Both leaf litter breakdown and associated aquatic hyphomycete diversity have been considered as useful indicators of the functional integrity of streams (Gessner and Chauvet, 2002) and as potential bioindicators of anthropogenic stress (Solé *et al*., 2008). In particular, reduced leaf litter breakdown and concurrent loss of aquatic hyphomycete diversity have been reported in numerous studies, notably under high concentrations of metals such as Al in acidified streams (Baudoin *et al*., 2008), Cd (Moreirinha *et al*., 2011), Ag (Pradhan *et al*., 2011), Zn (Fernandes *et al*., 2009; Niyogi *et al*., 2009) or even in the context of multiple (i.e. Cu and Zn) contaminants (Duarte *et al*., 2008). These observed decreases in species richness under anthropogenic stress have traditionally been assessed using microscopic methods based on the morphological identification of conidia (asexual spores), which varies between species.

Recently developed molecular methods allow investigation of fungal communities regardless of their life history stage, including mycelia, which constitute the metabolically active part of fungal biomass on leaves (Bärlocher, 2007; 2010; Krauss *et al*., 2011). Molecular fingerprinting of microbial communities, namely terminal restriction fragment length polymorphism analysis and denaturing gradient gel electrophoresis (DGGE), first showed that the traditional method (conidial identification)

underestimates fungal diversity on leaves (Nikolcheva *et al*., 2003). Subsequently, molecular approaches using taxon-specific fungal primers (Nikolcheva and Bärlocher, 2004) and sequencing (Seena *et al*., 2008) or comparison of fungal clone libraries (Harrop *et al*., 2009) revealed a consistent contribution of non-aquatic hyphomycete species to leaf-associated microbial assemblages.

Some previously cited studies that showed a loss of leaf-associated sporulating aquatic hyphomycete diversity under anthropogenic disturbance concurrently used DGGE to estimate fungal richness on leaves and revealed contrasting phylotype richness depending on situations, i.e. negative impact with exposure to Cd (Moreirinha *et al*., 2011) and to Cu and Ag (Pradhan *et al*., 2011), but no effects under Cu and/or Zn stress (Duarte *et al*., 2008; Fernandes *et al*., 2009). Conversely, phylotype richness always exceeded that of sporulating aquatic hyphomycete in these studies, confirming the underestimation of fungal diversity when assessed only by traditional methods. Despite the differences observed, no studies have attempted to identify taxonomic changes occurring in altered sites through molecular techniques.

In addition, most studies of the effects of anthropogenic stress on leaf-associated fungi have been restricted to the benthic compartment of streams. Nevertheless, recent studies have also reported the occurrence of aquatic hyphomycetes (Bärlocher *et al*., 2006) and their significant involvement in leaf decomposition in the hyporheic zone of streams (Cornut *et al*., 2010), where large amounts of organic matter may be stored (Jones, 1997; Cornut *et al*., 2012a). Bärlocher and colleagues (2006) even hypothesized that this stream compartment, which is intimately connected with the benthic zone through exchanges of water, organic matter and nutrients (Boulton *et al*., 1998), could be a long-term reservoir for dispersal of aquatic hyphomycetes. Therefore, investigations of leaf colonization in the hyporheic and benthic zones may provide a more global view of fungal decomposer diversity involved in stream ecosystem functioning.

We recently reported the alteration of leaf litter decomposition in the benthic and hyporheic zones of streams subjected to anthropogenic acidification (Cornut *et al*., 2012b), and particularly, its negative impact on macroinvertebrate shredders and sporulating aquatic hyphomycete communities. We found that the diversity of these latter communities was significantly reduced in both zones along the gradient of acidification and Al concentrations, providing interesting data that could be used for comparison with molecular analyses of leaf-associated fungal assemblages.

The aim of this study was to identify the effects of an anthropogenic stress on fungal community structure and diversity associated with decaying leaves in the benthic and hyporheic zones of streams by combining molecular analyses and results obtained by traditional method. During the initial stages of leaf decomposition, we followed leaf colonization in both zones of five low-order streams subjected to different levels of Al mobilized by anthropogenic acidification. The structure and diversity of fungal communities on leaves assessed by DGGE were then compared with those previously evaluated through conidial identification (Cornut *et al*., 2012b). To identify differences in community composition between the reference non-impacted stream and the most heavily impacted one, fungal communities from both zones were compared based on analysis of 18S rRNA gene clone libraries.

Results

Surface and interstitial water characteristics and leaf decomposition

Streams ranged across a gradient of increasing Al concentrations, from LM, i.e. the reference circumneutral stream, to CL, i.e. the most impacted one. Most of the physicochemical variables were quite similar between the benthic and hyporheic zones within each stream (Table 1). Although the streams were well oxygenated, two-way ANOVA (stream × zone) revealed that oxygen saturation was significantly lower in the hyporheic zones (mean oxygen saturation range = 83.2–90.7%) than in the benthic zones (mean oxygen saturation range = 96.6–100%) of streams ($F_{1,40} = 97.3$, $P < 0.001$). After 4 weeks,

Table 1. Main physicochemical parameters over the 4-week study period, leaf mass loss after 4 weeks and cumulative richness of both sporulating aquatic hyphomycetes species and DGGE phylotypes associated with decaying alder leaves exposed in the benthic and hyporheic zones of the five streams across the Al gradient. Values are the means of physicochemical variables ($n = 5$) and leaf mass loss ($n = 4$).

	Benthic					Hyporheic				
	LM	GV	MB	RV	CL	LM	GV	MB	RV	CL
pH	7.3	6.4	7.0	6.2	4.6	7.4	6.4	6.9	6.3	4.7
ANC (μeq l^{-1})	446	55	210	27	–27	486	60	302	43	–21
Total Al (μg l^{-1})	31	35	100	113	658	140	74	200	424	745
O_2 (%)	99.3	96.6	97.8	99.9	100.0	86.9	84.4	83.2	85.1	90.7
Leaf mass loss (%)	67.9	32.5	56.9	44.2	23.1	36.7	19.4	28.7	24.6	15.5
No. of sporulating aquatic hyphomycete species	18	18	16	12	12	12	8	7	7	4
No. of DGGE phylotypes	21	22	22	17	21	24	21	22	20	21

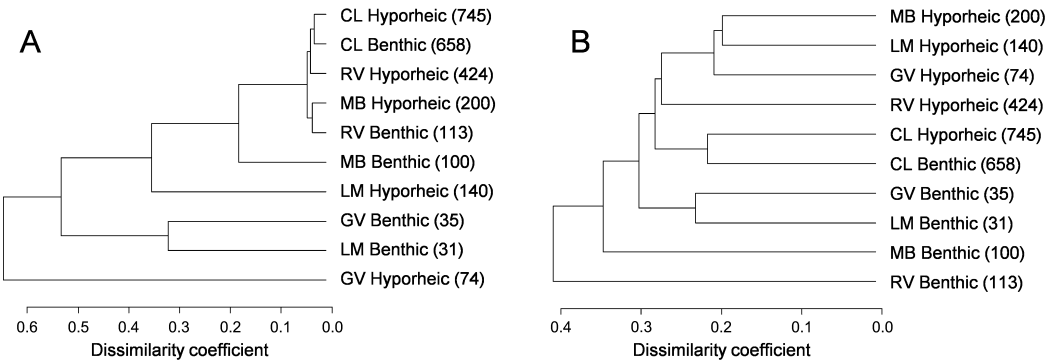

Fig. 1. Cluster dendrograms of sporulating aquatic hyphomycete assemblages (A) and fungal communities investigated by DGGE (B) over the four sampling dates on leaves incubated in the benthic and hyporheic zones of the five streams. Values in brackets are mean total Al concentrations ($\mu g\ l^{-1}$) in streams during the study.

leaf mass loss differed significantly among streams (two-way ANOVA, $F_{4,30} = 92.8$, $P < 0.001$), being highest in LM (67.9% and 36.7% of leaf mass loss in the benthic and hyporheic zones respectively) and lowest in the most impacted stream CL (23.1% and 15.5% in the benthic and hyporheic zones respectively) (Table 1). Leaf mass loss also differed among compartments of streams, being significantly lower in the hyporheic zone (two-way ANOVA, $F_{1,30} = 267.8$, $P < 0.001$). However, the difference between zones was reduced for the most impacted stream CL (significant interaction zone $\times$ stream, $F_{4,30} = 13.3$, $P < 0.001$).

Fungal assemblages

Conidial identification revealed a total of 31 species of sporulating aquatic hyphomycetes on leaf litter during the 4-week experiment, whereas 38 phylotypes were observed by DGGE (data not shown). The aquatic hyphomycete richness on leaves was negatively affected by the Al gradient and was lower in the hyporheic than in the benthic zone (Table 1). Sporulating species richness ranged from 18 in LM and GV to 12 in RV and CL in the benthic zone and from 12 in LM to 4 in CL in the hyporheic zone. Higher fungal richness was revealed by DGGE analyses, and this pattern was independent of the Al gradient and the compartment of the stream (Table 1).

Cluster analyses of aquatic hyphomycete assemblages clearly revealed that communities on leaves grouped together in impacted streams (Fig. 1A), whereas DGGE analyses of fungal assemblages showed that communities from hyporheic zones and from both zones of CL were separated from the others (Fig. 1B).

Conidial identification indicated that *Flagellospora curvula* (FLCU) was the dominant species in both zones of the most impacted streams, CL and RV, contributing to more than 95% of the conidial production in these streams (Fig. 2). Together with FLCU, *Tetrachaetum elegans* (THEL) was the codominant species in the benthic zone of LM and GV, and its contribution decreased across the Al gradient in both zones. However, the THEL contribution was lower in the hyporheic zone of LM and GV, where *Heliscus lugdunensis* (HELU) was the codominant species with FLCU.

Conidial biomass production differed significantly between stream zones, being lower in the hyporheic one,

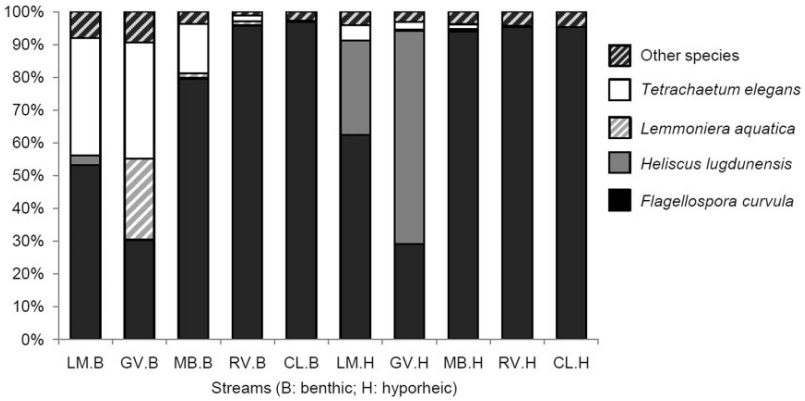

Fig. 2. Contribution of the leaf-associated aquatic hyphomycete species to conidial production (%) for leaves exposed in the benthic and hyporheic zones of the five streams across the Al gradient. Species contributing less than 5% to total conidial production were grouped together.

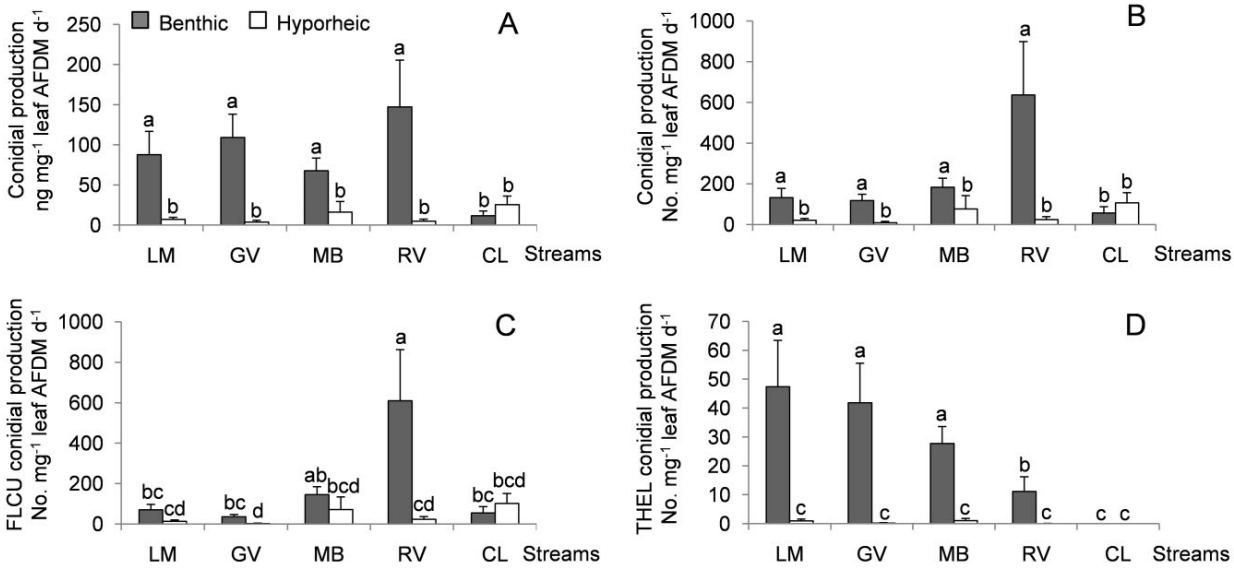

Fig. 3. Conidial biomass (A), total conidial (B) production, FLCU (C) and THEL (D) specific conidial productions over the four sampling dates for leaves exposed in the benthic and hyporheic zones of the five streams across the Al gradient. Values are the means ± 1 SE ($n = 16$). Different letters are statistically different (two-way ANOVA $P < 0.05$ followed by Tukey's test).

except for CL (Fig. 3A and Table 2). The differences observed were primarily due to a significant decrease of conidial production in the hyporheic zone of streams (Fig. 3B and Table 2) and a higher production of FLCU conidia in the benthic zone of RV when compared with the hyporheic zone (Fig. 3C). In the benthic zones, a significant decrease of THEL conidial production was also observed across the Al gradient (Fig. 3D).

Fungal diversity assessed through clone libraries

To identify differences in the community composition in both benthic and hyporheic zones between the reference non-impacted stream (LM) and the most heavily impacted stream (CL), PCR amplifications of the 18S rRNA genes were performed. Overall, 63, 64, 53 and 56 sequences were obtained for the LM benthic, LM hyporheic, CL benthic and CL hyporheic samples respectively.

When a 99% sequence similarity cut-off was applied, 36 distinct OTUs were found on litter incubated in the benthic and hyporheic zones of the LM and CL streams (Table 3). A total of 16 and 15 OTUs were found in the benthic and hyporheic zones of LM, respectively, while 19 OTUs were found in both zones of CL.

Clone sequences related to the Ascomycetes (mainly orders such as Pleosporales, Helotiales, Capnodiales and Taphrinales) were distributed in 24 OTUs (OTU1-24) and contributed to between 70% and 79% of the total sequences of the four clone libraries (Fig. 4).

In the benthic zone of LM, 39% of the clone sequences were identified as belonging to the group of aquatic hyphomycetes, whereas lower proportions were found in

Table 2. Two-way ANOVAs of conidial biomass production, conidial production, FLCU and THEL specific conidial productions from leaves incubated for 4 weeks in the benthic and hyporheic zones of the five streams.

Two-way ANOVA		Stream	Zone	Stream × zone
	df	4	1	4
Conidial production (biomass)	*F*	1.9	105.4	6.4
	P	0.120	**< 0.001**	**< 0.001**
Conidial production (No.)	*F*	1.7	82.0	3.0
	P	0.157	**< 0.001**	**0.021**
FLCU conidial production	*F*	4.1	52.0	2.8
	P	**0.004**	**< 0.001**	**0.030**
THEL conidial production	*F*	24.9	202.7	16.1
	P	**< 0.001**	**< 0.001**	**< 0.001**

Significant results are indicated in bold (level of significance is $P < 0.05$).

Table 3. Distribution of 18S rRNA gene clones affiliated with OTUs at 99% sequence similarity cut-off for leaves exposed in the benthic and hyporheic zones of the non-impacted reference stream LM and of the most impacted stream CL.

	LM		CL				
	Benthic	Hyporheic	Benthic	Hyporheic	Fungal order	Nearest BLAST hit (Accession No.)	% Similarity
Ascomycetes							
OTU1	2	6	3	3	*Taphrinales*	*Taphrina tosquinetii* (AJ496252.1)	100
OTU2	0	0	0	2	*Taphrinales*	*Taphrina wiesneri voucher* OSC:100 047 (NG_013152.1)	99
OTU3	6	18	13	18	*Pleosporales*	*Phoma* sp. Zzz202 (HQ696111.1)	100
OTU4	2	2	0	1	*Pleosporales*	*Phaeosphaeria caricicola* strain CBS 603.86 (GQ387529.1)	100
OTU5	4	3	1	0	*Pleosporales*	*Alternaria alternata* strain HDJZ-zwm-34 (JN673371.1)	100
OTU6	1	0	1	0	*Pleosporales*	*Pleosporales* sp. PG170709B1 isolate PG170709B3 (JN397390.1)	99
OTU7	4	0	0	0	*Helotiales*	*Goniopila monticola* strain FNP 1 (AY357277.1)	100
OTU8	1	0	0	0	*Dothideales*	*Aureobasidium* sp. B23 (HQ696089.1)	99
OTU9	2	0	2	5	*Hypocreales*	*Flagellospora curvula* 130–1655 and 180–1662 (KC691983-KC691984)	100
OTU10	4	0	0	1	*Helotiales*	*Alatospora acuminata* strain 102–280 (AY357261.1)	100
OTU11	1	0	3	2	*Helotiales*	*Botryotinia fuckeliana* strain DAOM 189076 (JN939020.1)	100
OTU12	0	0	1	0	*Helotiales*	*Satchmopsis brasiliensis* strain CPC 11017 (DQ195809.1)	99
OTU13*	13	2	0	1	*Helotiales*	*Tetrachaetum elegans* strain 105–326 (AY357281.1)	100
OTU14	0	0	1	0	*Pezizales*	*Phialea strobilina* strain CBS 643.85 (EF596820.1)	99
OTU15	0	0	1	1	*Helotiales*	*Tricladium patulum* strain 139–1063 (AY357285.1)	99
OTU16	0	0	0	1	*Pleosporales*	*Anguillospora filiformis* strain CCM F-20687 (AY178825.1)	99
OTU17	0	0	2	0	*Diaporthales*	*Sirococcus conigenus* strain CBS 113.75 (EU754115.1)	99
OTU18	0	0	0	1	*Diaporthales*	*Sirococcus conigenus* strain CBS 113.75 (EU754115.1)	99
OTU19	2	1	0	0	*Hypocreales*	*Fusarium* sp. Zzz612 (HQ696108.1)	100
OTU20	0	1	0	0	*Hypocreales*	*Nectria lugdunensis* strain CS-950 (AY357278.1)	99
OTU21	0	1	0	0	*Hypocreales*	*Fusarium* sp. Zzz612 (HQ696108.1)	99
OTU22	8	16	7	4	*Capnodiales*	*Cladosporium* sp. B01 (HQ696097.1)	100
OTU23	0	0	1	0	*Capnodiales*	*Cladosporium* sp. YK16 (JN546120.1)	99
OTU24	0	0	1	0	*Capnodiales*	*Cladosporium* sp. YK16 (JN546120.1)	99
Basidiomycetes							
OTU25	4	3	10	7	*Cystofilobasidiales*	*Itersonilia perplexans* (AB072228.1)	100
OTU26	0	2	1	1	*Tremellales*	*Cryptococcus skinneri* (AB032646.1)	99
OTU27	0	1	1	1	*Tremellales*	*Cryptococcus heveanensis* (AB032635.1)	99
OTU28	2	0	0	0	*Tremellales*	*Cryptococcus* sp. FYB-2007a strain AS 2.2653 (EF363152.1)	99
OTU29	0	0	1	0	*Filobasidiales*	*Bullera taiwanensis* (AB072234.1)	100
OTU30	0	0	0	1	*Tremellales*	*Cryptococcus terricola* strain RUB001 (JN938686.1)	99
Chytridiomycetes							
OTU31	0	0	2	3	Not assigned	Uncultured Chytridiomycota clone T3P1AeH07 (GQ995262.1)	98
OTU32	0	0	1	0	Not assigned	Uncultured Chytridiomycota clone T3P1AeH07 (GQ995262.1)	99
OTU33	0	3	0	0	*Chytridiales*	*Chytridiales* sp. JEL187 (AY635825.1)	100
OTU34	7	1	0	0	*Rhizophydiales*	*Rhizophydium* sp. JEL138 (AF164266.1)	96
Zygomycetes							
OTU35	0	0	0	1	*Mortierellales*	*Mortierella angusta* strain CBS 293.61 (HQ667443.1)	99
Other Eukaryotes					Taxonomic group		
OTU36	0	4	0	2	*Rhizaria*	Cercozoa sp. WA28p82t6LS (EU709189.1)	100
Total no. of clones	63	64	53	56			
Total no. of OTUs	16	15	19	19			

**Lemonniera terrestris* strain ccm-F125 (AY204607.1) and *Lemonniera aquatica* (AY204605.1) also match sequences of this OTU at 100% similarity.

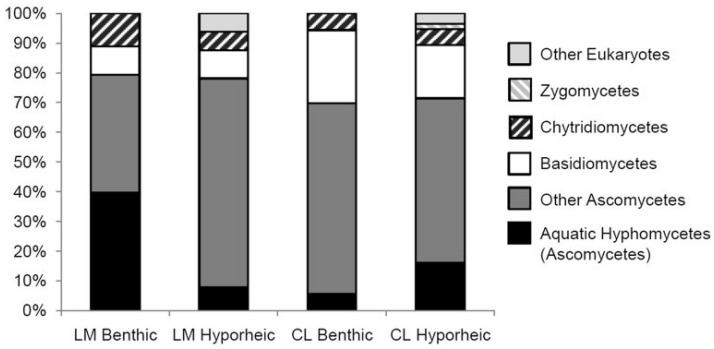

Fig. 4. Taxonomic distribution of the nearest BLAST hits from 18S rRNA gene clone libraries of leaves exposed in the benthic and hyporheic zones of the non-impacted stream LM and the most impacted stream CL.

the hyporheic zone of LM (8%) and in CL (6% and 16% in the benthic and hyporheic zones respectively) (Fig. 4). The results showed that sequences close to FLCU and belonging to OTU9 (Table 3) contributed to 3% and 0% of the total sequences in the benthic and hyporheic zones of LM, respectively, and to 4% and 9% in the benthic and hyporheic zones of CL respectively. In OTU13 (Table 3), sequences that were close to THEL, the species codominating conidial production in the benthic zone of LM, accounted for 17% and 3% of the total sequences in the benthic and hyporheic zones of LM, respectively, and for 0% and 2% in the benthic and hyporheic zones of CL respectively. Sequences that had nearest blast hits to *Lemonniera terrestris* (LETE) and *Lemonniera aquatica* (LEAQ) were also found in OTU13 accounted for 3% of the clone sequences from the benthic zone of LM.

In both zones of CL and the hyporheic zone of LM, 25–32% of the clone sequences were related to *Phoma* sp. (OTU3), while only 10% were found in the benthic zone of LM (Table 3). Sequences close to *Cladosporium* sp. (OTU22-24) accounted for 13% and 25% of the total clone sequences in the benthic and hyporheic zones of LM, respectively, and for 17% and 7% in the benthic and hyporheic zones of CL respectively.

A total of 6 OTUs (OTU25-30) most closely matched Basidiomycetes, and these sequences accounted for 9% (LM, hyporheic) to 25% (CL, benthic) of the total clone sequences (Fig. 4), most of which were closely related to *Itersonilia perplexans* (OTU25, order Cystofilobasidiales) (Table 3). Clone sequences were also identified as belonging to the Chytridiomycetes (OTU31-34), which comprised between 5% and 11% of the total sequences (Fig. 4). A few other clone sequences related to Zygomycetes (OTU35) and to other eukaryotes (OTU36) were also identified in our clone libraries.

Discussion

Traditional versus molecular analyses

For the first time, this study combined traditional and molecular approaches to provide new insights into leaf-associated fungal communities in the benthic and hyporheic zones of streams subject to a gradient of anthropogenic disturbance. Molecular analyses using DGGE and sequencing of clone libraries indicated that the microscopic method underestimates the fungal diversity on leaves in both zones, particularly in impacted sites. Notably, our results showed that previously observed differences between both methods (e.g. see Nikolcheva *et al.*, 2003; Duarte *et al.*, 2008; 2010) also apply to the hyporheic zone, where they are even more pronounced. Despite this, comparison of fungal assemblages by conidial identification and DGGE revealed that the traditional method enabled better discrimination between impacted and non-impacted sites. However, it would be interesting to compare those differences in fungal assemblages by using other sets of PCR primers because the gene region targeted can also play an important role in diversity studies (e.g. see Smit *et al.*, 1999).

We noticed that the analyses of clone libraries revealed the presence of sequences close to aquatic hyphomycetes (e.g. *Goniopila monticola*) not detected by conidial identification. These results could be due to the fact that some species are growing on leaves without producing conidia or to the poor representation of aquatic fungi in sequence databases. Indeed, many clone sequences have not revealed a perfect identity with database sequences, which precluded an unequivocal identification, similar to results previously obtained for OTU9 before FLCU sequencing. We observed that this species that dominates conidial production (up to 96%) has never contributed more than 9% of total sequences from clone libraries, revealing marked differences between sporulation and molecular analyses for the characterization of fungal communities as previously reported by Seena and colleagues (2008) on maple, linden and oak leaves. In contrast to the sporulation analyses, the results also showed that fungal richness assessed by molecular analyses was not depressed across the AI gradient, regardless of the stream compartment. Phylotype richness evaluated by DGGE was slightly higher than OTU richness assessed through clone library analyses, which

was supported by some sequences grouping into the same OTU being differentiated by DGGE. For example, sequences of both THEL and LETE were grouped into the same OTU at a 99% similarity cut-off, but gave distinct phylotypes on DGGE profiles when assessed with the specific DGGE method used in this study (data not shown). This was likely due to divergences of sequences within an OTU by up to 1%, i.e. five nucleotides, because the sequence size used for our phylogenetic analyses was slightly higher than 500 bp.

Acidification effects on benthic and hyporheic sporulating aquatic hyphomycete assemblages

Sporulating aquatic hyphomycete richness was severely reduced in the hyporheic zone of streams, falling from 12 to only 4 species in the most impacted stream (CL) over the 4-week study period. Various abiotic and biotic factors could affect the diversity of aquatic hyphomycetes and their ability to sporulate in the hyporheic zone of streams. While differences in dissolved oxygen concentrations never exceeded 15% between zones, lower oxygen concentrations in the hyporheic zone could have a direct effect on the sporulation of aquatic hyphomycetes (Medeiros *et al*., 2009), and/or exacerbate the previously observed effects of Al on the metabolism of aquatic hyphomycetes. In a previous study, Chergui and Pattee (1988) also demonstrated that sporulating aquatic hyphomycete richness and leaf breakdown were reduced because of lower dissolved oxygen concentrations and lower water velocity in part of a river network. Leaf decomposition becomes slower in the hyporheic zone, which also slows down fungal succession, but this could also result from the inability of conidia to easily access leaf litter. Lower turbulence, which may affect sporulation (Webster and Towfik, 1972; Sanders and Webster, 1980), and low sediment porosity could slow down conidia dispersal and thus delay fungal colonization in the hyporheic zone of streams.

The important dominance of FLCU in impacted streams did not result from increasing FLCU conidial production, but rather from decreasing conidial production of other species. Specifically, THEL conidial production was negatively affected across the Al gradient. Moreover, HELU and FLCU were the codominant species in the hyporheic zone of less-impacted streams LM and GV, whereas THEL and/or LEAQ were codominant with FLCU in their benthic counterpart. In another area (Montagne Noire, SW France), Cornut and colleagues (2010) reported similar trends for the contribution of these species to conidial production in both zones. The specific morphology and size of their conidia indicate that species producing smaller conidia (i.e. FLCU and HELU) can likely disperse more readily within the sediment. Metabolic constraints due to elevated Al concentrations in impacted streams and lower oxygen concentrations in the hyporheic zone could also affect aquatic hyphomycete ability to produce large tetraradiate conidia (e.g. THEL and LEAQ), as indicated by their lower conidial biomass production.

New insights from molecular evidence

As previously observed in other molecular studies (Nikolcheva and Bärlocher, 2004; Seena *et al*., 2008), sequencing of clone libraries showed that Basidiomycetes and Chytridiomycetes may contribute significantly to leaf-associated fungal assemblages, which were largely dominated by Ascomycetes. Sequences close to other eukaryotes were also found in our clone libraries as reported by Seena and colleagues (2008), but sequences that resulted from this lack of primer specificity were in the minority. Surprisingly, clone library sequencing showed that the contribution of aquatic hyphomycetes to fungal communities was much higher than in previous studies (Seena *et al*., 2008; Harrop *et al*., 2009). Nevertheless, continuing advances in the characterization of microbial strains from environmental samples have resulted in more exhaustive databases with the potential for more precise identifications. An important contribution of terrestrial fungi (genera such as *Phoma*, *Cladosporium*, *Itersonilia* and *Taphrina*) was observed in both zones of CL and in the hyporheic zone of LM, indicating that these phyllosphere colonizers could persist longer on leaves as well in the hyporheic zone of streams as in impacted streams. These fungi are considered to be less efficient leaf decomposers than aquatic hyphomycetes in freshwater systems (Bärlocher and Kendrick, 1974). However, in addition to the potential reduction of both sporulation and conidia dispersal in hyporheic zones and impacted streams, they could outcompete aquatic hyphomycetes and thereby delay their colonization and growth on leaves.

Leaf ergosterol content is commonly assessed to estimate fungal living biomass associated with decaying leaves. Cornut and colleagues (2012b) confirmed previous findings of no effects of acidification on ergosterol leaf content and by extension on fungal biomass on leaves (Dangles and Chauvet, 2003; Baudoin *et al*., 2008; Simon *et al*., 2009; Clivot *et al*., 2013). A mean ergosterol content of 5.1 mg per gram of fungal biomass was found for numerous and diverse species (Djajakirana *et al*., 1996), and this was close to the level of 5.5 found by Gessner and Chauvet (1993) for aquatic hyphomycetes. However, acidification could have an impact on other fungal species that may contain variable amounts of ergosterol through its effects on leaf colonizer assemblages. Notably, we observed that the contribution to clone libraries of

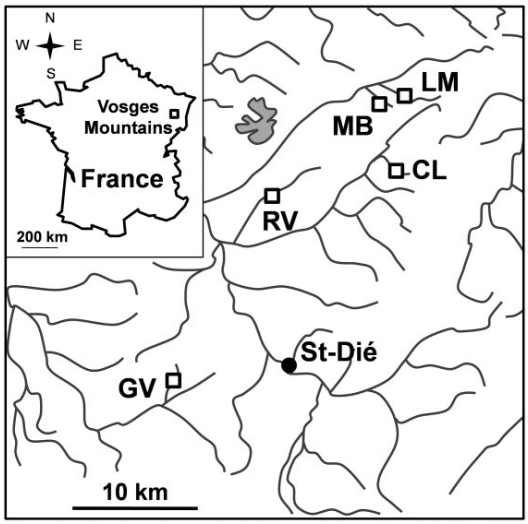

Fig. 5. Location of the five study sites in the Vosges Mountains, north-eastern France.

Chytridiomycetes, which do not contain ergosterol (Gessner *et al.*, 1991; Gessner and Newell, 2002), is slightly higher on leaves in the benthic zone of LM than in CL. Moreover, higher contributions of Basidiomycetes have been observed in both zones of CL when compared with those of LM, and Pasanen and colleagues (1999) reported high amount of ergosterol (37–42 mg per gram of fungal biomass) in some species affiliated with this phylum. Therefore, it is possible that fungal biomass on leaves could have been underestimated in non-impacted streams and overestimated in impacted streams, potentially minimizing the observed effects of acidification on mycelial growth. Accordingly, further molecular analyses and isolation of other fungal strains to assess their specific ergosterol contents are needed.

Conclusion

This study showed that molecular responses were complementary with the results obtained from a traditional approach. Indeed, sporulating aquatic hyphomycete diversity and contribution of this fungal group to clone libraries appeared to be major factors associated with leaf processing. This underlines the major role of aquatic hyphomycetes in leaf decomposition, and shows that constraints prevailing in the hyporheic zone and/or linked to anthropogenic disturbances could alter the efficiency with which they colonize and process plant matter.

To date, few molecular studies have attempted to characterize leaf-associated fungi. Therefore, further investigations are needed to better understand the contribution of each fungal taxon and what factors drive microbial assemblages and activities on decaying leaves. More attention to terrestrial fungi is also required owing to their significant presence on leaves in impacted streams as well as in hyporheic zones. Even if sporulation analysis underestimates leaf-associated fungal diversity, we confirmed that this method may serve as a valuable bioindicator of anthropogenic disturbance. The combination of both methods and its extension to investigations of the hyporheic zone of streams thus has the potential to deepen our knowledge of interactions between microorganisms and their role in the functioning of ecosystems, particularly when subject to human-induced alterations.

Experimental procedures

Site description

This study focused on the first 4 weeks of the litter bag experiment presented by Cornut and colleagues (2012b), which was conducted in the Vosges Mountains (north-eastern France) from mid-November 2008 to January 2009 in a forested area sensitive to acidification. Five first- and second-order streams subjected to different levels of acidification and along an Al gradient were selected. A map showing the location of the five study sites is presented in Fig. 5. The site characteristics are described in Cornut and colleagues (2012b).

Field experiment and leaf litter processing

Leaves of alder (*Alnus glutinosa* L.), one of the most common riparian tree species across our study sites, were collected from trees at abscission in October 2008, air-dried and stored at room temperature. Leaves (3.00 ± 0.03 g mean air-dry mass) were packed into plastic net bags (15×10 cm, 5 mm mesh), and 32 leaf bags were exposed in the benthic and hyporheic (i.e. buried 15–20 cm below the sediment surface) zones of each stream. Four replicate bags were randomly retrieved from each zone of the five streams after 7, 14, 21 and 28 days and placed in individual plastic bags with their respective stream water. Sets of five and ten 12 mm diameter discs were cut from the leaves, avoiding the central vein. The set of five leaf discs was frozen at −80°C until processing for DNA extraction, while the set of 10 discs was immediately used for sporulation experiments. Remaining leaf litter was oven-dried for 24 h at 105°C to constant mass and then weighed. Portions of dry leaf material were ignited in a muffle furnace for 4 h at 550°C to determine the ash-free dry mass (AFDM). Leaf mass loss was calculated by subtracting the AFDM of the remaining leaf litter from the initial AFDM. To accomplish this, the litter of four unexposed litter bags was used to determine the leaf initial AFDM. Leaf mass loss was then expressed as a percent loss of the initial AFDM.

Physical and chemical analyses

Dissolved oxygen concentrations in surface and interstitial waters were measured in each stream (Multi 350i, WTW, Weilheim, Germany). Surface and interstitial waters were collected from each stream at each of the four sampling dates and when leaf bags were initially introduced into the streams. The sampling protocol is described in detail in Cornut and colleagues (2012b). Stream pH was determined in the laboratory using a microprocessor pH meter (pH 3000, WTW). Acid-neutralizing capacity was measured by Gran's titration. Total Al concentrations (after acidification with HNO_3) were determined by atomic absorption spectrophotometry (AAnalyst 100; Perkin Elmer, Courtaboeuf, France and SpectrAA-300, Varian, Les Ulis, France).

The main physicochemical parameters during the experiment are detailed in Table 1. Briefly, La Maix (LM) and Gravelle (GV) are circumneutral (mean pH of 7.3) and moderately acidic (mean pH of 6.4), respectively, exhibiting low mean total Al concentrations in the water column (31 and 35 $\mu g\ l^{-1}$ respectively over the experimental period). Menombru and Ravines (RV) are circumneutral (mean pH of 7.0) and moderately acidic (mean pH of 6.2), respectively, with intermediate Al concentrations (100 and 113 $\mu g\ l^{-1}$ respectively). Courbeligne (CL) is the most heavily impacted stream, exhibiting low pH (mean of 4.6) and high Al concentrations (658 $\mu g\ l^{-1}$).

Sporulation and conidial identification

The protocol for sporulation experiments is detailed in Cornut and colleagues (2012b). Briefly, 10 leaf discs were incubated at 10°C during 48 h in aerated microcosms (Suberkropp, 1991) in 40 ml of filtered water from their respective streams. Conidial suspensions were then filtered (5 μm pore size filter), after being preserved with formalin (2.6% final concentration). Conidia on the filter were stained with Trypan blue (Bärlocher, 2005), after which they were identified and counted under the microscope (× 200). Conidial production was expressed as the number of conidia released per milligram leaf AFDM per day or as total conidial biomass released per mg leaf AFDM per day. Conidial biomass released was calculated using the individual conidial mass for the species mentioned in Chauvet and Suberkropp (1998) and those listed in Bärlocher and Schweizer (1983) with a conversion factor of 500 fg per μm^3 (Gessner and Chauvet, 1994). The average value of 200 pg per conidium was applied to the other species (Gessner, 1997).

For each stream and compartment, means of the four sampling dates were calculated using the relative contribution of each aquatic hyphomycete species to the total conidial production to compare fungal assemblages on leaves. Bray–Curtis distance matrices were generated (Bray and Curtis, 1957). Cluster analysis of aquatic hyphomycete assemblages were performed using the unweighted pair group method with arithmetic mean and illustrated with dendrograms (Legendre and Legendre, 1998).

DNA extraction and amplification

For molecular analysis, three leaf discs from each of the four replicates were pooled together for each sampling date. The 12 leaf discs were then finely ground with a micro-pestle, and total DNA from each composite sample was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). DNA concentrations were quantified after extraction using a Nanodrop ND-1000 spectrophotometer (Labtech, Palaiseau, France). Fungal partial 18S rRNA gene fragments were amplified using a two-step polymerase chain reaction (PCR) protocol (Oros-Sichler *et al.*, 2006). The first amplification used primers NS1 (5′-GTAGTCATATGCTTGT CTC-3′) and EF3 (5′-TCCTCTAAATGACCAAGTTTG-3′). The second amplification was performed using primers NS1 and FR1-GC (5′- CCCCCGCCGCGCGCGGCGG GCGGGGCGGGGGCACGGGCCGAICCATTCAATCGG TAIT-3′). PCR mixtures (100 μl) contained 6 U of Taq DNA polymerase (5 PRIME, Hamburg, Germany), 1x Taq buffer (5 PRIME), 200 μM of each deoxyribonucleotide triphosphate (dNTP), 0.5 μM of each primer and 50 ng of extracted DNA as template for the first PCR and a dilution of the first PCR amplicons (1:500) was used as template for the second amplification. The first PCR amplification protocol consisted of 5 min at 94°C, 25 cycles of 30 s at 94°C, 45 s at 47°C and 3 min at 72°C, followed by a final extension of 10 min at 72°C. The second step was identical to the first, except that the number of cycles was lowered to 20, and the annealing temperature was 48°C.

DGGE

The size of the amplification products was checked on 1% (w/v) agarose gel and separated using the DCODE Mutation Detection System (Bio-Rad, Hercules, CA, USA). For each stream and compartment, separate DGGE samples were run for each of the four sampling dates. A volume of 15 μl per sample was loaded onto 6% (w/v) polyacrylamide gels in 1× TAE buffer with a denaturing gradient of 25–40% [100% denaturant corresponds to 40% (v/v) formamide and 7 M urea]. The gels were run in 1× TAE buffer at 180 V and 58°C for 16 h and then stained with SYBR Green I. The gels were imaged with a STARION FLA-9000 scanner (Fujifilm Life Sciences FSVT, Courbevoie, France).

GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was used to normalize and align DGGE profiles.

To accomplish this, internal control samples were loaded onto each gel, amplification products of some of the studied samples being used as migration markers for alignment of DGGE profiles. For each stream and compartment, means of the four sampling dates were calculated using the band relative intensity to compare fungal assemblages on leaves. Bray–Curtis distance matrices were generated, and cluster analysis of DGGE mean profiles were performed as described above for aquatic hyphomycete assemblages. For each condition, the phylotype richness was calculated as the total number of bands over the 4-week period.

Cloning and sequencing of amplified 18S rRNA gene

Equal DNA quantities of the PCR products from the four sampling dates were pooled into a composite sample for each stream and each zone, after which they were cloned using a Clone JET PCR Cloning Kit (Fermentas, Villebon sur Yvette, France), and 72 individual colonies were sequenced by GATC Biotech (Konstanz, Germany) using the NS1 primer. Sequences were checked, and only high-quality sequences of approximately 530 bp were kept for phylogenetic analyses. All selected sequences were deposited in GenBank under accession numbers KC691747-KC691982.

Flagellospora curvula *18S rRNA gene sequence*

Flagellospora curvula dominated the conidial production (number of conidia) in benthic and hyporheic zones of CL and LM. However, in contrast to other species that are frequently observed in these streams, the 18S rRNA gene of FLCU had not been sequenced at the time that this study was conducted. To identify sequences of our clone library belonging to this species, 18S rRNA genes of two FLCU strains (FLCU 130–1655 and FLCU 180–1662) were sequenced. The single conidial isolates of these two FLCU strains were obtained from foam samples originating from two streams of southwestern France, Le Lampy (43°25′07″N; 2°11′15″E) and Les Montauds (43°29′14″N; 2°15′43″E), respectively. Cultures were grown and maintained on 2% malt-agar plates, after which both strains were cultivated in 2% malt liquid medium. DNA extraction from mycelia and amplification of the partial 18S rRNA gene were then conducted as described above for leaf samples. PCR products were sequenced by GATC Biotech using NS1 primer, and two sequences of 975 bp, which were identical between the two FLCU strains, were deposited in GenBank under accession numbers KC691983-KC691984.

Phylogenetic analyses

Multiple sequence alignments were performed using CLUSTALW (Larkin *et al*., 2007) at the default settings. Distance matrices were constructed using DNAdist (default parameters) in the PHYLIP package (Felsenstein, 2005). Operational taxonomic units (OTUs) were defined at a 99% sequence similarity cut-off using the MOTHUR software (Schloss *et al*., 2009). Sequences were individually compared with those deposited in the GenBank database using the BLAST algorithm (Altschul *et al*., 1990). Each OTU was affiliated with the fungal order according to the best match score obtained between sequences constituting the OTU and GenBank database. The name of the species whose sequence gave the nearest BLAST hit score among the sequences constituting each OTU is reported in Table 3. The relative abundances of fungal groups were defined by calculating the sequenced clone proportion of total sequences from each condition.

Statistical analysis

Differences in physicochemical variables, percentage of leaf mass loss and the rate of conidial production (logarithmically transformed data) were tested using two-way analysis of variance (ANOVA) with stream and zone as factors. Differences among groups were identified by post hoc multiple-comparisons (Tukey's HSD test).

The R Software was used for all statistical analyses (R Development Core Team, 2008). For this study, the level of significance was set at $P = 0.05$.

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