



# Leaf Litter decomposition in streams subjected to global change: the role of heterotrophic microbial communities

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Florent Rossi. Leaf Litter decomposition in streams subjected to global change: the role of heterotrophic microbial communities. Ecology, environment. Université Clermont Auvergne [2017-2020], 2018. English. NNT : 2018CLFAC101 . tel-02895270

**HAL Id: tel-02895270**

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ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE -  
AGRONOMIE - ENVIRONNEMENT

## THESE

présentée pour obtenir le grade de

### DOCTEUR D'UNIVERSITE

*Spécialité : Ecologie*

Par « **ROSSI Florent** »

**D.E.A. ou Master**

**Biologie et Environnement (BE) option Fonctionnement et Restauration des  
Milieux Aquatiques Continentaux (FREMAC)**

**Leaf litter decomposition in streams subjected to global  
change: the role of heterotrophic microbial communities**

Soutenue publiquement le 17 décembre 2018, devant la commission d'examen.

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## Extended abstract

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Human activity through industry, urbanization and agriculture, has led to the production and release of a large amounts of chemical compounds (including pesticides and pharmaceuticals) into the biosphere. One of the problematic related to the xenobiotic compounds fate is their transfer to aquatic ecosystems and the alteration of diversity and activity of microbial communities. Microbial communities associated with immersed leaf-litter can be impacted by these compounds, and in turn, alter global processes such as the carbon and nutrient cycling in the stream ecosystem. Accordingly, this thesis work aims to assess the effects of realistic chemical contamination on microbial leaf-litter decomposition process in streams.

The first chapter of this thesis was focused on the comparison of microbial decomposition activity in alder leaves in six watersheds presenting different land uses (agricultural, urbanized, forested) over four seasons (spring, summer, autumn, winter). The effect of the gradient of contamination on microbial organic matter processing from upstream to downstream sections in each watershed was also assessed. Monitoring revealed that microbial decomposition of leaves was slightly higher in contaminated watersheds (agricultural and urbanized) in comparison with control ones (forested), probably because of the compensation effect by nutrients over xenobiotics. However, this compensation mechanism was partial since fungal biomass accumulated in leaves was greatly reduced in contaminated watersheds. Overall, this highlights microbial communities being more efficient for leaf decomposition in polluted watersheds than in the less contaminated ones, which is probably explained by changes in microbial community structure.

The second chapter of this thesis aimed to evaluate *in vitro* the specific interactions between nutrients (nitrogen and phosphorus) and pesticides (herbicide and fungicide, alone or in mixture) exposure on microbial communities during leaf-litter decomposition. High nutrient concentrations (eutrophic conditions) tended to exacerbate the effects of pesticides on leaf decomposition rates suggesting that the compensatory mechanism of nutrients over pesticides observed in the previous part is probably concentration dependent and does not always apply to aquatic microbial communities. Moreover, a stimulation in laccase activity was observed when microbial communities were exposed to the fungicide, suggesting a role of this enzyme in detoxification mechanisms. However, the fact that such stimulation was not observed when exposed to the mixture of both pesticides (herbicide and fungicide) suggest that the interaction between these two molecules impaired the ability of microbial communities to display proper

stress response. These results constitute the first evidence of the potential interaction between an herbicide and a fungicide on leaf-associated microbial communities functioning.

In the third chapter of this thesis, the resistance and resilience abilities of fungal communities associated with submerged leaf-litter were evaluated *in vitro* using a translocation experiment between an upstream (less contaminated) and downstream (more contaminated) sites of an agricultural stream. As observed in the previous chapters, leaf-associated fungal communities appeared sensitive to variations in water chemical contamination, and especially in terms of extracellular laccase activity and fungal community structure. Interestingly, removing water contamination stress through the translocation of microbial communities from the more contaminated to the less contaminated channels resulted in a full recovery in terms of laccase activity and fungal community structure that can be explained by species transfer from the less contaminated to the more contaminated communities. These results highlight the importance of headwater stream fungal communities in supporting diversity for fungal communities in the more downstream sections.

In the last chapter of this thesis, we aimed to further understand the effects of nutrients and pesticides on the regulation of microbial extracellular laccase activity. Here, the response of such activity on microbial communities associated with leaf-litter and exposed to growing concentrations of both nitrogen ( $\text{N-NO}_3$ ) and a fungicide (tebuconazole) was assessed *in vitro* (dose-response tests). We observed that extremely high nitrogen concentrations stimulated extracellular laccase activity whereas the fungicide had no effects on this activity. These activity results showed opposite trends to what has been observed in previous chapters of this thesis, where laccase activity tended to be negatively affected by nitrates concentration especially in the less contaminated stream sections. However, we suspect different mechanisms to be involved between these different studies. In the dose-response tests, the observed stimulation in laccase activity can be explained by a stress response probably caused by an osmotic stress to microbes due to extremely high nitrogen concentrations. In contrast and given the involvement of extracellular laccase in nitrogen uptake, inhibition of such activity in the field can reflect a repression by the substrate, probably because microbial communities already met their nutritional demands on nitrogen.

In conclusion, the results of this thesis work showed that xenobiotics (including pesticides and pharmaceuticals) at environmentally relevant concentrations only have a limited effect on microbial decomposition of leaf-litter in stream ecosystems. Such low effect of the xenobiotics can be explained by the high plasticity and resilience capacity of microbial communities,

coupled with their potential to produce extracellular enzymes that can be deployed for stress defense purposes.

**Keywords:**

Chemical contamination, nutrients, xenobiotics, microbial communities, leaf-litter, organic matter decomposition, laccase activity.



## Résumé étendu

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L'activité humaine, au travers de l'industrie, l'urbanisation ou encore l'agriculture, a conduit à la production et au relargage d'une grande quantité de produits chimiques (dont les xénobiotiques) dans la biosphère. Une des problématiques liée aux composés xénobiotiques est leur transfert vers les écosystèmes aquatiques et l'altération de la diversité et des activités microbiennes. Ces communautés peuvent être impactées par ces composés, potentiellement affectant en retour les grands processus écosystémiques tels que le cycle du carbone. Ce travail de thèse vise donc à évaluer les effets d'une contamination chimique réaliste sur les processus de décomposition microbiens de la matière organique dans les écosystèmes lotiques via l'utilisation de la litière de feuilles comme descripteur.

Une première partie de ces travaux de thèse a été consacrée à décrire et à comparer la décomposition microbienne (bactéries et champignons) de la litière de feuilles au travers de la structure des communautés microbiennes (structure génétique et biomasse) et les activités associées (activités enzymatiques extracellulaires) dans six bassins versants présentant des occupations des sols différentes (urbanisés, agricoles et forestier) et au cours de quatre saisons (printemps, été, automne et hiver). L'effet du gradient de contamination depuis l'amont vers aval sur ce processus a également été évalué. La décomposition microbienne s'est avérée légèrement plus élevée pour les bassins versants agricoles et urbanisés par rapport aux bassins versants forestier (contrôle), probablement en raison de l'effet de la compensation des nutriments sur les xénobiotiques. Cependant, cette compensation ne s'est avérée que partielle, la biomasse fongique ayant été considérablement réduite dans les bassins versants contaminés. Ces résultats mettent en évidence une meilleure efficacité des communautés microbiennes pour la décomposition des feuilles dans les bassins versants présentant une contamination chimique, probablement par l'intermédiaire de changements au niveau de la structure des communautés microbiennes.

La deuxième partie de ces travaux de thèse consistait à évaluer *in vitro* les interactions entre l'eutrophisation des cours d'eau et des concentrations réalistes en pesticides (herbicides et fongicides, seuls ou en mélange) sur l'activité microbienne de décomposition de la litière de feuilles. Les fortes concentrations en nutriments (condition eutrophe) ont eu tendance à exacerber les effets des pesticides sur les taux de décomposition microbiens, suggérant que l'effet compensatoire des nutriments sur les pesticides observé dans la partie précédente dépend de la concentration et ne s'applique pas toujours aux communautés microbiennes aquatiques.

De plus, une stimulation de l'activité laccase a été observée chez les communautés microbiennes exposées au fongicide, ce qui suggèrent l'utilisation de cette enzyme en tant que mécanisme de détoxification. Cependant, le fait que cette stimulation n'ait pas été observée en présence du mélange de pesticides (herbicide et fongicide) suggère que l'interaction entre ces deux composés peut altérer la capacité des communautés microbiennes à se défendre contre le stress. Ces résultats constituent la première description de l'interaction potentielle entre un herbicide et un fongicide sur les communautés microbiennes associées à la litière de feuilles.

Par la suite, les capacités de résistance et de résilience des communautés fongiques associées à la litière ont été évaluées *in vitro* par une expérience de translocation, utilisant de l'eau provenant à la fois des sites amont (contrôles) et aval (contaminés) d'un cours d'eau. Comme observé dans le chapitre précédent, les communautés fongiques associées aux feuilles ont montré une certaine sensibilité aux variations de la contamination chimique, notamment sur l'activité laccase extracellulaire et la structure de la communauté. La suppression du stress engendré par la translocation des communautés microbiennes depuis les canaux contaminés vers les canaux contrôles a permis un rétablissement complet de l'activité laccase et de la structure de la communauté, qui peut notamment s'expliquer par des transferts d'espèces. Globalement, ces résultats appuient l'importance des cours d'eau de tête de bassin versant dans le maintien des processus de décomposition de la diversité au sein des écosystèmes lotiques.

Dans le dernier chapitre de cette thèse, nous avons voulu approfondir les connaissances sur la régulation de l'activité laccase par les nutriments et les pesticides. La réponse de cette activité chez les communautés microbiennes associées à la litière de feuilles et exposées à des concentrations croissantes en azote ( $\text{N-NO}_3$ ) et en fongicide (tébuconazole) a été évaluée *in vitro* (tests doses-réponses). Ces résultats montrent que des concentrations extrêmes en azote provoquent une stimulation de l'activité laccase extracellulaire, tandis qu'aucune variation de cette activité n'a été observée en présence du fongicide. Ces résultats d'activité montrent une tendance opposée aux observations de nos études précédentes, qui montrent une activité laccase extracellulaire qui tend à être négativement affectée par l'azote (nitrates), et en particulier dans les sections préservées des cours d'eau. Cependant, différents mécanismes semblent être impliqués entre ces différentes études. La stimulation de l'activité laccase observée lors des tests dose-réponse peut être expliquée par une réponse au stress probablement causée par le stress osmotique résultant des concentrations extrêmes en azote. À l'inverse, et sachant que l'activité laccase extracellulaire est impliquée dans l'acquisition d'azote, l'inhibition de cette

activité sur le terrain peut refléter une répression par le substrat, probablement dû au fait que les communautés microbiennes répondaient déjà à leurs besoins nutritionnels en azote.

En conclusion, les résultats de cette thèse ont montré que les xénobiotiques (incluant les pesticides et les produits pharmaceutiques) à des concentrations réalistes n'ont qu'un effet limité sur la décomposition microbienne de la litière de feuilles dans les écosystèmes des cours d'eau. Ce faible effet des xénobiotiques peut s'expliquer par la grande plasticité et la capacité de résilience des communautés microbiennes, associé à leur potentiel de production d'enzymes extracellulaires pouvant être utilisées à des fins de défense contre le stress.

**Mots clés:**

Contamination chimique, nutriments, xénobiotiques, communautés microbiennes, litière de feuilles, décomposition de matière organique, activité laccase.



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# **General introduction**

Earth is mainly covered with water. While most of this water constitutes the oceans (>96% of total water in earth), a much smaller portion (ca. 3%) is in the form of freshwater. Freshwaters include glaciers, groundwater, atmospheric water, lakes and rivers. Despite freshwater ecosystems represent no more than 0.01% of global water resources (Shiklomanov, 1993a), they support at least 100 000 species (corresponding to 6% of described species in the biosphere) and thus represent invaluable ecological value (Dudgeon *et al.*, 2006). In addition to their ecological importance, freshwater ecosystems also hold substantial socioeconomic values. Human health and well-being deeply rely on freshwater resources to satisfy body water requirements. Humans also need water for society functioning. This includes water demand for agriculture, industry, energy, navigation, fishing or recreation. However, this massive water demand is not without environmental consequences. Freshwaters pollution resulting from human activities has become a widespread phenomenon over the last decades. Overexploitation of water resources for industrial and domestic purposes coupled with agriculture intensification have led to the mass input of both nutrients (including nitrogen and phosphorus) and toxicants (including pesticides which are generally spread in the form of cocktails of molecules) threatening functions and services of many freshwater ecosystems (Trewavas, 2002; Vörösmarty *et al.*, 2010). In addition to this, human population is expected to reach more than 8 billion individuals by 2025. Given the increase in human population and resource use (Brown & Kane, 1995; Brown, 2000; Roy *et al.*, 2012), demands on water will intensify (Postel, Daily & Ehrlich, 1996), and forecasts predict an additional increase of water withdrawals by almost 20% by 2025. In this context, protecting biodiversity and functions of stream ecosystems has become essential and will certainly permit to better protect and manage water resources for human consumption.

Within freshwater ecosystems, rivers and streams account for the most impacted ecosystems on the planet. This is especially the case of headwater streams, whose small size in comparison with the amount of pollution received, makes them especially sensitive to disruption. Although they represent a tiny proportion of available freshwater stocks on earth, these ecosystems offer an enormous array of habitats for microbial, plant and animal life (Meyer *et al.*, 2007), thus representing a huge reservoir of biological species. Several authors have suggested that headwater streams are pivotal for maintaining functions (i. e. organic matter decomposition) and ‘health’ of river networks (Meyer & Wallace, 2001; Gomi, Sidle & Richardson, 2002; Bernhardt *et al.*, 2005; Lowe & Likens, 2005; Wipfli, Richardson & Naiman, 2007) and that they are important areas for maintaining biodiversity (Meyer & Wallace, 2001; Gomi *et al.*,

2002; Heino, 2005; Lowe & Likens, 2005; Meyer *et al.*, 2007; Richardson & Danehy, 2007). Besides, headwater streams provide a huge array of ecosystem services. This includes, provisioning services (provide goods such as drinking water), regulation services (control various processes such as floods or erosion), supporting services (water and nutrient fluxes, biodiversity conservation) and cultural services (provide spiritual, moral and aesthetic benefits) among the most important ones (Limburg, 2009).

Forested headwater streams in temperate regions are characterized by small forested catchments whose riparian forest provides shadow upon surface water, greatly limiting autotrophic production. Biological communities in these ecosystems largely rely on allochthonous organic matter inputs such as plant litter. The decomposition of plant leaf-litter is a pivotal mechanism in headwater streams since it permits to i) better link terrestrial organic matter production to aquatic ecosystems production and ii) allows the transfer of matter and energy through the aquatic food web and contribute to many biogeochemical cycles (N, C, P, ...) (Cummins, 1974; Gessner & Chauvet, 1994; Webster & Meyer, 1997). The decomposition of leaf litter in streams involves abiotic factors, such as leaching, followed by the joint action of microorganisms and macroinvertebrates (Mann, 1975a; Gessner, Chauvet & Dobson, 1999; Gaudes *et al.*, 2009). While macroinvertebrates are responsible of most of the leaf mass loss, microorganisms, and especially aquatic fungi, (Suberkropp & Klug 1976, Mann 1975; Gessner, Chauvet & Dobson 1999; Gaudes *et al.* 2009) can contribute up to 25-45% of leaf mass loss (Bärlocher & Kendrick, 1975; Hieber & Gessner, 2002; Baldy *et al.*, 2007a). They play also an indirect role by conditioning leaf-litter in streams, subsequently enhancing its palatability for macroinvertebrate feeders.

In the context of streams' contamination by human activities, the influence of water chemical pollution on stream ecosystem functions (i. e. organic matter decomposition) has become a central research issue. A better understanding of the effect of chemical contamination on heterotrophic microbial communities' biodiversity and activity would provide valuable informations for stream managers and societies in their challenge for stream ecosystems preservation.

This thesis work provides original research on the effect of stream water chemical contamination, including xenobiotics and nutrients, on the resistance and resilience of microbial communities' structure and decomposition activity. To answer this main objective, both field and microcosm experiments were combined in order to assess i) the sensitivity of microbial

communities to realistic stream water chemical contamination gradients in the field, ii) the potential interaction between nutrients (phosphorus and nitrate) and pesticides (herbicide and fungicide) on microbial communities, iii) the resilience of these communities when removing chemical contamination is removed and iv) the potential use of microbial enzymatic response as biomarker of chemical contamination exposure in streams.

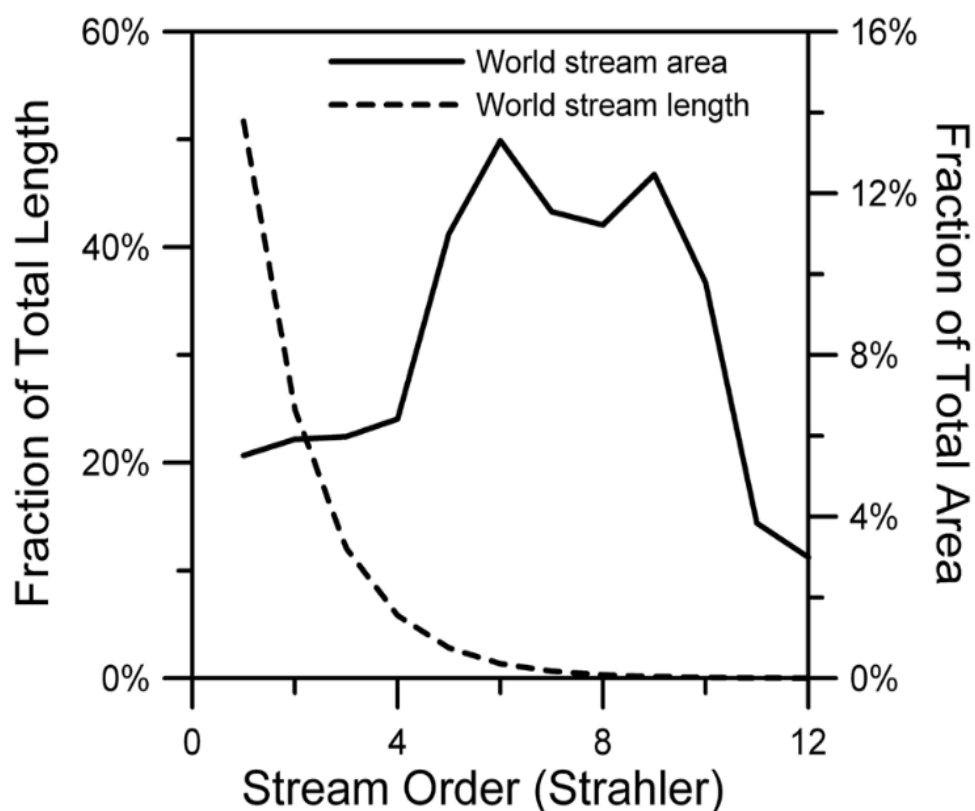
## **State of the art**

## 1. Temperate forested streams

Streams originate from overland flow and from soil saturated zones according to the watershed geology, topography, and vegetation, as well as to the precipitation types (Bravard & Petit, 2009). One characteristic of fluvial systems is the directed motion of water masses (Sukhodolov, Kozerski & Rhoads, 2009), which begins with upstream sources to the downstream large rivers, to finally reach seas and oceans. From this particular layout, Strahler (1957) developed the widely accepted classification of streams, known as the ‘stream order’ concept. According to this classification, streams can roughly be grouped into 3 categories: headwaters streams (Strahler order 1 to 3), medium-sized streams (Strahler 4 to 6) and large rivers (Strahler > 6) (Vannote *et al.*, 1980). While each of these categories vary from each other in terms of physical and chemical properties, they remain interconnected and thus influence each other. This notion, known as the *river continuum* concept was first proposed by Vannote *et al.* in 1980. The *river continuum* concept proposes that understanding the biological strategies and dynamics of river systems requires consideration of the longitudinal gradient of physical and chemical factors from upstream to downstream as well as the surroundings of the watershed. From the upstream part of the stream to its downstream extent, the physical variables within a stream system present a continuous gradient of physical and chemical conditions inherent to the stream, including width, depth, velocity, flow, temperature, and entropy gain. Due to exceptions to the model, particularly in arid lands and grasslands, where streams are dammed, the *river continuum* concept has suffered several criticisms over the last decades (Montgomery, 1999; Benda *et al.*, 2004; Fisher & Sponseller, 2009). Particularly, this model fails in integrating parameters such as temporal variability, natural disconnection of the streams, and physical disturbances (storms, fires, floods) that can modify the linear perspective inherent to the *river continuum* concept (Reeves *et al.*, 1995; Poff, 1997; Rice, Greenwood & Joyce, 2001). This led to the emergence of new theories such as the *river discontinuum*, the network dynamic hypothesis (NDH) or the *process domain concept* (PDC) that try to integrate the patchiness, the stochastic disturbances and the landscape-scale pattern of streams (Montgomery, 1999; Poole, 2002; Benda *et al.*, 2004). Nonetheless, the fact that river ecosystems exhibit continuous change in a downstream direction is still largely accepted.

Headwater streams are generally long and thin and have a high surface to volume ratio (Fisher & Sponseller, 2009) as shown in Figure 1. As a consequence, they are highly influenced by exchanges from the adjacent terrestrial systems and especially from the surrounding riparian vegetation, providing shadow upon the water surface but also allowing for inputs of a large

amount of allochthonous detritus during the fall (Vannote *et al.*, 1980). As stream depth and width increase, the contribution of allochthonous organic matter decreases and the opening of the canopy cover permits light to reach surface water. Following that succession pattern, stream ecosystems functioning evolve from a more heterotrophic (upstream) to a more autotrophic (downstream) gradient. The zone through which the stream shifts from a heterotrophic-dominated to an autotrophic-dominated metabolism, depend on the degree of shading and is commonly fixed at order 3, although this seems highly stream-dependent (Minshall, 1978). Given the fact that headwater streams represent the vast majority of the hydrographic network in terms of linear (reaching up to 60% to 75% of world stream length Fig.1), (Hansen, 2001; Benda *et al.*, 2005; Leopold, Wolman & Miller, 2012) and that litter decomposition can prevail up to tens of kilometers from headwaters (Webster, 2007), allochthonous organic matter inputs from the riparian forest are of central importance in the functioning and integrity of stream ecosystems.



**Figure 1:** Fraction of World River and stream area and length related to stream order (Strahler). (Figure from Downing *et al.* (2012)).



## 2. Forested streams functioning

### i. Origin and type of organic matter inputs

As mentioned above, headwater forested streams are highly influenced by organic matter inputs from terrestrial origin, which represent the largest part of organic matter sources for in-stream organisms. For example, up to 99% of the energy input can be imported from the terrestrial surroundings in the tiny first order streams, with only 1% derived from stream photosynthesis by mosses and algae (Fisher & Likens, 1973; Cummins, 1974). Organic matter comes from vertical inputs (directly from the trees) or lateral inputs (from the surrounding river bank) (Elosegi & Pozo, 2005), though these inputs may vary considerably from one stream to another due to differences in riparian species vegetation and/or stream order. Reported plant litter inputs (including vertical and lateral inputs) in 33 north American streams ranging from order 1 to 9 with different cover types (mixed deciduous forest, coniferous forest, shrub cover) showed values ranging 0 in a clear-cut stream to  $4363 \text{ g m}^{-2} \text{ yr}^{-1}$  in a mixed deciduous forest (Benfield, 1997). Besides, the study of Connors & Naiman (1984) showed that annual litter inputs per unit of stream surface area decreased exponentially with increasing stream order (from  $307\text{-}534 \text{ g m}^{-2} \text{ yr}^{-1}$  in first order stream (81-95% of organic carbon supply) to  $15\text{-}13 \text{ g m}^{-2} \text{ yr}^{-1}$  in sixth-order stream (5-15% of organic carbon supply)) while autochthonous organic matter production showed the opposite trend ( $20\text{-}34 \text{ g m}^{-2} \text{ yr}^{-1}$  production in first order (5-19% of organic carbon supply) to  $96\text{-}168 \text{ g m}^{-2} \text{ yr}^{-1}$  in sixth-order stream (85-95% of organic carbon supply)). Similarly, direct annual litter inputs in European deciduous forested streams and rivers, are estimated to range from 42 to  $719 \text{ g m}^{-2}$ , depending on stream order (Weigelhofer & Waringer, 1994; Abelho & Graça, 1998a). Other sources of variation in litter inputs include climate, vegetation, type of soils, age of trees, and geomorphological characteristics of streams (Abelho, 2001a). For instance, most of the litter inputs occurs in autumn in temperate deciduous forests representing up to 79% to the annual inputs (Abelho & Graça, 1998a), whereas most of the inputs in Australian eucalyptus forest (31-75%) occurs in spring or summer (Abelho & Graça, 1996a; Grigg & Mulligan, 1999a). Another parameter influencing organic matter disponibility is leaf retention, which mostly rely on stream geomorphology. The retention of leaves in a stream channel can vary considerably depending on both hydrology and substrate-related features (Abelho, 2001a). For example, retentiveness in first order streams is estimated to the first 3-5 meters against 17 meters in third-order streams (Webster *et al.*, 1994; Larrañaga *et al.*, 2003).

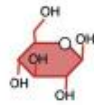
Alloctonous organic matter inputs are various and include leaves, logs, branches, floral parts, nuts, fruits, spores, among others (Abelho, 2001a). However, leaves often represent the major part of total alloctonous inputs, ranging from 41 to 98% of it. Main alloctonous organic matter inputs occurs in autumn in temperate streams and materials can remain stored from some weeks to some months in the benthos thus providing food and energy to the trophic web. After reaching the stream, leaves are progressively degraded and transformed into particulate, fine and/or dissolved organic matter to be finally transformed into carbon dioxide and nutrients (Cummins, 1974). Hence, leaf litter decomposition is an important component in stream nutrient cycling and is achieved by the action of both biotic and abiotic factors. Leaf chemical composition is one of the most important factors controlling the decomposition process.

## ii. Chemical composition of leaves

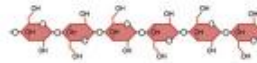
Leaves are among the most important organs in vascular plants. There is a huge variety of leaves displaying different shape, structure and chemical composition from one species to another. These variations depend on many factors such as plants adaptation to climate, light and nutrient availability, herbivory or competition with other plants (Evert, 2006). However, leaves are typically broad flat and thin, and hold a major role in plant photosynthesis as well as in water and chemical energy storage. Leaves are composed of several types of complex polymers (e. g. cellulose, hemicellulose, pectin and lignin) embedding a large quantity of nutrients (e. g. nitrogen; Fig. 2). Current knowledge suggest that common features exist in plant cell walls composition and structure among different groups of the *Plantae* Kingdom (Fig.3) (Sarkar, Bosneaga & Auer, 2009). Plant cell walls are overall composed of cellulose microfibrils that form the mechanical framework of the wall, and a matrix that forms cross-links among microfibrils and fills the space between fibrillar frameworks (McCann, 1991; Carpita & Gibeaut, 1993; O'Neill & York, 2003; Somerville *et al.*, 2004). In the secondary walls of plant cells, hemicellulose form covalent links with lignin. The tight association between cellulose, hemicellulose and lignin confers thus high resistance to the tissue and renders its breakdown difficult (Sarkar *et al.*, 2009). In non-grass angiosperms (Fig.3 A), a high amount of hemicelluloses (fucoside xyloglucan (XG), xylans and some mannans) and structural proteins can be found. The primary walls have high amounts of pectins (homogalacturonans (HG), rhamnogalacturonan (RG) I and II) while the secondary walls have high amounts of lignins with guaicyl (G) and syringyl (S) units.

## Cellulose

Glucose



Cellulose



Cellulose microfibril



## Hemicelluloses

Glucose



Xylose



Galactose



Arabinose



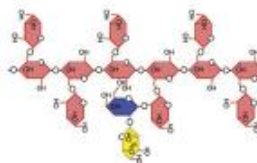
Mannose



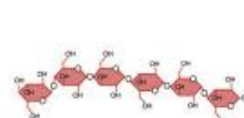
Fucose



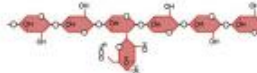
Fucoside xyloglucan



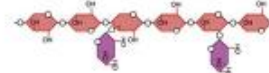
Mixed-linkage glucan



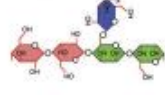
4-O-methylglucuronoxylan



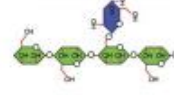
Arabinoxylan



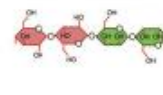
Glucogalactomannan



Galactomannan

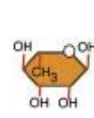
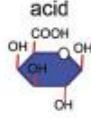


Glucomannan

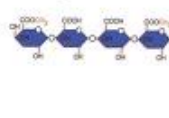


## Pectins

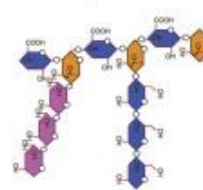
Galacturonic acid Rhamnose



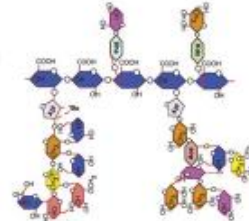
Homogalacturonan



Rhamnogalacturonan I

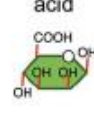
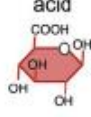


Rhamnogalacturonan II



## Uronic acids

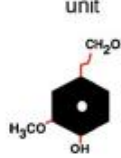
Glucuronic acid Mannuronic acid



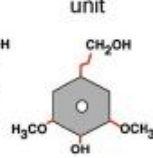
Unidentified polymers

## Lignins

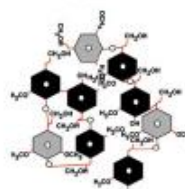
Gualacyl unit



Syringyl unit



Lignin GS



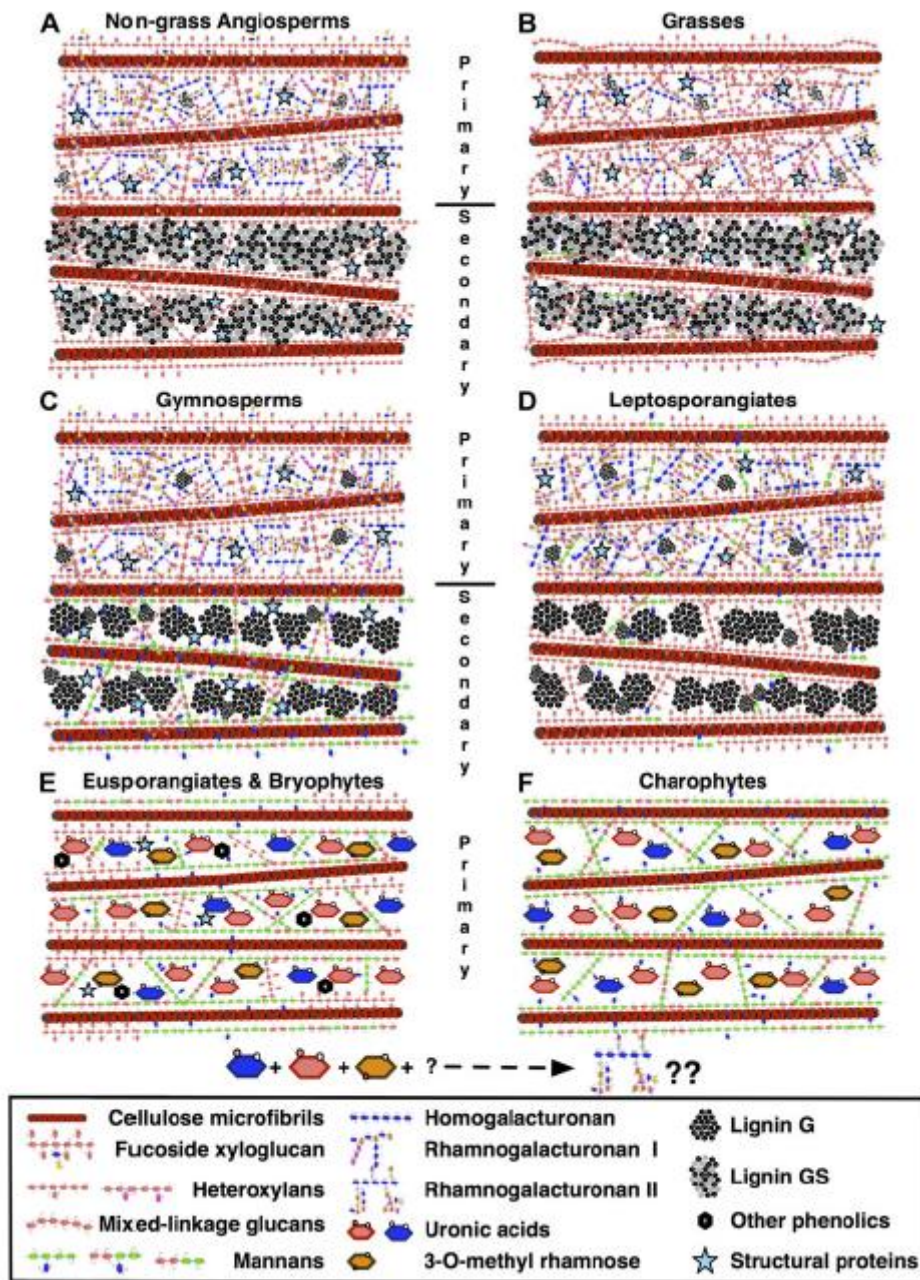
Lignin G



**Figure 2:** Chemical structure of the predominant building blocks of plant cell walls. Left panel: monomers. Right panel: subunit of the respective polymers (Figure from Sarkar, Bosneaga & Auer, 2009).

Cellulose and hemicellulose are the main constituents of leaves, although variations in the content between these two compounds have been observed among species. For instance, cellulose content may range from 13.8 % of leaf Dry Mass (DM) for *Alnus glutinosa* leaves to 32.2% of leaf DM in the case of *Fagus sylvatica* (Gessner, 2005). Both cellulose and hemicellulose are polysaccharides consisting in the assemblage of several hundred to many thousands sugar monomers. In the case of cellulose ( $(C_6H_{10}O_5)_n$ ), the only monomer is the D-glucose whose association forms a linear chain, whereas xylose, galactose, arabinose, mannose, and fucose can also be found in hemicellulose (Fig. 2, Klemm *et al.*, 2005; Scheller & Ulvskov, 2010). Lignin content in leaves is generally lower than that of cellulose or hemicellulose, although this may vary with species, ranging from 6.8 % of leaf DM (*Fraxinus excelsior*) to 30.9 % of leaf DM (*Platanus hybrida*). In the case of *Alnus glutinosa*, reported lignin content ranges between 8 to 12 % of leaf DM (Chauvet, 1987; Gessner, 2005). In contrast with cellulose and hemicellulose, generally considered as labile, lignin is much more recalcitrant. Lignin is a complex aromatic heteropolymer formed by the association of three main monolignols precursors: p-coumaryl, coniferyl and sinapyl alcohols, differing within each other by their degree of methoxylation (Río Andrade *et al.*, 2017). Schematically, the lignin molecule can be viewed as a three dimensional amorphous polymer whose precursors association produces two differently linked guaiacyl (G) and syringyl (S) lignin units, generating a huge variety of structures within the polymer (Fig. 2). Hence, unlike other biopolymers, lignin contains no readily hydrolysable bond recurring at periodic intervals along a linear backbone, providing high resistance to degradation (Singh *nee* Nigam & Pandey, 2009).

Aquatic ecologists often consider the chemical content of the leaves as the amount of nutrients available to feed decomposer and/or consumer organisms. Since decomposer microorganisms recover leaf nutrients for their growth, C N and P content in leaves are very important descriptors of



**Figure 3:** Simplified 2D representation of general cell wall composition in the different groups of Kingdom Plantae, including non-grass angiosperms (A), grasses (B), gymnosperms (C), leptosporangiates (D), eusporangiates, bryophytes (E) and charophytes (F). All groups have cellulose microfibrils. Note: Spatial distribution, orientation, size and proportion of polymers in this diagram are not per scale. (Figure from Sarkar *et al.* (2009)).

their chemical quality and consequently good proxies to estimate microbial decomposition rates (Enríquez, Duarte & Sand-Jensen, 1993; Graça, Bärlocher & Gessner, 2005). Slight variations in nutrient content within leaves from the same species or from the same sampling sites have been reported. Dawson & Funk (1981) observed variations in the nitrogen content from alder leaves directly collected from the trees ranging between 1.5 to 3% of the total dry mass according to the season. Such differences appears to be driven by environmental factors, such as temperature, rainfall levels, light availability or soils nutrients (Wright, Reich & Westoby, 2001). Apart from these slight differences within the same species, huge variations in nutrient content are often observed between species. Generally, leaves nutritional quality are expressed as nutrient molar ratios (i. e. C:N, N:P and C:P). For example, oak leaves show high C:N ratio (ranging 46.3 - 93.6) but moderate C:P ratio (ranging 167-596), whereas alder leaves show low C:N ratio (19.27) but moderate C:P ratio (673). Accordingly, the rate at which the litter is decomposed generally depends on litter intrinsic characteristics (Ferreira *et al.*, 2016). In a general way, leaves displaying low C:N and C:P ratios are decomposed faster than leaves displaying higher C:N and C:P ratios. Similarly, leaves toughness is an important descriptor to assess leaf-litter decomposition, and rates tend to be inversely proportional to the initial lignin concentration of the leaf.

Forests surrounding rivers and streams usually include several riparian species. Accordingly, the influence of species diversity in mixed species litter decomposition has been investigated. In soil ecosystems, the presence of certain labile leaf species such as alder tends to accelerate the overall breakdown of mixed species litter (Briones & Ineson, 1996). However, most of the studies in stream ecosystems failed to find a general relationship between leaf species richness and breakdown rate (Swan & Palmer, 2004). Overall, mixing different leaf species leads to non-additive effects on breakdown rates, with no differences observed between the single species conditions and the mixed ones (Kominoski *et al.*, 2009; Kominoski, Marczak & Richardson, 2011; Ferreira, Encalada & Graça, 2012).

### iii. Leaf litter decomposition processes

#### 1) Abiotic factors

The very first step in leaf litter decomposition in streams is purely abiotic: leaching. According to the literature, leaching occurs within 24h after immersion of leaves in stream water and may contribute up to 33% of the leaf mass loss (Petersen & Cummins, 1974; Benfield, 1996; Gessner *et al.*, 1999). Although this mechanism is not completely understood, it is generally assumed that simple organic compounds such as reducing sugars, amino acids and phenolic substances as well as nutrients (i. e. phosphorus and potassium) are simply leached out from the leaf surface to end up in the stream water (Jensen, 1974; Suberkropp *et al.*, 1976; Gessner and Schwoerbel, 1989). Leaching can vary depending on the way that leaves reach the stream water (i.e. vertical or lateral inputs), which may further affect abiotic and/or biotic decomposition processes. For example, leaves from vertical inputs (falling directly into the stream water) will lose P gradually over more than two weeks, whereas leaves from lateral inputs (coming from the surrounding riverbanks) lose the same amount of P within a couple of days. This is because tissues in natural air-dried leaves are already heavily damaged (Gessner, 1991). Moreover, air-drying of leaves amplify leaf mass loss during the leaching phase. In the study of Gessner and Schwoerbel, (1989), pre-dried *Alnus* leaves lost ca. 20% of their total mass within 24 hours whereas non pre-dried leaves did not experience significant mass loss over a period of 6.5 days. Accordingly, this could be attributed to retention mechanisms present in the plant to retain soluble cell components such as proteins and pigments. Cuticle and epicuticular waxes serves as an efficient barrier to water-soluble cell compounds by directly impeding permeation. The chemical constitution of the waxes and the morphological structure of the leaf surface have thus great importance in the leaf surface wettability (Tukey Jr, 1970; Jeffree, 1986). Pre-drying of leaves irreversibly damages the cuticular cover, enhancing permeation, and resulting in the observed differential mass losses between fresh and air-dried leaves. Since most of studies in litter decomposition use pre-dried leaves enclosed in mesh bags, leaching is perhaps less widespread in the environment than commonly assumed. However, to which extent these practices influence the estimation of actual in-stream leaf decomposition is currently unknown.

Physical fragmentation is the other main abiotic parameter involved in leaf litter decomposition. It results from abrasion exerted by the flowing water and frictions against rocks and transported sediments and contributes to the release of fine particulate organic matter into stream water (Witkamp & Frank, 1969; Hodkinson, 1975; Ferreira *et al.*, 2006). Non-disturbed headwater



streams are generally constituted by a succession of geomorphic units provoking an alternance of water velocities and depths within the stream section (Malavoi & Souchon, 2002). The nature and succession pattern of these geomorphic units is inherent to the stream and depend on many factors such as stream order, elevation, water flow and velocity or the nature of the bed substrata. This heterogeneity within the stream channel associated with the hydromorphological characteristics in the reach have been shown to highly influence leaf-litter decomposition (Bastias Álamo, 2018). In their work, the authors recorded a 3 fold variation in terms of decomposition rates (ranging from  $0.0076^{-1}$  to  $0.0222 \text{ day}^{-1}$ ) between study reaches, mostly explained by differences in terms of water velocity. Overall, this indicates that streambed hydromorphological heterogeneity may highly influence leaf-litter processing in streams, and that this parameter should be considered to avoid bias in decomposition rates estimation.

## 2) Biotic decomposition

Within the next hours after leaves fall into the stream, they will be colonized by microorganisms. These microorganisms are mostly heterotrophic and form communities attached to the leaves embedded in a complex matrix of extracellular polymeric substances called biofilms (Carrias & Sime-Ngando, 2009). Microbial decomposition of leaf-litter involves the combined action of a wide range of microorganisms (Slater & Lovatt, 1984). While it is widely accepted that bacteria and fungi are the main contributors due to their extracellular enzyme capabilities, other microorganisms may act indirectly, such as protists and photosynthetic algae, that will be discussed later on in this manuscript. The contribution of microbes to leaf litter decomposition is highly variable but remains non-negligible, generally ranging between 25% and 45% of the total leaf mass loss (Bärlocher & Kendrick, 1975; Hieber & Gessner, 2002; Baldy *et al.*, 2007a). However, microbial decomposition has already been shown to greatly exceed that of macroinvertebrate shredders. This is especially the case in disturbed streams where high concentrations of pesticides and nutrients and/or stream hydromorphological modifications (i.e. resulting in a loss of habitats) may alter the diversity and richness of aquatic invertebrates. In the study of Pascoal *et al.* (2005a), reported that microbial decay rates in a polluted river (obtained from fine mesh bags) represented more than 75% of the total decomposition rates (obtained from coarse mesh bags) mostly because of the poor



representativeness of macroinvertebrates along the stretches of their study sites. Besides, microbial communities play also an indirect role by conditioning the leaf substrate for macroinvertebrate consumers. Leaves maceration by microbial enzymatic activities combined with the accumulation of microbial biomass of high nutritional value has been described to enhance leaf decomposition by macroinvertebrates. Thus, microbial degradation is considered as a key mechanism determining leaf breakdown rates in streams.

## Fungi

Fungi are the dominant group colonizing decaying leaves and wood substrates in streams. Estimation of fungal biomass using ergosterol (a lipid specific to cell membranes of higher fungi) concentration as proxy, revealed that fungi account for 95–99% of the total microbial biomass in decaying leaves (Gessner & Chauvet, 1994; Hieber & Gessner, 2002; Pascoal & Cássio, 2004; Duarte *et al.*, 2010). Up to now, about 2000 freshwater fungal species have been described in the literature, including representatives of all major fungal taxonomic groups (Chytridiomycota, Zygomycota, Basidiomycota and Ascomycota, (Gulis, Kuehn & Suberkropp, 2009a)). However, the most commonly found on decaying leaves in streams are hyphomycetes. These fungi, also known as Ingoldian fungi, comprise over 300 species, most of them belonging to the Ascomycota phylum (Webster, 1992; Shearer *et al.*, 2007; Gulis, Kuehn & Suberkropp, 2009b). Aquatic hyphomycetes are anamorphic microscopic fungi with mycelial growth and hyphae developing on or within the leaf substrate (Gulis *et al.*, 2009b). Their specificity lies in their adaptation to stream environments through the production of conidia (asexual spore of fungi), allowing them to easily colonize new substrates after their dispersion in water (Fig.4). However, their whole life cycle in streams is still poorly understood. Moreover, there is no evidence yet on the exact phylogenetic origin of aquatic hyphomycetes. A recent publication using molecular phylogeny approaches highlighted the great affinity of aquatic hyphomycetes with soil fungi, and especially the Leotiomycetes class of Ascomycota phylum (Baschien *et al.*, 2013). Besides, certain studies have shown that aquatic hyphomycetes may reach leaves before they enter the stream (Bandoni, 1972), highlighting the amphibian behavior of certain species. These results reinforce the hypothesis that aquatic hyphomycetes are natural descendants of terrestrial fungi who evolved to adapt to aquatic environments.

Aquatic fungi, including hyphomycetes, also dominate leaf-associated microbial communities in terms of activity. Specifically, their contribution to the overall leaf carbon loss ranges

between 15 and 38% (Hieber & Gessner, 2002; Pascoal & Cássio, 2004). Fungi are very active at the early stage of leaves decomposition, whereas their biomass and activity tend to decline during later stages due to grazing by macroinvertebrates. At these later stages, fungi become more active in spore production and release them into the water column to colonize new substrates (Gulis *et al.*, 2009b). Fungi are mostly associated with the decomposition of coarse particulate organic matter (1-100 mm) in streams. They decompose organic matter using a large panel of extracellular enzymes, hydrolyzing and/or oxidizing the main constituents of the leaves (previously described in section 2). These enzymes include proteolytic, phospholytic, cellulolytic (cellulases, hemicellulases) and ligninolytic enzymes (laccase, phenol oxidase, peroxidase), which transform high molecular weight polymeric compounds such as cellulose, hemicellulose, and lignin into smaller molecular weight compounds that can be easily assimilated by the fungal cell (Abdullah *et al.*, 1989; Chandrashekar & Kaveriappa, 1991; Abdel-Raheem & Shearer, 2002; Romani, Artigas & Ylla, 2012). Overall, fungi are recognized as the main producers of extracellular ligninolytic enzymes and thus act as key players in the decomposition of lignified recalcitrant compounds. According to the model of Berg (1986), litter decomposition begins with the degradation of easily assimilable non-lignified compounds whereas later stages involve the degradation of the most recalcitrant fraction, concomitant with the higher ligninolytic activity (Artigas, Romani & Sabater, 2004a). Following that pattern, Nikolcheva, Bourque & Bärlocher (2005) observed a decrease in fungal diversity in the later stages of leaf-litter decomposition, indicating that some fungal strains are more efficient in the decomposition of lignified compounds.

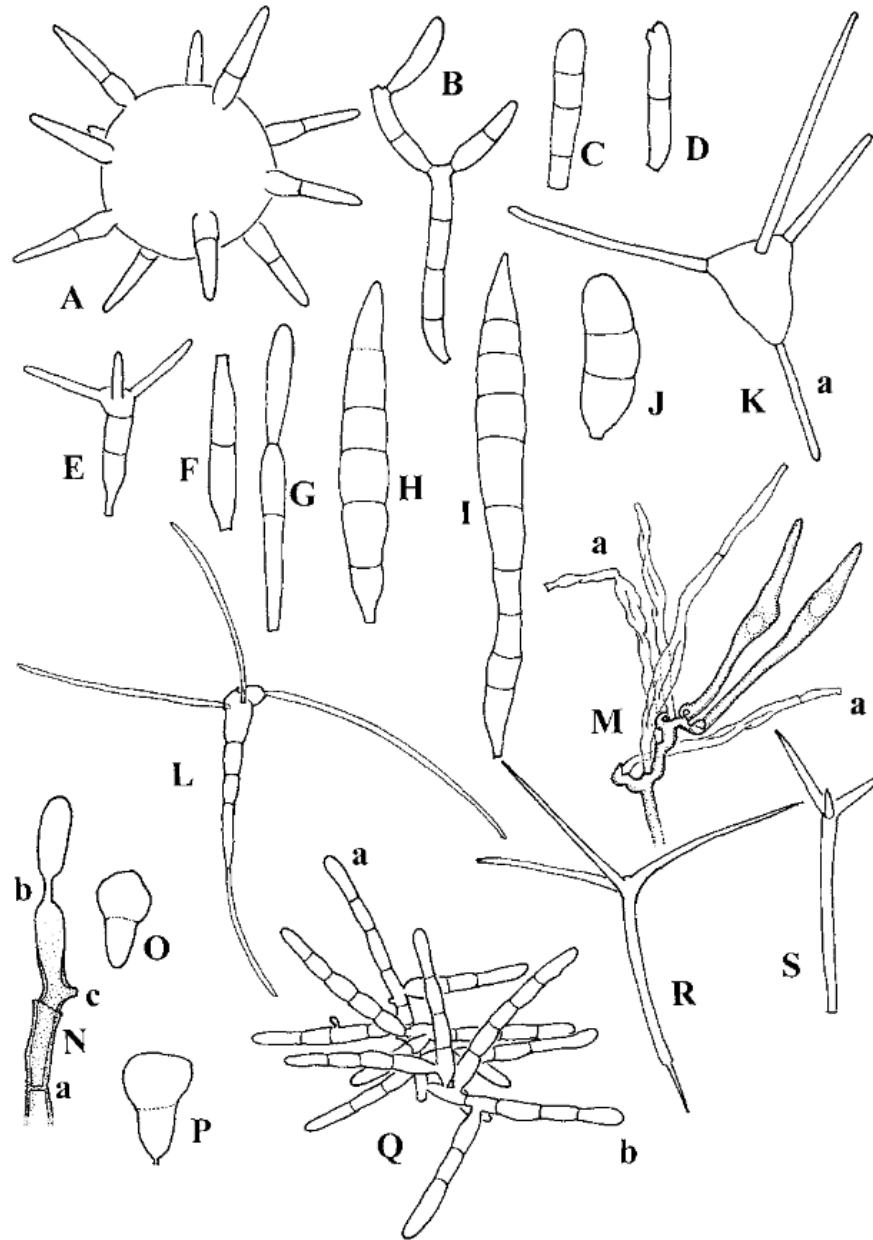


Figure 4: Examples of conidia produced by aquatic fungi. (Figure from Descals, (2005)).

### Microbial extracellular enzymatic activities

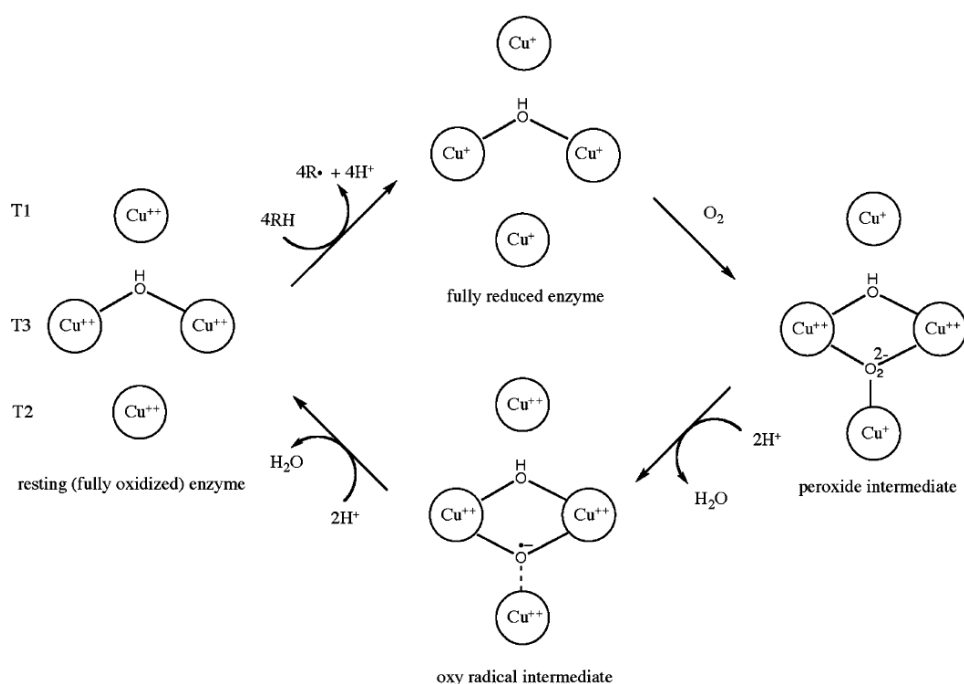
As stated above, one essential function of heterotrophic microbial communities is the processing and recovery of key nutrients from the leaves (Caldwell, 2005). Such activity requires the utilization of extracellular enzymes to degrade the lignin barrier and to process complex organic compounds into assimilable subunits (such as sugars, amino acids). Hence, the enzymatic degradation of biopolymers requires the synergistic action of several classes of enzymes, and each of these classes can be linked with the production of assimilable P, C or N sources for microorganisms. Accordingly, microbial demand can be associated with the elemental stoichiometry of microbial biomass in relation to the environmental nutrient availability (Sinsabaugh, Hill & Shah, 2009). Hence, an equilibrium between C:N:P activity ratios of 1:1:1 is indicative of an equilibrium between the elemental composition of available organic matter, microbial biomass and nutrient assimilation (Romaní *et al.*, 2013). Following that basis, the theory of “coenzymatic stoichiometry” developed by Sinsabaugh, Hill & Shah (2009), suggests that microbial nutrient demand can also be reflected on the extracellular enzymatic activities, whose regulation is highly dependent on the available substrate present on the leaf or in stream water.

There are several classes of enzymes, each of which is involved in a specific nutrient cycle. The most abundant compounds that are generally associated with the carbon cycle are cellulose, hemicellulose and lignin. The main enzymes involved in the decomposition of cellulose and hemicellulose of submerged leaves are cellulases (López-Mondéjar *et al.*, 2016). Most of them (EC 3.2.1.4) possess an hydrolytic activity which consists in the cleavage of the  $\beta$ -1,4 bond in the polysaccharide chain. Cellulases are classified according to their mode of action as endocellulases (cleaving internal bonds of the cellulose chain), exocellulases or cellobiohydrolases (acting on the reducing or non-reducing ends of cellulose chains) and  $\beta$  - glucosidases (converting cellobiose into glucose monomers) (Baldrian & Valášková, 2008a; Horn *et al.*, 2012a). Production of cellulases and hemicellulases have been reported in fungi, however, it is generally assumed that bacteria are the main contributor to polysaccharide decomposition (Romaní *et al.*, 2006a). The complete enzymatic decomposition of lignin requires several oxidative enzymes that occur more or less simultaneously. Most of these ligninolytic enzymes belong to the class of polyphenol oxidases and include lignin peroxidases (EC 1.11.1.14) and laccases (EC 1.10.3.2). Laccase are perhaps the largest class of enzymes within the polyphenol oxidases family belonging to the multi copper enzymes. These are proteins containing four copper active sites that catalyzes each one-electron oxidation of four

reducing substrate molecules concomitant with the four-electron reduction of molecular oxygen to water (Piontek, Antorini & Choinowski, 2002; Wong, 2009, Fig.5). In addition to lignin, laccase can oxidize many other phenolic compounds (Baldrian, 2006).

The main compounds that are generally associated with the nitrogen cycle are proteins and peptides and the enzymes involved in the degradation of such compounds are proteases (EC 3.4). Proteases constitute a huge group of enzymes that break down protein molecules through peptide bond hydrolysis to produce amino acids (Eskin & Shahidi, 2012). Proteases include endo and exopeptidases depending on their site of action which in turn include aminopeptidases (EC 3.4.14) which act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide (Souza *et al.*, 2015).

Enzymes involved in the phosphorus cycle are phosphatases. Organisms can only assimilate dissolved phosphate and therefore phosphatase activity plays a fundamental role in the transformation of P into available forms (Margalef *et al.*, 2017). There is a huge variety of distinct phosphatases which differ from each other by their substrate specificity and sequence homology in catalytic domains. However, all phosphatases catalyze the same general hydrolysis reaction that uses water to cleave a phosphoric acid monoester into a phosphate ion and an alcohol (Dighton, 1983).



**Figure 5:** Catalytic cycle of laccase. (Figure from (Wong, 2009))

## Bacteria

The second microbial group present in decomposing submerged leaves is bacteria. Despite they represent less than 5% of the microbial biomass in leaves, bacteria can be as active as fungi when regarding literature reports (Hieber & Gessner, 2002). In contrast with fungi, who can penetrate the leaves tissues thanks to their hyphae, most of bacteria are restricted to colonize leaf surfaces, and eventually the surface of the fungal hyphae (Gulis & Suberkropp, 2003). There is a huge diversity of bacteria inhabiting streams. Reported bacterial species richness on leaves often exceed those of fungi by a factor 2 to 5, generally reaching 30 to 40 different species, although similar richnesses have already been reported (Das, Royer & Leff, 2007; Mykrä, Tolkkinen & Heino, 2017). However, still little is known about the real bacterial diversity in streams. Based on the amplification of the 16S rRNA genes, Methé, Hiorns & Zehr (1998) reported that *Proteobacteria* (mostly  $\alpha$  and  $\beta$ ) constitutes the major phylum in freshwater habitats, usually representing more than 50% of the total number of sequences. Other main phyla include *Bacteroidetes*, *Actinobacteria* and *Acidobacteria* (Heino *et al.*, 2014). Interestingly, new generation sequencing technics (such as direct sequencing of the 16S rRNA

gene) allowed to highlight distinct microbial assemblages between freshwater and soil ecosystems suggesting that the vast majority of bacterial species are exclusive to freshwaters (Logue, Bürgmann & Robinson, 2008). On the other hand, many of the freshwater clades appear to have widespread geographic distributions (Yannarell & Kent, 2009).

Although bacteria have faster growth and turnover rates comparing to fungi, they are considered as less important for plant litter decomposition, especially because they lack the enzymatic machinery that breaks down the plant fibers (Romaní *et al.*, 2006a). Contribution of bacteria to overall leaf carbon loss rarely exceeds 15% (Pascoal & Cássio, 2004). As for fungi, bacteria are capable to synthesize a large array of extracellular enzymes. However, bacteria are mostly associated with the decomposition of simple polymeric compounds resulting from fungal primo-degradation (Allison & Vitousek, 2005). The above statement remains confusing since ligninolytic activity has also been described for bacteria (Kumar & Chandra, 2018). Then, bacteria are supposed to play a key role in the decomposition of fine particulate and dissolved organic matter, explaining their higher contribution in later stages of leaves decomposition in streams (Baldy, Gessner & Chauvet, 1995).

### **Benthic microalgae and protists**

Although we observed that autotrophic production in forested streams was globally minor compared to the heterotrophic one, this does not necessarily means that primary producers are unimportant for heterotrophic microbial communities functioning (Danger *et al.*, 2013). Primary producers can colonize the surface of leaf substrates and have been shown to participate indirectly to the decomposition of leaves. Resulting from their photosynthetic activity, algae can release substantial amounts of labile carbon exudates permitting to accelerate leaves decomposition by heterotrophs. The stimulation of the decomposition of recalcitrant compounds by high-quality organic matter resources is called the “priming effect”. The priming effect has been widely studied in soils (Kuzyakov, 2010) but less frequently in aquatic ecosystems (Guenet *et al.*, 2010; Kuehn *et al.*, 2014). These labile algal-based exudates could facilitate the coexistence between fungi and bacteria and among fungal species by contributing to create new ecological niches (Danger *et al.*, 2013). This is especially the case for bacteria that lack the enzymatic machinery to degrade leaves’ complex polymers (e.g. lignin) and thus are highly dependent on fungi. Unfortunately, most studies addressing the priming effect in

heterotrophic microbial communities are laboratory microcosm approaches which complicate the extrapolation of this phenomenon to the field.

Heterotrophic protists are also associated to decaying leaves in streams (Gaudes *et al.*, 2009). This group of unicellular eukaryotes includes ciliates, amoeba and heterotrophic flagellates, (Sanders, 2009). The way how protists affect leaf decomposition has been poorly investigated. However, heterotrophic protists, which are the smallest consumers of microbes and especially bacteria, may act as top-down controls of bacterial abundance and carbon flow (Stuart, Lucas & Newell, 1981; Fenchel, 1986; Berninger, Finlay & Kuuppo-Leinikki, 1991; Finlay & Esteban, 1998). Protist grazing on bacteria would enhance leaf decomposition rates because of higher bacterial turnover in response to grazing pressure by consumers, stimulating overall microbial decomposition (Sherr & Sherr, 1994; Ribblett, Palmer & Wayne Coats, 2005).

### Macroinvertebrates

Stream macroinvertebrate communities include aquatic insects belonging to the Ephemeroptera, Plecoptera, Odonata, Diptera and Trichoptera orders (Tachet, Bournaud & Richoux, 1991), as well as other invertebrates taxa such as crustaceans (Gammarid amphipods) and mollusks (Wallace & Eggert, 2009). The main functional feeding groups involved in the fragmentation of submerged leaves in streams (i.e. release of particulate and fine organic matter from leaves) are shredders and grazers-scrappers. Typical shredder organisms are Gammarid amphipods, many Plecoptera, Trichoptera and some Tipulid dipterans that feed mostly on coarse particulate organic matter (leaves). Conversely, typical grazer organisms are Ephemeroptera (mayflies) that feed almost exclusively on the periphyton, plant roots, and to a lesser extent on leaf litter (Graça, 2001a; Winkelmann *et al.*, 2007).

Macroinvertebrates are considered as the main contributors to leaf mass loss in streams (i. e. 64% and 51 % for alder and willow leaves in temperate streams, respectively, (Hieber & Gessner, 2002)). Their contribution to leaf decomposition is tightly dependent on population densities, leading to some cases in which no differences between microbial and macroinvertebrate decomposition are observed (Graça, 2001a). In addition, macroinvertebrate shredders show feeding preferences depending on the leaf species (Canhoto & Graça, 1995; Schulze & Walker, 1997). For example, shredders tend to preferentially feed on alder leaves (mean consumption of  $0.044 \text{ mg mg DW}^{-1} \text{ day}^{-1}$ ) rather than eucalyptus leaves (mean consumption of  $0.01 \text{ mg mg DW}^{-1} \text{ day}^{-1}$ , (Webster & Benfield, 1986; Canhoto & Graça, 1995,



1996)). Such differences appear to be driven by leaf chemical composition (including leaf toughness, nutrient content, and the presence of indigestible/defensive chemical compounds). Indeed, freshwater macroinvertebrates typically lack the enzymatic machinery to digest cellulose and lignin (Bärlocher, 1985; Suberkropp & Wallace, 1992; Graça, Maltby & Calow, 1993; Rong, Sridhar & Bärlocher, 1995). This highlights the high dependence of macroinvertebrates to the microbial conditioning of leaves, the latter providing micronutrients not present in leaf tissues to the former (Cargill *et al.*, 1985). Consequently, microbial colonization of leaves influences macroinvertebrate shredders feeding behavior since they preferentially feed on conditioned leaves (Kiran, 1996). As an illustration, shredders assimilation efficiencies are considerably higher when ingesting microorganisms (50-92%) than when just ingesting plant detritus (6-35%) (Graça, 2001b). However, it seems not to be the case for all fungal species. For example, *Alatospora acumunata*, *Clavariopsis aquatica*, or *Flagellospora curvula* were reported as species preferred by *Gammarus fossarum* whereas *Tetracladium marchalianum*, appeared rejected by the later (Bundschuh *et al.*, 2011a). Although the basis of such selection is not clear, several authors advanced that such differences may come from the production of distasteful compounds by some fungal species (Arsuffi & Suberkropp, 1989). Indeed, fungi can produce a variety of secondary metabolites involved in microbe-microbe interactions for defensive purpose, which may repel macroinvertebrates at the same time.

### 3. River ecosystems and human activity

Anthropogenic activity, through agriculture, urbanization, industrial activities or transports, poses a great threat to water resources quality and quantity. This contamination is especially controversial when affecting headwater stream ecosystems that are considered as reservoirs of biodiversity and responsible of pivotal functions for river downstream sections. The mass inputs of a large amount of chemical compounds, including xenobiotics, stream eutrophication by nutrients from fertilizers uses and bed hydrophormological modifications to facilitate human activities provoke massive damages on stream biodiversity and the ecosystem services they provide (Harding *et al.*, 1998; Sala *et al.*, 2000). Sadly, freshwater ecosystems are among the most endangered ecosystems in the world and it is generally agreed that the intensity and rate of

recent perturbations have no precedent since the beginning of the Holocene, 11500 years ago (Mann, Bradley & Hughes, 1998; Barnett, Pierce & Schnur, 2001; Barnett *et al.*, 2004). Europe makes no exception to this finding. Most of European rivers and streams are highly impacted by eutrophication, and this has been recognized of significant environmental concern since the late 1980s up to nowadays (European Environment Agency, 2010, Leaf, 2017). Moreover, more than a half of the monitored rivers in Europe are contaminated by at least one pesticide compound (WISE WFD Database, Malaj *et al.* (2014)), excluding pharmaceuticals.

Variable (units)	Oligotrophic	Mesotrophic	Eutrophic
mean benthic chlorophyll (mg m <sup>-2</sup> )	<20	20–70	>70
maximum benthic chlorophyll (mg m <sup>-2</sup> )	<60	60–200	>200
suspended chlorophyll (µg L <sup>-1</sup> )	<10	10–30	>30
Total N (µg L <sup>-1</sup> )	<700	700–1500	>1500
Total P (µg L <sup>-1</sup> )	<25	25–75	>75

**Table 1:** Suggested trophic boundaries for rivers and streams (from Dodds et al. 1998). Note: these were based on current nutrient distributions in the United States at the time, not corrected for anthropogenic influence (an unknown proportion of the sites used to create these distributions were true reference sites). (Data from Dodds & Smith (2016))

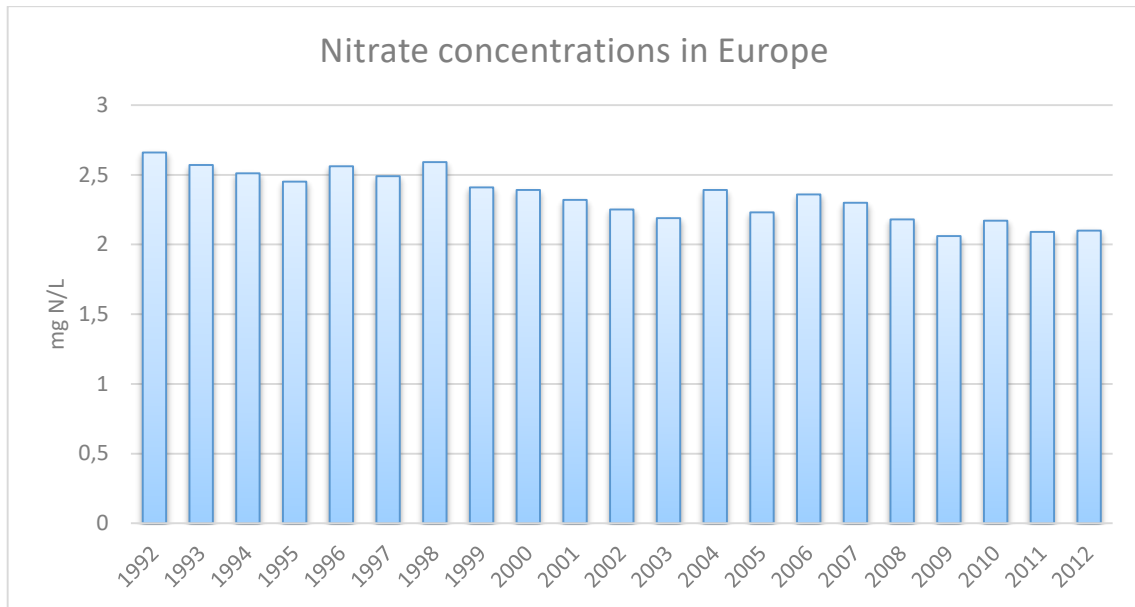
#### iv. Effects of eutrophication

Eutrophication, from the Greek eutrophos, literally “well-nourished”, can be defined as the increase in the rate of supply of organic carbon to an ecosystem, stimulated by inputs of mineral nutrients such as nitrogen and/or phosphorus (Richardson & Jørgensen, 1996). This concept was first introduced for lake ecosystems by Naumann in 1931 (as cited in Hutchinson, 1967) to describe the increase in autotrophic production due to nutrients by phytoplankton (Dodds & Cole, 2007). This concept has been adapted to headwater streams, where eutrophication levels must take into account mineral nutrient concentrations and autochthonous organic matter production (Dodds, 2007). Dodds & Smith, (2016) suggested a new classification system for rivers and streams trophic boundaries that we will further use in this thesis manuscript as basis for stream classification (Table 1).

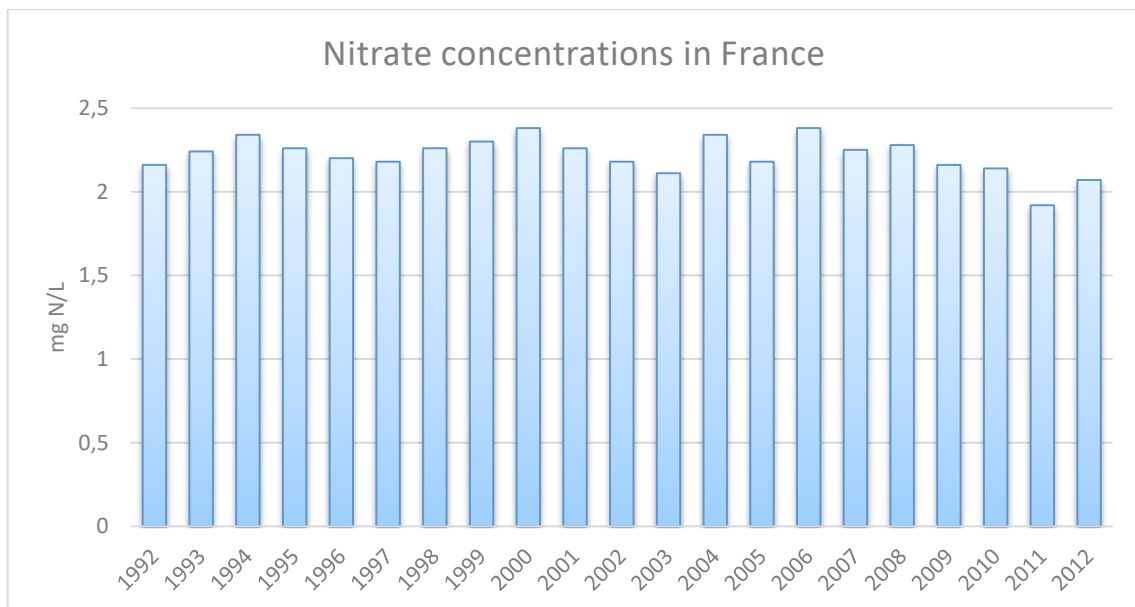
Eutrophication is a natural phenomenon, and each stream has its own trophic baseline, which depends on the region, climate, riparian vegetation, and benthic substrata composition. However, human activities have greatly accelerated this phenomenon by large nutrient inputs in streams over the last decades. We can distinguish direct and indirect causes for stream eutrophication derived from human activities. Direct causes are characterized by point-source and chronic source inputs of nutrients (nitrogen and phosphorous) and dissolved organic carbon in stream water. These pollution sources are derived from agriculture, waste water treatment plants, domestic waste, among others. Indirect causes include modifications of stream hydromorphology such as channelization and afforestation. Channelization decreases water residence time and afforestation increases nutrient inputs. Interestingly, seasonal patterns in nutrient concentrations (especially nitrates) in temperate streams subjected to agricultural pressure revealed that nitrate concentration is maximal in winter and minimal in summer. These inter seasonal variations in nitrate concentrations can be explained by i) a higher nitrate leaching due to the lower nutrient retention by the riparian vegetation in winter (Arheimer, Andersson & Lepistö, 1996; Holloway & Dahlgren, 2001; Ocampo, Sivapalan & Oldham, 2006; Molénat *et al.*, 2008) and ii) higher in-stream nitrogen uptake and denitrification rates in summer comparing to winter (Peterson *et al.*, 2001; Mulholland *et al.*, 2008, 2009).

Europe is particularly concerned by nutrient contamination in streams and rivers, recording decreasing but high nitrate concentrations between 1992 and 2012. Average nitrate concentrations in 2012 in European rivers were about 2 mg L<sup>-1</sup>, (Fig.6A). In France, a similar pattern can be observed (Fig 6B), with half of the regions (mostly in northern France) exhibiting average nitrate concentrations of 10 mg L<sup>-1</sup>. Nutrient data available since 1992 confirm that nitrate concentrations in French rivers remained stable and that this is the main parameter responsible for their eutrophication. In contrast to nitrate, phosphate concentrations in European streams and rivers show a progressive decrease over the last 20 years (Fig.7A). In 2012, average phosphorus concentrations display values ranging 50-75 µg L<sup>-1</sup>. Phosphate concentrations in French rivers have also decreased over the last 20 years, recording concentrations of 48 µg L<sup>-1</sup> in 2012 (Fig.7A). This observed pattern of nutrient concentrations variations in streams and rivers can mostly be explained by fertilizer sales (Fig.8). While nitrogen fertilizer uses remains stable over time, phosphorus fertilizers uses have greatly decreased in the last 20 years. Despite the efforts of the European Water Framework Directive to improve surface water quality in terms of nutrient concentrations, most of rivers in France (*idem* for Europe) are still considered as eutrophic regarding Dodds & Smith, (2016) classification (Table 1).

A



B



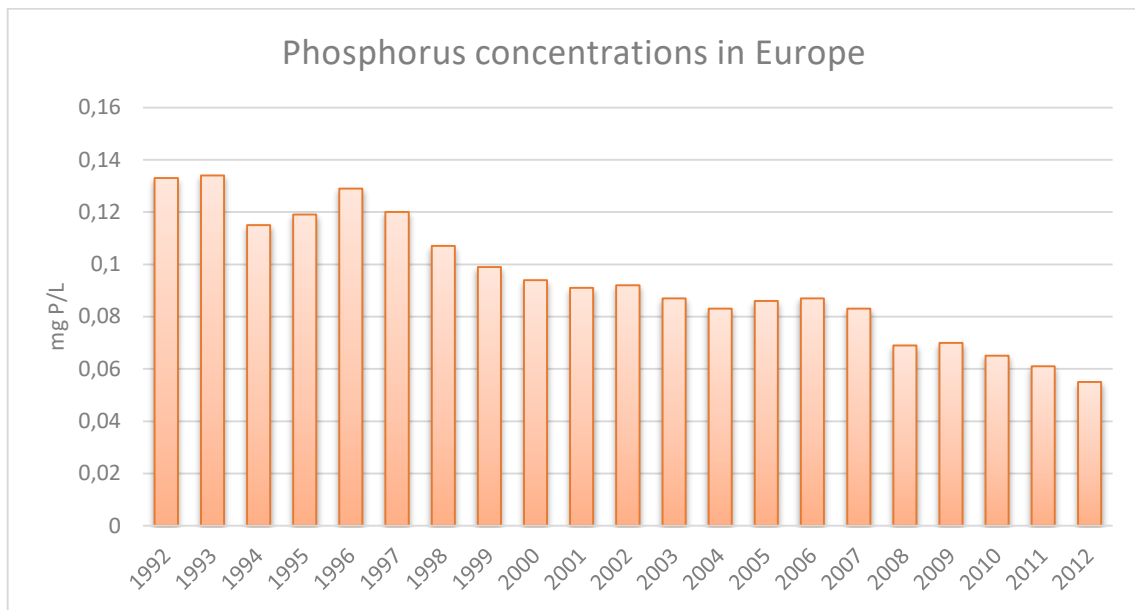
**Figure 6:** A Average nitrate concentrations in rivers and stream in Europe between 1992 and 2012. Values are means. B Average nitrate concentrations in rivers and stream in France between 1992 and 2012. (Source: Waterbase - Rivers provided by European Environment Agency (EEA)).

Nowadays, streams and rivers eutrophication has become a major problem worldwide, and has many consequences for both aquatic communities biodiversity and ecosystem processes

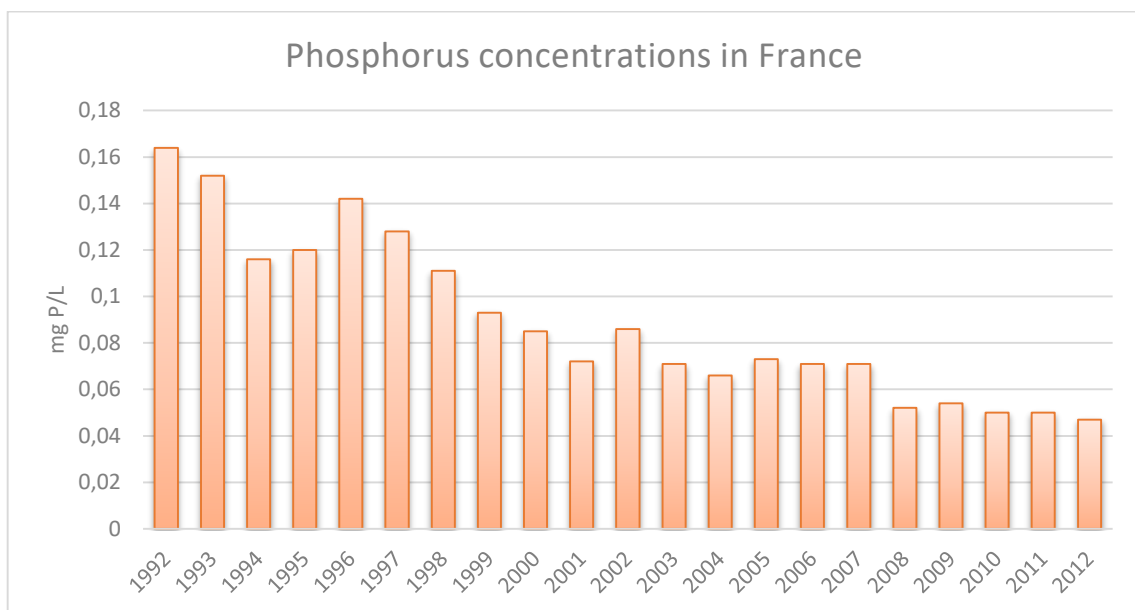
(Vitousek *et al.*, 1997a b; Nijboer *et al.*, 2004). Although there is considerable controversy regarding which nutrient (nitrogen or phosphorus) influences the most litter decomposition, it is overall accepted that nutrient enrichment accelerates leaf mass loss, stimulates microbial activity and enhances fungal biomass accumulation (Artigas, Romaní & Sabater, 2004b; Hagen, Webster & Benfield, 2006; Magbanua *et al.*, 2010). (Ferreira *et al.*, 2015) conducted a meta-analysis study including 840 case studies and showed that overall nutrient enrichment by both nitrate and phosphate stimulated leaf-litter decomposition rates by approximately 50%. Interestingly, this study also revealed that stimulation was lower when nitrate or phosphate were added separately. Nutrient addition has also been reported to modify C:N and C:P molar ratios of the immersed leaves. In most cases, this can be explained by a modification of the nutrient molar ratios of the attached microbial communities. However, Kominoski *et al.* (2015) also observed a reduction of leaf C:N and C:P molar ratios associated to a higher carbon uptake by microorganisms to compensate dissolved nitrogen and phosphorus addition.

Besides microbial stimulation, various studies observed a negative effect of nutrients on litter decomposition which depends on the concentration and nature of amendments (Baldy *et al.*, 2002; Duarte *et al.*, 2009). Indeed, inhibition of leaf decomposition rates may occur when dissolved nutrient concentrations reach toxic levels (Lecerf *et al.*, 2006; Ferreira & Chauvet, 2011). For instance, high nitrite ( $\text{NO}_2^-$ ), ammonium ( $\text{NH}_4^+$ ) and ammonia ( $\text{NH}_3$ ) concentrations (above 103, 1800 and 15.6  $\mu\text{g L}^{-1}$ , respectively) in eutrophic streams have been shown to decrease fungal diversity and biomass, as well as their potential for decomposing leaf litter (Baldy *et al.*, 2002; Duarte *et al.*, 2009). Nitrogen concentration increases have been shown to decrease certain fungal extracellular enzyme activities (Sinsabaugh, 2010). This seems particularly the case of the ligninolytic activities, mostly because of their involvement in nutrients uptake by aquatic fungi (Osono, 2007; Baldrian & Valášková, 2008a; Horn *et al.*, 2012a; Walker & White, 2017a). When nutrient amendments stimulate litter decomposition, eutrophication might narrow the window of opportunity for allochthonous organic matter decomposition leading to changes in stream ecosystem functioning (i. e. increasing autotrophy, Sabater *et al.*, 2011). Moreover, another important issue that needs to be taken into account when investigating contaminated streams functioning today, is the presence of micropollutants, possibly interacting (positively and/or negatively) with nutrients.

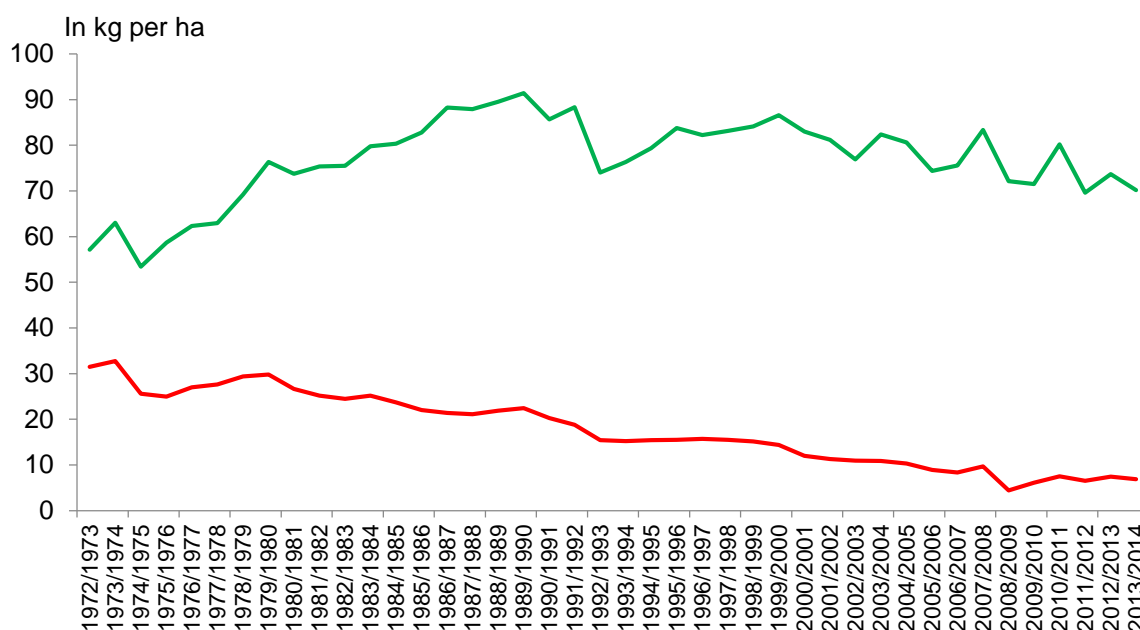
A



B



**Figure 7:** A Average phosphorus concentrations in rivers and stream in Europe between 1992 and 2012. Values are means. B Average phosphorus concentrations in rivers and stream in France between 1992 and 2012. (Source: Waterbase - Rivers provided by European Environment Agency (EEA)).



**Figure 8:** Evolution of nitrogen (green) and phosphorus (red) fertilizers delivered per hectare (ha) of fertilizable area between 1972 and 2014 in France. (Sources: Unifa, enquête sur les livraisons d'engrais; Maaf/SSP).

#### v. Effects of micropollutants

Micropollutants constitute a large family of organic, mineral and/or biological substances of anthropic origin, present at low concentrations (from ng/L to µg/L) in the environment, and being able to display severe ecotoxicological effects on living organisms and/or ecosystems integrity. Micropollutants include pharmaceuticals, pesticides, personal care products, steroid hormones, industrial chemicals, and many other emerging compounds such as nanoparticles. In this manuscript, we will focus on pharmaceutical and pesticide molecules effects on microbial litter decomposition in streams.

## Pharmaceuticals

Pharmaceuticals contamination in streams received growing interest from societies, stream managers and scientists over the last decades. There are approximately 4000 pharmaceutical molecules on the market belonging to many different families, including antibiotics, beta-blockers, anxiolytics, anti-hypertensors, anti-inflammatory, analgesics, and stimulators, among others (Monteiro & Boxall, 2010). These compounds have been essentially developed for human or veterinary uses, though a large number are also used in agriculture to fight against plant diseases (Rosi-Marshall & Royer, 2012). Water chemical analyses have revealed growing concentrations of pharmaceuticals in many stream ecosystems across the world over the last decades (Kolpin *et al.*, 2002). Main pollution sources include livestock farming and hospital effluents. Drugs supplied to humans and animals can be only partially metabolized and end up in the environment (Bundschuh *et al.*, 2009). Due to an incomplete elimination of these compounds by actual wastewater-treatment plants, residues of many pharmaceutical products can reach surface waters. Antibiotic compounds such as trimethoprim, ciprofloxacin or sulfamethoxazole are the most frequently detected, with concentrations ranging from 10 to 2000 ng/L (Nikolaou, Meric & Fatta, 2007). However, occurrence of antibiotics in stream waters is highly variable among countries, regions and/or seasons.

Up to now, few studies have focused on the effects of antibiotics on leaf-litter decomposition in streams, investigating for effects on microbial communities and macroinvertebrates. Because of their intrinsic mode of action, antibiotics (bactericides and fungicides) are expected to cause adverse effects to aquatic biological communities, especially microbes (bacteria and fungi) (Haritash & Kaushik, 2009; Minguez *et al.*, 2016). However, reported effects on these communities appears almost insignificant at environmentally relevant concentrations (Bundschuh *et al.*, 2009, 2017; Jonsson *et al.*, 2015). Only very high concentrations of the antibiotic ciprofloxacin (above 100 µg/L) were reported to induce changes in aquatic microbial community structure, but not on macroinvertebrate community structure (Maul *et al.*, 2006). Very little literature is available on the effect pharmaceutical compounds on leaf litter decomposition, other than antibiotics. To our knowledge, only Hughes, Kay & Brown (2016) tested the effect of beta-blockers (propranolol), anti-inflammatory (diclofenac, ibuprofen, mefenamic acid) alone or in mixture with an antibiotic (erythromycin) on leaf-litter decomposition. Results revealed that exposure to these pharmaceuticals in mixture at either 7.2 µg L<sup>-1</sup> or 7.2 mg L<sup>-1</sup> did not influence litter breakdown by microbes and/or macroinvertebrates, or leaf colonization by macroinvertebrates. Hughes *et al.* (2016) concluded that release of

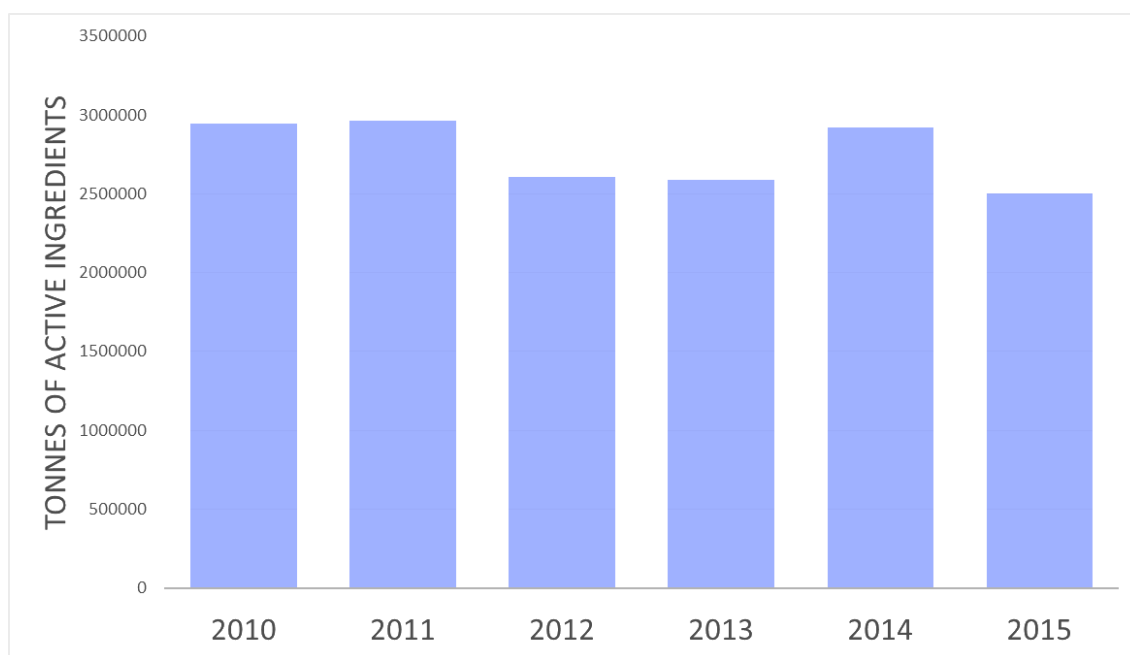


sewage effluents do not represent a risk to leaf-litter breakdown in streams, though this statement must be taken with great caution. However, streams and rivers are often contaminated by complex mixtures of xenobiotic compounds (including pharmaceuticals and pesticides). To which extent these various compounds may interact with each other and impact aquatic organisms remains unknown.

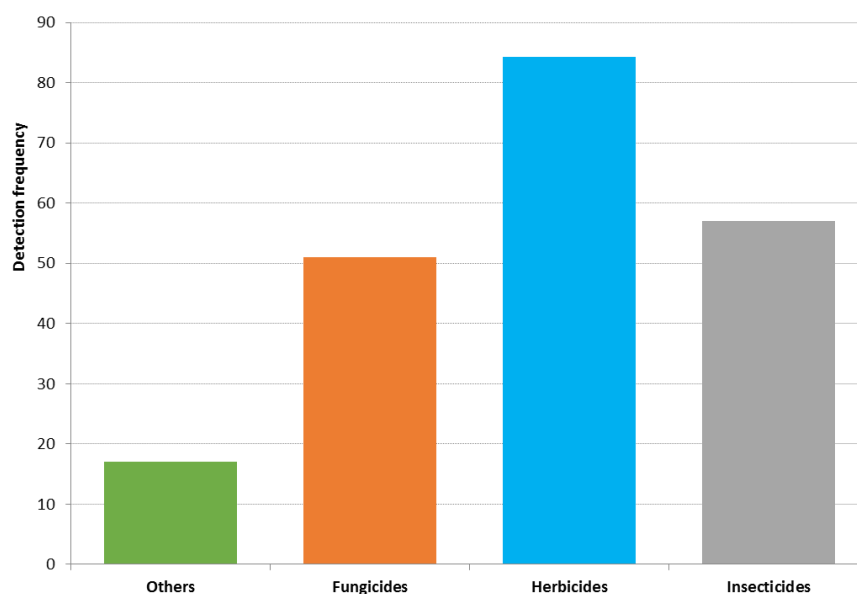
## Pesticides

Pesticides constitute another class of contaminants present in stream ecosystems at concentrations often higher than those of pharmaceuticals. Pesticide compounds include herbicides, fungicides, insecticides, nematicides, plant growth regulators, among others. While they can be used in a wide range of applications including pathogen and weed control, they mostly originate from agricultural practices. Changes in agricultural practices to increase agricultural yields (i.e. use of cocktail of pesticide molecules instead of high doses of a unique molecule) have led to use mixtures of different pesticide compounds. In 2015, pesticide consumption worldwide was estimated to 250,000 tons of active ingredients, and this consumption does not seem to significantly decrease since 2010 (Fig.9) (Food and Agriculture Organization of the United Nation, FAOSTAT). Among this huge quantity of pesticides used, half of the detected compounds in surface waters are herbicides followed by fungicides and insecticides in similar proportions (Fig.10). Pesticide spreading often occurs during spring and summer, depending on the type of crops, and is often used in several applications. While a small part of pesticides applied is intercepted by the vegetal cover, most of them reach soils. The major pathway of pesticides input in streams consists in runoff from soils during rainfall events (Leu *et al.*, 2004; Rabiet *et al.*, 2010; Bereswill *et al.*, 2012). Other pesticide transport pathways exist including i) wind drift during spreading ii) percolation from soils to groundwater and iii) exchange between groundwater and stream water (Barbash *et al.*, 2001; Kolpin, Thurman & Linhart, 2001; Schulz, 2004). Although contamination of surface waters by pesticide compounds is expected to be transitory, and mostly during spreading periods, many parameters influence their inputs in streams. Briefly, soil physical and chemical properties, as well as the intrinsic physical and chemical characteristics of the pesticide molecules, and/or the presence of adjuvants may affect its transport to streams (Krogh *et al.*, 2003). For instance, several pesticide compounds (including the herbicides atrazine and diuron) are considered as very recalcitrant, adsorbing to surface sediments where they can remain for several years. In surface waters, the high frequency of detection and high concentrations of pesticides are expected to

strongly affect litter decomposition processes (Beketov *et al.*, 2013). In France, more than 50% of rivers and streams are contaminated by at least one pesticide compound at concentrations above the threshold for drinking water ( $0.5 \mu\text{g L}^{-1}$ ), and only 48% are in good chemical status regarding pesticide contamination (Fig.11). Among pesticides, herbicides are the most detected compounds in French streams, with a detection frequency in water samples above 80%, far from insecticides (57%) and fungicides (51%) detection frequencies.



**Figure 9:** Global pesticides consumption in Europe between 2010 and 2015. Values are expressed as tonnes of active ingredients. (Source: Food and Agriculture Organization of the United Nation, FAOSTAT).



**Figure 10:** Detection frequency of the main family of pesticides compounds in French rivers and streams in 2012.

The effects of pesticides on leaf-litter decomposition in streams have been extensively studied over the last decades. Most of these studies have been focused on fungicides impact given their potential direct effects on aquatic fungal communities contributing to litter decomposition. Fungicides include many compound families each with a specific mode of action (i. e. inhibiting of amino acids synthesis, cell division or sterol biosynthesis (Van den Brink *et al.*, 2007; Maltby, Brock & van den Brink, 2009). In most cases, exposure to fungicides lead to a decrease in microbial decomposition rates of leaves explained by i) a decrease in fungal biomass, ii) changes in microbial community structure, and iii) eventual decrease in enzymatic activities (Zubrod *et al.*, 2011a, 2015b a; Bundschuh *et al.*, 2011a; Artigas *et al.*, 2012). Indirect effects of fungicides (i. e. tebuconazole) have been shown to affect macroinvertebrates due to decreases in leaf nutritional quality (i.e. decrease in fungal biomass and/or changes in fungal community structure) suggesting a potential influence of the fungicide on the entire stream food web (Zubrod *et al.*, 2011a; Bundschuh *et al.*, 2011a). Very little literature is available on herbicides' and insecticides' effects on leaf-associated microbial communities, probably because their effects are supposed to be negligible. Insecticides have been shown to strongly impact macroinvertebrate decomposers, provoking mortality or changes in their feeding behavior (Schäfer *et al.*, 2007). Whether microbial communities exposed to insecticides could propagate its toxicity to macroinvertebrates remain and open question. Concerning herbicides' impact, they have been shown to directly impact autotrophic organisms in the periphyton, reducing their

diversity and chlorophyll content (Villeneuve, Larroudé & Humbert, 2011a; Pesce, Bouchez & Montuelle, 2012a). Since microalgae increase leaf-litter decomposition through the priming effect, herbicides in streams could indirectly impair leaf microbial decomposition. This is, however, just a hypothesis and remains to be tested.

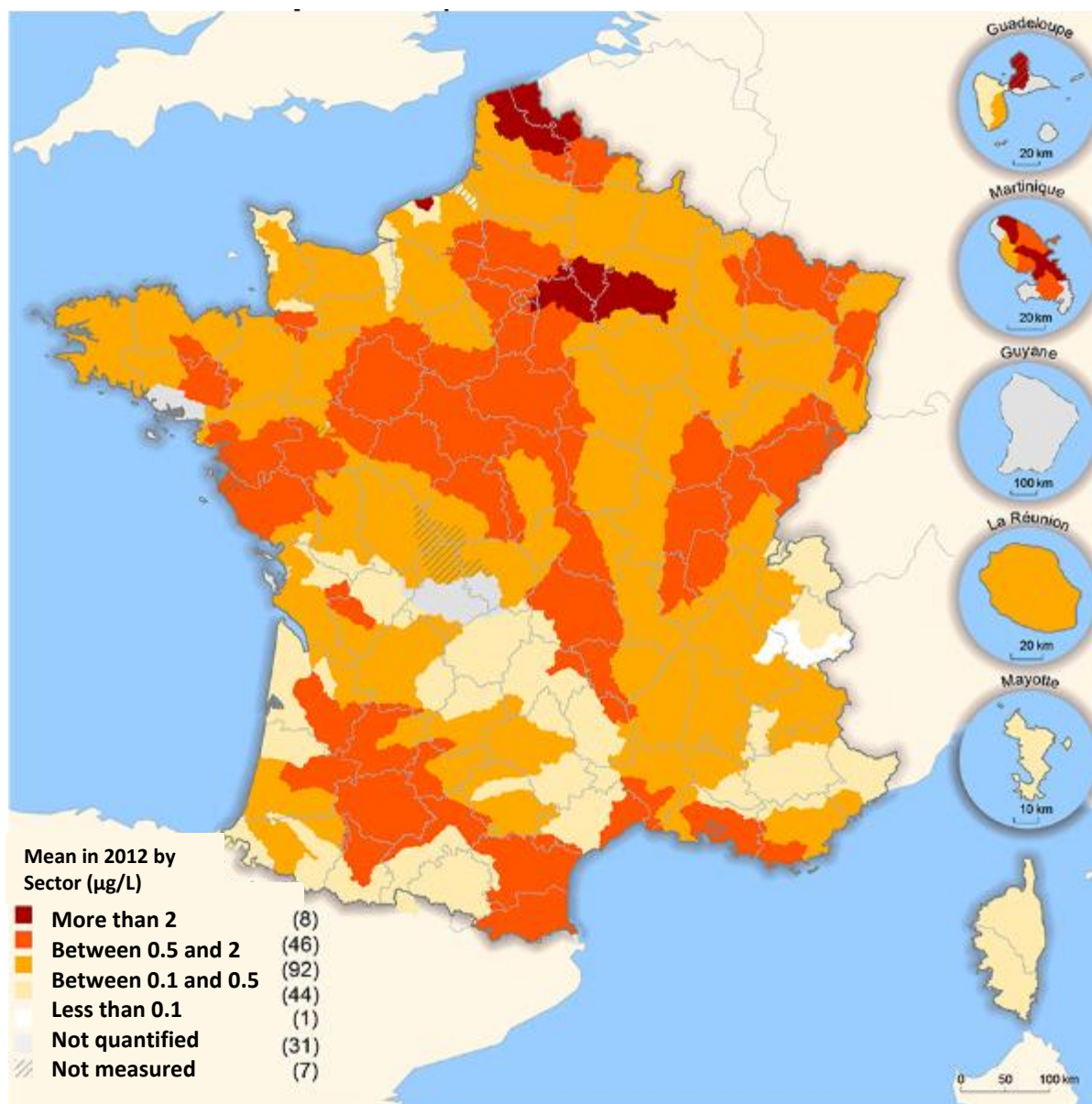


Figure 11: Average concentrations of pesticides in French streams in 2012. (Sources: Agences et offices de l'eau, 2015 (SOeS, 2015a))

## vi. Water framework directive and ecological status of streams

Pressure on freshwater resources is intensifying rapidly with climate change, population growth, continuing economic development and the expansion of biofuel crops, raising the concern of both governmental and non-governmental organizations. Thus on 23 October 2000, the Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy was adopted by all European countries under the name of Water Framework Directive (WFD) with one goal: “Getting Europe's waters cleaner”. This include general protection of the aquatic ecosystems, specific protection of unique and valuable habitats, protection of drinking water resources, and protection of water for recreative activities. To reach these objectives, a general requirement for ecological protection (good ecological status), and a general minimum chemical standard (good chemical status) were introduced with a classification system (High, good, moderate, poor and bad). Good chemical status refers to the compliance of stream waters with all the quality standards established for chemical substances at European level. This includes information on thresholds for many substances (including nutrients, heavy metals and xenobiotics) and a list a 45 priority substances presenting a significant risk to aquatic environments. Good ecological status is defined as the state of the ecosystem in the absence of disturbance, and mainly refers to the quality of the biological communities or their deviation from what would be expected in a system without perturbations (reference system). This assessment relies on the integrity of the trophic chain, from primary producers (microalgae, particularly Diatoms) to macroinvertebrates and fishes. In addition, to achieve the sustainable use of pesticides across Europe, a framework for Community action was established (Directive 2009/128/EC). For example, in France, the WFD was accompanied with the Ecophyto management plan I (Agence BIO). Initiated in 2008, this management plan had as objective to decrease by 50% the use of phytosanitary compounds in France by 2018. Now in 2018, the objectives of the Ecophyto plan I have not been reached and a second management plan has been launched (Ecophyto II) postponing Ecophyto I objectives to 2025 (Agence BIO).

Although the quality of surface waters was greatly improved over the last 50 years, the WFD has still not delivered its main objectives of non-deterioration of water status and the achievement of good status for all EU waters, originally planned for 2015. The European Environmental Agency reveals that 57% of European rivers still present a moderate, poor or bad ecological status regarding chemical contamination (wise WFD database, 2017). In

addition, fertilizer (including N and K<sub>2</sub>O) and pesticide consumption across Europe tends to increase or at least, remain stable (FAOSTAT). Moreover, there is a serious lack of knowledge on the number and nature of chemical compounds present in streams and rivers accross the world. Advances in environmental chemistry have highlighted the presence of numerous xenobiotic compounds present in surface waters. This complex contamination complicates ecotoxicological studies since researchers have to deal not only with parent compounds' effects but also with their metabolites issued from abiotic (phototransformation) or biotic (microroganisms) transformation. In some cases, the toxicity of metabolites can be greater than that of parent compounds (Carles, 2016).

In front of this evidence, it has become obvious that indicators utilized by the WFD are insufficient. Current indices of the biological quality of surface waters are based on the presence/absence of diatom, macroinvertebrate and fish sensitive species as well as their densities, and they neglected functional ecosystem descriptors. Moreover, current indices do not assess heterotrophic microbial communities, which are at the basis of the stream food webs supporting allocthonous and/or authochthonous organic matter recycling. This thesis will therefore assess whether microbial leaf-litter decomposition is a good descriptor to assess stream functional integrity (Gessner & Chauvet, 2002; Young, Matthaei & Townsend, 2008) in the context of complex chemical contamination.



## **Structure and Objectives of the thesis**



As current knowledge assessing chemical contamination effects in leaf-litter decomposition in streams is increasing, several research questions still remain unanswered. The following research gaps derived from published studies in aquatic ecotoxicology have inspired this thesis work:

1. Many studies have assessed the effects of xenobiotic compounds and nutrients on leaf decomposition, but most of them were conducted in the laboratory using toxicant concentrations far above environmentally relevant ones (Zubrod *et al.*, 2011b). Although laboratory experiments in microcosms confer a large array of advantages (various physico-chemical parameters can be controlled, easy to set-up, fast response, and cheap) they seriously lack ecological representability since they are simplified systems. Then, to which extent results obtained using microcosm approaches can be extrapolated to what is happening at the stream ecosystem level?
2. In contaminated streams, xenobiotics are often present in complex mixtures and tend to co-occur with high nutrient concentrations. However, most studies tested the effect of nutrients and pesticides separately on leaf-associated microbial communities (Gulis & Suberkropp, 2003; Dimitrov *et al.*, 2014). The environmental representativity of these studies can be questionable given the proved interaction between nutrients and xenobiotics on microbes. Few studies have tried to test such interaction, and only between nutrients and a fungicide or between a fungicide and an insecticide (Flores *et al.*, 2014; Dawoud *et al.*, 2017). Since stream waters in Europe are essentially contaminated by herbicides (in terms of both detection frequency and concentration level), it would be interesting to investigate the specific interaction between herbicides, fungicides, and nutrients on leaf-associated microbial communities.
3. Several studies have assessed the impact of a stress by water chemical contamination on microbial leaf-litter decomposition in streams (Rasmussen *et al.*, 2012), but studies are missing on the resilience capacity of these communities after this stress is removed. This is particularly relevant in the context of the WFD and the French Ecophyto programm. Can contaminated stream ecosystems recover from chronic exposure to chemical contamination of anthropogenic origin?
4. Extracellular enzymes are necessary for microbes to transform and/or mineralize organic matter for their self-consumption. Yet, assessing the extracellular enzymatic potential of aquatic microbial communities *in situ* is complex due to the water chemical contamination. Indeed, enzymatic activities have been considered as weakly specific

physiological descriptors of stream water contamination (Sabater *et al.*, 2006), mainly because of their multiple roles in fungal physiology. However, recent findings suggest that laccase enzymatic activity might be involved in the detoxification mechanisms employed by aquatic fungi against pesticide exposure. Can laccase activity be used as bioassessment tool to assess pesticide exposure in streams?

Thus, the objectives of this thesis are:

i) to compare natural microbial communities structure and activity during leaf-litter decomposition in less contaminated (upstream sites) and more contaminated (downstream sites) stream sections within different watershed types (agricultural, urbanized, and forested). And, to assess which of environmental parameters (nutrients, conductivity, oxygen, pH, among others) and/or xenobiotics (pharmaceuticals and pesticides) influence the most litter decomposition process (chapter 1)

We hypothesized that watersheds exposed to high agricultural and urban pressures (greater input of pesticides and pharmaceuticals) would be negatively affected in terms of microbial decomposition activity compared to forested watersheds, though this effect could be compensated by the co-occurrence with high nutrient concentrations.

ii) to assess the effect of an interaction between stream eutrophication and pesticide contamination on leaf-associated microbial decomposer communities. Two different nutrient conditions were selected (mesotrophic and eutrophic) and combined with three pesticide treatments (fungicide alone, herbicide alone and the mixture of both) to seek for interactive effects on microbial communities using a microcosm approach. In addition, multi-contamination effect on leaf palatability for macroinvertebrate consumers was also assessed in this experiment (chapter 2)

We hypothesized that fungicide exposure (alone or in mixture with the herbicide) would significantly affect fungal communities associated with alder leaves, while the effects of the herbicide alone would be expected to be very limited due to its specific mode of action. Besides, we expected that nutrient increase in the eutrophic condition would minimize the effects of pesticides on microbial communities' biomass and activity and further mask pesticide effects on leaf palatability for the macroinvertebrate *Gammarus fossarum*.

iii) to determine the resistance and recovery abilities of leaf-associated microbial communities regarding water chemical contamination using a translocation experiment (chapter 3)

We hypothesized that microbial communities would be impaired by chemical exposure due to the translocation from upstream to downstream, suggesting a low resistance potential. On the other hand, the recovery potential of leaf-associated fungal communities suggests that such communities could recover after stress removal caused by the translocation from downstream to upstream, probably because of species immigration processes from the preserved sites.

iv) to investigate the response of microbial laccase activity to stream water chemical contamination. Laccase activity data obtained from chapters 1, 2, and 3 were combined with dose-response experiments performed in the laboratory in the aim to determine whether laccase activity is a good biomarker of stream water chemical contamination (chapter 4)

We hypothesized that exposure to fungicides would increase microbial laccase activity by triggering detoxification mechanisms, while high nitrate availability is expected to balance this effect and reduce laccase activity.

# **Chapter 1: Stimulation or inhibition: Leaf microbial decomposition in streams subjected to complex chemical contamination**



The very first step of this thesis work was to understand microbial decomposition of leaf-litter by comparing a range of streams subjected to different types of chemical contaminations. One of the advantages of the Puy-de-Dôme region (Centre France) is the availability of a high diversity of streams and rivers, including highly preserved upstream sections at moderate altitudes ( $\approx 800\text{m}$ ) and contaminated downstream sections at lower altitudes ( $\approx 400\text{m}$ ). The selection of the study sites was performed in agreement with “Eaux et Produits Phytosanitaires en Auvergne-Rhône-Alpes” ex Phyt’Eauvergne (<http://www.eauetphyto-aura.fr>), a group in charge of the pesticide monitoring in surface and groundwaters from the Auvergne-Rhône Alpes region in France. Based on previous data from Phyt’eauvergne (obtained over a 5 year-period) and the prevailing land use, we selected a total of 6 watersheds (right column):

Urbanized	Artière
	Charlet
	Jauron
Agricultural	Lembronnet
	Couze
Forested	Veyre

The classification of the surveyed streams was performed according to the study of (Rasmussen *et al.*, 2012). Each stream fell into one of these categories (i.e. forested, urbanized and agricultural) according to its prevailing land use.

All the catchment areas of these streams belong to the Allier basin, the Allier belongs to the upstream part of the large Loire basin which flows to the Atlantic Ocean. For each of the six streams, a downstream contaminated site (close to the confluence with the Allier river) and an upstream pristine site (close to the stream source) were selected (Fig.12). While the choice of different watershed types allowed us to compare the effect of land use contamination on microbial decomposition of leaves, the use of the two sites (i.e. upstream and downstream) allowed us to have insights on the gradient of contamination within each watershed. All these six watersheds and associated upstream/downstream sections ( $n = 12$ ) were monitored seasonally to assess the variations in chemical contamination (spring, summer, autumn, winter) between June 2016 and march 2017 as well as the spatial and temporal variability in microbial leaf-litter decomposition.



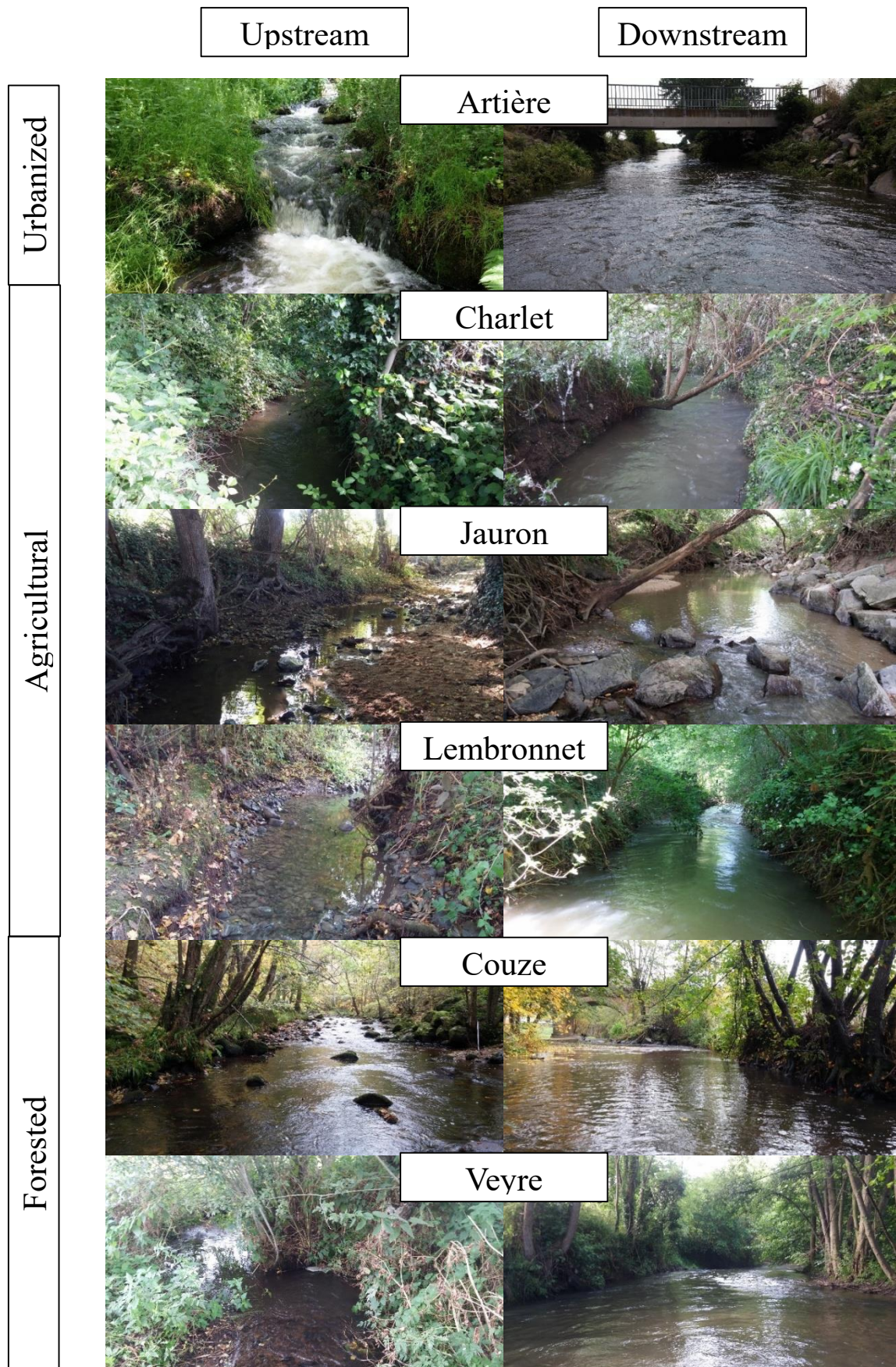


Figure 12: Pictures of the 12 study sites used in the field experiment



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# Stimulation or inhibition: Leaf microbial decomposition in streams subjected to complex chemical contamination

Florent Rossi\*, Clarisse Mallet, Christophe Portelli, Florence Donnadieu, Frédérique Bonnemoy, Joan Artigas

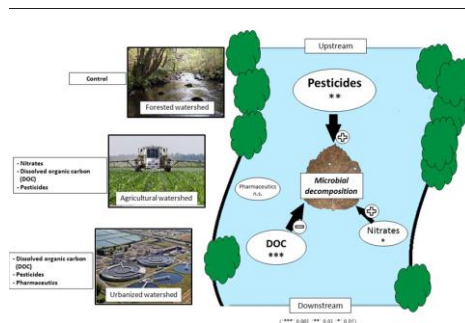
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## HIGHLIGHTS

- Water contamination coincided with high leaf decay rates but low fungal biomass.
- Pesticides toxicity was the second main factor influencing leaf decomposition rates.
- Microbial communities can adapt to xenobiotic stress to maintain ecosystem function.
- Chemical contamination may decrease leaf nutritional value for invertebrate feeders.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

## Article history:

Received 28 June 2018  
Received in revised form 14 August 2018  
Accepted 15 August 2018  
Available online 17 August 2018

Editor: D. Barcelo

## Keywords:

Watershed types  
Leaf decomposition  
Fungi  
Bacteria  
Enzymatic activities

## ABSTRACT

Leaf litter decomposition is a key mechanism in headwater streams, allowing the transfer of nutrients and energy into the entire food web. However, chemical contamination resulting from human activity may exert a high pressure on the process, possibly threatening the structure of heterotrophic microbial communities and their decomposition abilities. In this study, the rates of microbial *Alnus glutinosa* (*Alnus*) leaf decay were assessed in six French watersheds displaying different land use (agricultural, urbanized, forested) and over four seasons (spring, summer, autumn, winter). In addition, for each watershed at each sampling time, both upstream (less-contaminated) and downstream (more-contaminated) sections were monitored. Toxicities (estimated as toxic units) predicted separately for pesticides and pharmaceuticals as well as environmental parameters (including nutrient levels) were related to microbial decay rates corrected for temperature and a range of fungal and bacterial community endpoints, including biomass, structure, and activity (extracellular ligninolytic and cellulolytic enzymatic activities).

Results showed that agricultural and urbanized watersheds were more contaminated for nutrients and xenobiotics (higher pesticides and pharmaceuticals predicted toxicity) than forested watersheds. However, *Alnus* decay rates were higher in agricultural and urbanized watersheds, suggesting compensatory effects of nutrients over xenobiotics. Conversely, fungal biomass in leaves was 2-fold and 1.4-fold smaller in urbanized and agricultural watersheds than in the forested watersheds, respectively, which was mostly related to pesticide toxicity. However, no clear pattern was observed for extracellular enzymatic activities except that  $\beta$ -glucosidase activity positively correlated with *Alnus* decay rates. Together, these results highlight microbial communities being more efficient for leaf decomposition in polluted watersheds than in less contaminated ones, which is probably explained by changes in microbial community structure. Overall, our study showed that realistic chemical contamination in stream ecosystems may affect the biomass of *Alnus*-

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associated microbial communities but that these communities can adapt themselves to xenobiotics and maintain ecosystem functions.

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## 1. Introduction

Human activities, through agriculture and urbanization, have led to the input of a large amount of xenobiotic compounds and their metabolites into the biosphere, with an additional thousand new compounds input each year (Hou et al., 2003; Vitousek et al., 1997). While their exact fate in the environment remains uncertain (Gómez et al., 2007; Li, 2014), a major fraction of these xenobiotic compounds can reach aquatic systems by spray drift, runoff or even being released from point source pollution such as waste water treatment plant effluents (Huber et al., 2000; Schulz, 2004). Such inputs contribute to the global pollution of freshwater and threaten aquatic ecosystem services and functions (Vörösmarty et al., 2010). Recent reports from the European Environmental Agency showed that only 40% of the European rivers and streams achieved good chemical status, and a more recent study showed that 2890 of 4510 monitored rivers in Europe were contaminated by at least one pesticide (WISE WFD Database, 2018; Malaj et al., 2014).

In this context, the assessment of aquatic ecosystem functions in contaminated areas has become a central issue for aquatic ecologists. Over the past two decades, efforts have been made to assess the effects of xenobiotics on aquatic organisms and the biogeochemical cycles with which they are associated (Hasenbein et al., 2015). However, assessing the impact of multistress contamination remains a challenge, particularly because of the confounding effects of environmental factors (Liess and Ohe, 2005). In addition, most of the studies in ecotoxicology have focused on the effects of pesticide molecules, but little is known about the potential effects of other compounds such as pharmaceuticals and their possible interactions with pesticides and/or dissolved nutrients.

In river ecosystems, the utilization of leaf litter decomposition as a tool to assess aquatic ecosystem integrity is relevant since it can be directly associated with an important ecosystem function (Gessner and Chauvet, 2002; Young et al., 2008). Indeed, decomposer activity is a key mechanism in headwater streams since it i) strengthens the link between terrestrial and aquatic ecosystems, ii) allows the transfer of matter and energy within the aquatic food web and, to a greater extent, iii) aids in various biogeochemical cycles (N, C, P, and S) (Cummins, 1974; Djukic et al., 2018; Gessner and Chauvet, 1994; Mora-Gómez et al., 2018; Webster and Meyer, 1997). While macroinvertebrates account for the major portion of leaf mass loss in streams (Suberkropp and Klug, 1976; Mann, 1975; Gessner et al., 1999; Gaudes et al., 2009), microorganisms have a non-negligible action, increasing leaf palatability for macroinvertebrates and directly contributing up to 25–45% of leaf mass loss (Baldy et al., 2007; Bärlocher and Kendrick, 1975; Hieber and Gessner, 2002). Such degradation activity in microbes is possible thanks to a large panel of extracellular hydrolytic and oxidative enzymes that they possess (Abdullah et al., 1989; Chandrashekar and Kaveriappa, 1991; Abdel-Raheem and Ali, 2004).

Up to now, reports on pesticide effects on aquatic microbial communities are contrasted. For instance, due to their direct mode of action, fungicides tends to affect the structure and decomposition activity of leaf-associated fungal communities more deeply than other pesticides (Artigas et al., 2012; Fernández et al., 2015; Flores et al., 2014; Zubrod et al., 2015), whereas environmentally relevant concentrations of more complex mixtures of pesticides (including herbicides and insecticides) have no effects (Münze et al., 2017; Piscart et al., 2009; Talk et al., 2016). Only Rasmussen et al. (2012) and Fernández et al. (2015)

reported a negative relationship between microbial litter decomposition and pesticide toxicity in agricultural exposed streams. In contrast, pharmaceutical effects on leaf-associated microbial communities are poorly understood. However, these molecules are of special concern regarding the European water framework directive (Commission Implementing 2015/495 to Directive 2008/105/EC) for freshwater ecosystem preservation, especially because of their persistence in the environment as well as their specific targeting of microbes (Haritash and Kaushik, 2009; Minguez et al., 2016). Among the very little research on this topic, studies on pharmaceuticals reported no effects of antibiotic mixtures and other compounds (antihistamine) at low concentrations (ca.  $2 \mu\text{g L}^{-1}$ , (Bundschuh et al., 2017; Jonsson et al., 2015)) on leaf-associated microbial communities, whereas very high concentrations (above  $100 \mu\text{g L}^{-1}$ ) provoked changes in community structure (Maul et al., 2006).

An integrative and realistic ecological risk assessment of aquatic ecosystems must consider the interactions between pesticides, pharmaceuticals and environmental parameters, including nutrients. Unfortunately, very few studies have dealt with such multistress scenarios and its potential effects on the decomposition activity of aquatic microbial communities. Only two studies tested the interactions between nutrient enrichment (nitrate and phosphate) and a mixture of fungicides in the laboratory (Feckler et al., 2017; Fernández et al., 2016). In both studies, fungicide effects on leaf microbial decomposition were suppressed with high nutrient exposure (above  $10 \text{ mg L}^{-1}$  for nitrates and  $0.35 \text{ mg L}^{-1}$  for phosphate), suggesting compensatory effects of nutrients that might be explained by adaptive mechanisms (i.e. changes in microbial community structure) linked with the emergence of more tolerant and competitive species. However, Feckler et al. (2017) also observed a 50% decrease in fungal biomass after a very high exposure to fungicides ( $600 \mu\text{g fungicides L}^{-1}$ ), suggesting that nutrient cannot compensate very high stress.

While studies assessing the effects of complex mixtures (including pesticides, pharmaceuticals and nutrients) are becoming more frequent, the main drawback remains the clear lack of field-based evidence concerning such multistress contamination effects on lotic ecosystems. Another drawback is the small number of studies assessing the variability of large spatial and temporal scales affecting these processes. In the present work, we thus compare microbial activity during leaf litter decomposition among 6 watersheds presenting different land uses (agriculture, urbanization, and forested) and gradients of contamination (less-contaminated upstream sites and more-contaminated downstream sites) over 4 seasons and investigate which environmental parameters (nutrients, conductivity, oxygen, pH, ...) or xenobiotics (pharmaceuticals and pesticides) cause the most microbial litter decomposition. For this purpose, the microbial community structure, leaf decay rates and associated extracellular enzymatic activities were assessed and related to the physical and chemical characteristics of the site. Temporal variability in terms of water chemical contamination (i.e. pesticide spreading periods and patterns of infectious diseases) was assessed by seasonal sampling. We hypothesized that i) the watershed contamination relative to agricultural and urban land uses (pesticides and pharmaceuticals) would negatively affect microbial decomposition activity but that this effect would be compensated by high nutrient concentrations and that ii) despite nutrients exerting a major effect on microbial decomposer activity and biomass, they could be outcompeted by the effects of specific pesticides (i.e. fungicides) and/or pharmaceuticals (e.g. antibiotics) directly targeting microbes during specific seasons (i.e. pesticide spreading in spring).



## 2. Materials and methods

### 2.1. Study sites and experimental design

The study was conducted between May 2016 and March 2017 on six French streams located in six different watersheds of Puy-de-Dôme region (Central France) and presenting different gradients of contamination. Watersheds were selected according to their similarities/differences in land use occupation and water chemical contamination levels. Past and present data from the six selected watersheds were obtained from the regional surveillance network Phyt'auvergne and the Loire-Bretagne water agency (Phyt'Auvergne, 2015). The six selected watersheds were Veyre, Couze, Charlet, Lembronnet, Jauron and Artière (Fig. 1). We established three watershed types according to the prevailing percentage of land use in the watershed: i) forested watersheds, including the Veyre and Couze (<20% both agriculture and urbanization), were used as references; ii) agricultural watersheds, including the Charlet, Lembronnet and Jauron watersheds (68%, 48% and 35% agricultural land use, respectively); and iii) one urbanized watershed (the

Artière, 42% urban land use) (Fig. 1). For each watershed, one less-contaminated 500 m-section upstream and one more-contaminated 500 m-section downstream were selected (12 sites in total). The downstream site of the urbanized watershed was located right after the waste water treatment plant of the city of Clermont-Ferrand, representing 425,000-population equivalent.

The study analyzed the biomass, structure, and activity of aquatic microbial communities during *Alnus* leaf decomposition for each site during each different season. The seasons were spring (May 10th to June 8th, 28 days of leaf immersion), summer (from August 10th to August 31st, 20 days), autumn (from September 27th to October 26th, 29 days) and winter (from January 31st to March 8th, 36 days). At each site and during each season, six fine mesh bags (0.5 mm mesh size, 1 L = 15 cm × 15 cm) were filled with pre-dried (72 h at room temperature) *Alnus glutinosa* (L.) Gaertn leaves. These leaves were harvested at the upstream site of the Artière in November 2015. Once filled, bags were immersed directly into the stream water for a residence time that varied between seasons and depended on the decomposition rate of leaves (residence times are detailed above). Six bags

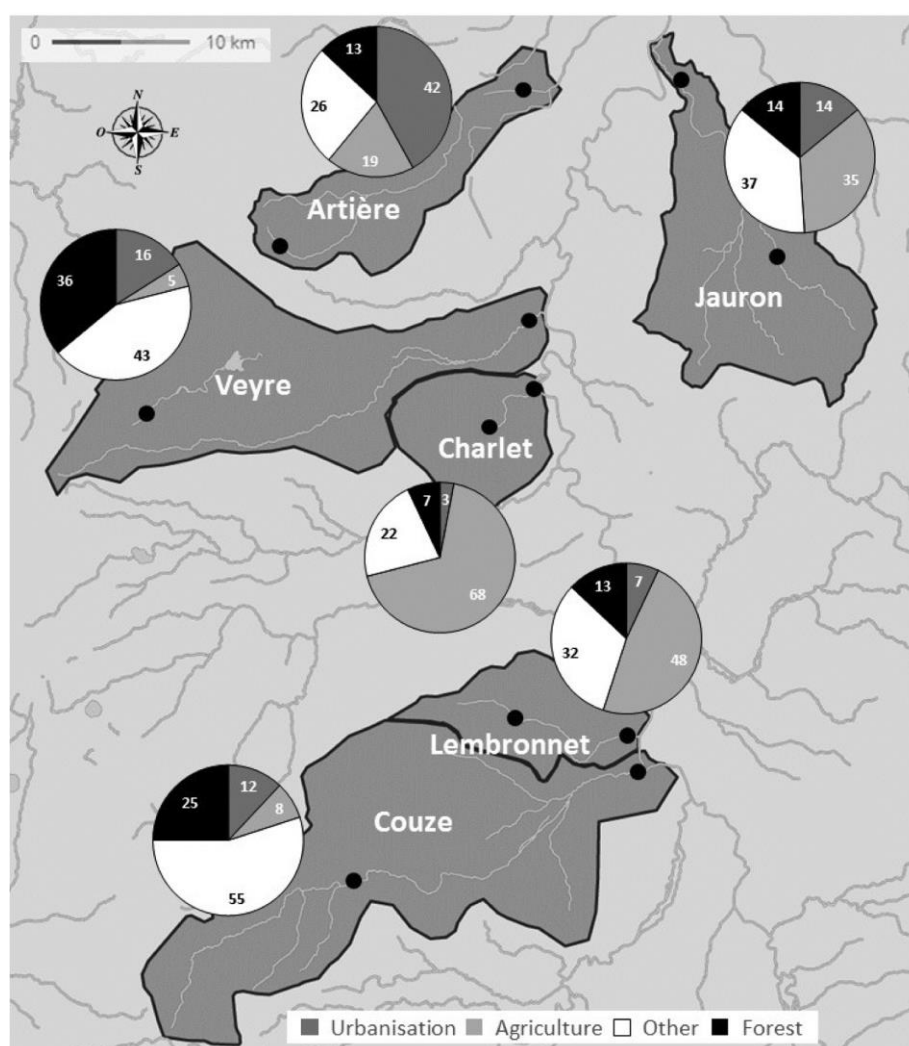


Fig. 1. Geographical location of the studied streams (Puy-de-Dôme region, central France). Gray areas represent the watersheds surface areas and dots represents the samplings sites. Land use occupation of each watersheds are represented as pie chart.

were immersed per site; three of these bags were filled with  $3 \pm 0.2$  g of *Alnus* and used for leaf mass loss measurements, and the other three bags were used for biological analyses (enzymatic activities, biomass and the structures of fungal and bacterial communities). At each sampling time (once per season), bags were retrieved, placed in a cooler (4 °C) and brought back to the laboratory for analyses. In parallel, water physical and chemical measurements were performed as explained below.

## 2.2. Water physical and chemical characteristics

Measurements of stream water characteristics were performed directly in the field using portable probes twice per season (at the laying and at their retrieval of bags, 6 values per season in total) and in triplicate (3 measurements over a 50 m section for each site), except for temperature, which was measured continuously using a data logger (HOBO pendant, ONSET, Bourne, USA). Chemical measurements consisted of determining dissolved oxygen levels (ProODO, YSI, USA), conductivity and pH (Orion 4-star, Thermo Fisher Scientific, USA), while physical measurements consisted of light (Testo 545, Testo AG, Germany) and flow (FlowTracker, SonTek/YSI Inc., USA) determinations. In addition, filtered (0.2 µm) water samples were taken in order to assess nitrate ( $\text{N-NO}_3$ , Nitrate Test kit, Merck, Darmstadt, Germany), phosphate ( $\text{P-PO}_4$ ) (Murphy and Riley, 1962), total nitrogen (TN) and dissolved organic carbon (DOC,  $\text{TOC-VCPH}$ , Shimadzu, Kyoto, Japan) concentrations.

The quality of dissolved organic matter (DOM) in stream water was assessed using two different fluorescence indices: the humification index (HIX, Zsolnay et al., 1999), and the biological index (BIX). These two indices were used as a proxy of the main DOM resource available for microbes in the stream and possibly affecting their activity (Ejarque et al., 2017). The HIX was calculated from emission spectra with an excitation wavelength of 254 nm as the emission curve area ranging from 435 to 480 nm divided by the emission curve area ranging from 300 to 345 nm ( $I_{\text{em}435-480}/I_{\text{em}300-345}$ ). For instance, high HIX values indicate the presence of complex molecules (i.e. aromatic compounds) in stream water (Huguet et al., 2009; Senesi et al., 1991). In addition, the BIX indicates the presence of the  $\beta$ -fluorophore, which is characteristic of autochthonous biological activity (Huguet et al., 2009). The BIX was calculated as the ratio of the emission intensity at 380 nm to the emission intensity at 430 nm ( $I_{\text{em}380}/I_{\text{em}430}$ ) with an excitation wavelength of 310 nm. For instance, BIX values  $\geq 1$  are characteristic of the release of fresh DOM into the water (indicating autochthonous organic matter production), whereas lower values (0.6–0.7) represent less autochthonous production.

In parallel, water samples (3 L) were directly taken from the stream and sent by refrigerated boxes to the CARSO Laboratory (COFRAC agreement number 1-1531) for pesticide and pharmaceutical measurements. The predicted toxicity for each family of xenobiotic compounds (pesticides and pharmaceuticals) was estimated according to the toxic unit (TU) concept (Sprague, 1971) using the following equation:

$$\text{TU} = \frac{C}{\text{EC}_{50}}$$

where  $C$  is the actual concentration of the compound in the water sample and  $\text{EC}_{50}$  is the median acute effect of this compound inhibiting 50% of the growth rate of a standard aquatic microbial species. Predicted toxicity of the water sample, noted as  $\Sigma\text{TUs}$ , was calculated both for upstream and downstream sites at each sampling time as the sum of all TU for all the compounds detected in the sample. Since toxicity data ( $\text{EC}_{50}$ ) for many xenobiotic compounds on aquatic fungi are not available in the literature (Maltby, 2009; Maltby et al., 2009), average  $\text{EC}_{50}$  values from microalgae (mostly *Scenedesmus subspicatus* and *Raphidocelis subcapitata* but also *Scenedesmus quadricauda*, *Navicula pelliculosa*, *Navicula seminulum*, *Anabaena flos-aquae*, *Coccolithus*

*huxleyi*, *Microcystis aeruginosa*) were used as surrogates (see Rasmussen et al., 2012).

## 2.3. Biomass and structure of microbial communities

The fungal biomass colonizing *Alnus* leaves was determined through ergosterol concentration determination using 15-leaf disks of 1 cm in diameter per sample and according to the protocol described in Rossi et al. (2017). Results were expressed in mg fungal carbon/g leaf dry weight ( $\text{DW}^{-1}$ ). Bacterial biomass was determined through bacterial density analyses using flow cytometry and according to a previous protocol (Borrel et al., 2012). Results were expressed in mg bacterial carbon/g  $\text{DW}^{-1}$  using the conversion factor from previous work (Bratbak, 1985).

The structure of fungal and bacterial communities associated with *Alnus* leaves was assessed using denaturing gradient gel electrophoresis (DGGE) according to the protocol described in Rossi et al. (2017). DGGE band (operational taxonomical unit, OTU) profiles were analyzed using Gelcompar II software (Applied Maths, Belgium) in order to obtain the fungal and bacterial OTU presence/absence matrix, and the OTU richness was then calculated for both fungal and bacterial communities.

## 2.4. Microbial activity

The microbial decay rates of *Alnus* leaves were determined as the leaf mass loss between the laying and retrieval of bags. At the end of each sampling season, bags containing  $3 \pm 0.2$  g of pre-dried *Alnus* leaves were retrieved, and leaves were rinsed with tap water in order to eliminate sediments. Leaves were then dried for 72 h at 60 °C to obtain a constant DW. Leaf decay rates were obtained after fitting data to an exponential decay model with the following equation:  $M_t = M_0 e^{-kt}$ , where  $M_0$  is the initial DW (g),  $M_t$  is the final DW (g) at time  $t$  and  $k$  is the decay rate coefficient (Petersen & Cummins, 1974). Decay rates were finally corrected for the accumulated temperature of stream water and expressed in  $\text{degree}^{-1} \text{day}^{-1}$ .

Microbial extracellular ligninolytic (phenol oxidase, peroxidase and laccase) and cellulolytic ( $\beta$ -glucosidase) enzymatic activities were measured using commercial substrate analogs (Sigma-Aldrich, St. Louis, Missouri, USA). Phenol oxidase (EC 1.14.18.1) and peroxidase (EC 1.11.1.7) activities were measured from three leaf disks per sample using 3,4-dihydroxy-L-phenylalanine (L-DOPA, 1.5 mM final concentration), and laccase (EC 1.10.3.2) activity was measured from three leaf disks per sample using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, 3 mM final concentration); all three sets of activities were assessed in acetate buffer (pH 4.65, Sigma-Aldrich, St. Louis, Missouri, USA). In the case of peroxidase, 0.2 mL of 0.3%  $\text{H}_2\text{O}_2$  was added (Sinsabaugh et al., 1994; Johannes and Majcherzyk, 2000). In addition,  $\beta$ -glucosidase (EC 3.2.1.21) activity was measured from one leaf disk per sample using 4-methylumbelliferyl  $\beta$ -D-glucopyranoside (0.3 mM final concentration, (Artigas et al., 2012)). After a 1-hour incubation in the dark and under agitation at a constant room temperature of 19 °C, phenol oxidase, peroxidase and laccase activities were determined spectrophotometrically (460 nm for peroxidase/phenol oxidase, 415 nm for laccase) using an Ultrospec 2000 (Pharmacia Biotech, Trowbridge, UK) whereas  $\beta$ -glucosidase activity was determined fluorometrically (360/460 nm excitation/emission) after the addition of 0.3 mL of 0.05 M glycine buffer (pH 10.4). All enzyme activities were expressed in  $\mu\text{mol}$  substrate transformed  $\text{h}^{-1} \text{mg}$  microbial (fungi + bacteria)  $\text{C}^{-1}$ .

## 2.5. Data analysis

### 2.5.1. Differences among watershed types and seasons

Differences in biological parameters (decay rates, microbial biomass, structure and enzymatic activities) among watershed types (urbanized, agricultural and forested; "type effect") and seasons (spring, summer, autumn and winter; "time effect") were evaluated by two-way

ANOVA. Differences in water physical and chemical parameters (pH, conductivity, light, flow, oxygen, DOC, TN, P-PO<sub>4</sub> concentration, N-NO<sub>3</sub> concentration, HIX, BIX, pesticide content and pharmaceutical content) among watershed types (urbanized, agricultural and forested; “type effect”), seasons (spring, summer, autumn and winter; “time effect”) and sites (upstream and downstream; “site effect”) were evaluated by three-way ANOVA.

### 2.5.2. Differences among upstream and downstream

In addition, upstream/downstream ratios (Rups/dws) were calculated for each biological parameter and used to compare differences between sites among types of watersheds. *t*-Tests were then performed on Rups/dws ratios to evaluate how different they are from a value of 1. A ratio of 1 indicates similar descriptor values at upstream and downstream sites. All data were transformed using the Box-Cox function prior to the ANOVA to meet assumptions of normality and homoscedasticity of data residuals (Box and Cox, 1964), and these approaches were complemented with post hoc multiple comparison tests (Tukey's test,  $P < 0.05$ ).

### 2.5.3. Relationship between biological and physical/chemical parameters

A First partial least square regression (PLSR, Type II, (Abdi, 2010; Carrascal et al., 2009) analysis was performed on the entire dataset to determine the relationships between biological and environmental parameters and predicted xenobiotic toxicity data (pesticides and pharmaceuticals). Environmental/xenobiotic data matrices included 11 parameters: DOC concentration, DOM quality (HIX and BIX), TN, N-NO<sub>3</sub> concentration, P-PO<sub>4</sub> concentration, dissolved oxygen, pH, conductivity and  $\Sigma$ TUs for both pesticides and pharmaceuticals. The biological data matrix included 9 parameters: decay rates (in degree<sup>-1</sup> day<sup>-1</sup>), enzymatic activities (phenol oxidase, peroxidase, laccase, and  $\beta$ -glucosidase), fungal and bacterial biomasses, and fungal and bacterial band richness.

In addition, a second PLSR (Type I) model using 3 components was performed on leaf decay rates only, in order to determine the weight (represented as coefficient) of each environmental/xenobiotic parameter in the variation in decay. The number of components was determined using cross validation with the jackknife method, and the model was validated through the root mean square error for prediction (RMSEP). All data from the matrices were transformed prior to analysis using a log10 transformation followed by standardization. PLSR Type 1 is a special case of PLSR Type II used for 1 dependent variable only, whereas PLSR Type II can be used for matrices of dependent variables. All statistical analyses were computed using R software, and the PLSR was performed using the pls package (<http://cran.r-project.org/>).

## 3. Results

### 3.1. Water physical and chemical characteristics

In total, 91 different pesticide compounds (in terms of concentration, mostly herbicides such as aminomethylphosphonic acid (AMPA), glyphosate and metolachlor) and 38 pharmaceutical compounds (mostly antihypertensives, anxiolytics and analgesics such as irbesartan, oxazepam and tramadol) were found in stream waters during our study. However, fungicide contribution to pesticide concentrations never reached >4% regardless of site. Regarding land use, statistical differences were observed in terms pesticides concentrations (ANOVA,  $P < 0.001$ ). Agricultural (Charlet, Jauron and Lembronnet) and urbanized (Artière) watersheds displayed significantly higher  $\Sigma$ TU pesticide values than forested (Veyre and Couze) watersheds (Tukey's test,  $P < 0.001$  each). These differences were consistent among the four sampling seasons (Table 1). In addition, pesticide toxicity showed differences between seasons (ANOVA,  $P < 0.001$ ), with the highest toxicity values recorded in spring and autumn ( $2.72 \cdot 10^{-2} \pm 5.76 \cdot 10^{-3}$  and  $3.92 \cdot 10^{-2} \pm 6.99 \cdot 10^{-3}$ , respectively) and the lowest in winter ( $3.96$

$10^{-3} \pm 1.87 \cdot 10^{-3}$ ). Concerning pharmaceuticals, differences were also observed between watershed types (ANOVA,  $P < 0.001$ ). Highest concentrations were recorded at the urbanized and agricultural watersheds and again coincided with significantly higher  $\Sigma$ TU pharmaceutical values at this sites than forested watersheds (Tukey's test,  $P < 0.001$  each; Table 1). Specifically,  $\Sigma$ TU pharmaceutical values were 212 and 20 times higher for urbanized and agricultural watersheds than the forested ones, respectively. However, no variation between seasons were observed for pharmaceutical toxicity (ANOVA,  $P = 0.098$ ).

As for xenobiotics, DOC, nutrient concentrations (TN, N-NO<sub>3</sub> and P-PO<sub>4</sub>) and conductivity presented differences between watershed types (ANOVA,  $P < 0.001$ ). Highest concentrations were recorded in agricultural and urbanized watersheds (Tukey's test,  $P < 0.001$  each), although agricultural watersheds displayed the highest TN and N-NO<sub>3</sub> concentrations (Tukey's test,  $P < 0.001$  each; Table 1). Similarly, conductivity was two times higher in agricultural and urbanized watersheds than in the forested ones (Tukey's test,  $P < 0.001$  each). In contrast, pH, DOC, and HIX and BIX indexes were relatively similar in the three types of watersheds (Table 1). Moreover, the HIX index was affected by seasons (ANOVA,  $P < 0.001$ ). Highest values were observed in spring ( $4.65 \pm 0.20$ ) and winter ( $4.10 \pm 0.19$ ), whereas lowest values were observed in summer ( $2.90 \pm 0.18$ ) and autumn ( $3.07 \pm 0.20$ ).

When the six watersheds were analyzed together, differences between upstream and downstream sites were also recorded in terms of pesticide toxicity, pharmaceutical toxicity, nutrient (DOC, TN, P-PO<sub>4</sub> and N-NO<sub>3</sub>) concentrations, conductivity and BIX (ANOVA,  $P < 0.001$  for each descriptor). Overall, downstream sites were characterized by greater pesticide and pharmaceutical toxicities, DOC, nutrient concentrations and BIX values. Specifically, the greatest differences were observed for the urbanized watershed Artière, which was characterized by a pristine upstream site and a heavily contaminated downstream site in terms of both nutrients (N-NO<sub>3</sub> and P-PO<sub>4</sub>) and xenobiotic toxicity (Table 1). In contrast, only a few differences were observed between the upstream and downstream sites of the three agricultural watersheds; specifically, pharmaceutical toxicity and P-PO<sub>4</sub> concentrations often displayed higher values at the downstream sites of these watersheds (Tukey's test,  $P < 0.001$  each).

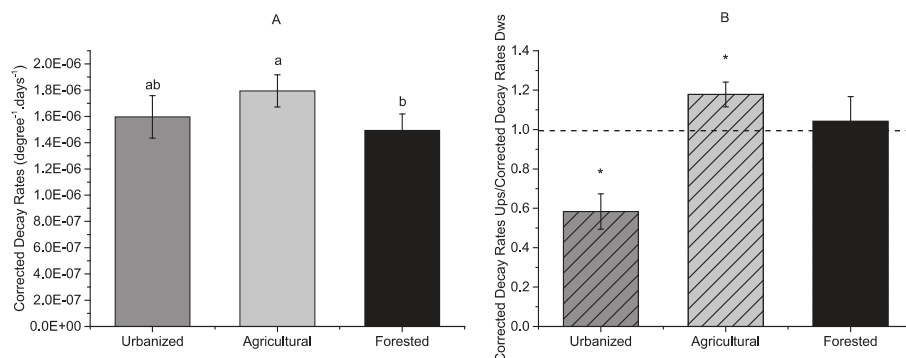
### 3.2. Microbial decomposition of leaf litter

*Alnus* microbial decay rates corrected for accumulated temperature (in degree<sup>-1</sup> day<sup>-1</sup>) showed significant differences among watershed types, although variation was subtle (ANOVA,  $P < 0.01$ ; Fig. 2.A). Higher values were recorded at the agricultural watersheds and lower values at the forested ones, whereas the urbanized watershed displayed intermediate values (Tukey's test,  $P < 0.01$ ). Differences were also observed between seasons (ANOVA,  $P < 0.001$ ). The highest decay rates were recorded in spring ( $2.70 \cdot 10^{-6} \pm 1.98 \cdot 10^{-7}$  degree<sup>-1</sup> day<sup>-1</sup>), especially for agricultural watersheds ( $3.18 \cdot 10^{-6} \pm 2.74 \cdot 10^{-7}$  degree<sup>-1</sup> day<sup>-1</sup>), and the lowest rates in winter were recorded for the forested watersheds ( $5.90 \cdot 10^{-7} \pm 6.92 \cdot 10^{-8}$  degree<sup>-1</sup> day<sup>-1</sup>). In addition, the Rups/dws ratio of decay rates responded differently according to watershed types (Fig. 2.B). While similar decay rates were observed in the upstream and downstream sites in forested watersheds (Rups/dws =  $1.04 \pm 0.12$ ; *t*-test,  $P = 0.37$ ), the decay rates of *Alnus* were 42% faster downstream than upstream in the urbanized watershed (Rups/dws =  $0.58 \pm 0.09$ ; *t*-test,  $P < 0.001$ ). Conversely, agricultural watersheds experienced 18% greater decay rates upstream than downstream (Rups/dws =  $1.18 \pm 0.06$ ; *t*-test,  $P < 0.01$ ).

Specific extracellular ligninolytic enzyme activities (corrected for microbial carbon) in *Alnus* leaves showed high variability (Fig. 3.A 1, 2, 3). Phenol oxidase activity showed no variation between watershed types (ANOVA,  $P = 0.06$ ), whereas peroxidase and laccase activities did (ANOVA,  $P < 0.001$  and  $P < 0.05$ , respectively). Specifically, peroxidase activity was approximately two times higher at the urbanized watershed (than at the forested and agricultural watersheds (Tukey's test,







**Fig. 2.** A Average microbial decay rates in degree days for each watershed type (mean of upstream and downstream decay rates at the four seasons  $\pm$  standard error of the mean,  $n = 24$  for urbanized,  $n = 72$  for agricultural and  $n = 48$  for forested). B Upstream over downstream decay rates in degree days for each watershed type ( $n = 12$  for urbanized,  $n = 36$  for agricultural and  $n = 24$  for forested). The dotted line represents a ratio of 1, indicating similar decay rates between upstream and downstream sites. Asterisk indicates significant differences with the dotted line ( $t$ -test,  $P < 0.05$ ).

$P < 0.05$ ). In contrast, laccase activity was slightly higher at the forested watersheds than at the urbanized and agricultural ones (Tukey's test,  $P < 0.05$ , respectively). Similarly,  $\beta$ -Glucosidase activity showed differences between watershed types (ANOVA,  $P < 0.001$ , Fig. 3.A 4). Specifically, values were two times higher in the urbanized watershed than in the agricultural and forested watersheds (Tukey's test,  $P < 0.01$  each). Temporal variations were also observed in laccase and phenol oxidase activities, although different patterns were observed (ANOVA,  $P < 0.001$  each). While laccase activity was two times higher in winter than in other seasons ( $0.94 \pm 0.15 \mu\text{mol ABTS}^+ \text{mg C}^{-1} \text{h}^{-1}$ ), phenol oxidase activity was higher in autumn ( $0.47 \pm 0.05 \mu\text{mol DIQC mg C}^{-1} \text{h}^{-1}$ ) and lower in spring ( $0.08 \pm 0.01 \mu\text{mol DIQC mg C}^{-1} \text{h}^{-1}$ ) (data not shown).

The Rups/dws values for ligninolytic activities were generally  $>1$ , indicating higher activity values at the upstream sites than at the downstream sites, although some exceptions were present (Fig. 3.B 1, 2, 3). For instance, phenol oxidase activity was higher upstream than downstream in agricultural watersheds (Rups/dws =  $1.38 \pm 0.19$ ;  $t$ -test,  $P < 0.05$ ), but not in the forested and urbanized watersheds. Peroxidase activity was higher upstream in urbanized (Rups/dws =  $3.24 \pm 0.92$ ;  $t$ -test,  $P < 0.05$ ) and forested (Rups/dws =  $6.06 \pm 1.61$ ;  $t$ -test,  $P < 0.01$ ) watersheds, whereas laccase activity was higher upstream only in forested watersheds (Rups/dws =  $2.04 \pm 0.33$ ;  $t$ -test,  $P < 0.01$ ). In contrast with ligninolytic activities,  $\beta$ -glucosidase activity was 41% higher at the downstream site than at the upstream site in the urbanized watershed (Rups/dws =  $0.59 \pm 0.14$ ;  $t$ -test,  $P < 0.01$ , Fig. 3.B 4).

### 3.3. Microbial biomass

Fungal biomass accumulated in *Alnus* leaves varied between watershed types (ANOVA,  $P < 0.001$ , Fig. 4.A). Highest fungal biomass values were recorded in forested watersheds, intermediate values in agricultural watersheds and lowest values in the urbanized watershed (Tukey's test,  $P < 0.001$ ). Temporal variations in fungal biomass accrual on leaves were also observed (ANOVA,  $P < 0.001$ ). Highest values were recorded in summer and winter ( $20.16 \pm 1.54$  and  $18.61 \pm 1.16 \text{ mg C g leaf DW}^{-1}$ , respectively), and the lowest values were recorded in autumn and spring ( $10.23 \pm 1.05$  and  $14.47 \pm 1.20 \text{ mg C g leaf DW}^{-1}$ , respectively). The Rups/dws ratio for fungal biomass was close to 1 for agricultural and forested watersheds but not for the urbanized one, indicating that the urbanized watershed had higher biomass upstream than downstream (Rups/dws =  $1.67 \pm 0.33$ ,  $t$ -test  $P < 0.05$ , Fig. 4.B).

The bacterial biomass also differed with watershed type, but these differences were less marked than those for fungi biomass (ANOVA,  $P$

$< 0.01$ , Fig. 4.A). Here, highest biomass values were recorded at the agricultural and urbanized watersheds and lowest at the forested ones (Tukey's test,  $P < 0.01$ ). Variations were also observed between seasons (ANOVA,  $P < 0.001$ ), with highest bacterial biomass recorded in summer ( $0.77 \pm 0.06 \text{ mg C g leaf DW}^{-1}$ ). The Rups/dws ratio for bacterial biomass was below 1 regardless of the watershed type, indicating higher biomass downstream than upstream (Fig. 4.B).

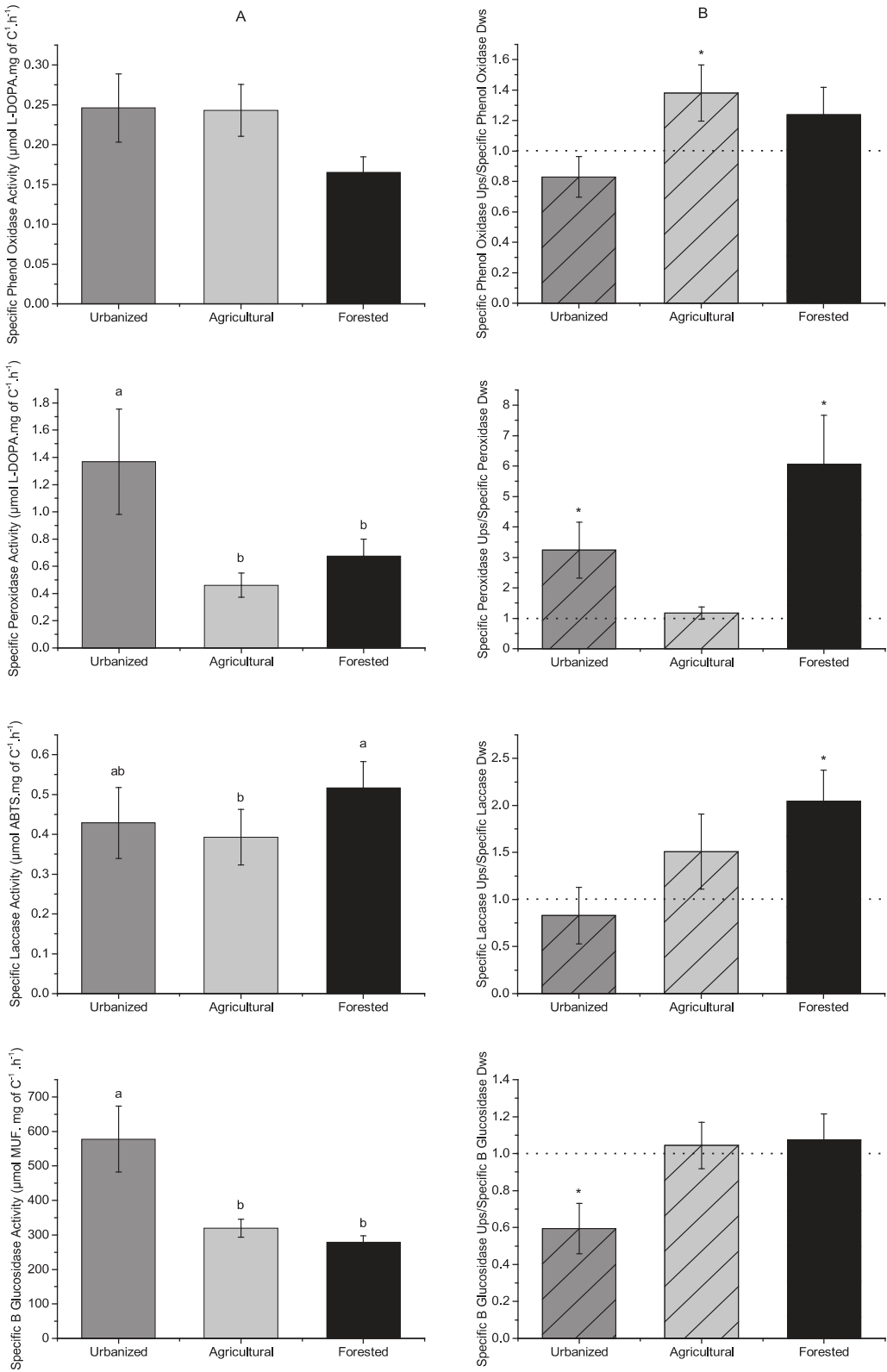
### 3.4. Microbial community structure

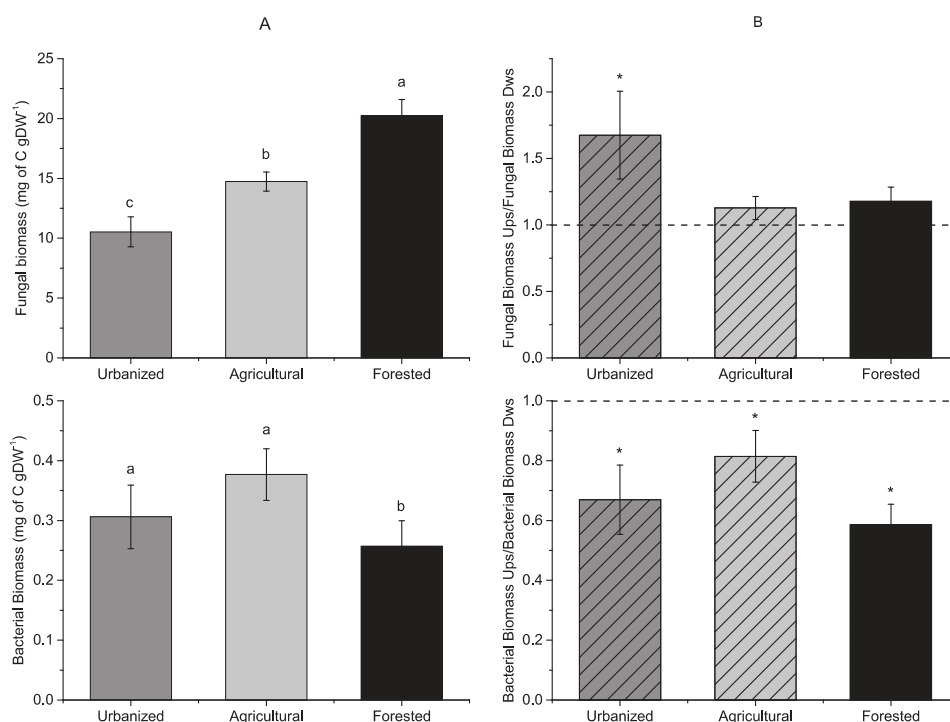
Fungal OTU richness, obtained from DGGE analyses, displayed differences between watershed type (ANOVA,  $P < 0.001$ , Fig. 5.A). Higher fungal OTU richness was observed for urbanized and agricultural watersheds, whereas lower values were observed for the forested watersheds (Tukey's test,  $P < 0.001$ ). In addition, the Rups/dws ratio for fungal band richness displayed values below 1 irrespective of the watershed type (Fig. 5.B). Overall, fungal OTU richness was higher downstream than upstream but more noticeable at the forested watersheds ( $t$ -test,  $P < 0.001$  each). In addition, variations were also observed between seasons (ANOVA,  $P < 0.01$ ), with the highest OTU numbers being observed in winter (Tukey's test,  $P < 0.001$ ).

Bacterial band richness displayed differences between watershed types (ANOVA,  $P < 0.001$ , Fig. 5.A). Here, the highest bacterial band richness was recorded at the agricultural and forested watersheds, and the lowest was recorded at the urbanized one (Tukey's test,  $P < 0.001$ ). Similarly to the ratio for fungal band richness, the Rups/dws ratio for bacterial band richness displayed values below 1, indicating higher bacterial richness downstream than upstream ( $t$ -test,  $P < 0.001$ , Fig. 5.B).

### 3.5. Relationship between microbial, environmental, and xenobiotic parameters

A correlation circle plot extracted from PLSR analysis displayed 2 axes that together represented 64% of the variance caused by environmental/xenobiotic parameters and 31% of that caused by biological parameters (Fig. 6). Component 1 (53% of the variance from environment/xenobiotics and 18% of that from biology) showed that laccase activity and fungal biomass were positively correlated with DOC and negatively correlated with water chemical contamination (including pesticide and pharmaceutical toxicity, BIX, DOC and nutrient (TN, P-PO<sub>4</sub> and N-NO<sub>3</sub>) concentrations). Conversely, microbial decay rates and bacterial biomass were positively correlated to the above-described water chemical contamination (especially pesticide toxicity) and varied with phenol oxidase and cellulolytic activity ( $\beta$ -glucosidase). Component 2 (11% of the variance from environment/xenobiotics and





**Fig. 4.** A Average fungal and bacterial biomass in decomposing leaf litter for each watershed type (mean of upstream and downstream decay rates at the four seasons  $\pm$  standard error of the mean,  $n = 24$  for urbanized,  $n = 72$  for agricultural and  $n = 48$  for forested). Lower case indicate significant differences between each type of watersheds ( $a > b > c$ ; Tukey test,  $P < 0.05$ ) B Upstream over downstream fungal and bacterial biomass for each watershed type ( $n = 12$  for urbanized,  $n = 36$  for agricultural and  $n = 24$  for forested). The dotted line represents a ratio of 1, indicating similar biomass between upstream and downstream sites. Asterisk indicates significant differences with the dotted line ( $t$ -test,  $P < 0.05$ ).

13% of that from biology) showed that pH, DOC and N-NO<sub>3</sub> concentrations were positively correlated with fungal and bacterial band richness and negatively correlated with peroxidase activity.

Standardized coefficients extracted from the PLSR analysis showed that *Alnus* decay rates that had been corrected for temperature were positively related to stream water conductivity, pesticide TUs, N-NO<sub>3</sub> concentration and BIX but negatively related to DOC concentrations and pH (Table 2). Based on the respective weights of each descriptor, the most important descriptors explaining the variation in microbial decay rates in *Alnus* leaves were stream water conductivity, pesticide toxicity, and DOC.

#### 4. Discussion

In this study, we assessed the multistress effects of complex water chemical contamination (including that from nutrients, pesticides and pharmaceuticals) on heterotrophic microbial communities decomposing *Alnus* leaves in stream ecosystems. Leaf litter decomposition in stream ecosystems is a process closely related to the availability of allochthonous organic matter, which is more abundant upstream and which is known to occur mostly in autumn, when the leaf litter stock in the stream is the highest (Mninishall, 1967). Here, our study examines data from monitoring water during four seasons in 3 different types of watersheds (urbanized, agricultural and forested). This extensive dataset allows a good overview for understanding the relationships

between water chemical contamination and microbial litter decomposition. In addition, the obtained microbial decay rates of *Alnus* leaves ranged between  $0.0022 \pm 0.00064 \text{ day}^{-1}$  and  $0.0399 \pm 0.00175 \text{ day}^{-1}$ , which coincides with values found in the field for temperate streams ( $k = 0.0054 \text{ day}^{-1}$ , (Chauvet, 1987);  $k = 0.0163\text{--}0.0247 \text{ day}^{-1}$ , (Brosed et al., 2016)).

Microbial decomposition of *Alnus* leaves appeared slightly affected by the type of watershed in our study. Higher decay rates (corrected for temperature) were recorded at both agricultural and urbanized watersheds (17% and 6% higher, respectively) than at forested watersheds despite that pesticide and pharmaceutical toxicities recorded in these first two watersheds was higher than those in the forested ones. Interestingly, this trend was closely related to N-NO<sub>3</sub> concentrations (Pearson,  $r = 0.19$ ,  $P < 0.05$ , data not shown), whose values were highest at the agricultural watershed and second highest at the urbanized watershed, suggesting a positive relationship with litter decay rates. Globally, such observations seems to support our first hypothesis, stating that nutrients (mostly nitrogen) can buffer the toxicity of xenobiotics to microbial litter decomposition, as already reported in the literature (Fernández et al., 2016). A similar but stronger pattern was observed when the samples from the upstream and downstream sites of the urbanized watershed (Artière) were compared. Here, the rates of microbial *Alnus* leaf decay were 42% higher downstream than upstream, suggesting that nutrients not only compensated for the effect of xenobiotics but appeared to enhance decomposition (Aristi et al., 2016). This

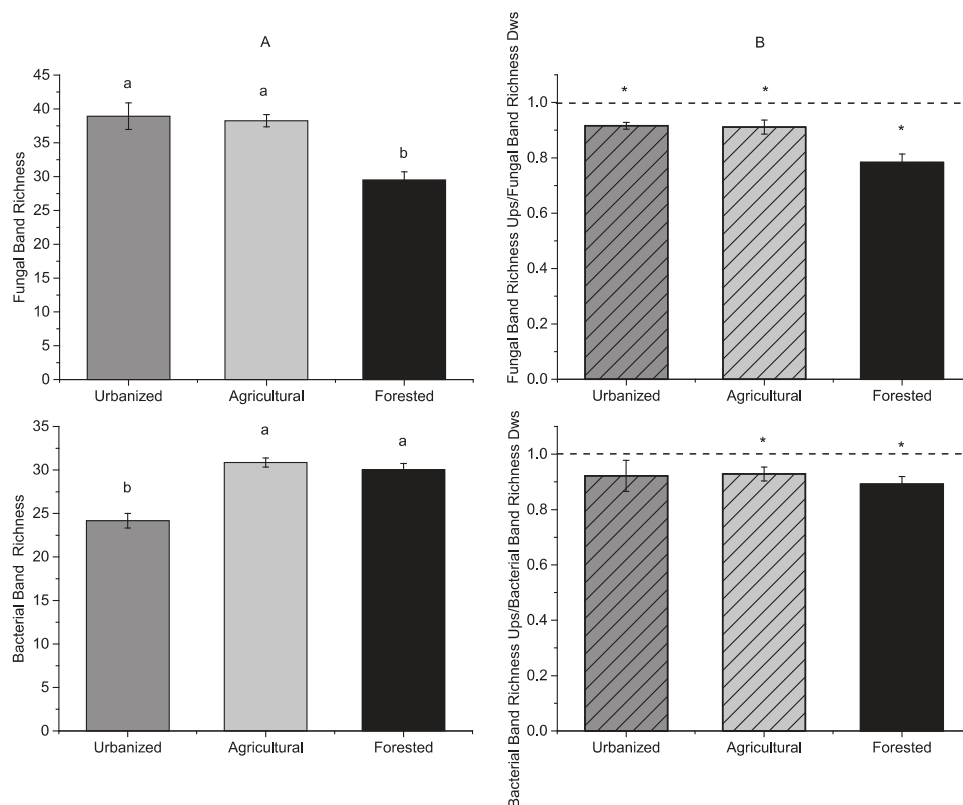
**Fig. 3.** A 1, 2, 3 Average specific ligninolytic activities (Phenol oxidase, peroxidase and laccase) and 4 specific  $\beta$ -glucosidase activity for each watershed type (mean of upstream and downstream decay rates at the four seasons  $\pm$  standard error of the mean,  $n = 24$  for urbanized,  $n = 69$  for agricultural and  $n = 48$  for forested). Lower case indicate significant differences between each type of watersheds ( $a > b$ ; Tukey test,  $P < 0.05$ ) B 1, 2, 3 Upstream over downstream specific ligninolytic activities and 4 specific  $\beta$ -glucosidase activity for each watershed type ( $n = 12$  for urbanized,  $n = 35$  for agricultural and  $n = 24$  for forested). The dotted line represents a ratio of 1, indicating similar enzymatic activities between upstream and downstream sites. Asterisk indicates significant differences with the dotted line ( $t$ -test,  $P < 0.05$ ).

compensatory effect of nutrients over xenobiotics observed in our study might be explained by adaptive mechanisms, such as changes in the structure of microbial communities associated with *Alnus* leaves (Fernández et al., 2016). As observed for microbial communities from river sediments, some microbial groups may have been favored by elevated nutrient concentrations in our study, provoking increases in their decomposition activity, possibly masking negative effects of pesticides on other microbial groups within the community (Widenfalk et al., 2004, 2008). However, such differences in litter decomposition were not observed between the upstream and downstream sites of agricultural and forested watersheds, most certainly because of the small differences in terms of nutrient concentrations in these sites.

In contrast to the effects of nutrients, the microbial decomposition of leaves was found to be negatively correlated by DOC in our PLSR analysis. This could indicate that microbial communities likely tend to use DOC instead of the leaf carbon because the decomposition of the latter is probably more expensive in terms of metabolism (Baldy et al., 2007). In comparison to the DOC, we found that the quality of DOM appeared to play a rather minor role. The degree of humification (HIX) did not come out as significant in our analysis, probably because of its values being lower (ranging from 2.59 to 5.65) in our study than those commonly reported in the literature (between 10 and 16, (Huguet et al., 2009)). Conversely, the origin of DOM (i.e. allochthonous versus autochthonous and reflected by the BIX index) appeared significantly correlated with *Alnus* decay rates (Fig. 6 and Table PLS). Interestingly, BIX values were higher at both urbanized and agricultural watersheds

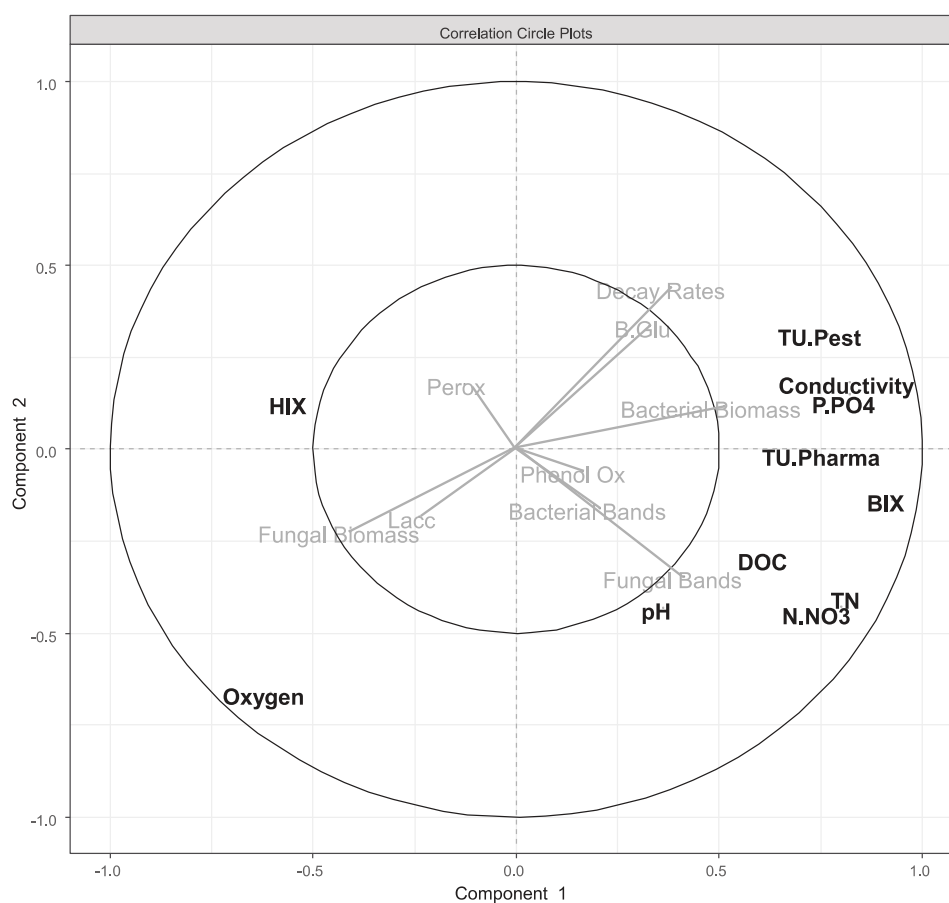
than at forested watersheds and were higher at downstream sites than upstream sites. This might be related to a greater primary production in these sections, which may be explained by greater nutrient and light availability due to a less dense riparian vegetation. According to these results, we hypothesize that the positive relationship between BIX and microbial decay rates is explained by a priming effect (Kuehn et al., 2014), which increases leaf litter decomposition in headwater streams (Danger et al., 2013) even in mesotrophic and/or eutrophic systems (Guenet et al., 2014).

Results from PLSR analysis highlighted that conductivity and the pesticide toxicity of stream water showed a strong positive relationship with the microbial decomposition of *Alnus* leaves. These results contrast with those of Rasmussen et al. (2012) and (Fernández et al., 2015), who attributed a reduction in microbial decay rates to the presence of pesticides in stream water. However, the lower concentrations and toxicity of pesticides (especially by fungicides) in our study could explain the absence of microbial responses to toxicants described in the studies cited above. A possible explanation for the greater decomposition efficiency in contaminated sites in our study can be the use of adaptation mechanisms (pollution-induced community tolerance) leading to more efficient microbial communities for *Alnus* leaves decomposition (Feckler et al., 2017). Thus, the strong positive weight of pesticide toxicity in the decomposition of leaf litter in our study is most certainly indirect. However, the present study did not allow us to draw solid conclusions regarding such mechanisms, since we did not control the exposure time of microbial communities to water contamination and



**Fig. 5.** A Average fungal and bacterial band richness in decomposing leaf litter for each watershed type obtained using DGGE (mean of upstream and downstream decay rates at the four seasons  $\pm$  standard error of the mean,  $n = 24$  for urbanized,  $n = 72$  for agricultural and  $n = 48$  for forested). Lower case indicate significant differences between each type of watersheds (a > b > c; Tukey test,  $P < 0.05$ ). B Upstream over downstream fungal and bacterial band richness for each watershed type ( $n = 12$  for urbanized,  $n = 36$  for agricultural and  $n = 24$  for forested). The dotted line represents a ratio of 1, indicating similar band richness between upstream and downstream sites. Asterisk indicates significant differences with the dotted line ( $t$ -test,  $P < 0.05$ ).





**Fig. 6.** Correlation circle plot extracted from the type 2 Partial Least Square (PLS) regression analysis combining physico-chemical and biological variables. Components 1 and 2 explain 64% of the total variance for physico-chemical variables and 31% for biological variables. Physical and chemical variables are represented in blue and consisted in dissolved organic carbon (DOC), total nitrogen (TN), nitrates (N.NO<sub>3</sub>), phosphate (P.PO<sub>4</sub>), conductivity, pH, oxygen, HIX (humification index), BIX (biological index), pesticides toxic units (TU.Pest) and pharmaceuticals toxic units (TU.Pharma). Biological variables are represented in orange and consisted in temperature corrected decay rates (Decay Rates), fungal biomass (Fungal Biomass), bacterial biomass (Bacterial Biomass), bacterial and fungal band richness (Fungal Bands and Bacterial Bands) and enzymatic activities: laccase (Lacc), phenol oxidase (Phenol.Ox), peroxidase (Perox) and B-glucosidase (B.Glu).

therefore adaptive mechanisms cannot be evidenced. In contrast with the relationship with pesticide toxicity, no significant relationship was found between pharmaceutical toxicity and the microbial decomposition of *Alnus* leaves. This supports current knowledge on the subject as

**Table 2**

Standardized coefficients and standard error of the mean extracted from partial least square regression analysis representing the weight of each physical and chemical variable explaining in temperature-corrected decay rates (Kj) variation. Coefficients were obtained from 3 PLSR components after validation using the root mean square error (RMSEP). Asterisks and P values indicates significant weight in the PLSR model (jackknife,  $P < 0.05$ ).

	Coefficients	Std error	P values
DOC	−0.262	0.035	4.151 10 <sup>−5***</sup>
TN	−0.131	0.093	0.192
P.PO <sub>4</sub>	0.008	0.109	0.941
N.NO <sub>3</sub>	0.107	0.037	0.018*
Oxygen	−0.113	0.072	0.151
Conductivity	0.406	0.107	0.004**
pH	−0.269	0.096	0.020*
HIX	0.016	0.051	0.757
BIX	0.132	0.056	0.044*
Pesticide toxicity	0.328	0.084	0.004**
Pharmaceutical toxicity	−0.141	0.064	0.056

no effect of pharmaceutical compounds were reported on microbial decomposition so far (Hughes et al., 2016). However, pharmaceutical toxicity (even at very low concentrations) showed a slight but non-negligible negative relationship with microbial decomposition in our study (Table 2). In addition, the results from xenobiotic toxicity must be considered with care due to i) the lack of toxicity data for aquatic fungi (toxic unit calculations were performed on toxicity data for microalgae), ii) the fact that xenobiotic concentrations were obtained from spot samplings not always representative of the cumulative contamination to which the microbial community is exposed and iii) the fact that the toxic unit concept assumes an additive model, even if interactions such as antagonism or synergism between xenobiotics occur (Zubrod et al., 2015). Altogether, these results highlights how hard it remains to assess the toxicity risks of pesticide and pharmaceutical for aquatic fungal communities, and especially in a multistress scenario involving many confounding factors that may interact with each other.

Regarding the link between microbial structural and functional parameters explaining *Alnus* litter decomposition, the highest *Alnus* decay rates (contaminated sites) were obtained in microbial communities exhibiting the lowest fungal biomass and the highest fungal and bacterial OTU richnesses. These results suggest that changes in the structure of microbial communities lead to a community with more

efficient decomposition ability when exposed to xenobiotic contamination. These observations might be explained by fungal community adaptation (Feckler et al., 2017; Pascoal et al., 2005) and a more efficient resource partitioning (Gessner et al., 2010; Lawrence and Barraclough, 2016) between microbial species, permitting the retention of a given function despite lower biomass (Fryar et al., 2001). However, it is important to take into account that fungal biomass and enzymatic activities recorded at the recovering of bags are time-specific measurements and therefore not representative of the whole decomposition process. In addition, linking litter decomposition activity with extracellular enzymes remains difficult given the high variability of these enzyme activities (Artigas et al., 2004; Rossi et al., 2017; Sinsabaugh et al., 2002), except for the  $\beta$ -glucosidase and laccase activities in our study. Here, the highest  $\beta$ -glucosidase activity values were recorded at the downstream site of the urbanized watershed, where fungal biomass was the lowest and bacterial biomass was relatively high, whereas a strong positive relationship was observed between  $\beta$ -glucosidase activity and decomposition rates (Fig. 6). In contrast, laccase activity tended to be highest in upstream sections, where fungal biomass accrual in leaves was also greater. According to the bacterial origin of  $\beta$ -glucosidase activity (Romani et al., 2006, 2012) and the fungal origin of laccase activity (Artigas et al., 2017), one could hypothesize that bacterial decomposition activity in submerged leaves could be greater than the decomposition activity of fungi in contaminated sites.

## 5. Conclusion

In the present in situ experiment, we observed that the chemical contamination of streams related to agriculture and urbanization tended to positively affect the decomposition of *Alnus* leaves by microbial communities and that this relation can be explained by the compensatory effects of nutrients (mostly nitrogen). In contrast, the biomass and species richness of fungi in leaves appeared very sensitive to xenobiotic contamination (including pesticides and pharmaceuticals), even at low concentrations. Altogether, these results tend to highlight microbial communities being more efficient in terms of leaf litter decomposition when exposed to stream water contamination, which can be explained by adaptive mechanisms accompanied by changes in the structures of microbe communities associated with leaves. Development from urbanization and new agricultural practices resulting from global change suggest that streams will become more polluted because of anticipated drastic increases in nutrients and micropollutants in water. Such a mixture, even at low xenobiotic concentrations, could thus accelerate microbial litter decomposition, leading to a narrowing of the ecological window of opportunity for higher trophic levels relying on these resources (i.e. macroinvertebrates and fish). We therefore suggest that both microbial biomass and decomposition activity should be assessed to improve ecological and ecotoxicological risk assessment in streams.

## Acknowledgements

This study was funded by the water agency of Loire-Bretagne through the EcoBioPest project. We also thank Fanny Perrière for her help in the water chemical analyses as well as Hermine Billard for the flux cytometry. Florent Rossi was funded by a PhD grant from the French Ministry of Higher Education and Research. This manuscript was edited for grammar, clarity, and consistency and by Wiley Editing Services.

## References

- Abdel-Raheem, A.M., Ali, E.H., 2004. Lignocellulolytic enzyme production by aquatic hyphomycetes species isolated from the Nile's delta region. *Mycopathologia* 157, 277–286.
- Abdi, H., 2010. Partial least squares regression and projection on latent structure regression (PLS regression). *Wiley Interdiscip. Rev. Comput. Stat.* 2, 97–106.

- Abdullah, S.K., et al., 1989. Extracellular enzymatic activity of aquatic and aero-aquatic colonial fungi. *Hydrobiologia* 174, 217–223.
- Aristi, I., Casellas, M., Elosegi, A., Insa, S., Petrovic, M., Sabater, S., Acuña, V., 2016. Nutrients versus emerging contaminants—or a dynamic match between subsidy and stress effects on stream biofilms. *Environ. Pollut.* 212, 208–215.
- Artigas, J., Romani, A.M., Sabater, S., 2004. Organic matter decomposition by fungi in a Mediterranean forested stream: contribution of streambed substrata. *Annales de Limnologie-International Journal of Limnology*, Cambridge Univ. Press, pp. 269–277.
- Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., Pesce, S., 2012. Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. *Aquat. Toxicol.* 122, 197–205.
- Artigas, J., Rossi, F., Gerphagnon, M., Mallet, C., 2017. Sensitivity of laccase activity to the fungicide tebuconazole in decomposing litter. *Sci. Total Environ.* 584, 1084–1092.
- Baldy, V., Gobert, V., Guérol, F., Chauvet, E., Lambrigt, D., Charcoset, J.-Y., 2007. Leaf litter breakdown budgets in streams of various trophic status: effects of dissolved inorganic nutrients on microorganisms and invertebrates. *Freshw. Biol.* 52, 1322–1335.
- Bärlocher, F., Kendrick, B., 1975. Leaf-conditioning by microorganisms. *Oecologia* 20, 359–362.
- Borrel, G., Colombet, J., Robin, A., Lehours, A.-C., Prangishvili, D., Sime-Ngando, T., 2012. Unexpected and novel putative viruses in the sediments of a deep-dark permanently anoxic freshwater habitat. *ISME J.* 6, 2119–2127.
- Box, G.E., Cox, D.R., 1964. An analysis of transformations. *J. R. Stat. Soc. Ser. B Methodol.* 21, 217–252.
- Bratbak, G., 1985. Bacterial biovolume and biomass estimations. *Appl. Environ. Microbiol.* 49, 1488–1493.
- Brosed, M., Lamothe, S., Chauvet, E., 2016. Litter breakdown for ecosystem integrity assessment also applies to streams affected by pesticides. *Hydrobiologia* 773, 87–102. <https://doi.org/10.1007/s10750-016-2681-2>.
- Bundschuh, M., Hahn, T., Gessner, M.O., Schulz, R., 2017. Antibiotic mixture effects on growth of the leaf-shredding stream detritivore *Gammarus fossarum*. *Ecotoxicology* 26, 547–554. <https://doi.org/10.1007/s10646-017-1787-2>.
- Carrascal, L.M., Galván, I., Gordo, O., 2009. Partial least squares regression as an alternative to current regression methods used in ecology. *Oikos* 118, 681–690. <https://doi.org/10.1111/j.1600-0706.2008.16881.x>.
- Chandrasekar, K.R., Kaveriappa, K.M., 1991. Production of extracellular cellulase by *Lunulospora curvula* and *Flagellospora penicillioidei*. *Folia Microbiol. (Praha)* 36, 249–255.
- Chauvet, E., 1987. Changes in the chemical composition of alder, poplar and willow leaves during decomposition in a river. *Hydrobiologia* 148, 35–44.
- Cummins, K.W., 1974. Structure and function of stream ecosystems. *Bioscience* 24, 631–641.
- Danger, M., Cornut, J., Chauvet, E., Chavez, P., Elger, A., Lecerf, A., 2013. Benthic algae stimulate leaf litter decomposition in detritus-based headwater streams: a case of aquatic priming effect? *Ecology* 94, 1604–1613. <https://doi.org/10.1890/12-0606.1>.
- Djukic, I., Kepfer-Rojas, S., Schmidt, I.K., Larsen, K.S., Beier, C., Berg, B., Verheyen, K., Calliman, A., Paquette, A., Gutiérrez-Girón, A., Humber, A., Valdecantos, A., Petraglia, A., Alexander, H., Augustaitis, A., Saillard, A., Fernández, A.C.R., Sousa, A.I., Lillebo, A.I., da Rocha Gripp, A., Francez, A.-J., Fischer, A., Bohner, A., Malyshev, A., Andrić, A., Smith, A., Stanisci, A., Seres, A., Schmidt, A., Avila, A., Probst, A., Ouin, A., Khuroo, A.A., Verstraeten, A., Palabral-Aguilera, A.N., Stefanski, A., Gaxiola, A., Mums, B., Bosman, B., Ahrends, B., Parker, B., Sattler, B., Yang, B., Jüräni, B., Erschbamer, B., Ortiz, C.E.R., Christiansen, C.T., Carol Adair, E., Meredieu, C., Mony, C., Nock, C.A., Chen, C.-L., Wang, C.-P., Baum, C., Rixen, C., Delire, C., Piscart, C., Andrews, C., Rebmann, C., Branquinho, C., Polyanskaya, D., Delgado, D.F., Wundram, D., Radeideh, D., Ordóñez-Regil, E., Crawford, E., Preda, E., Tropina, E., Groner, E., Lucot, E., Hornung, E., Gacia, E., Lévesque, E., Benedito, E., Davydov, E.A., Ampoorer, E., Bolzan, F.P., Varela, F., Kristöfel, F., Maestre, F.T., Maunoury-Danger, F., Hofhansl, F., Kitz, F., Sutter, F., Cuesta, F., de Almeida Lobo, F., de Souza, F.L., Berninger, F., Zehetner, F., Wohlfahrt, G., Vourlitis, G., Carreño-Rocabado, G., Arena, G., Pinha, G.D., González, G., Canut, G., Lee, H., Verbeeck, H., Auge, H., Pauli, H., Nacro, H.B., Bahamonde, H.A., Feldhaar, H., Jäger, H., Serrano, H.C., Verheyden, H., Bruelheide, H., Meesenburg, H., Jungkunst, H., Jactel, H., Shibata, H., Kurokawa, H., Rosas, H.L., Rojas Villalobos, H.L., Yesilonis, I., Melece, I., Van Halder, I., Quirós, I.G., Makelele, I., Senou, I., Fekete, I., Mihal, I., Ostonen, I., Borovská, J., Roales, J., Shogeir, J., Lata, J.-C., Theurillat, J.-P., Probst, J.-L., Zimmermann, J., Vijayanathan, J., Tang, J., Thompson, J., Doležal, J., Sanchez-Cabeza, J.-A., Merlet, J., Henschel, J., Neirynck, J., Knops, J., Loehr, J., von Oppen, J., Porláskdóttir, J.S., Löffler, J., Cardoso-Mohedano, J.-G., Benito-Alonso, J.-L., Torezan, J.M., Morina, J.C., Jiménez, J.J., Quinde, J.D., Alatalo, J., Seebler, J., Stadler, J., Kriiska, K., Coulibaly, K., Fukuzawa, K., Szlavetz, K., Gerhátová, K., Lajtha, K., Kappel, K., Jennings, K.A., Tielbörger, K., Hoshizaki, K., Green, K., Yé, L., Pazianoto, L.H.R., Dienstbach, L., Williams, L., Yahdjian, L., Brigham, L.M., van den Brink, L., Rustad, L., Zhang, L., Morillas, L., Xiankai, L., Carneiro, L.S., Di Martino, L., Villar, L., Bader, M.Y., Morley, M., Lebouvier, M., Tomaselli, M., Sternberg, M., Schaub, M., Santos-Reis, M., Glushkova, M., Torres, M.G.A., Giroux, M.-A., de Graaff, M.-A., Pons, M.-N., Bauters, M., Mazón, M., Frenzel, M., Didion, M., Wagner, M., Hamid, M., Lopes, M.L., Apple, M., Schädler, M., Weih, M., Gualmini, M., Vadeboncoeur, M.A., Bierbaumer, M., Danger, M., Liddell, M., Mirtl, M., Scherer-Lorenzen, M., Růžek, M., Carbognani, M., Di Musciano, M., Matsushita, M., Zhiyanski, M., Puşcaş, M., Barna, M., Ataka, M., Jiangming, M., Alsafran, M., Carnol, M., Barsoum, N., Tokuchi, N., Eisenhauer, N., Lecomte, M., Filipova, N., Hölzel, N., Ferlian, O., Romero, O., Pinto, O.B., Peri, P., Weber, P., Vittoz, P., Turturanu, P.D., Fleischer, P., Macreadie, P., Haase, P., Reich, P., Petřík, P., Choler, P., Marmonier, P., Muriel, P., Ponette, Q., Guariento, R.D., Canessa, R., Kiese, R., Hewitt, R., Rönn, R., Adrian, R., Kanka, R., Weigel, R., Gatti, R.C., Martins, R.L., Georges, R., Meneses, R.I., Gavilán, R.G., Dasgupta, S., Wittlinger, S., Pujalon, S., Freda, S., Suzuki, S., Charles, S., Gogo, S., Drollinger, S., Mereu, S., Wipf, S., Trevathan-Tackett, S., Löfgren, S., Stoll, S., Trogisch, S., Hoerber, S., Seitz, S., Glatzel, S., Milton, S.J., Dousset, S., Mori, T., Sato, T.,

- Ise, T., Hishi, T., Kenta, T., Nakaji, T., Michelan, T.S., Camboulive, T., Mozdzer, T.J., Scholten, T., Spiegelberger, T., Zechmeister, T., Kleinebecker, T., Hiura, T., Enoki, T., Ursu, T.-M., di Cella, U.M., Hamer, U., Klaus, V.H., Régo, V.M., Di Cecco, V., Busch, V., Fontana, V., Piscová, V., Carbonell, V., Ochoa, V., Bretagnolle, V., Maire, V., Farjalla, V., Zhou, W., Luo, W., McDowell, W.H., Hu, Y., Utsumi, Y., Kominami, Y., Zaika, Y., Rozhkov, Y., Kotroczó, Z., Tóth, Z., 2018. Early stage litter decomposition across biomes. *Sci. Total Environ.* 628–629, 1369–1394. <https://doi.org/10.1016/j.scitotenv.2018.01.012>.
- Ejarque, E., Freixa, A., Vazquez, E., Guarch, A., Amalfitano, S., Fazi, S., Romani, A.M., Butturini, A., 2017. Quality and reactivity of dissolved organic matter in a Mediterranean river across hydrological and spatial gradients. *Sci. Total Environ.* 599–600, 1802–1812. <https://doi.org/10.1016/j.scitotenv.2017.05.113>.
- Feckler, A., Goedkoop, W., Kanschak, M., Bundschuh, R., Kennigott, K.G.J., Schulz, R., Zubrod, J.P., Bundschuh, M., 2017. History matters: heterotrophic microbial community structure and function adapt to multiple stressors. *Glob. Chang. Biol.* <https://doi.org/10.1111/gcb.13859>.
- Fernández, D., Voss, K., Bundschuh, M., Zubrod, J.P., Schäfer, R.B., 2015. Effects of fungicides on decomposer communities and litter decomposition in vineyard streams. *Sci. Total Environ.* 533, 40–48.
- Fernández, D., Tummala, M., Schreiner, V.C., Duarte, S., Pascoal, C., Winkelmann, C., Mewes, D., Muñoz, K., Schäfer, R.B., 2016. Does nutrient enrichment compensate fungicide effects on litter decomposition and decomposer communities in streams? *Aquat. Toxicol.* 174, 169–178. <https://doi.org/10.1016/j.aquatox.2016.02.019>.
- Flores, L., Banjac, Z., Farré, M., Larrañaga, A., Mas-Martí, E., Muñoz, I., Barceló, D., Elosegi, A., 2014. Effects of a fungicide (imazalil) and an insecticide (diazinon) on stream fungi and invertebrates associated with litter breakdown. *Sci. Total Environ.* 476–477, 532–541. <https://doi.org/10.1016/j.scitotenv.2014.01.059>.
- Fryar, S.C., Yuen, T.K., Hyde, K.D., Hodgkiss, I.J., 2001. The influence of competition between tropical fungi on wood colonization in streams. *Microb. Ecol.* 41, 245–251. <https://doi.org/10.1007/s002480000075>.
- Gaudes, A., Artigas, J., Romani, A.M., Sabater, S., Muñoz, I., 2009. Contribution of microbial and invertebrate communities to leaf litter colonization in a Mediterranean stream. *J. N. Am. Benthol. Soc.* 28, 34–43. <https://doi.org/10.1899/07-131.1>.
- Gessner, M.O., Chauvet, E., 1994. Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology* 75, 1807–1817.
- Gessner, M.O., Chauvet, E., 2002. A case for using litter breakdown to assess functional stream integrity. *Ecol. Appl.* 12, 498–510.
- Gessner, M.O., Chauvet, E., Dobson, M., 1999. A perspective on leaf litter breakdown in streams. *Oikos* 377–384.
- Gessner, M.O., Swan, C.M., Dang, C.K., McKie, B.G., Bardgett, R.D., Wall, D.H., Hättenschwiler, S., 2010. Diversity meets decomposition. *Trends Ecol. Evol.* 25, 372–380.
- Gómez, M.J., Pazos, F., Guijarro, F.J., de Lorenzo, V., Valencia, A., 2007. The environmental fate of organic pollutants through the global microbial metabolism. *Mol. Syst. Biol.* 3. <https://doi.org/10.1038/msb4100156>.
- Guenet, B., Danger, M., Harraut, L., Allard, B., Jauset-Alcala, M., Bardoux, G., Benest, D., Abbadie, L., Lacroix, G., 2014. Fast mineralization of land-born C in inland waters: first experimental evidences of aquatic priming effect. *Hydrobiologia* 721, 35–44. <https://doi.org/10.1007/s10750-013-1635-1>.
- Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *J. Hazard. Mater.* 169, 1–15.
- Hasenbein, S., Lawler, S.P., Geist, J., Connon, R.E., 2015. The use of growth and behavioral endpoints to assess the effects of pesticide mixtures upon aquatic organisms. *Ecotoxicology* 24, 746–759. <https://doi.org/10.1007/s10646-015-1420-1>.
- Hieber, M., Gessner, M.O., 2002. Contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates. *Ecology* 83, 1026–1038.
- Hou, B.K., Wackett, L.P., Ellis, L.B.M., 2003. Microbial pathway prediction: a functional group approach. *J. Chem. Inf. Comput. Sci.* 43, 1051–1057. <https://doi.org/10.1021/ci034018f>.
- Huber, A., Bach, M., Frede, H.G., 2000. Pollution of surface waters with pesticides in Germany: modeling non-point source inputs. *Agric. Ecosyst. Environ.* 80, 191–204.
- Hughes, S.R., Kay, P., Brown, L.E., 2016. Impact of anti-inflammatories, beta-blockers and antibiotics on leaf litter breakdown in freshwaters. *Environ. Sci. Pollut. Res.* 23, 3956–3962. <https://doi.org/10.1007/s11356-015-5798-3>.
- Huguet, A., Vacher, L., Relexans, S., Saubusse, S., Froidefond, J.-M., Parlanti, E., 2009. Properties of fluorescent dissolved organic matter in the Gironde Estuary. *Org. Geochem.* 40, 706–719.
- Johannes, C., Majcherzyk, A., 2000. Laccase activity tests and laccase inhibitors. *J. Biotechnol.* 78, 193–199.
- Jonsson, M., Ershammar, E., Fick, J., Brodin, T., Klaminder, J., 2015. Effects of an antihistamine on carbon and nutrient recycling in streams. *Sci. Total Environ.* 538, 240–245. <https://doi.org/10.1016/j.scitotenv.2015.08.061>.
- Kuehn, K.A., Francoeur, S.N., Findlay, R.H., Neely, R.K., 2014. Priming in the microbial landscape: periphytic algal stimulation of litter-associated microbial decomposers. *Ecology* 95, 749–762.
- Lawrence, D., Barraclough, T.G., 2016. Evolution of resource use along a gradient of stress leads to increased facilitation. *Oikos* 125, 1284–1295. <https://doi.org/10.1111/oik.02989>.
- Li, W.C., 2014. Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil. *Environ. Pollut.* 187, 193–201. <https://doi.org/10.1016/j.envpol.2014.01.015>.
- Liess, M., Ohe, P.C.V.D., 2005. Analyzing effects of pesticides on invertebrate communities in streams. *Environ. Toxicol. Chem.* 24, 954–965.
- Malaj, E., Peter, C., Grote, M., Kühne, R., Mondy, C.P., Usseglio-Polatera, P., Brack, W., Schäfer, R.B., 2014. Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. *Proc. Natl. Acad. Sci.* 111, 9549–9554.
- Maltby, L., 2009. 4: Heterotrophic Microbes. *River Biota Divers. Dyn.* 45.
- Maltby, L., Brock, T.C., van den Brink, P.J., 2009. Fungicide risk assessment for aquatic ecosystems: importance of interspecific variation, toxic mode of action, and exposure regime. *Environ. Sci. Technol.* 43, 7556–7563.
- Mann, K.L.L., 1975. Detritus formation from eelgrass (*Zostera marina* L.): the relative effects of fragmentation, leaching, and decay. *Limnol. Oceanogr.* 20, 924–934.
- Maul, J.D., Schuler, L.J., Belden, J.B., Whiles, M.R., Lydy, M.J., 2006. Effects of the antibiotic ciprofloxacin on stream microbial communities and detritivorous macroinvertebrates. *Environ. Toxicol. Chem.* 25, 1598. <https://doi.org/10.1897/05-441R.1>.
- Minguez, L., Pedelucq, J., Farcy, E., Ballandonne, C., Budzinski, H., Halm-Lemeille, M.-P., 2016. Toxicities of 48 pharmaceuticals and their freshwater and marine environmental assessment in northwestern France. *Environ. Sci. Pollut. Res.* 23, 4992–5001. <https://doi.org/10.1007/s11356-014-3662-5>.
- Minshall, G.W., 1967. Role of allochthonous detritus in the trophic structure of a woodland springbrook community. *Ecology* 139–149.
- Mora-Gómez, J., Duarte, S., Cássio, F., Pascoal, C., Romani, A.M., 2018. Microbial decomposition is highly sensitive to leaf litter emersion in a permanent temperate stream. *Sci. Total Environ.* 621, 486–496. <https://doi.org/10.1016/j.scitotenv.2017.11.055>.
- Münze, R., Hannemann, C., Orlinskiy, P., Gunold, R., Paschke, A., Foit, K., Becker, J., Kaske, O., Paulsson, E., Peterson, M., Jernstedt, H., Kreuger, J., Schüürmann, G., Liess, M., 2017. Pesticides from wastewater treatment plant effluents affect invertebrate communities. *Sci. Total Environ.* 599–600, 387–399. <https://doi.org/10.1016/j.scitotenv.2017.03.008>.
- Murphy, J., Riley, J., 1962. A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* 27, 31–36.
- Pascoal, C., Cássio, F., Marvanová, L., 2005. Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Arch. Für Hydrobiol.* 162, 481–496.
- Phyt'Auvergne, 2015. Eau et produits phytosanitaires – Qualité des eaux en Auvergne 2004 – 2015 Phyt'Auvergne – June 2015.
- Piscart, C., Genot, R., Doledec, S., Chauvet, E., Marmonier, P., 2009. Effects of intense agricultural practices on heterotrophic processes in streams. *Environ. Pollut.* 157, 1011–1018. <https://doi.org/10.1016/j.envpol.2008.10.010>.
- Rasmussen, J.J., Wiberg-Larsen, P., Baattrup-Pedersen, A., Monberg, R.J., Kronvang, B., 2012. Impacts of pesticides and natural stressors on leaf litter decomposition in agricultural streams. *Sci. Total Environ.* 416, 148–155. <https://doi.org/10.1016/j.scitotenv.2011.11.057>.
- Romani, A.M., Fischer, H., Mille-Lindblom, C., Tranvik, L.J., 2006. Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. *Ecology* 87, 2559–2569.
- Romani, A.M., Artigas, J., Ylla, I., 2012. Extracellular enzymes in aquatic biofilms: microbial interactions versus water quality effects in the use of organic matter. *Microb. Biofilms Curr. Res. Appl.* 153–174.
- Rossi, F., Artigas, J., Mallet, C., 2017. Structural and functional responses of leaf-associated fungal communities to chemical pollution in streams. *Freshw. Biol.* 62, 1207–1219.
- Schulz, R., 2004. Field studies on exposure, effects, and risk mitigation of aquatic nonpoint-source insecticide pollution. *J. Environ. Qual.* 33, 419–448.
- Senesi, N., Miano, T.M., Provenzano, M.R., Brunetti, G., 1991. Characterization, differentiation, and classification of humic substances by fluorescence spectroscopy. *Soil Sci.* 152, 259–271.
- Sinsabaugh, R.L., Osgood, M.P., Findlay, S., 1994. Enzymatic models for estimating decomposition rates of particulate detritus. *J. N. Am. Benthol. Soc.* 160–169.
- Sinsabaugh, R.L., Carreiro, M.M., Repert, D.A., 2002. Allocation of extracellular enzymatic activity in relation to litter composition, N deposition and mass loss. *Biogeochemistry* 60, 1–24.
- Sprague, J.B., 1971. Measurement of pollutant toxicity to fish—III: sublethal effects and “safe” concentrations. *Water Res.* 5, 245–266.
- Suberkropp, K., Klug, M.J., 1976. Fungi and bacteria associated with leaves during processing in a woodland stream. *Ecology* 707–719.
- Talk, A., Kublik, S., Uksa, M., Engel, M., Berghahn, R., Welzl, G., Schlöter, M., Mohr, S., 2016. Effects of multiple but low pesticide loads on aquatic fungal communities colonizing leaf litter. *J. Environ. Sci.* 46, 116–125. <https://doi.org/10.1016/j.jes.2015.11.028>.
- Vitousek, P.M., Mooney, H.A., Lubchenco, J., Melillo, J.M., 1997. Human domination of Earth's ecosystems. *Science* 277, 494–499.
- Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R., et al., 2010. Global threats to human water security and river biodiversity. *Nature* 467, 555–561.
- Webster, J.R., Meyer, J.L., 1997. Organic matter budgets for streams: a synthesis. *J. N. Am. Benthol. Soc.* 16, 141–161.
- Widenfalk, A., Svensson, J.M., Goedkoop, W., 2004. Effects of the pesticides captan, deltamethrin, isoprothuron and pirimicarb on the microbial community of a freshwater sediment. *Environ. Toxicol. Chem.* 23, 1920. <https://doi.org/10.1897/03-345>.
- Widenfalk, A., Bertilsson, S., Sundh, I., Goedkoop, W., 2008. Effects of pesticides on community composition and activity of sediment microbes – responses at various levels of microbial community organization. *Environ. Pollut.* 152, 576–584. <https://doi.org/10.1016/j.envpol.2007.07.003>.
- WISE WFD Database (Water Framework Directive Database), 2018. European waters: chemical status of surface water bodies. <https://www.eea.europa.eu/themes/water/water-assessments/chemical-status-of-surface-water-bodies> (July).
- Young, R.G., Matthaei, C.D., Townsend, C.R., 2008. Organic matter breakdown and ecosystem metabolism: functional indicators for assessing river ecosystem health. *J. N. Am. Benthol. Soc.* 27, 605–625.
- Zsolnay, A., Baigar, E., Jimenez, M., Steinweg, B., Saccomandi, F., 1999. Differentiating with fluorescence spectroscopy the sources of dissolved organic matter in soils subjected to drying. *Chemosphere* 38, 45–50.
- Zubrod, J.P., Englert, D., Feckler, A., Koksharova, N., Kanschak, M., Bundschuh, R., Schnetzer, N., Englert, K., Schulz, R., Bundschuh, M., 2015. Does the current fungicide risk assessment provide sufficient protection for key drivers in aquatic ecosystem functioning? *Environ. Sci. Technol.* 49, 1173–1181. <https://doi.org/10.1021/es5050453>.

### Main conclusions of the study

Water chemical contamination in urbanized and agricultural watersheds coincided with slightly higher leaf decay rates, but lower fungal biomass accumulation, in comparison with that of forested watersheds.

The potential toxicity of the pesticide compounds found in stream water was among the main factor influencing leaf decay rates, partially explaining the observed differences between watershed types. Thus we hypothesized that microbial communities were more efficient in leaf-litter decomposition at pesticide-contaminated waters probably because of the compensatory effect of nutrients over pesticides.

Overall, leaf-associated microbial communities can adapt themselves to a moderate stress by xenobiotics and maintain pivotal ecosystem functions such as the organic matter decomposition.

Based on the results of this study, the association between high microbial decomposition but lower fungal biomass colonizing leaves in agricultural and urbanized watersheds suggest that chemical contamination of stream waters may decrease leaf nutritional value for higher trophic level invertebrate feeders.



## **Chapter 2: Interactive effects of pesticides and nutrients on microbial communities responsible of litter decomposition in streams**



As shown in the previous chapter, water chemical contamination from urbanized and agricultural watersheds tended to slightly stimulate microbial decomposition of alder leaves, and we associated this effect to the compensation by nutrients. However, regarding the positive effect of pesticide toxicity (TUs) and nitrate concentrations on leaf decomposition in our statistical PLSR analysis, it remains hard to discriminate which of them had the most significant effect on the activity of microbial communities. Obtaining clear biological trends from field studies always remains a challenge due to i) the confounding effects of many environmental variables, ii) the variability within streams sites and iii) the temporal scale, making the interpretation rather difficult (Liess and Ohe, 2005). In order to clearly distinguish the effect of nutrient from the effect of xenobiotics, we decided to simplify the model by performing a microcosm experiment. For this study, we collaborated with the MAEP research team (Milieux Aquatiques, Ecologie et Pollutions) from the IRSTEA (Institut national de Recherche en Sciences et Technologies pour l'Environnement et l'Agriculture) of Lyon, who shared with us their artificial stream channels platform (Fig.13).

Based on the field experiment and on the classification of (Dodds & Smith, 2016) (Table 1), two different nutrient concentrations were selected: i) mesotrophic ( $0.8 \text{ mg L}^{-1} \text{ N-NO}_3$  and  $0.05 \text{ mg L}^{-1} \text{ P-PO}_4$ ) similar to that found in the Couze stream, and ii) eutrophic ( $8 \text{ mg L}^{-1} \text{ N-NO}_3$  and  $0.5 \text{ mg L}^{-1} \text{ P-PO}_4$ ) about half the concentrations observed in the Charlet stream. For this experiment, we selected two pesticide compounds: the fungicide tebuconazole (TBZ) and the herbicide S-metolachlor (S-met). The choice of these compounds was mostly based on their occurrence in surface waters as well as their mode of action.

Metolachlor is an herbicide that belongs to the chloroacetanilide family. It inhibits the fatty acid elongation (FAE1) synthase, a key enzyme involved in the elongation of very-long fatty acids in plants, impairing cell division and development in susceptible plants (Götz & Böger, 2004; Paul *et al.*, 2006). S-metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-[(1S)-2-methoxy-1-methylethyl] acetamide) is enriched with the S-isomeric form of metolachlor (up to 80%), conferring the compound an increase in its herbicidal activity (Fig.14). This enrichment resulted in a substantial reduction of application doses (up to 40%) for similar activity and physical and chemical properties comparing to metolachlor. Despite that, S-metolachlor is frequently detected in french surface waters, with a detection frequency of 56.5% in 2015. According to the water agency, S-metolachlor it is the 5<sup>th</sup> pesticide compound the most frequently detected in French surface waters, behind AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), glyphosate, atrazine and metolachlor (Fig.15). However, given the fact that metolachlor

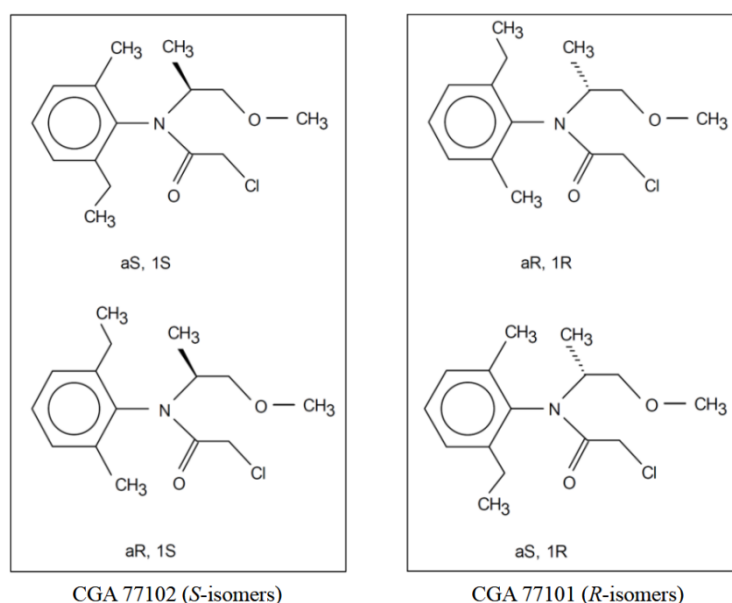
was banned in France since 2003, metolachlor concentrations reported in Fig.15 probably partly refers to S-metolachlor whose distinction with the former has not been performed. Besides, metolachlor is a racemic mixture of both S and R forms. Hence, S-metolachlor concentrations in French streams and rivers are certainly underestimated and S-metolachlor could thus be the most detected herbicide in streams in France.

Tebuconazole ((RS)-1-p-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol) is a fungicide that belongs to the triazole family (Fig.16). It inhibits the sterol C-14  $\alpha$ -demethylation of 24-methylenedihydrolanosterol (which is a precursor of ergosterol, a key component of the fungal cell membrane) thus limiting the development of fungal biomass (Copping & Hewitt, 2007). This compound is frequently detected in French surface waters, reaching 11% detection frequency in French rivers (NAIADES database, EauFrance, 2010-2015) and especially in vineyard areas where tebuconazole is the 7<sup>th</sup> most used compound (Table 2).



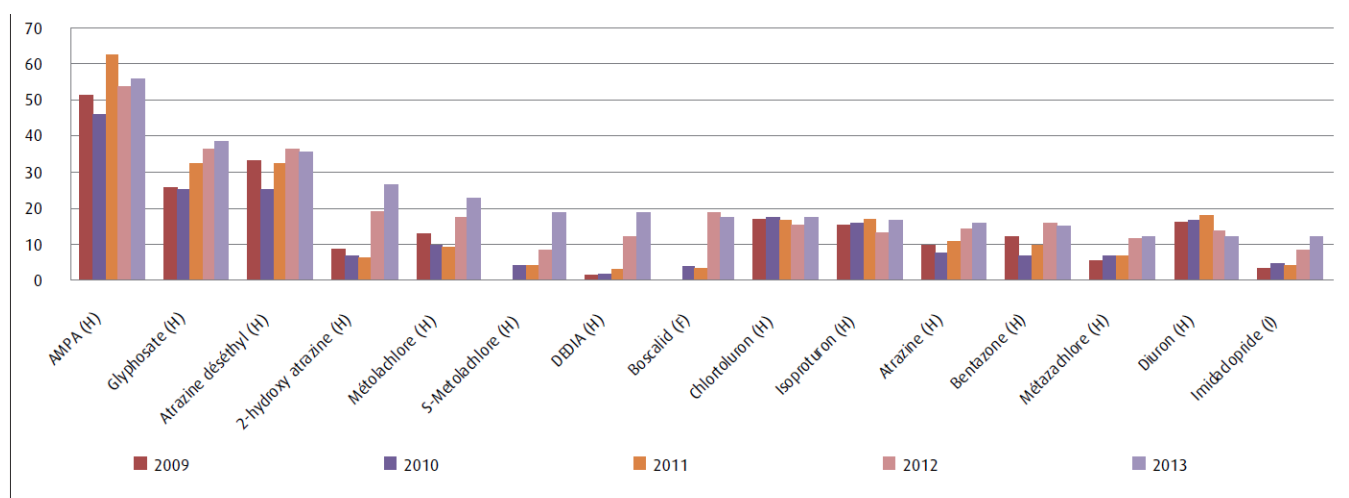
Figure 13: Artificial stream channels used in the chapter 2.

The toxicity of S-metolachlor toward aquatic organisms is still poorly described in the literature, most studies being performed in soil ecosystems. However, current knowledge indicates that environmentally relevant concentrations of S-metolachlor present low to moderate potential threat to non-target algae (including cyanobacteria and macrophytes) (Peterson et al., 1994). Only a few studies revealed significant effects, such as changes in the structure of diatom communities accompanied with frustule deformation at concentrations  $\geq 5 \mu\text{g L}^{-1}$  (Roubeix et al., 2011) and inhibition of cell reproduction in *Scenedesmus vacuolatus* by 50% after a 6h exposure to very high concentrations ( $598 \mu\text{g L}^{-1}$ ). Effects of S-metolachlor were also reported on microbial activity from freshwater sediments, characterized by stimulation of phosphorus fluxes but inhibition of nitrogen ones. No effects of this compound have been reported on heterotrophic microbial communities associated with leaf-litter in streams.



**Figure 14:** Isomeric forms (S- and R-) of the metolachlor. (Figure from Carles, (2016))





**Figure 15:** Detection frequency of the main herbicide compounds found in French rivers and streams between 2009 and 2013. (Source: agences de l'eau; ineris, BNVD. Traitements : SOeS, 2015)

In contrast to S-metolachlor, the effects of tebuconazole on aquatic fungi are well documented. Dijksterhuis *et al.*, (2011), reported that NOEC (no observed effect concentration) of tebuconazole on seven non target aquatic fungi for growth were lower than the previously reported HC<sub>5</sub> (hazardous concentration), and ranged between 0.008 mg L<sup>-1</sup> and 0.5 mg L<sup>-1</sup>, indicating significant effect of this fungicide. In addition, tebuconazole has been shown to decrease fungal biomass associated with submerged leaf litter, decreasing at the same time microbial leaf decomposition (Bundschuh *et al.*, 2011; Zubrod *et al.*, 2011, 2015a; Artigas *et al.*, 2012). This impairment in leaf fungal biomass was also shown to negatively affect macroinvertebrate feeding activity, probably because of a decrease in leaf nutritional quality (Zubrod *et al.*, 2011a; Bundschuh *et al.*, 2011a).

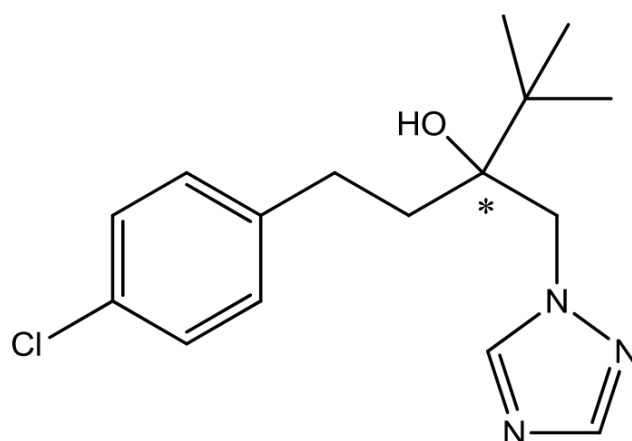


Figure 16: Molecular structure of the tebuconazole.

Table 2: Principal active substances used in vineyards in 2006 (expressed in % of treated areas). (Source: Agreste)

Active substances	Action	% of treated area
Glyphosate (isopropylamine salt)	Herbicide	70
Flopet	Fungicide	68
Micronized sulfur	Fungicide	56
Aluminium fosetyl	Fungicide	53
Mancozeb	Fungicide	51
Cymoxanil	Fungicide	43
Tebuconazole	Fungicide	37
Zinc metiram	Fungicide	36
Copper(II) sulfate	Fungicide	35
Chlorpyrifos	Insecticide, acaricide	27
Copper(II) hydroxide	Fungicide	27
Dinocap	Fungicide	26
Dimethomorph	Fungicide	25
Flufenoxuron	Insecticide, acaricide	25
Myclobutanil	Fungicide	24
Spiroxamine	Fungicide	23
Copper oxychloride	Fungicide	22
Diuron	Herbicide	21
Quinoxifen	Fungicide	20
Aminotriazole	Herbicide	20



# Interactive Effects of Pesticides and Nutrients on Microbial Communities Responsible of Litter Decomposition in Streams

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## OPEN ACCESS

### Edited by:

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### Specialty section:

This article was submitted to  
Microbiotechnology, Ecotoxicology  
and Bioremediation,  
a section of the journal  
Frontiers in Microbiology

**Received:** 29 June 2018

**Accepted:** 24 September 2018

**Published:** 17 October 2018

### Citation:

Rossi F, Pesce S, Mallet C,  
Margoum C, Chaumot A, Masson M  
and Artigas J (2018) Interactive  
Effects of Pesticides and Nutrients on  
Microbial Communities Responsible  
of Litter Decomposition in Streams.  
Front. Microbiol. 9:2437.  
doi: 10.3389/fmicb.2018.02437

Global contamination of streams by a large variety of compounds, such as nutrients and pesticides, may exert a high pressure on aquatic organisms, including microbial communities and their activity of organic matter decomposition. In this study, we assessed the potential interaction between nutrients and a fungicide and herbicide [tebuconazole (TBZ) and S-metolachlor (S-Met), respectively] at realistic environmental concentrations on the structure (biomass, diversity) and decomposition activity of fungal and bacterial communities (leaf decay rates, extracellular enzymatic activities) associated with *Alnus glutinosa* (*Alnus*) leaves. A 40-day microcosm experiment was used to combine two nutrient conditions (mesotrophic and eutrophic) with four pesticide treatments at a nominal concentrations of 15  $\mu\text{g L}^{-1}$  (control, TBZ and S-Met, alone or mixed) following a  $2 \times 4$  full factorial design. We also investigated resulting indirect effects on *Gammarus fossarum* feeding rates using leaves previously exposed to each of the treatments described above. Results showed interactive effects between nutrients and pesticides, only when nutrient (i.e., nitrogen and phosphorus) concentrations were the highest (eutrophic condition). Specifically, slight decreases in *Alnus* leaf decomposition rates were observed in channels exposed to TBZ (0.01119 days<sup>-1</sup>) and S-Met (0.01139 days<sup>-1</sup>) than in control ones (0.01334 days<sup>-1</sup>) that can partially be explained by changes in the structure of leaf-associated microbial communities. However, exposition to both TBZ and S-Met in mixture (MIX) led to comparable decay rates to those exposed to the pesticides alone (0.01048 days<sup>-1</sup>), suggesting no interaction between these two compounds on microbial decomposition. Moreover, stimulation in ligninolytic activities (laccase and phenol oxidase) was observed in presence of the fungicide, possibly highlighting detoxification mechanisms employed by microbes. Such stimulation was not observed for laccase activity exposed to the MIX, suggesting antagonistic interaction of these two compounds on the ability of microbial communities to cope with stress by xenobiotics. Besides, no effects of the treatments

were observed on leaf palatability for macroinvertebrates. Overall, the present study highlights that complex interactions between nutrients and xenobiotics in streams and resulting from global change can negatively affect microbial communities associated with leaf litter, although effects on higher trophic-level organisms remains unclear.

**Keywords:** microcosm, stressors interaction, leaf litter, fungal communities, microbial ecotoxicology, macroinvertebrates

## INTRODUCTION

Freshwaters pollution resulting from human activities has become a widespread phenomenon over the last decades. Among pollution sources, agriculture intensification coupled with changes in practices to increase agricultural yields have led to the massive inputs of both nutrients (including nitrogen and phosphorus) and toxicants (including pesticides which are generally used in mixture) in many stream ecosystems (Trewavas, 2002; Vörösmarty et al., 2010). Accordingly and despite the effort of the European Water Framework directive (WFD) to improve surface water chemical quality, more than 56% of streams in Europe are still qualified as moderate ecological status or worst regarding chemical contamination (WISE WFD database, 2017). This reality copes with the still increasing fertilizers (including  $\text{NO}_3$ ,  $\text{P}_2\text{O}_5$  ad  $\text{K}_2\text{O}$ ) and pesticides consumption across Europe (FAOSTAT). In this context, the evaluation of the threat posed by such a chemical multi-contamination on aquatic organisms and stream ecosystems integrity and the ecological functions and services they provide has become a central preoccupation for societies, stream managers and scientists (Brosed et al., 2016).

Among aquatic organisms that can be found in headwater stream ecosystems, heterotrophic microbial communities have a pivotal role. Mainly composed by fungi (representing up to 98% of the total microbial biomass), microbial communities in leaves also host viruses, bacteria and protists. Thanks to a large panel of extracellular enzymatic activities (Chandrashekar and Kaveriappa, 1991; Abdel-Raheem and Ali, 2004), including ligninolytic (laccase, phenol oxidase) and cellulolytic ( $\beta$ -glucosidase, cellobiohydrolase) enzymes, fungi and bacteria directly participates to the mineralisation of leaf litter (up to 45% of the total leaf mass loss). Thus, they have an important role in the recycling of organic matter in stream ecosystems (Cummins, 1974; Gessner and Chauvet, 1994; Webster and Meyer, 1997). In addition, microorganisms have been shown to stimulate leaf consumption by macroinvertebrates by (i) softening leaf tissues through their extracellular enzymes activity and ii) increasing the nutritional quality and palatability of the leaves (Suberkropp et al., 1983; Baldy et al., 2007). Overall, heterotrophic microbial communities play a non-negligible role in many biogeochemical cycles in addition of being at the base of the trophic networks in stream ecosystems (Webster and Meyer, 1997).

However, pesticides inputs may cause serious threat on such communities, possibly impairing microbial decomposition activity, thus, unbalancing the entire food web. This is particularly the case of fungicides, whose direct effects on fungi, including aquatic hyphomycetes, have already been described (Yang et al., 2011). In polluted streams, one of the most frequently

detected fungicide is the tebuconazole (TBZ) (e.g., 11% of French rivers, NAIADES database, EauFrance, 2010–2015). Belonging to the azole family, TBZ ( $\text{C}_{16}\text{H}_{22}\text{ClN}_3\text{O}$ ) is a triazole fungicide (Richardson, 2009) that inhibits the sterol C-14  $\alpha$ -demethylation of 24-methylenedihydrolanosterol (which is a precursor of ergosterol, a key component of the fungal cell membrane) thus limiting the development of fungal biomass (Copping and Hewitt, 2007). Accordingly, it was previously shown that TBZ exposure can have negative effects on biomass, structure and extracellular enzymatic activities of fungal communities, overall leading to a decrease in the decomposition rates of leaf litter (Bundschuh et al., 2011; Zubrod et al., 2011, 2015; Artigas et al., 2012). Moreover, indirect effects of tebuconazole have been also shown to affect higher trophic level organisms such as macroinvertebrates due to the decrease of leaf nutritional quality (i.e., decrease in fungal biomass and/or changes in microbial community structure), suggesting a potential influence at the stream food web (Bundschuh et al., 2011; Zubrod et al., 2011). In addition to the fungicides, heterotrophic microbial communities are also chronically exposed to herbicides which are prevalent in these ecosystems. The most frequently detected herbicide in river ecosystems since the last 10 years is the S-metolachlor (S-Met) (e.g., 24% of French rivers, NAIADES database, EauFrance, 2010–2015). Belonging to the chloroacetanilide family, S-Met ( $\text{C}_{15}\text{H}_{22}\text{ClNO}_2$ ) is the S isomeric form of the herbicide metolachlor. Mostly used for maize treatment, this herbicide inhibits the fatty acid elongation 1 (FAE1) synthase, a key enzyme involved in the elongation of very-long fatty acids in plants (Götz and Böger, 2004; Paul et al., 2006). Although its effects on aquatic heterotrophic microbial communities colonizing leaf-litter are unknown, S-Met has been shown to induce changes in the structure of diatom communities accompanied with frustule deformation at concentrations  $\geq 5 \mu\text{g L}^{-1}$  (Roubeix et al., 2011) and to inhibit cell reproduction of the green algae *Scenedesmus vacuolatus* by 50% after a 6h exposure to very high concentrations ( $598 \mu\text{g L}^{-1}$ ).

Given the fact that both pesticides and nutrients generally occur at the same time in stream ecosystems (Pesce et al., 2008), it appears essential to assess their interaction in order to understand the consequences at the microbial community level (Segner et al., 2014). Only a few studies tested the interactive effects between nutrients and fungicides on leaf-associated microbial communities, and the available knowledge suggest that the microbial stimulation by addition of nutrients can compensate fungicide toxicity (Fernández et al., 2016; Gardeström et al., 2016). Similarly, some studies tested the interactions between two different types of pesticides on leaf-associated microbial communities (Flores et al., 2014; Dawoud et al., 2017). In the

case of the fungicide imazalil and the insecticide diazinon, Flores et al. (2014) observed that the effect of a mixture of both pesticides led to similar effects than the fungicide alone. On the other hand, (Dawoud et al., 2017) demonstrated that both insecticide (lindane) and fungicide (azoxystrobin) exposure lead to a stimulation of fungal sporulation, but that this stimulation was not observed in the presence of both pesticides in mixture. Together, these results highlights that interactions, such as synergism, between different pesticides may occur, although this seems tightly linked to the type of pesticide compound tested as well as their concentrations. Besides, such differences could also be explained by differences in terms of nutrient concentrations in the media, as (Flores et al., 2014) used stream water whereas Dawoud et al. (2017) used standard test medium M7 (OECD, 1998) for their exposure conditions. However, to our knowledge, no study evaluated the interactions between nutrients and a cocktail of different types of pesticides on leaf-associated microbial communities and the implication of such exposure on the palatability of leaves for macroinvertebrates.

Accordingly, the present study aims to assess (i) the interactions between nutrient increase, namely nitrate and phosphate, relative to stream eutrophication and the presence of TBZ and S-Met alone or in mixture on microbial decomposer communities associated to submerged leaves and (ii) how such multi-contamination further affect leaf palatability for macroinvertebrate consumers. In order to answer these two objectives, we conducted a microcosm experiment in a  $2 \times 4$  full factorial design using laboratory artificial streams, with nutrient condition as the first factor (mesotrophic versus eutrophic) and pesticide treatment as the second one (control, TBZ alone;  $15 \mu\text{g L}^{-1}$ , S-Met alone;  $15 \mu\text{g L}^{-1}$  and MIX;  $15 \mu\text{g L}^{-1}$  of each compounds) over 40 days exposure. Chosen concentrations of pesticides were based on values observed on the field (Berenzen et al., 2005; Kalkhoff et al., 2012) and already used in laboratory experiments (Artigas et al., 2012; Donnadieu et al., 2016; Pesce et al., 2016). Stressors effects were assessed on leaf decomposition rates and a range of extracellular enzymatic activities associated (laccase, phenol oxidase,  $\beta$ -glucosidase, leucine-aminopeptidase and alkaline phosphatase), as well as on the biomass and structure of fungal and bacterial communities. Indirect effects of contamination were investigated on the feeding rates of the macroinvertebrate *Gammarus fossarum*, a shredder amphipod which represents the dominant macroinvertebrate species, in terms of biomass, in many lotic ecosystems (MacNeil et al., 1997). Macroinvertebrates were fed using leaves previously exposed for 40 days to each of the treatments described above.

We firstly hypothesized that the fungicide exposure (alone or in mixture with the herbicide) would significantly affect fungal communities associated with *Alnus* leaves, while the effects of the herbicide alone would be expected to be very limited due to the lack of direct toxicity to fungi and bacteria. In addition, we hypothesized that nutrient increase relative to the eutrophic condition could minimize the effects of pesticides on leaf-associated microbial communities and therefore mask pesticide effects on leaf palatability for the macroinvertebrate *Gammarus fossarum*.

## MATERIALS AND METHODS

### Microbial Inoculum

Leaf-associated fungal and bacterial communities were obtained from the Couze d'Ardes River, a well preserved mesotrophic third-order forested stream, draining a basin surface area of 21476 ha (mostly forest and prairies) in the Puy-de-Dôme region (Centre France). Freshly fallen *Alnus glutinosa* (L.) Gaertn leaves were harvested in October 2016, brought back to the laboratory and dried at room temperature for 72 h. Leaves (ca. 3 g of dried leaves) were placed into three litter bags of 0.5 mm mesh size ( $1 \times w = 15 \times 15$  cm) and immersed in the upstream section of the Couze d'Ardes River during 4 weeks, from September 25 to October 26 2016, to allow microbial colonization. After in-stream colonization, litter bags were retrieved and transported back to the laboratory where leaves were cleaned with filtered stream water ( $0.2 \mu\text{m}$ ), cut into small circles (1 cm in diameter) and placed into nine sterile 250 mL flasks (3 flasks per retrieved bag). Each flask was filled with 200 mL of stream water diluted to 1/10 with demineralized water and contained 15 pre-colonized leaf disks (Gessner and Chauvet, 1993). Fungal mycelia sporulation was achieved by incubations at  $10^\circ\text{C}$  under agitation at 180 rpm (Artigas et al., 2008). After 5 days, water suspension and leaf disks from the same replicates were pooled by transferring the content into three new sterile flasks (1 per replicate, 600 mL per flask) and used as inoculum for the subsequent microcosm experiment (75 mL of inoculum per stream channel).

### Experimental Design

The experiment was performed in microcosm under controlled conditions of temperature ( $20 \pm 2^\circ\text{C}$ ) and photoperiod (13 h light: 11 h dark). In total, 24 artificial glass stream channels were set up ( $63 \text{ cm} \times 11 \text{ cm} \times 4 \text{ cm} = l \times w \times h$ ) following a  $2 \times 4$  factorial design, with nutrient condition as first factor (mesotrophic and eutrophic) and pesticide treatment as second one (Control, TBZ, S-Met, and MIX) in triplicates. Each channel was independent and had a separate 20 L tank, filled with 10 L of water from the corresponding condition (see details below) which supplied water through an individual aquarium pump (MJ 750, NEWA,  $1.5 \text{ L} \cdot \text{min}^{-1}$ ). Drilled ground water diluted to 1/3 with demineralized water was used for the experiment and distributed in two different 200 L tanks filled up to 100 L. Nutrient conditions were obtained by adding monobasic potassium phosphate ( $\text{K}_2\text{HPO}_4$ ) and sodium nitrate ( $\text{NaNO}_3$ ) to reach nominal concentrations of  $8 \text{ mg L}^{-1}$   $\text{N-NO}_3$  and  $0.5 \text{ mg L}^{-1}$   $\text{P-PO}_4$  for the eutrophic condition and  $0.8 \text{ mg L}^{-1}$   $\text{N-NO}_3$  and  $0.05 \text{ mg L}^{-1}$   $\text{P-PO}_4$  for the mesotrophic one. Nutrient enriched water was then distributed into 8 smaller tanks of 30 L (4 for either mesotrophic and eutrophic conditions), and pesticide contaminated treatments were obtained by adding either TBZ, S-Met or both from a  $5 \text{ mg L}^{-1}$  stock solution diluted in water (Sigma-Aldrich, Germany) to reach nominal pesticide concentrations of  $15 \mu\text{g L}^{-1}$  TBZ or/ and  $15 \mu\text{g L}^{-1}$  S-Met in the corresponding condition. Those 30 L tanks were finally used to supply water to each triplicate channels per each pesticide treatment.



In each channel, a total of 15 bags containing *Alnus glutinosa* leaves was disposed as follows: 6 small bags containing each 60 leaf disks (1 cm diameter) for biological analyses (extracellular enzymatic activities, fungal and bacterial biomass, fungal and bacterial community structure), 2 bags containing each 40 leaf disks (2 cm diameter) for *Gammarus* feeding rates, 1 bag containing 60 leaf disks (2 cm diameter) for adsorbed pesticides and leaf nutrient content analyses and 6 larger bags containing each 2 pre-weighted leaves for leaf mass loss determination. The experiment started in November 3 until December 13 2016 (6 weeks duration in total). After 4 days acclimation of leaves in all 24 channels under mesotrophic water (plus microbial inoculums) without pesticides, water from the channels was replaced to fit the eight experimental conditions described above. Samplings for biological analyses were performed every week by taking one of the small bags cited above and water was renewed weekly during the entire experiment using the above described procedure (6 water renewals during the whole experiment).

### Water and Leaves Characteristics

Physical and chemical parameters of stream water, including dissolved nutrients, TBZ, and S-Met concentrations were measured before and after each water renewal to evaluate nutrient and pesticide dissipation. Light was measured continuously for each condition using data loggers (HOBO® Pendant Temperature/Light, ProSensor), whereas temperature, pH, conductivity and dissolved oxygen were measured in each channel before and after each water renewal using portable probes (WTW). Concentrations of orthophosphates (P-PO<sub>4</sub>), nitrates (N-NO<sub>3</sub>), nitrites (N-NO<sub>2</sub>) and ammonium (N-NH<sub>4</sub>) were determined using ionic chromatography (930 Compact IC Flex, Metrohm) following standard methods for anions (NF EN ISO 10304-1; AFNOR, 2009) and cations (NF EN ISO 14911; AFNOR, 1999). Dissolved organic carbon (DOC) concentrations were measured in water following standard method (NF EN 1484; AFNOR, 1997) using an elemental analyzer (Multi N/C 3100, Analytik Jena). Prior to analyze DOC, inorganic carbon was removed by purging acidified (pH = 1 with HCl) samples with a CO<sub>2</sub>-free gas. Dissolved TBZ and S-Met concentrations were determined by direct injection of 20 µL of water samples previously filtered using a 0.2 µm polyester filter (MACHEREY-NAGEL) into an Ultra-High-Performance Liquid Chromatography (UHPLC) system (Shimadzu Nexera®) coupled to a triple quadrupole mass spectrometer (API 4000, LC/MS/MS system, AB Sciex). The chromatographic separation was performed on a Waters HSS T3 1.8 µm 2.1 × 100 mm column. Limit of quantification was 0.05 µg L<sup>-1</sup> for both pesticides.

Chemical composition of *Alnus* leaves (C, N, and P content) and adsorbed pesticides (TBZ and S-Met) were assessed at the end of the microcosm experiment (Day 40). Total carbon and nitrogen of the leaves were measured by dry combustion using CNS elemental analyzer (Flash2000, ThermoFisher Scientific). Total phosphorus was analyzed after microwave-acid mineralization in *aqua regia* (NF EN ISO 15587-1; AFNOR, 2002) using inductively coupled plasma

optical emission spectrometry (ICP-OES, 700 Series, Agilent Technologies) following standard method (EN ISO 11885, 2009). Results were obtained in mg per kg<sup>-1</sup> of leaf dry mass (DM)<sup>-1</sup> and converted into C:N, N:P, and C:P molar ratios. Adsorbed pesticides were extracted from leaves using the QuEChERS protocol (Payá et al., 2007) and pesticide were quantified using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS-MS). Results were expressed in ng per g leaf DM<sup>-1</sup> with a limit of quantification for both the TBZ and S-Met of 8 ng g leaf DM<sup>-1</sup>.

### Microbial Biomass and Community Structure

Ergosterol concentration was used as a proxy to estimate fungal biomass associated with leaves (Gessner and Schmitt, 1996). Briefly, lipids were extracted from previously lyophilized leaves (15 leaf disks of 1 cm in diameter per sample) after incubation in 0.14 M KOH methanol at 80°C. Extracts were purified, concentrated using solid-phase extraction (tC18 cartridges, Sep-Pak Vac RC, 500 mg, Waters) and then analyzed using a high pressure liquid chromatography system (Lachrom L-7400, Merck-Hitachi) equipped with an ODS-2 Hypersil column (250 × 4.6 mm, 5 µm particle diameter; Thermo Scientific). Ergosterol was detected at 282 nm according to its specific absorbance spectrum. Ergosterol concentration was converted into fungal carbon using the conversion factor proposed by Gessner and Chauvet (1993) and Baldy and Gessner (1997).

Bacterial biomass was estimated using bacterial density according to the protocol of Borrel et al. (2012). Briefly, one leaf disk (1 cm diameter) was sampled at each sampling time and for each channel and fixed into 1 mL of 2% paraformaldehyde (storage at 4°C) until analysis using a BD FACS Calibur flow cytometer (Becton Dickinson Bio-sciences) (see Pesce et al., 2016). Bacterial density was converted to bacterial biomass using the conversion factor of (Bratbak, 1985).

The structure of fungal and bacterial communities associated to *Alnus* leaves was assessed using Automated Ribosomal Intergenic Spacer Analysis (ARISA) as described in Artigas et al. (2012). Total DNA extraction was performed from 5 leaf disks of 1 cm using the FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, United States) according to the manufacturer's instructions. Fungal community was characterized by amplification of the ITS1-5.8S-ITS2 region using the primers 2234C (5'-GTTTCGGTAGGTGAACCTGC-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3'). Bacterial community was characterized by amplification of the 16S-23S ribosomal intergenic spacer region using the primers S-D-Bact-1522-b-S-20 (5'-185 TGCGGCTGGATCCCCTCCTT-3') and L-DBact-132-a-A-18 (5'-CCGGGTTTCCCCATTCGG-3'). PCR mixture and conditions are described in Baudoin et al. (2010). ARISA was performed using an Agilent 2100 Bioanalyzer with a DNA 1000 kit (Agilent Technologies) following the manufacturer's instructions. ARISA profiles (**Supplementary Figures S3, S4**) were then analyzed using the Gelcompare2 software (Applied Maths, Belgium) in order to obtain a fungal and bacterial OTUs presence/absence matrix.

## Microbial Activity

Decay rates of *Alnus* leaves ( $k$ ) were evaluated as the leaf mass loss during the experiment. At each sampling time and in each channel, 1 bag containing 2 pre-weighted *Alnus* leaves was sampled and brought to the oven (70°C for 48h) in order to obtain dry mass (DM). Decay rates were then obtained after fitting data to an exponential decay model according to the following equation:  $M_t = M_0 e^{-kt}$ , where  $M_0$  is the initial DM (g),  $M_t$  is the final DM (g) at time  $t$ , and  $k$  is the leaf decay coefficient (Petersen and Cummins, 1974).

Microbial enzymatic potential was estimated through the measurement of 5 extracellular enzyme activities: phenol oxidase (EC 1.14.18.1), laccase (EC 1.10.3.2),  $\beta$ -glucosidase (EC 3.2.1.21), alkaline phosphatase (EC 3.1.3.1) and leucine-aminopeptidase (EC). Phenol oxidase and laccase activities were measured from five leaf disks per sample, using 3,4-Dihydroxy-L-phenylalanine (L-DOPA, 1.5 mM final concentration) and 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS, 3 mM final concentration) respectively, in acetate buffer (pH 4.65, Sigma-Aldrich, St. Louis, MO, United States), as described in Sinsabaugh et al. (1994) and Johannes and Majcherczyk (2000).  $\beta$ -glucosidase, leucine-aminopeptidase and alkaline phosphatase activities were measured from 1 leaf disk per sample using the corresponding methylumbelliferyl and amido-4-methylcoumarin substrate analog (0.3 mM final concentration for each activity assay), as described in Artigas et al. (2012). After 1.5 h incubation in the dark and under agitation at 20°C, phenol oxidase and laccase activities were measured spectrophotometrically (460 nm and 415 nm, respectively) using an Ultrospec 2000 (Pharmacia Biotech, Trowbridge, United Kingdom) whereas  $\beta$ -glucosidase, leucine-aminopeptidase and alkaline phosphatase were determined fluorometrically (360/460 nm excitation/emission, Biotek synergy HT fluorometer) after addition of 0.3 mL glycine buffer (pH 10.4). All enzyme activity measurements were conducted under substrate saturating conditions. Area under the curve (AUC) was calculated for each enzyme and divided by the AUC for microbial biomass in order to obtain one value of integrated specific enzymatic activity for each nutrient condition and pesticide treatment. Activity values were expressed in  $\mu\text{mol substrate h}^{-1}$  mg of microbial C $^{-1}$ .

## Gammarus fossarum Feeding

Feeding rates of *Gammarus fossarum* were assessed as described in Pesce et al. (2016). Briefly, *Gammarus* were harvested from the River Le Pollon in France (45°57'25.8"N 5°15'43.6"E) and acclimatized to laboratory conditions as previously described (Coulaud et al., 2011). In the laboratory, organisms were kept in 30 L tanks continuously supplied with drilled groundwater (500  $\mu\text{S/cm}$ ) and under constant aeration for 10 days. Photoperiod (16 h light: 8 h dark) was maintained and the temperature was kept at  $12 \pm 1^\circ\text{C}$ . Organisms were fed *ad libitum* with alder leaves (*Alnus glutinosa*), previously conditioned for  $6 \pm 1$  days in water. At the end of the microcosm experiment, bags containing 2 cm leaf disks were recovered and displayed into plastic beakers (8 disks per beaker) filled with 500 mL of

oxygenated water (12°C) and containing 12 *Gammarus* males with homogenous body size ( $\sim 10$  mm). The experiment was performed in triplicate for each replicate ( $n = 9$ ) and included a control (leaves without *G. fossarum*) to estimate leaf mass loss unrelated to *Gammarus* consumption. In parallel, daily water renewals were performed and dead *Gammarus* were recorded and removed if any to avoid bias in feeding rates estimation. Only 3 deaths occurred over the whole set of replicates (864 gammarids). The experiment was stopped after 28 h when leaf consumption achieved approximately 50% in at least one replicate, and the surface of remaining leaf disks was scanned numerically using SigmaScan® Pro v5.0 imaging software (Systat Software). Results were expressed in % of leaf consumed per *Gammarus* and per day and converted into ingested fungal C (mg Fungal C *Gammarus* $^{-1}\text{Day}^{-1}$ ) using the measurements of fungal C on leaves (see above).

## Statistical Analyses

Differences between nutrient conditions and between pesticide treatments for water physical and chemical characteristics (pH, oxygen, conductivity, temperature, TBZ and S-Met), leaf composition (C:N, C:P, and N:P, adsorbed TBZ and S-Met), decomposition rates, specific enzymatic activities and leaf palatability for macroinvertebrates were assessed using a Kruskal–Wallis non parametric test followed by pairwise comparison test using Tukey contrast ( $P < 0.05$ ). In addition, fungal and bacterial biomass colonization on *Alnus* leaves was fitted to a logistic growth model using the following equation:

$$y = \frac{A}{1 + \frac{A - y_0}{y_0} e^{-4W \max x / A}}$$

Where  $y_0$  is the fungal biomass (mg of fungal carbon g Leaf DM $^{-1}$ ) at day 0,  $A$  is the fungal biomass at day 40 and  $W_{\max}$  is the maximum growth rate (day $^{-1}$ ). Parameters from the model were then compared within each-other using the Kruskal–Wallis non-parametric test followed by pairwise comparison test using Tukey contrast ( $P < 0.05$ ). In addition, two non-metric dimensional scaling (NMDS) for either bacteria or fungi were used to assess communities' structure based on Bray–Curtis dissimilarity matrices. First NMDS was performed including all sampling times, whereas second NMDS was performed on last sampling time only (day 40). Each NMDS was accompanied by permutational multivariate analysis of variance (PERMANOVA) using nutrient condition as first factor and treatment as second one. All statistical analyses were computed using the R software and NMDS and PERMANOVA analyses were performed using metaMDS and Adonis functions from the vegan package (Oksanen et al., 2008) followed by pair-wise *post hoc* multiple comparisons.

## RESULTS

### Water and Leaves Characteristics

Average initial phosphate, nitrate and S-Met concentrations measured in water right after each water renewal were relatively

**TABLE 1** | Water physical and chemical characteristics measured after each water renewals in experimental stream channels exposed to fungicide (TBZ), herbicide (S-Met), both fungicide + herbicide (MIX) or non-exposed (Ctrl) in either mesotrophic or eutrophic nutrient condition.

	Mesotrophic				Eutrophic			
	Ctrl	TBZ	S-Met	MIX	Ctrl	TBZ	S-Met	MIX
P-PO <sub>4</sub> (mg L <sup>-1</sup> )		0.05 ± 0.01				0.46 ± 0.01		
N-NO <sub>3</sub> (mg L <sup>-1</sup> )		0.66 ± 0.01				7.89 ± 0.03		
TBZ water (μg L <sup>-1</sup> )	<0.05	9.87 ± 0.30	<0.05	11.39 ± 0.85	<0.05	10.67 ± 0.75	<0.05	11.64 ± 0.86
S-Met water (μg L <sup>-1</sup> )	<0.05	<0.05	16.26 ± 1.22	14.28 ± 0.54	<0.05	<0.05	17.21 ± 1.39	15.79 ± 0.96
TBZ leaf (μg gDM <sup>-1</sup> )	<0.008	1.22 ± 0.13	<0.008	1.16 ± 0.09	<0.008	0.68 ± 0.04	<0.008	0.71 ± 0.06
S-Met leaf (μg gDM <sup>-1</sup> )	<0.008	<0.008	1.37 ± 0.19	1.05 ± 0.10	<0.008	<0.008	0.64 ± 0.05	0.50 ± 0.04
Temperature (°C)	19.9 ± 0.3	20.1 ± 0.3	18.4 ± 1.8	20.2 ± 0.2	20.2 ± 0.2	20.3 ± 0.2	20.4 ± 0.2	20.3 ± 0.2
Conductivity (μS)	167 ± 1	170 ± 2	168 ± 1	168 ± 1	208 ± 9*	205 ± 9*	206 ± 9*	205 ± 9*
Oxygen (mg L <sup>-1</sup> )	8.3 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	8.2 ± 0.1	8.3 ± 0.2	8.3 ± 0.2	8.6 ± 0.4	8.4 ± 0.2
pH	7.7 ± 0.1	7.7 ± 0.1	7.7 ± 0.1	7.8 ± 0.1	7.8 ± 0.1	7.9 ± 0.1	7.9 ± 0.2	7.9 ± 0.2

Values are means ± standard error of the mean over the all experiment ( $n = 5$  for pesticides and  $n = 6$  for nutrients, temperature, conductivity, oxygen, and pH). Asterisks indicates significant differences between nutrient conditions for the considered treatment (Kruskal–Wallis,  $P < 0.05$ ).

close to the expected nominal concentrations (Table 1). In contrast, initial TBZ concentrations were about one-third lower than expected. No statistical differences were observed for both initial TBZ and S-Met concentrations between treatments (alone or MIX) and the subsequent water renewals. Similarly, no statistical differences were observed for initial N-NO<sub>3</sub> and P-PO<sub>4</sub> concentrations within each nutrient condition and between further water renewals. In addition, water temperature, dissolved oxygen concentration and pH displayed similar values between treatments (Kruskal–Wallis,  $P > 0.05$ , Table 1), while water conductivity displayed higher values in eutrophic conditions (Kruskal–Wallis,  $P < 0.001$ ) probably because of the higher nutrient (phosphate and nitrate) concentrations.

Nutrient dissipation, calculated as the difference after and before each water renewal in stream channels (once per week), revealed that both nitrate and phosphate were completely dissipated in the mesotrophic condition (Table 2), but not in the eutrophic one (average dissipation percentage of 65.4% for N-NO<sub>3</sub> and 93.1% for P-PO<sub>4</sub>). In the eutrophic condition, P-PO<sub>4</sub> dissipation was greater than that of N-NO<sub>3</sub> revealing that leaf-associated microbial community's used more P than N from water. This difference was also reflected in leaves nutrient content at the end of the experiment (Kruskal–Wallis,  $P < 0.01$ , Figure 1). Specifically, lower C:P and N:P ratios were recorded in eutrophic channels, which show the greater P accumulation in leaves and therefore confirms the greater P dissipation from water. Pesticide treatments did not significantly influence N-NO<sub>3</sub> and P-PO<sub>4</sub> dissipation (Kruskal–Wallis,  $P = 0.18$  and  $P = 0.13$  respectively), although nitrate dissipation for the eutrophic condition was about 13–22% greater in the MIX in comparison with Ctrl, and S-Met and TBZ alone (Table 2).

Concerning pesticides in water, about 39–70% of each pesticide were dissipated every week in all pesticide-treated channels (Table 2). For each pesticide, no differences in dissipation were observed between nutrient conditions and pesticide treatments (i.e., Alone vs. MIX) with some exceptions. In eutrophic channels, S-Met dissipation was slightly greater in the MIX treatment (69% dissipation) compared to S-Met

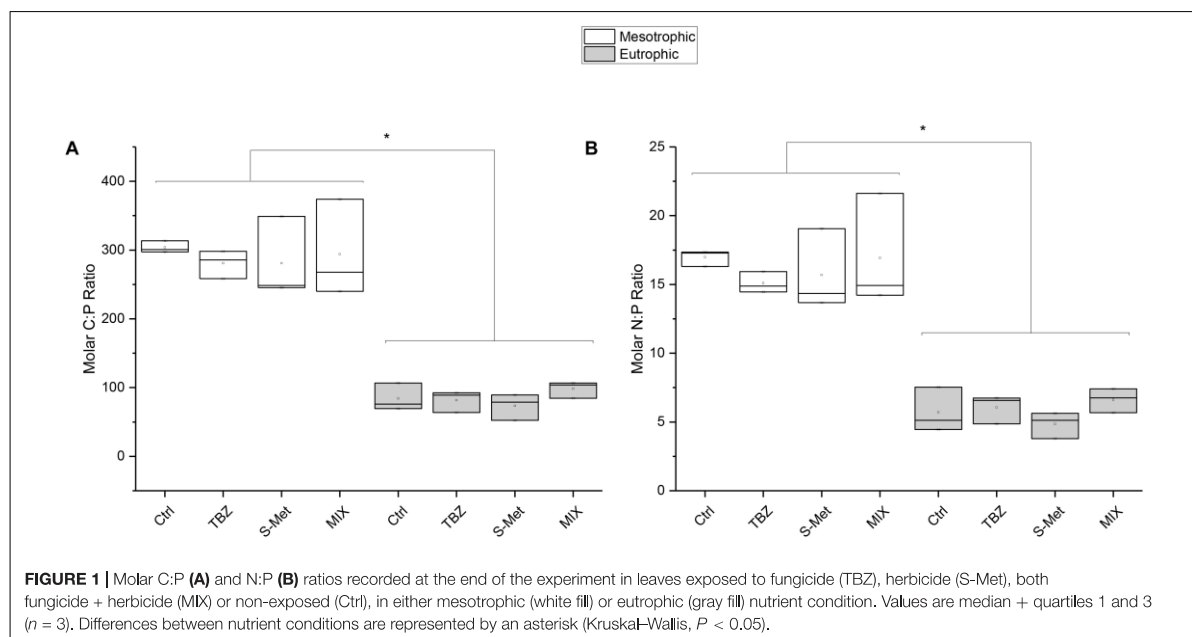
alone (56%) (Kruskal–Wallis,  $P < 0.05$ ). Pesticide sorption was assessed at the end of the experiment and only a small part of TBZ and S-Met weekly supplied to channels was adsorbed onto *Alnus* leaves (less than 5%, Table 2). Comparatively, pesticide adsorption was higher in mesotrophic channels compared with the eutrophic ones (Kruskal–Wallis,  $P < 0.05$ ). Exposure of pesticides alone or in MIX did not have an effect on their adsorption to leaves.

## Microbial Leaf-Litter Decomposition

*Alnus* leaves decomposition displayed differences between both nutrient conditions (Kruskal–Wallis,  $P < 0.001$ ) and pesticide treatments (Kruskal–Wallis,  $P < 0.01$ , Table 3 and Supplementary Table S1). On average, leaf decay rates in eutrophic channels were 34% higher compared to those in mesotrophic channels. In addition, decay rates were on average 17% higher in control than in pesticide-treated channels (including TBZ, S-Met, and MIX) in the eutrophic condition, whereas no differences between pesticide treatments and control were observed under mesotrophic conditions. No differences were observed for *Alnus* decay rates in TBZ and S-Met alone compared to MIX, regardless of the nutrient condition.

Specific extracellular enzymatic activities (i.e., cumulated enzymatic activity corrected by cumulated microbial biomass) displayed variation between the tested conditions and the enzyme considered (Figure 2). Specifically, differences in ligninolytic (laccase and phenol oxidase, Figures 2A,B) and peptidase (leucine-aminopeptidase, Figure 2C) activities were observed between both nutrient conditions and pesticide treatments, whereas no between-treatment difference were observed for β-glucosidase and alkaline phosphatase (data not shown). Ligninolytic activities (phenol oxidase and laccase) were higher in eutrophic than in mesotrophic conditions (Kruskal–Wallis,  $P < 0.001$  each). Exclusively for the eutrophic condition, phenol oxidase activity was enhanced in both TBZ and MIX treatments (pairwise,  $P < 0.001$  each) while laccase activity increased in the presence of TBZ (pairwise,  $P < 0.001$ ), but not in the MIX treatment (pairwise,  $P = 0.90$ ). As for ligninolytic activities,





**TABLE 2 |** Average percentage of nutrients and pesticides dissipation as well as adsorbed pesticides on leaves recorded at the end of the experiment in experimental stream channels exposed to fungicide (TBZ), herbicide (S-Met), both fungicide + herbicide (MIX) or non-exposed (Ctrl) in either mesotrophic or eutrophic nutrient condition.

	Mesotrophic				Eutrophic			
	Ctrl	TBZ	S-Met	MIX	Ctrl	TBZ	S-Met	MIX
P-PO <sub>4</sub>	100	100	100	100	93.80 ± 2.05	98.01 ± 1.13	88.26 ± 2.23	92.46 ± 3.24
N-NO <sub>3</sub>	100	100	100	100	64.99 ± 5.90*	57.98 ± 4.84*	63.89 ± 7.37*	74.53 ± 0.87*
TBZ	–	38.87 ± 2.88	–	45.80 ± 2.12	–	43.56 ± 2.93	–	51.19 ± 1.28
S-Met	–	–	54.68 ± 0.99	50.16 ± 1.89	–	–	56.45 ± 0.77	69.40 ± 0.39
Adsorbed TBZ (μg g of leaf DM <sup>-1</sup> )	–	1.22 ± 0.13	–	1.17 ± 0.09	–	0.68 ± 0.04*	–	0.72 ± 0.06*
Adsorbed S-Met (μg g of leaf DM <sup>-1</sup> )	–	–	1.37 ± 0.19	1.05 ± 0.10	–	–	0.64 ± 0.05*	0.50 ± 0.04*

Values are means ± standard error of the mean over the all experiment including all replicates ( $n = 15$  for pesticides and nutrients dissipation in water and  $n = 3$  for adsorbed TBZ and S-Met on leaves). Asterisks indicates significant differences between nutrient conditions for the considered treatment (Kruskal-Wallis,  $P < 0.05$ ).

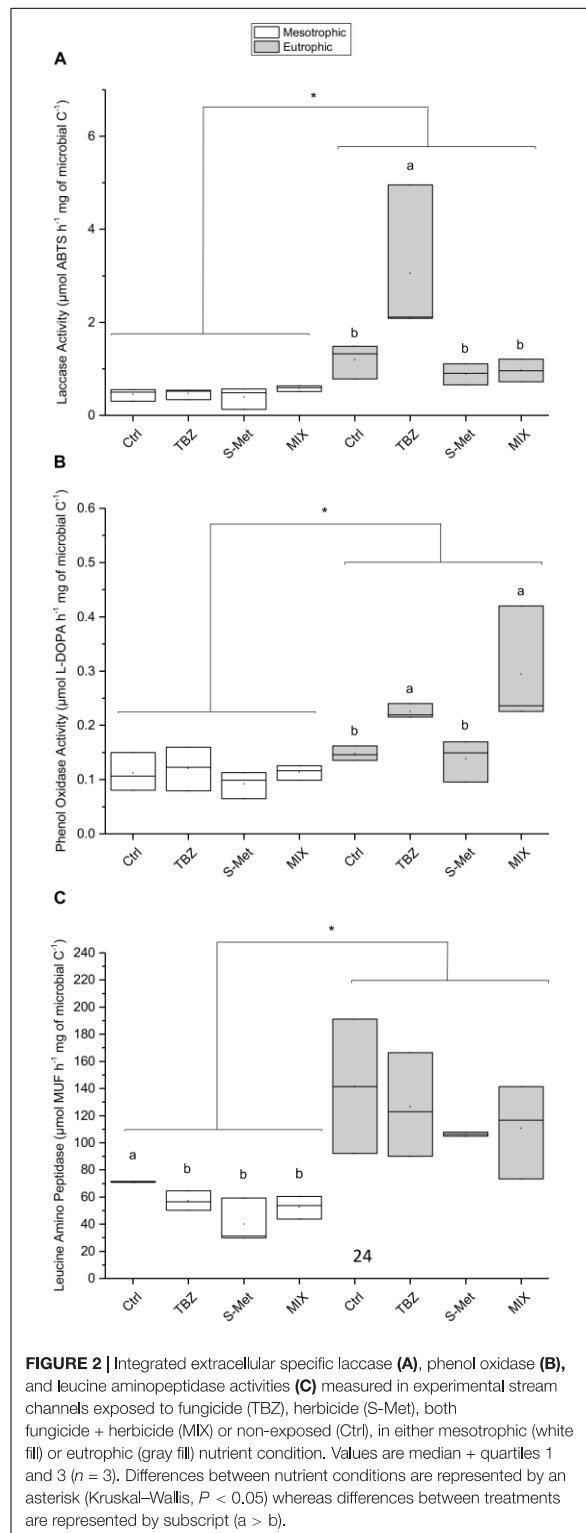
**TABLE 3 |** Alnus leaves decomposition rates ( $K$ , means ± standard error of the mean) expressed in day<sup>-1</sup> measured in experimental stream channels exposed to fungicide (TBZ), herbicide (S-Met), both fungicide + herbicide (MIX) or non-exposed (Ctrl), in either mesotrophic or eutrophic nutrient condition.

	Ctrl		+TBZ		+S-Met		+MIX	
	$K$	$R^2$	$K$	$R^2$	$K$	$R^2$	$K$	$R^2$
Eutrophic	$13.34 \times 10^{-3}$ ± $1.52 \times 10^{-3}$	0.94	<b><math>11.19 \times 10^{-3}</math></b> ± $1.23 \times 10^{-3}$	0.95	<b><math>11.39 \times 10^{-3}</math></b> ± $1.34 \times 10^{-3}$	0.94	<b><math>10.48 \times 10^{-3}</math></b> ± $1.27 \times 10^{-3}$	0.93*
Mesotrophic	$8.81 \times 10^{-3}$ ± $1.91 \times 10^{-3}$	0.81	$8.69 \times 10^{-3}$ ± $1.79 \times 10^{-3}$	0.83	$7.49 \times 10^{-3}$ ± $1.52 \times 10^{-3}$	0.83	$7.38 \times 10^{-3}$ ± $1.39 \times 10^{-3}$	0.85

Values were obtained by fitting average leaf mass loss (in %) over the experiment for each treatment to an exponential decay model and goodness of fit was represented by  $R^2$ . Bold values indicate significant differences between treatments and control within nutrient condition whereas asterisk indicates significant differences between nutrient conditions.

leucine aminopeptidase was higher in the eutrophic condition (Kruskal-Wallis,  $P < 0.001$ ) but effects of pesticide treatments were only observed in the mesotrophic condition. Under the

mesotrophic nutrient condition, lower peptidase activity was recorded in all pesticide exposed channels (including TBZ, S-Met, and MIX) compared to control (pairwise,  $P < 0.05$  each) with a



reduction that was more important in channels exposed to S-met (average reduction of 44.8% compared to control).

## Microbial Biomass and Communities' Structure

Fungal biomass colonizing *Alnus* leaves displayed differences between nutrient conditions (Kruskal–Wallis,  $P < 0.001$ ). Globally, higher fungal biomass was accumulated at the end of the experiment in the eutrophic condition compared to the mesotrophic one (A, Table 4 and Supplementary Figure S1 and Table S2), despite overall lower fungal growth rate ( $W_{max}$ ) under eutrophic conditions than in mesotrophic conditions. Stationary phase for eutrophic channels (Supplementary Figure S1) was reached around 35 days or more than 40 days for the control treatment, except for the MIX treatment which occurred much earlier (around 25 days). No differences between pesticide treatments were observed in terms of maximal biomass (A, Table 4). However, maximal growth rate in control channels displayed lower values in comparison with the pesticides exposed ones ( $W_{max}$ , pairwise,  $P = 0.001$ ). In contrast with eutrophic channels, stationary phase for mesotrophic channels (Supplementary Figure S1) was reached much earlier (around 10 days). Again, no differences were observed under those nutrient conditions between pesticide treatments for maximal biomass neither for maximal growth rates.

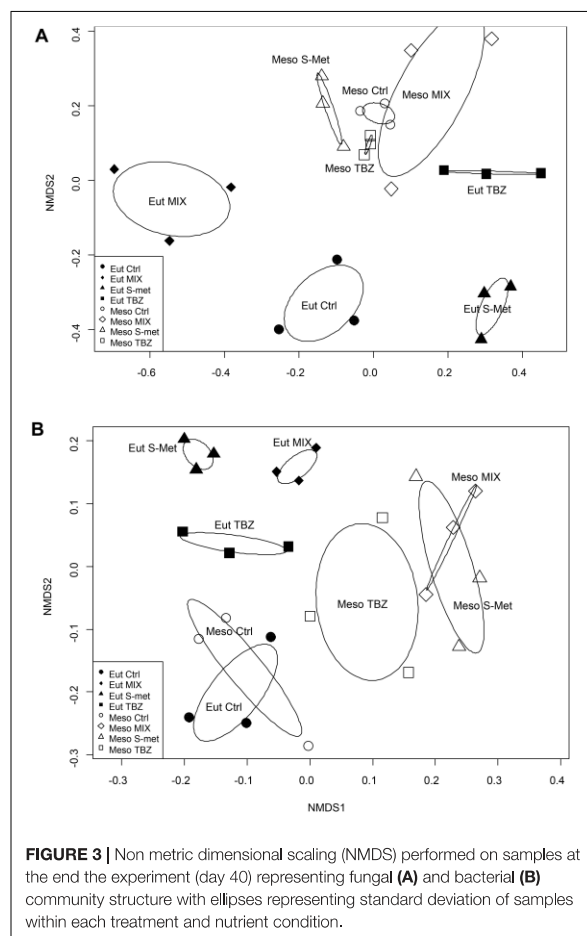
The fungal community structure appeared to mostly vary between sampling dates rather than according to nutrient and/or pesticide treatments (Supplementary Figure S3A). However, nutrient conditions also influenced the structure of these communities leading to significant differences between mesotrophic and eutrophic conditions the end of the study (PERMANOVA,  $P < 0.001$ , Figure 3A). Here, no differences were observed in terms of OTUs richness but in terms of OTUs replacement leading to changes in community structure between nutrient conditions. Differences were also observed at the end of the experiment between pesticide treatments regardless of the nutrient condition (PERMANOVA,  $P < 0.001$ ) although such differences were much more marked for the eutrophic condition (Figure 3A). Again, differences were explained by OTUs replacement rather than by shifts in OTUs richness.

Bacterial biomass was very low in comparison with that of fungi, representing less than 1% of the total microbial biomass colonizing *Alnus* leaves (Supplementary Figure S2). Similarly to fungi, bacteria displayed higher biomass values in the eutrophic condition compared to the mesotrophic one, although differences were less marked than for fungi (Kruskal–Wallis,  $P < 0.001$ ). Again, no differences were observed between pesticide treatments regardless of the nutrient condition. As for fungi, the structure of bacterial communities appeared to be mostly influenced by the sampling time (Supplementary Figure S3B). Significant differences were also observed between nutrient conditions at the end of the study (PERMANOVA,  $P < 0.001$ , Figure 3B), although these differences were less marked than those for fungi. In addition, differences were observed between pesticide treatments regardless of the nutrient condition (PERMANOVA,  $P < 0.001$ ) although still less marked in the case of the mesotrophic condition than the eutrophic one (Figure 3B). As for fungi,

**TABLE 4 |** Parameters extracted from the measured fungal biomass after fitting to a logistic growth model in experimental stream channels exposed to fungicide (TBZ), herbicide (S-Met), both fungicide + herbicide (MIX) or non-exposed (Ctrl), in either mesotrophic or eutrophic nutrient condition.

		$Y_0$ (mg of fungal C gDM <sup>-1</sup> )	$A$ (mg of fungal C gDM <sup>-1</sup> )	$W_{max}$ (Day <sup>-1</sup> )	$R^2$	$p$ -value
Mesotrophic	Ctrl	7.28 ± 0.60	18.86 ± 0.27	2.82 ± 0.38	0.99	1.08 <sup>E</sup> -6
	TBZ	7.35 ± 1.62	20.05 ± 0.74	2.49 ± 0.79	0.93	4.60 <sup>E</sup> -5
	S-Met	7.28 ± 2.25	18.65 ± 1.01	5.75 ± 13.85	0.84	1.97 <sup>E</sup> -4
	MIX	7.62 ± 1.60	20.54 ± 0.77	1.95 ± 0.64	0.93	4.35 <sup>E</sup> -5
Eutrophic	Ctrl	10.26 ± 1.71	<b>35.61 ± 3.26</b>	<b>0.89 ± 0.18</b>	0.96	5.40E-5
	TBZ	9.36 ± 2.44	<b>33.16 ± 2.26</b>	<b>1.35 ± 0.39*</b>	0.93	1.45E-4
	S-Met	9.34 ± 3.40	<b>34.58 ± 3.37</b>	<b>1.35 ± 0.52*</b>	0.89	5.18E-4
	MIX	8.88 ± 2.22	<b>30.57 ± 1.91</b>	<b>1.32 ± 0.38*</b>	0.94	1.20E-4

Values are means ± standard error of the mean returned by the origin software ( $n = 3$ ).  $Y_0$  represent the initial fungal biomass (day 0),  $A$  represent the maximal fungal biomass (day 40) and  $W_{max}$  represent maximal fungal growth rate. Values in bold represent differences between nutrient conditions (Kruskal–Wallis,  $P < 0.05$ ) and asterisk represent differences between treatments and control within each nutrient conditions (Tukey,  $P < 0.05$ ).



differences between nutrient conditions and pesticide treatments were mostly explained by OTUs replacement. However, control for both mesotrophic and eutrophic condition display lower OTUs richness compared to pesticides exposed treatments (both TBZ, S-Met and MIX).

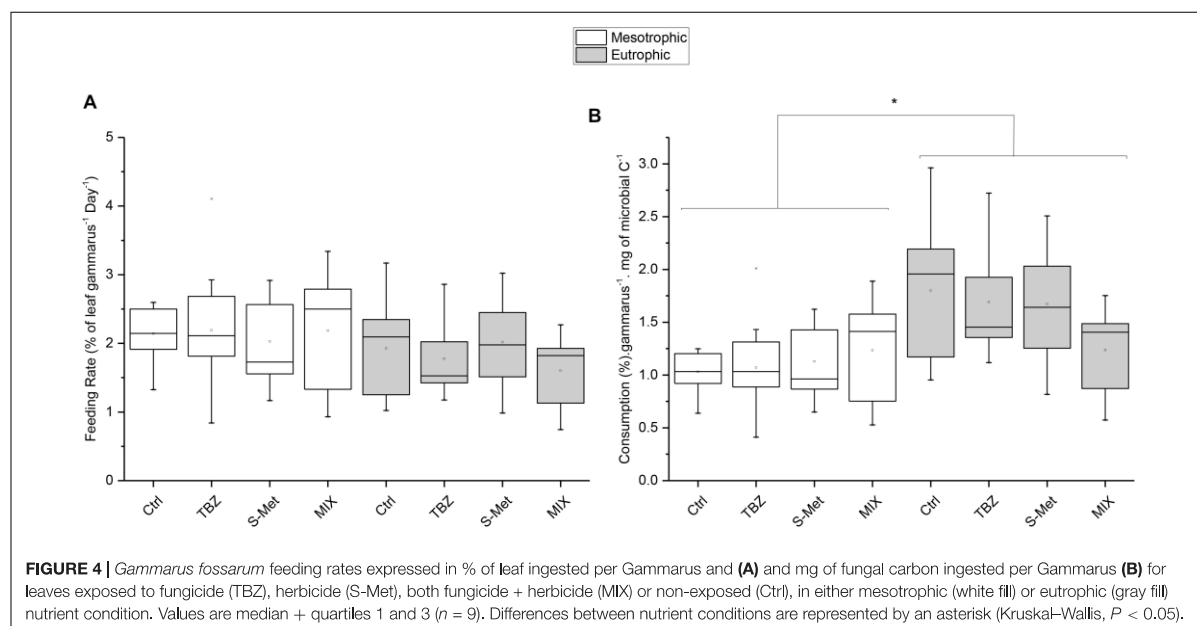
## Macroinvertebrate Feeding Activity

*Gammarus* feeding rates expressed as percentage of leaf consumption per individuals were neither affected by nutrients nor by pesticide treatments (Kruskal–Wallis,  $P = 0.13$  and  $P = 0.73$ ; respectively) (Figure 4A). Nevertheless, feeding rates corrected by mg of fungal carbon ingested per individual (Figure 4B) were significantly higher in eutrophic conditions compared to the mesotrophic ones (Kruskal–Wallis,  $P < 0.01$ ). No statistical differences were observed between pesticide treatments within each nutrient condition, although values for corrected feeding rates seemed substantially lower in the MIX treatment (TBZ + S-Met) compared to other pesticide treatments (and especially control) under eutrophic conditions (Figure 4B).

## DISCUSSION

In the present study, we assessed the effects of the interaction between nutrients (Nitrogen and Phosphorus) and pesticides (TBZ and S-Met, alone or in mixture) on the structure and activity of heterotrophic microbial communities associated with leaf litter. As already observed by Gulis and Suberkropp (2003), nutrients appeared as the main factor driving the activity and shaping the biomass and structure of the studied communities. However, significant effect of the pesticide treatments were observed, although rather minor in comparison with those of nutrients.

In mesotrophic condition, almost no effect of the pesticide treatments were observed on microbial communities, which contrast with our first hypothesis and with previous studies assessing the ecotoxicological effects of similar TBZ concentrations (Bundschuh et al., 2011; Artigas et al., 2012; Donnadieu et al., 2016). Only leucine aminopeptidase activity was slightly lower in treated channels than in control ones suggesting a potential direct but non-specific effects of pesticides' toxicity on bacteria, known as the main producer of this enzyme (Romani et al., 2006). This lack of microbial functional and structural response to TBZ might be explained by the fixed experimental conditions. Indeed, every week all dissolved nutrients were rapidly consumed by microorganisms. Hence, microbial communities may have suffered chronic nutrient



deficiency in the mesotrophic channels, limiting biomass accumulation and possibly masking pesticide effects in our study.

Significant effects of the pesticide treatments were observed in eutrophic conditions, which contrast with our second hypothesis as we expected nutrient availability to buffer the effects of pesticide toxicity in leaf-associated microbial communities (Aristi et al., 2016; Fernández et al., 2016). The slight decrease in *Alnus* decay rates exposed to TBZ (−16%), S-Met (−15%) and MIX (−22%) might suggest that nutrient concentrations (i.e.,  $\text{N-NO}_3$  and  $\text{P-PO}_4$ ) were not sufficient to compensate pesticide toxicity. Another explanation could be that co-occurrence between pesticides and high nutrients concentrations may have negative effects on microbial decomposition. In the study of Fernandes et al. (2009) assessing the interaction between a range of zinc ( $0.03 \text{ mg L}^{-1}$ ,  $0.98 \text{ mg L}^{-1}$ , and  $9.8 \text{ mg L}^{-1}$ ) and phosphate ( $0.05 \text{ mg L}^{-1}$ ,  $0.2 \text{ mg L}^{-1}$ , and  $0.5 \text{ mg L}^{-1}$ ) concentrations, strongest inhibition in *Alnus* decomposition were observed in leaves exposed to both zinc and high phosphate concentrations. Although mechanisms behind remain unclear, the author explained this by changes in fungal community structure. In our study, the changes in both fungal and bacterial community structure exposed to pesticides might explain the observed differences in terms of decomposition activity (Swan and Palmer, 2004; Gessner et al., 2010). Similarly, such changes in fungal community structure might explain the lower fungal growth rates in the control channels than in the pesticide treated ones (Zubrod et al., 2015). Since specific traits of certain species are known to have a greater influence on leaf decomposition (Duarte et al., 2006), high nutrient exposure in our study may have increased the contribution of some fungal species, which were at the same time more sensitive to pesticide exposure. However, this is a hypothesis that needs to be tested.

Overall, our results show that nutrients exacerbated the effects of pesticides on leaf decay rates, and that the previously described compensatory effect of nutrients on pesticide toxicity (Fernández et al., 2016; Gardeström et al., 2016) is probably toxicant and/or concentration dependent and does not always apply on aquatic microbial communities.

Interestingly, TBZ and S-met in the MIX treatment displayed different kind of interactions in eutrophic channels in our study. Specifically, antagonistic-type interaction was observed between those two compounds on leaf decay rates as no differences were observed between TBZ, S-Met and MIX treatments. Similarly, the fact that laccase activity was stimulated in channels exposed to TBZ but not in channels exposed to the MIX (TBZ + S-Met) in the eutrophic condition also highlight interaction between those two compounds on laccase activity. The observed effects on laccase activity suggest that perhaps (i) MIX exposure had negative effect on specific microorganisms producers of laccase or (ii) that antagonistic-type interaction occurred between TBZ and S-Met on this enzymatic activity. On the contrary, no interaction between TBZ and S-Met was observed on phenol oxidase activity. Regarding the literature, ligninolytic enzyme activity stimulation has been linked to toxicity mitigation of phenolic molecules (Baldrian, 2006; Sinsabaugh, 2010; Karas et al., 2011). Thus, the observed stimulation in TBZ treatments (for both phenol oxidase and laccase) could reflect detoxification mechanisms set up by fungi, possibly resulting in TBZ transformation in order to reduce its toxicity (Junghanns et al., 2005; Artigas et al., 2017). Whatever the hypothesis considered, our results show that the interaction between different pesticide compounds, even if they present low potential threat to non-target organisms, may impair the ability of microbial communities to display proper stress response (Dawoud et al., 2017).

The interaction between nutrient conditions and pesticide treatments (TBZ and S-Met alone) did not affect shredder feeding rates. In the context of TBZ exposure, this result contrasts with the study of Bundschuh et al. (2011) but is consistent with that of Pesce et al. (2016). These inter-study differences may be explained by differences in TBZ concentrations supplied, which were substantially higher in the study of Bundschuh et al. (2011) (50 and 500  $\mu\text{g L}^{-1}$ ) than in the study of Pesce et al. (2016) and the present one. The lack of effect of previous exposure of leaves to pesticides on feeding rates, and that whatever the tested treatment, can be explained by the lack of significant differences in term of fungal biomass between pesticide treatments, which is one of the main parameter influencing *Gammarus* feeding (Bundschuh et al., 2011; Zubrod et al., 2011). Besides, only small quantities of pesticides were found adsorbed on the exposed leaves, thus probably resulting in a low toxic effect to *Gammarus* macroinvertebrates. Overall, our results show that indirect effects of environmentally relevant concentrations of pesticides on leaf palatability for macroinvertebrates can be negligible.

## CONCLUSION

The present experiment showed that nutrients were the main parameter driving the structure and functioning of heterotrophic microbial communities colonizing *Alnus* leaf litter. However, we found that environmentally relevant concentrations of a fungicide and a herbicide, alone and/or in mixture, were sufficient to induce changes in the structure of microbial communities leading to a slight decrease in their decomposition activity, although no effects on leaf palatability for macroinvertebrates were observed. Interestingly, such effects were only visible under eutrophic conditions, suggesting antagonistic-type interaction between nutrients and xenobiotic on leaf decomposition.

## REFERENCES

- Abdel-Raheem, A. M., and Ali, E. H. (2004). Lignocellulolytic enzyme production by aquatic hyphomycetes species isolated from the Nile's delta region. *Mycopathologia* 157, 277–286. doi: 10.1023/B:MYCO.0000024178.62244.7c
- Aristi, L., Casellas, M., Elosegi, A., Insa, S., Petrovic, M., Sabater, S., et al. (2016). Nutrients versus emerging contaminants—or a dynamic match between subsidy and stress effects on stream biofilms. *Environ. Pollut.* 212, 208–215. doi: 10.1016/j.envpol.2016.01.067
- Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., et al. (2012). Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. *Aquat. Toxicol.* 122, 197–205. doi: 10.1016/j.aquatox.2012.06.011
- Artigas, J., Romani, A. M., and Sabater, S. (2008). Effect of nutrients on the sporulation and diversity of aquatic hyphomycetes on submerged substrata in a Mediterranean stream. *Aquat. Bot.* 88, 32–38. doi: 10.1016/j.aquabot.2007.08.005
- Artigas, J., Rossi, F., Gerphagnon, M., and Mallet, C. (2017). Sensitivity of laccase activity to the fungicide tebuconazole in decomposing litter. *Sci. Total Environ.* 584, 1084–1092. doi: 10.1016/j.scitotenv.2017.01.167
- Baldrian, P. (2006). Fungal laccases—occurrence and properties. *FEMS Microbiol. Rev.* 30, 215–242. doi: 10.1111/j.1574-4976.2005.00010.x
- Baldy, V., and Gessner, M. O. (1997). Towards a budget of leaf litter decomposition in a first-order woodland stream. *Décomposition des litières dans une petite*

## AUTHOR CONTRIBUTIONS

ClM, JA, and SP designed the experimental design for this study. FR, ClM, JA, and SP participated in the redaction and discussion of the article. ChM performed all pesticides measurements (in water and adsorbed with leaves) whereas MM performed all nutrients measurements (carbon, nitrogen, and phosphorus in water and adsorbed with leaves). AC took care of the palatability tests for the macroinvertebrates. All authors were involved in the critical reading of the paper, with special contribution of ClM, JA, and SP.

## ACKNOWLEDGMENTS

This study was funded by the Water Agency Loire-Bretagne through the EcoBioPest project. The authors thank Bernadette Volat, Christophe Rosy, Bernard Motte, and Anaïs Charton for their help with the experimental setup and for microbial analysis. The authors also thank Celine Guillemain, Myriam Arhror, Corinne Brosse-Quilgars, and Loïc Richard from the LAMA team in Irstea for chemical analyses as well as Hervé Quéau and Nicolas Delorme for technical assistance on feeding rate measurements. FR was funded by a Ph.D. grant from the French Ministry of Higher Education and Research.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.02437/full#supplementary-material>

rivière forestière: essai de bilan. *C. R. Acad. Sci.-Ser. III-Sci. Vie* 320, 747–758. doi: 10.1016/S0764-4469(97)84824-X

- Baldy, V., Gobert, V., Guerold, F., Chauvet, E., Lambrigt, D., and Charcosset, J.-Y. (2007). Leaf litter breakdown budgets in streams of various trophic status: effects of dissolved inorganic nutrients on microorganisms and invertebrates. *Freshw. Biol.* 52, 1322–1335. doi: 10.1111/j.1365-2427.2007.01768.x
- Baudoin, E., Lerner, A., Mirza, M. S., El Zemrany, H., Prigent-Combaret, C., Jurkevich, E., et al. (2010). Effects of *Azospirillum brasilense* with genetically modified auxin biosynthesis gene ipdC upon the diversity of the indigenous microbiota of the wheat rhizosphere. *Res. Microbiol.* 161, 219–226. doi: 10.1016/j.resmic.2010.01.005
- Berenzen, N., Lentzen-Godding, A., Probst, M., Schulz, H., Schulz, R., and Liess, M. (2005). A comparison of predicted and measured levels of runoff-related pesticide concentrations in small lowland streams on a landscape level. *Chemosphere* 58, 683–691. doi: 10.1016/j.chemosphere.2004.05.009
- Borrel, G., Colombet, J., Robin, A., Lehours, A.-C., Prangishvili, D., and Sime-Ngando, T. (2012). Unexpected and novel putative viruses in the sediments of a deep-dark permanently anoxic freshwater habitat. *ISME J.* 6, 2119–2127. doi: 10.1038/ismej.2012.49
- Bratbak, G. (1985). Bacterial biovolume and biomass estimations. *Appl. Environ. Microbiol.* 49, 1488–1493.
- Brosed, M., Lamothe, S., and Chauvet, E. (2016). Litter breakdown for ecosystem integrity assessment also applies to streams affected by pesticides. *Hydrobiologia* 773, 87–102. doi: 10.1007/s10750-016-2681-2



- Bundschuh, M., Zubrod, J. P., Kosol, S., Maltby, L., Stang, C., Duester, L., et al. (2011). Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. *Aquat. Toxicol.* 104, 32–37. doi: 10.1016/j.aquatox.2011.03.010
- Chandrashekar, K. R., and Kaveriappa, K. M. (1991). Production of extracellular cellulase by *Lunulospora curvula* and *Flagellospora penicillioidea*. *Folia Microbiol.* 36, 249–255. doi: 10.1007/BF02814357
- Copping, L. G., and Hewitt, H. G. (2007). *Chemistry and Mode of Action of Crop Protection Agents*. London: Royal Society of Chemistry.
- Coulaud, R., Geffard, O., Xuereb, B., Lacaze, E., Quéau, H., Garric, J., et al. (2011). *In situ* feeding assay with *Gammarus fossarum* (Crustacea): modelling the influence of confounding factors to improve water quality biomonitoring. *Water Res.* 45, 6417–6429. doi: 10.1016/j.watres.2011.09.035
- Cummins, K. W. (1974). Structure and function of stream ecosystems. *BioScience* 24, 631–641. doi: 10.2307/1296676
- Dawoud, M., Bundschuh, M., Goedkoop, W., and McKie, B. G. (2017). Interactive effects of an insecticide and a fungicide on different organism groups and ecosystem functioning in a stream detrital food web. *Aquat. Toxicol.* 186, 215–221. doi: 10.1016/j.aquatox.2017.03.008
- Donnadieu, F., Besse-Hoggan, P., Forestier, C., and Artigas, J. (2016). Influence of streambed substratum composition on stream microbial communities exposed to the fungicide tebuconazole. *Freshw. Biol.* 61, 2026–2036. doi: 10.1111/fwb.12679
- Duarte, S., Pascoal, C., Cássio, F., and Bärlocher, F. (2006). Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. *Oecologia* 147, 658–666. doi: 10.1007/s00442-005-0300-4
- Fernandes, I., Duarte, S., Cássio, F., and Pascoal, C. (2009). Mixtures of zinc and phosphate affect leaf litter decomposition by aquatic fungi in streams. *Sci. Total Environ.* 407, 4283–4288. doi: 10.1016/j.scitotenv.2009.04.007
- Fernández, D., Tummala, M., Schreiner, V. C., Duarte, S., Pascoal, C., Winkelmann, C., et al. (2016). Does nutrient enrichment compensate fungicide effects on litter decomposition and decomposer communities in streams? *Aquat. Toxicol.* 174, 169–178. doi: 10.1016/j.aquatox.2016.02.019
- Flores, L., Banjac, Z., Farré, M., Larrañaga, A., Mas-Martí, E., Muñoz, I., et al. (2014). Effects of a fungicide (imazalil) and an insecticide (diazinon) on stream fungi and invertebrates associated with litter breakdown. *Sci. Total Environ.* 47, 532–541. doi: 10.1016/j.scitotenv.2014.01.059
- Gardeström, J., Ermold, M., Goedkoop, W., and McKie, B. G. (2016). Disturbance history influences stressor impacts: effects of a fungicide and nutrients on microbial diversity and litter decomposition. *Freshw. Biol.* 61, 2171–2184. doi: 10.1111/fwb.12698
- Gessner, M. O., and Chauvet, E. (1993). Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. *Appl. Environ. Microbiol.* 59, 502–507.
- Gessner, M. O., and Chauvet, E. (1994). Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology* 75, 1807–1817. doi: 10.2307/1939639
- Gessner, M. O., and Schmitt, A. L. (1996). Use of solid-phase extraction to determine ergosterol concentrations in plant tissue colonized by fungi. *Appl. Environ. Microbiol.* 62, 415–419.
- Gessner, M. O., Swan, C. M., Dang, C. K., McKie, B. G., Bardgett, R. D., Wall, D. H., et al. (2010). Diversity meets decomposition. *Trends Ecol. Evol.* 25, 372–380. doi: 10.1016/j.tree.2010.01.010
- Götz, T., and Böger, P. (2004). The very-long-chain fatty acid synthase is inhibited by chloroacetamides. *Z. Naturforsch. C* 59, 549–553. doi: 10.1515/znc-2004-7-818
- Gulis, V., and Suberkropp, K. (2003). Effect of inorganic nutrients on relative contributions of fungi and bacteria to carbon flow from submerged decomposing leaf litter. *Microb. Ecol.* 45, 11–19. doi: 10.1007/s00248-002-1032-1
- Johannes, C., and Majcherczyk, A. (2000). Laccase activity tests and laccase inhibitors. *J. Biotechnol.* 78, 193–199. doi: 10.1016/S0168-1656(00)00208-X
- Junghanns, C., Moeder, M., Krauss, G., Martin, C., and Schlosser, D. (2005). Degradation of the xenoestrogen nonylphenol by aquatic fungi and their laccases. *Microbiology* 151, 45–57. doi: 10.1099/mic.0.27431-0
- Kalkhoff, S. J., Vecchia, A. V., Capel, P. D., and Meyer, M. T. (2012). Eleven-year trend in acetanilide pesticide degradates in the Iowa River. *Iowa. J. Environ. Qual.* 41, 1566–1579. doi: 10.2134/jeq2011.0426
- Karas, P. A., Perruchon, C., Exarhou, K., Ehaliot, C., and Karpouzias, D. G. (2011). Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. *Biodegradation* 22, 215–228. doi: 10.1007/s10532-010-9389-1
- MacNeil, C., Dick, J. T., and Elwood, R. W. (1997). The trophic ecology of freshwater *Gammarus* spp. (Crustacea: Amphipoda): problems and perspectives concerning the functional feeding group concept. *Biol. Rev.* 72, 349–364. doi: 10.1017/S0006323196005038
- Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Simpson, G. L., Solymos, P., et al. (2008). *The Vegan Package. Community Ecology Package-Forge R-Project Org Projects Vegan*. Available at: <http://r-forge.r-project.org/projects/vegan/>
- Paul, S., Gable, K., Beaudoin, F., Cahoon, E., Jaworski, J., Napier, J. A., et al. (2006). Members of the *Arabidopsis* FAE1-like 3-ketoacyl-CoA synthase gene family substitute for the elop proteins of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 281, 9018–9029. doi: 10.1074/jbc.M50772.2000
- Payá, P., Anastassiades, M., Mack, D., Sigalova, I., Tasdelen, B., Oliva, J., et al. (2007). Analysis of pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) pesticide multiresidue method in combination with gas and liquid chromatography and tandem mass spectrometric detection. *Anal. Bioanal. Chem.* 389, 1697–1714. doi: 10.1007/s00216-007-1610-7
- Pesce, S., Fajon, C., Bardot, C., Bonnemoy, F., Portelli, C., and Bohatier, J. (2008). Longitudinal changes in microbial planktonic communities of a French river in relation to pesticide and nutrient inputs. *Aquat. Toxicol.* 86, 352–360. doi: 10.1016/j.aquatox.2007.11.016
- Pesce, S., Zoghalmi, O., Margoum, C., Artigas, J., Chaumot, A., and Foulquier, A. (2016). Combined effects of drought and the fungicide tebuconazole on aquatic leaf litter decomposition. *Aquat. Toxicol.* 173, 120–131. doi: 10.1016/j.aquatox.2016.01.012
- Petersen, R. C., and Cummins, K. W. (1974). Leaf processing in a woodland stream. *Freshw. Biol.* 4, 343–368. doi: 10.1111/j.1365-2427.1974.tb00103.x
- Richardson, S. D. (2009). Water analysis: emerging contaminants and current issues. *Anal. Chem.* 81, 4645–4677. doi: 10.1021/ac9008012
- Romaní, A. M., Fischer, H., Mille-Lindblom, C., and Tranvik, L. J. (2006). Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. *Ecology* 87, 2559–2569. doi: 10.1890/0012-9658(2006)87[2559:IOBAFO]2.0.CO;2
- Roubeix, V., Mazzella, N., Méchin, B., Coste, M., and Delmas, F. (2011). Impact of the herbicide metolachlor on river periphytic diatoms: experimental comparison of descriptors at different biological organization levels. *Ann. Limnol. Int. J. Limnol.* 47, 239–249. doi: 10.1051/limn/2011009
- Segner, H., Schmitt-Jansen, M., and Sabater, S. (2014). Assessing the impact of multiple stressors on aquatic biota: the receptor's side matters. *Environ. Sci. Technol.* 48, 7690–7696. doi: 10.1021/es405082t
- Sinsabaugh, R. L. (2010). Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biol. Biochem.* 42, 391–404. doi: 10.1016/j.soilbio.2009.10.014
- Sinsabaugh, R. L., Osgood, M. P., and Findlay, S. (1994). Enzymatic models for estimating decomposition rates of particulate detritus. *J. North Am. Benthol. Soc.* 13, 160–169. doi: 10.2307/1467235
- Suberkropp, K., Arsuffi, T. L., and Anderson, J. P. (1983). Comparison of degradative ability, enzymatic activity, and palatability of aquatic hyphomycetes grown on leaf litter. *Appl. Environ. Microbiol.* 46, 237–244.
- Swan, C. M., and Palmer, M. A. (2004). Leaf diversity alters litter breakdown in a Piedmont STREAM. *J. North Am. Benthol. Soc.* 23, 15–28. doi: 10.1899/0887-3593(2004)023<0015:LDLBI>2.0.CO;2
- Trewavas, A. (2002). Malthus foiled again and again. *Nature* 418:668. doi: 10.1038/nature01013
- Vörösmarty, C. J., McIntyre, P. B., Gessner, M. O., Dudgeon, D., Prusevich, A., Green, P., et al. (2010). Global threats to human water security and river biodiversity. *Nature* 467, 555–561. doi: 10.1038/nature09440
- Webster, J. R., and Meyer, J. L. (1997). Organic matter budgets for streams: a synthesis. *J. North Am. Benthol. Soc.* 16, 141–161. doi: 10.2307/1468247

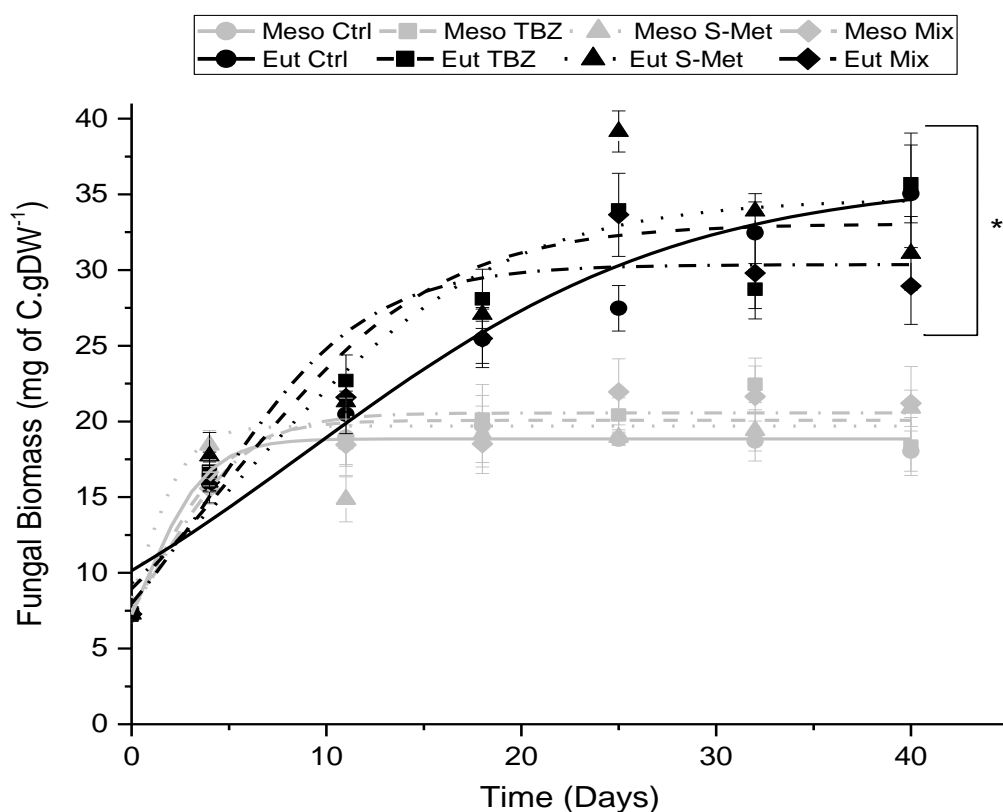
- Yang, C., Hamel, C., Vujanovic, V., and Gan, Y. (2011). Fungicide: modes of action and possible impact on nontarget microorganisms. *ISRN Ecol.* 2011:130289. doi: 10.5402/2011/130289
- Zubrod, J. P., Bundschuh, M., Feckler, A., Englert, D., and Schulz, R. (2011). Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. *Environ. Toxicol. Chem.* 30, 2718–2724. doi: 10.1002/etc.679
- Zubrod, J. P., Englert, D., Feckler, A., Koksharova, N., Korschak, M., Bundschuh, R., et al. (2015). Does the current fungicide risk assessment provide sufficient protection for key drivers in aquatic ecosystem functioning? *Environ. Sci. Technol.* 49, 1173–1181. doi: 10.1021/es5050453

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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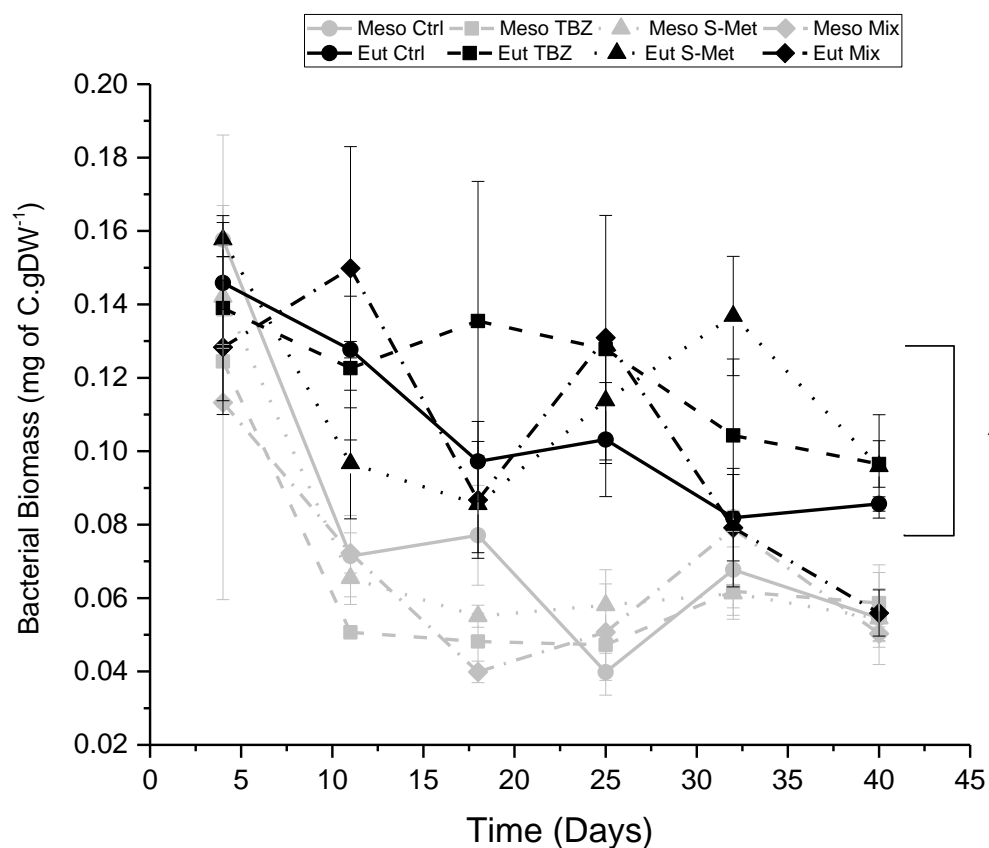
#### 4. Chapter 2: Supplementary Material

**Figure 1 SI:** Fungal biomass expressed in mg of carbon per g of leaf DM in experimental stream channels exposed to fungicide (TBZ), herbicide (S-Met), both fungicide + herbicide (MIX) or non-exposed (Ctrl), in either mesotrophic (grey) or eutrophic (black) nutrient condition. Lines represents fitting to a logistic growth model whose parameter can be found in table 4. Values are mean + standard error of the mean ( $n = 3$ ) and lines represents fitting to a logistic growth model (see material and methods). Asterisk represent differences between nutrient conditions (Tukey,  $P < 0.05$ ).

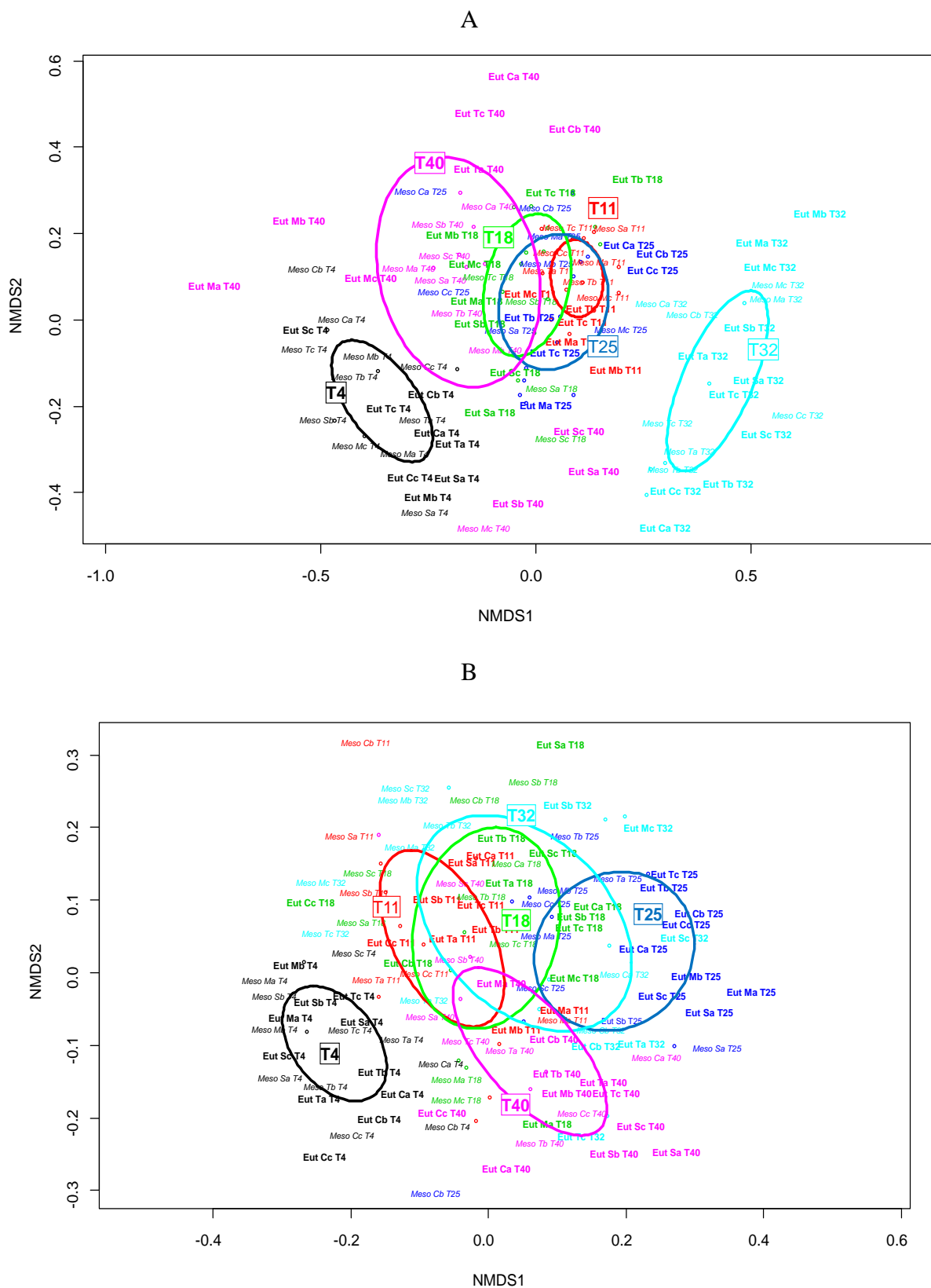




**Figure 2 SI:** Bacterial biomass expressed in mg of carbon per g of leaf DM in experimental stream channels exposed to fungicide (TBZ), herbicide (S-Met), both fungicide + herbicide (MIX) or non-exposed (Ctrl), in either mesotrophic (grey) or eutrophic (black) nutrient condition. Values are mean + standard error of the mean (n = 3). Asterisk represent differences between nutrient conditions (Tukey,  $P < 0.05$ ).

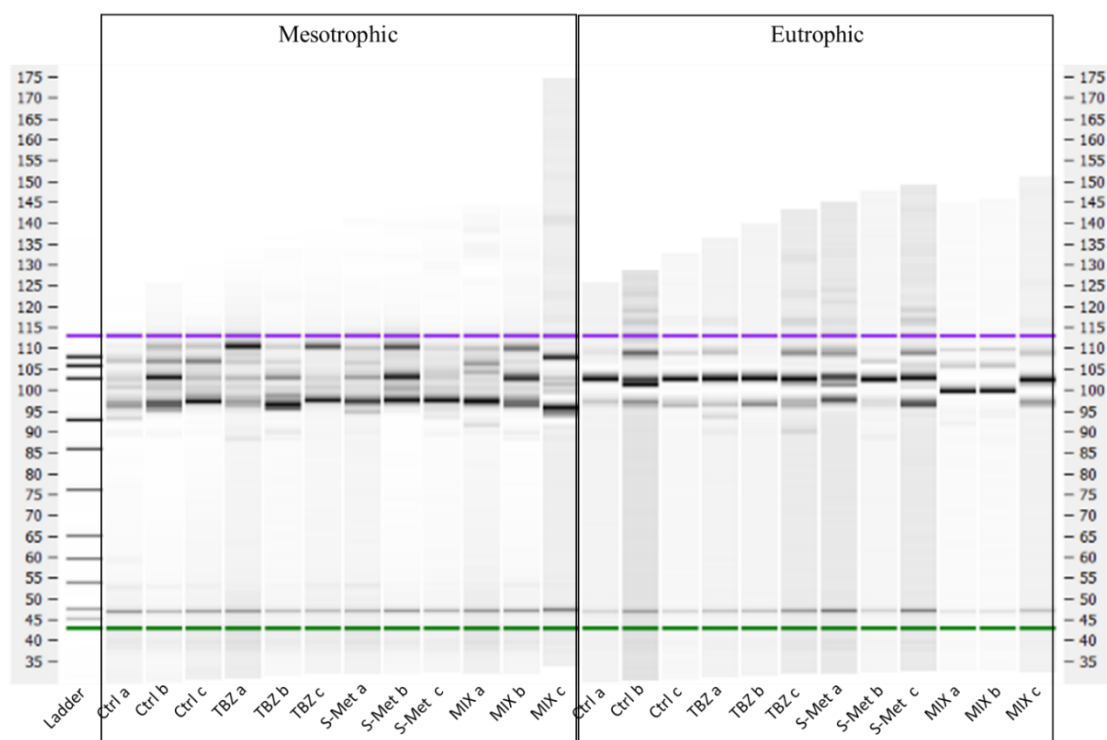


**Figure 3 SI:** Non metric dimensional scaling (NMDS) performed on all samples including all sampling times and representing fungal (A) and bacterial (B) community structure with ellipses representing standard deviation of samples for the time. Samples from eutrophic channels are represented in bold, whereas samples from mesotrophic ones are represented in italic.

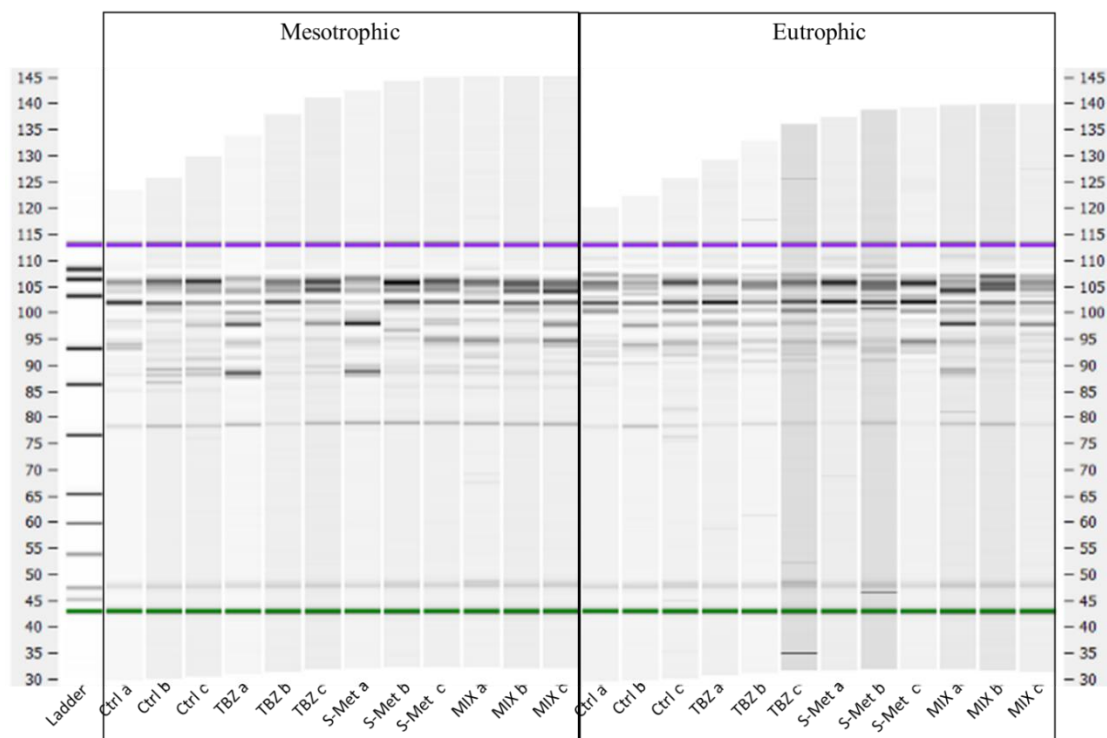


**Figure 4 SI:** ARISA (Automated Ribosomal Intergenic Spacer Analysis) profiles obtained for fungal (A) and bacterial (B) communities at T40.

A



B



**Table 1 SI:** Average remaining dry mass of *Alnus* leaves (in %) used for the fitting procedure and measured in experimental stream channels exposed to fungicide (TBZ), herbicide (S-Met), both fungicide + herbicide (MIX) or non-exposed (Ctrl), in either mesotrophic or eutrophic nutrient condition.

Time (days)	Mesotrophic				Eutrophic			
	Ctrl	TBZ	S-Met	MIX	Ctrl	TBZ	S-Met	MIX
0	100	100	100	100	100	100	100	100
4	84.00905	83.76125	85.58392	86.48583	87.36658	85.9886	87.17876	90.27008
11	78.97287	78.93959	83.40579	82.04049	84.66865	84.31554	82.53983	83.81644
18	75.55303	80.46878	78.44845	81.58783	80.3215	77.85278	77.74488	80.33156
25	76.16988	74.35288	77.15282	77.68286	71.80628	70.02546	67.56479	75.76603
32	73.54492	69.12353	76.57678	74.70157	66.59252	67.92357	68.66945	74.26985
40	64.02093	67.80269	69.42975	70.97451	52.35511	61.94293	62.05282	60.02563

**Table 2 SI:** Average fungal biomass colonizing *Alnus* leaves (in mg of fungal C per gDM) used for the fitting procedure and measured in experimental stream channels exposed to fungicide (TBZ), herbicide (S-Met), both fungicide + herbicide (MIX) or non-exposed (Ctrl), in either mesotrophic or eutrophic nutrient condition.

Time (days)	Mesotrophic				Eutrophic			
	Ctrl	TBZ	S-Met	MIX	Ctrl	TBZ	S-Met	MIX
0	7.28184	7.28184	7.28184	7.28184	7.28184	7.28184	7.28184	7.28184
4	16.44622	16.38867	18.44028	15.58348	15.93228	16.7107	17.72412	15.97419
11	18.97615	18.59193	14.85667	18.46485	20.47482	22.69966	21.29056	21.59514
18	19.71642	20.15524	19.1345	18.51965	25.43287	28.09981	27.07358	25.487
25	18.82591	20.42543	18.95344	21.95523	27.47612	33.96279	39.15522	33.64912
32	18.70557	22.45179	19.40803	21.64307	32.46375	28.73702	33.89941	29.80131
40	18.03808	18.35064	20.87283	21.19883	35.04788	35.6956	31.10662	28.94499

### Main conclusions of the study

In contrast with our first hypothesis, results showed that nutrients exacerbated the effects of pesticides on leaf decay rates. We thus suggest that the compensatory effect of nutrients on pesticide toxicity is probably concentration dependent and does not always apply on aquatic microbial communities.

Our results highlight antagonism between the S-metolachlor and the teboconazole on laccase activity in eutrophic conditions. Given the potential involvement of this activity in xenobiotic stress defense, this might suggest that the interaction between different pesticide compounds may impair the ability of microbial communities to display proper stress response.

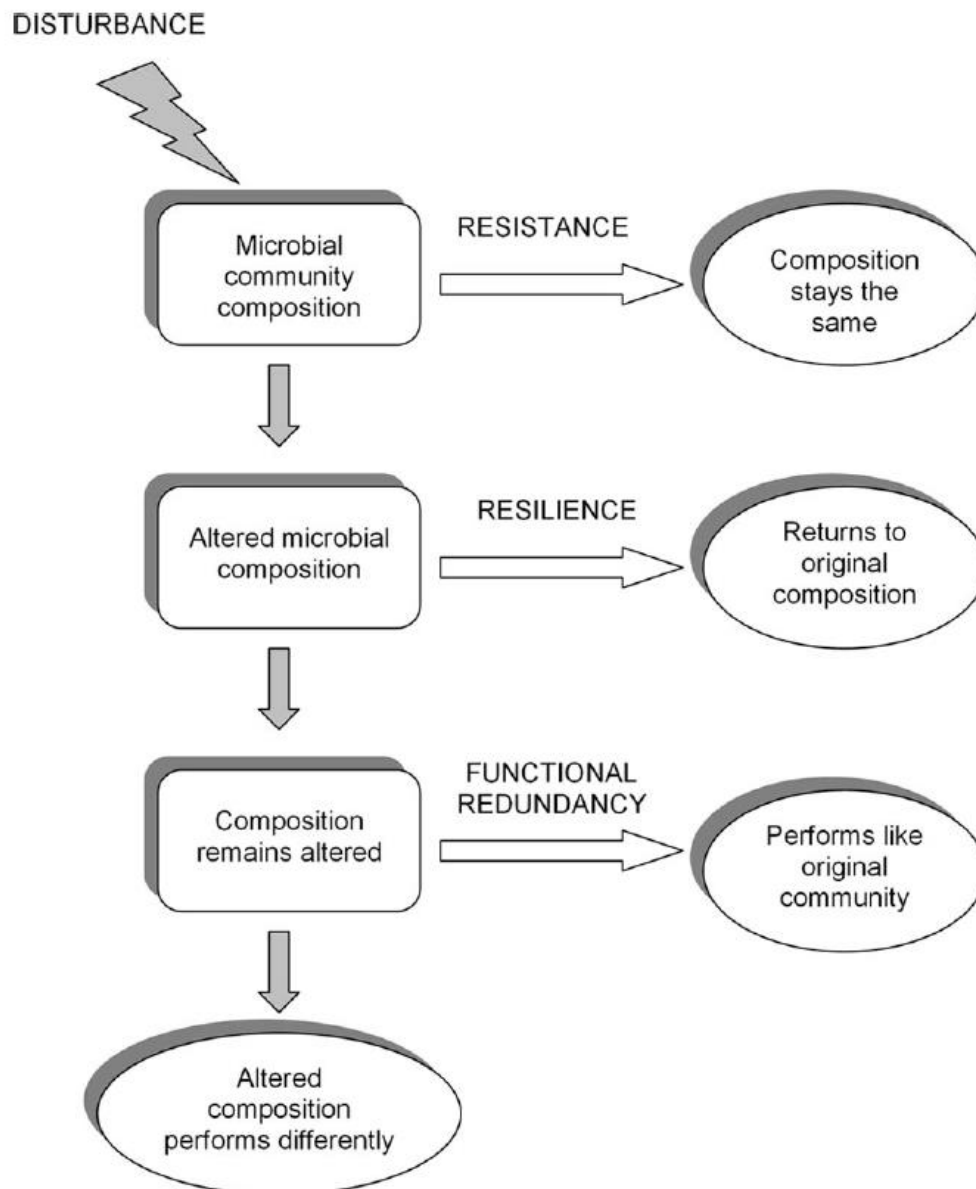
However, no effect on macroinvertebrate feeding activity was observed suggesting that environmentally realistic pesticide concentrations have negligible effects on leaf palatability for macroinvertebrates.

## **Chapter 3: Structural and functional responses of leaf-associated fungal communities to chemical pollution in streams**



After 18 years of the launch of the Water Framework Directive, all the European countries adhered to reach the good ecological and chemical status of their surface and ground water bodies in 2015. Although the deadline has been delayed to 2027 and although this objective appears far from being achieved, one could imagine that stream and river contamination will progressively decrease in the next decades. As observed in the previous studies (Chapters 1 and 2), pesticide contamination does not necessarily affect microbial decomposition activity, at least in a detectable manner, but impair biomass and structure of these communities. One question that remains unanswered is whether these communities will be able to recover from chronic xenobiotic exposure or not.

The different mechanisms induced by disturbances on the structure of microbial communities are perfectly illustrated by (Allison & Martiny, 2008) (Figure 17). According to this figure, resistance is when community structure remains unchanged (or almost) after stress exposure. Factors that contribute to the resistance of microbes include biofilm permeability, species plasticity, detoxification mechanisms, dormance... (Evans & Hofmann, 2012; Lear, 2016). If a community contains sensitive species, these species are likely to be affected by the stress eventually leading to community structure alterations such as the loss or the reduction in the abundance of some species. Resilience is defined as the ability of the community to return to its original structure. Factors influencing resilience includes the fast growth rate of microbes, allowing them to quickly recover, physiological flexibility or evolution through mutations and migration (Tobor-Kaplon *et al.*, 2005). However, the ability of microbial communities to recover from a stress appears highly dependent on the intensity and frequency of the disturbance (Tlili *et al.*, 2016). In this chapter we thus aim to assess the resistance and recovery of leaf-associated fungal communities exposed to a realistic chemical contamination from the Auzon River (central France) using laboratory microcosms.



**Figure 17:** A schematic of how disturbance can change microbial composition and thereby affect ecosystem processes versus when disturbance would not have this effect (when the microbial community is resistant, resilient, or functionally redundant). (Figure from Allison & Martiny, (2008))



# Structural and functional responses of leaf-associated fungal communities to chemical pollution in streams

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## Funding information

Fédération des Recherches en Environnement; French Ministry of Higher Education and Research

## Abstract

1. The decomposition of allochthonous plant litter is a key ecological process in forested streams, involving the lignolytic activities of aquatic fungi. In this study we investigated the relationship between water pollution (by a mixture of nutrients, metals and pesticides) and the biomass, structure and enzyme activities of leaf-associated fungal communities.
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 agricultural basin of the Auzon stream (Puy-de-Dôme region, Centre France). The resistance as well as the ability of these fungal communities to recover from pollution was also assessed through a translocation experiment.
3. Results showed a lower laccase activity and higher band richness in fungal communities from the downstream site comparing to the upstream site, which can partially be explained by the greater pesticide toxicity and concentration of dissolved organic carbon of downstream waters.
4. Specifically, a negative relationship between laccase activity and nitrate concentration was observed, irrespectively of the studied site, whereas the relationship between nitrate and phenol oxidase/peroxidase appeared much weaker.
5. The translocation experiment evidenced (1) a fast decrease in laccase activity concomitant with a shift in fungal structure after stress exposure, and (2) a recovery ability in terms of laccase activity and fungal community structure 2 weeks after stress removal.
6. This study underlines the sensitivity of leaf-associated fungal communities in 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 use of laccase as an indicator of chemical pollution of streams.

## KEYWORDS

aquatic fungi, chemical stress, ligninolytic enzymes, translocation

## 1 | INTRODUCTION

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 fuelled

with allochthonous organic matter coming from the riparian forest (Mninsall, 1967). These organic matter inputs, mostly in the form of leaf litter, can represent up to 90% of the total organic matter in streams (Cummins, 1974; Mninsall, 1967; Triska, 1970; Wallace & Webster, 1996). Consequently, the decomposition of leaf litter

becomes a central process in these ecosystems since it (1) intervenes in various biogeochemical cycles (C, P, N and S; Webster & Meyer, 1997), (2) provides mineral nutrients to the entire stream food web (Gessner & Chauvet, 1994), and (3) returns CO<sub>2</sub> to the atmosphere (Gholz, Wedin, Smitherman, Harmon, & Parton, 2000). Leaf litter decomposition process in streams is performed by the action of both physical (abrasion, leaching and/or fragmentation) and biological factors; the latter including the action of microorganisms and macroinvertebrates (Gaudes, Artigas, Romani, Sabater, & Muñoz, 2009; Gessner, Chauvet, & Dobson, 1999; Mann, 1975). While macroinvertebrates shredder taxa account for the major portion of the leaf mass loss in streams (Suberkropp & Klug, 1976), it has been estimated that microorganisms (including fungi and bacteria) can contribute up to 25%–45% of the leaf mass loss, even if these estimations vary depending on leaf species and sites studied (Baldy et al., 2007; Hieber & Gessner, 2002). Although the contribution of each microbial group on leaf litter decomposition remains hard to evaluate, especially because of the difficulty to separate them from the leaf matrix, it is widely accepted that microbial decomposition is mostly attributed to the action of aquatic fungi (Canhoto, Gonçalves, & Bärlocher, 2016; Krauss et al., 2011). Indeed, aquatic fungi (mostly the group of aquatic hyphomycetes) represent the major microbial biomass colonising the leaf substratum (>95% of the total microbial biomass; Hieber & Gessner, 2002), and possess a large panel of extracellular hydrolytic and oxidative enzymes such as cellulases, hemicellulases and/or laccases (Abdel-Raheem & Ali, 2004; Abdullah et al., 1989; Chandrashekar & Kaveriappa, 1991) capable to breakdown leaf structural compounds (e.g. cellulose, pectin, lignin).

Chemical pollution of streams by heavy metals, nutrients and xenobiotics can alter aquatic fungal communities and their decomposition abilities (Artigas et al., 2012), and by extension the entire ecosystem functioning (Duarte, Pascoal, Alves, Correia, & Cássio, 2008; Maltby, Brock, & van den Brink, 2009). Amongst those pollutions, the effects of nutrients on aquatic fungal communities 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, and activity (Artigas, Romani, & Sabater, 2004; Hagen, Webster, & Benfield, 2006; Magbanua, Townsend, Blackwell, Phillips, & Matthaei, 2010), some studies also observed a negative effect of the nutrients, depending on the concentration and nature of the amendments (Baldy, Chauvet, Charcosset, & Gessner, 2002; Duarte, Pascoal, Garabétian, Cássio, & Charcosset, 2009b). For 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 (Baldy et al., 2002; Duarte, Pascoal, Garabétian, et al., 2009b). Effects of metals (cadmium, copper, and zinc) are commonly associated with reductions in fungal species richness, biomass, sporulation rates, and leaf decay rates (Duarte, Pascoal, & Cássio, 2009a; Krauss et al., 2001; Zubrod et al., 2015). Recently, field experiments have attempted to assess the effects of pesticides on microbial litter decomposition in agricultural streams (Fernández, Voss, Bundschuh, Zubrod, & Schäfer, 2015; Krauss et al., 2003;

Rasmussen, Wiberg-Larsen, Baattrup-Pedersen, Monberg, & Kronvang, 2012). In most of the cases, the exposure to pesticides lead to decline in 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 of action would determine its potential impact on target and non-target leaf microorganisms (Maltby et al., 2009). This is the case of fungicides, being more toxic to aquatic fungi than other pesticides, due to their direct action on fungal physiology (Artigas et al., 2012; Fernández et al., 2015; Zubrod et al., 2015) but also indirectly affecting bacteria co-existing with fungi (Artigas et al. 2014). Up to now, however, the effects of multi-stressors, including pesticides and environmental stressors (e.g. temperature, droughts, nutrients...) in leaf-associated fungal communities are weakly studied in the literature (Fernández et al., 2015). Besides, Piscart et al. (2011) did not find any correlation between pesticide pollution and microbial decay rates of leaves in a stream draining vineyard area in France. The absence of correlations was partially explained by the low temperatures recorded in their experiment which may have masked pesticides impact on microbial litter decomposition.

Translocation experiments are suitable approaches to assess such multi-stress impacts on stream microbial communities (including aquatic fungal communities). Indeed, this approach, which consist on transferring biological communities from polluted sites to pristine sites and vice versa (i.e. Ivorra et al., 1999; Proia et al., 2013), permits to identify resistance/resilience mechanisms set up by microbial communities to cope stress (Duarte, Pascoal, & Cássio, 2009a; Sridhar, Bärlocher, Krauss, & Krauss, 2005). At present, various studies have performed translocation experiments on periphytic biofilms (mostly targeting diatom communities dynamics; Ivorra et al., 1999; Morin, Lambert, Artigas, Coquery, & Pesce, 2012). To the best of our knowledge, two studies have performed translocation experiments to assess metal pollution on leaf-associated fungal communities (Duarte, Pascoal, & Cássio, 2009a; Sridhar et al., 2005) but none regarding the overall influence of nutrients, metals and pesticides. Studies on metals showed that copper, zinc, and manganese exposure stopped litter decomposition and declined sporulation rates. However, these studies also showed that fungal communities were able to recover when stress by metals was removed after translocation from a polluted site to a cleaner one.

This study investigates the sensitivity of leaf-associated fungal communities to stream water pollution (by a mixture of nutrients, metals, and pesticides) by comparing communities from upstream (less polluted) and downstream (more polluted) sites of an agricultural stream (the Auzon, Puy-de-Dôme region, Central France). Our main objective is to assess the resistance and recovery abilities of leaf-associated fungal communities to water pollution using a translocation experiment in laboratory microcosms. As base hypothesis we presume that stream water pollution observed downstream (higher nutrients, metals, and pesticides concentrations) would promote shifts in the structure and activity of leaf-associated fungal communities comparing to communities from the upstream site (less polluted). As second hypothesis, we expected that chemical stress

exposure due to 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 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 to upstream.

## 2 | MATERIALS AND METHODS

### 2.1 | 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 6,074 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 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 variations throughout the watershed lead to an upstream section mostly occupied by forests and prairies and a downstream section devoted to agriculture (38% of the total watershed surface area, mainly cereal crops) and some dispersed urbanizations (10%). According to the 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 reaching concentration peaks of aminomethylphosphonic acid (AMPA), glyphosate, and S-metolachlor above 1 µg/L (Phyt'eauvergne 2014).

Fungal communities were obtained from the upstream and downstream sites of the Auzon in October 2014 using *Alnus glutinosa* (*Alnus*) leaf species as substratum. Freshly fallen leaves were harvested from the upstream site, dried 72 hr at room temperature, and placed in a total of six bags (1 mm of mesh size), each containing 10 g of the pre-dried leaves. 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 3 weeks of colonisation in-stream, litter bags were retrieved and transported to the laboratory where leaves were 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 100 ml of filtered (0.2 µm) stream water from each respective site (Gessner & Chauvet, 1993). Fungal mycelia growth and sporulation was achieved by incubations at 19°C under agitation at 180 rpm (Artigas, Romani, & Sabater, 2008). After 48 hr, water suspension and leaf disks that were crushed and well mixed, were transferred into sterilised flasks and used as inoculum for the subsequent microcosm experiment.

### 2.2 | Microcosm experiment and sampling

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

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 randomly chosen leaf packs from (1) upstream to downstream channels (Ups → Dws) and (2) downstream to upstream channels (Dws → Ups). One week after (7 days, week 3) the first translocated samples were sampled. Translocated samples were used to assess the impact (resistance) and the recovery (resilience) of leaf-associated fungal communities to stream water pollution. Fungal communities having remained permanently in upstream and 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 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 enzyme activities (laccase, peroxidase and phenol oxidase), leaf dry weight, and lignin content. While extracellular enzyme activity measurements were performed the same sampling date, samples for fungal community structure, ergosterol concentration and lignin content were stored frozen at  $-20^\circ\text{C}$  until analyses. Additionally, water samples from each channel were taken at each sampling time to determine nutrients (nitrate, phosphate, and dissolved carbon), multi-residues (including pesticides) and metals (cadmium, copper, manganese, zinc) concentrations.

### 2.3 | Water physical and chemical parameters

In each stream channel, water temperature, dissolved oxygen, conductivity and pH were measured at each sampling time using

portable probes (ProODO YSI, Cond340i WTW, pHMeter CG818, respectively). Soluble reactive phosphorus (Murphy & Riley, 1962) and nitrate concentrations (Nitrat-Test kit, Merck, Darmstadt, Germany) in water were measured spectrophotometrically. Total dissolved carbon (TDC), dissolved organic carbon (DOC) and inorganic carbon (DIC) as well as nitrogen concentrations were measured using a total organic carbon analyzer (TOC-<sub>VCPN</sub>, Shimadzu, Kyoto, Japan). In parallel, water samples 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 pesticides in water samples was estimated according to the toxic units (TU) concept (Sprague, 1971). TU for each pesticide and metabolite compound was calculated according to the following equation:

$$TU = \frac{C_i}{EC_{50i}}$$

where  $C_i$  is the actual concentration of the pesticide in the water sample and  $EC_{50i}$  is the median acute effect of this pesticide inhibiting 50% of the growth rate of standard aquatic microbial species. Predicted toxicity of the pesticide cocktail, noted as  $\Sigma TUs$ , was also 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 aquatic fungi are not available in the literature (Maltby et al., 2009),  $EC_{50}$  values of microalgae (*Scenedemus subspicatus*, *Scenedesmus abundans*, *Pseudokirchneriella subcapitata* and *Chlamydomonas angulosa*) were used as surrogates (see Rasmussen et al., 2012).

## 2.4 | Biomass and structure of fungal communities

Ergosterol concentration determination was used as proxy for fungal biomass in leaves (Gessner & Schmitt, 1996). Lipids were extracted from leaves (fifteen leaf disks per sample) after incubation in 0.14 M KOH methanol at 80°C during 30 min. Extracts were purified and concentrated using solid-phase extraction (tc18 cartridges, Sep-Pak Vac RC, 500 mg, Waters) and then analysed by a high pressure liquid chromatography system (Lachrom L-7400, Merck-Hitachi, Tokyo, Japan) equipped with an ODS-2 Hypersil column (250 × 4.6 mm, 5 µm particle diameter; Thermo Scientific, San Jose, CA, U.S.A.) and using methanol as the mobile phase at a flow rate of 1.4 mL/min. Ergosterol was detected at 282 nm and quantified according to an ergosterol standard (ergosterol purity ≥95%, Sigma-Aldrich, St. Louis, MO, U.S.A.) ranging from 0 to 200 µg/mL. Results were expressed in µg ergosterol per g of leaf dry mass (DM).

Total DNA extraction was performed from five leaf disks per sample using the FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, U.S.A.) following the manufacturer's instructions with some minor modifications. Briefly, two homogenisation steps (FastPrep, speed 6.0 for 40 s) were applied instead of one in order to improve fungal mycelium extraction from the leaf matrix. Extracted DNA was quantified using a Nanodrop (Nanodrop<sup>TM</sup> 2000, Wilmington, DE, U.S.A.), amplified by polymerase chain reaction (PCR) targeting the Internal Transcribed Spacer (ITS) region 2 of the fungal rRNA and

then analysed using denaturing gradient gel electrophoresis (DGGE) method. PCR mix was composed of 2.5 µL of MgCl (50 mM), 0.5 µL of BSA (50 mg/mL), 1 µL dNTPs (10 mM), 0.3 µL of Taq polymerase (5 U/µL), 2 µL of each primers (10 pmol/µL), 5 µL of 10X buffer and 4 µL of DNA (10 ng/L), completed with H<sub>2</sub>O to a final volume of 50 µL. Primers, purchased from MWG Biotech, were the forward ITS 3, with a GC tail attached in 5' (5'- CGC CCG CCG CGC CCC GCG CCC GCG CCG CCC CCG CCC CGC ATC GAT GAA GAA - 3') and the reverse ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC 3') (White, Bruns, Lee & Taylor, 1990). PCR conditions were set at 95°C for 5 min, followed by 35 steps (30 s at 95°C, 45 s at 55°C and 1 min at 72°C) and a final elongation phase of 7 min at 72°C. PCR products were then checked on 1% agarose gel (size expected ranging 200–450 bp) and quantified using the DNA Quantification Kit, Fluorescence Assay (Sigma-Aldrich) before loading into DGGE. DGGE was run with 500 ng of PCR product per sample, using the TIGEK 2401-220 device (CBS Scientific, San Diego, NC, U.S.A.) in 1X TAE buffer for 16 hr, with a temperature of 59°C and a voltage of 90 V. Gels contained 7.5% (w/v) of polyacrylamide and a linear urea-formamide denaturing gradient ranging of 25%–48% (100% = 7 M urea and 40% (v/v) formamide). At the end of the electrophoresis, gels were stained in 1X TAE buffer containing 1/20,000 dilution of Gel star (Lonza, Rockland, U.S.A.) and digitised using a BioSpectrumAC Imaging System (UVP, Upland, CA, U.S.A.). DGGE bands profiles were then analysed 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.

## 2.5 | 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 0–4 in the different experimental conditions. The percent loss in dry mass (DM, oven-weight after 48 hr 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 and 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 min. 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:

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

where  $A$  is the absorbance of the sample at 280 nm,  $\epsilon$  is the absorption coefficient for lignin ( $\epsilon_{\text{lignin}} = 20 \text{ g}^{-1} \text{ L cm}^{-1}$ ),  $l$  is the path



length of the beam in cm and C is the concentration of the sample in g of DM per L.

## 2.6 | Extracellular enzyme activities

Phenol oxidase (EC 1.14.18.1), peroxidase (EC 1.11.1.7), and laccase (EC 1.10.3.2) activities were measured in leaf samples (three leaf disks per sample) using 3,4-Dihydroxy-L-phenylalanine (L-DOPA) and 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) commercial substrates analogues (Sigma-Aldrich). All enzyme activities were conducted under saturating conditions of substrate (previously determined from substrate saturating curves) and according to the protocols of Sinsabaugh, Osgood, and Findlay (1994) and Johannes and Majcherzyk (2000). Phenol oxidase and peroxidase activities assays consisted in dark incubations of three leaf disks per replicate at 19°C under 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 assay received an addition of 0.2 ml of 0.3% H<sub>2</sub>O<sub>2</sub>. After 1 hr incubation, spectrophotometric readings at 460 nm were performed for phenol oxidase and peroxidase using the Ultrospec 2000 (Pharmacia Biotech, Trowbridge, U.K.). Net peroxidase activity was obtained after subtraction of phenol oxidase activity. Laccase activity was determined from three leaf disks per replicate, following the oxidation of 2 ml ABTS (3 mM, final concentration) in 0.1 M of citrate-phosphate buffer (pH 4.5) with 2 ml of water from the corresponding channel at 19°C, in the dark and under agitation (150 rpm) for 1 hr. Absorbance was then determined at 420 nm. All enzyme activities were corrected by control, performed with water from the corresponding site (no leaf disks), and expressed in  $\mu\text{mol substrate hr}^{-1} \text{ g leaf DM}^{-1}$ .

## 2.7 | 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 0–4) and site (upstream, downstream) as well as their interactions. Differences between fungal community descriptors (ergosterol concentration, fungal band richness and extracellular enzyme activities) and litter characteristics (lignin content) were assessed using a first two-way ANOVA on upstream and downstream samples for the whole experiment (factors time: weeks 0–4; factors condition: upstream and downstream) and a second two-way ANOVA on all samples at week 3 and 4 corresponding to the translocation experiment (factors time: week 3 and 4; factors condition: upstream, downstream, translocation upstream to downstream, and translocation downstream to upstream). All data were transformed using the 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 hoc multiple comparison tests (Tukey's test,  $p < .05$ ).

From the fungal fingerprints obtained after DGGE analyses, the fungal band presence/absence matrix was transformed to Euclidean distance, and fungal communities 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 relationships between water chemical and fungal community parameters. As described for ANOVA tests, a first RDA (RDA<sub>UpDw</sub>) was performed on upstream and downstream samples for the whole experiment, whereas the second RDA (RDA<sub>Transloc</sub>) was performed on translocated and non-translocated samples at weeks 3 and 4 only. Briefly, fungal community descriptors data matrices were composed of five parameters: ergosterol concentration, fungal band richness and extracellular enzyme activities (phenol oxidase, peroxidase, and laccase). Water physicochemical data matrices included 15 parameters: dissolved oxygen, pH, conductivity, and concentrations of metals (Cu, Cd, Mn, Zn), phosphate, nitrate, dissolved carbon and nitrogen concentrations and  $\Sigma\text{TUs}$ . 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 and 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,  $p = .05$ ).

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/>).

## 3 | RESULTS

### 3.1 | Physical and chemical characteristics of stream water

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 relatively low concentrations (below 0.05  $\mu\text{g/L}$ ) except for the herbicide glyphosate and its metabolite the aminomethylphosphonic acid (AMPA) (ranging 0.10–1.40  $\mu\text{g/L}$ , irrespectively of the studied site), the aminotriazole and the diuron. While only three molecules were detected at the upstream site (glyphosate, AMPA, and lindane), the whole 17 pesticide compounds were detected downstream. Potential pesticide toxicity calculations, represented as the  $\Sigma\text{TUs}$ , were on average (weeks 1–4) thirty times higher downstream ( $35.96 \times 10^{-3} \pm 4.78 \times 10^{-3}$ ) compared to upstream ( $1.14 \times 10^{-3} \pm 0.39 \times 10^{-3}$ ) (ANOVA, Site effect,  $p < .0001$ ). This higher pesticide toxicity observed downstream can be attributed to both the number of pesticide compounds and the presence of diuron ( $\text{TU} = 2.06 \times 10^{-2}$ ), oxadiazon ( $\text{TU} = 1.81 \times 10^{-3}$ ) and terbutryne ( $\text{TU} = 3.13 \times 10^{-3}$ ), contributing to the highest TU observed downstream (Table 1). In addition, heavy metals (Cu, Zn, Mn, Cd) displayed very low concentrations at both sites (Table 1) though their concentrations were significantly higher

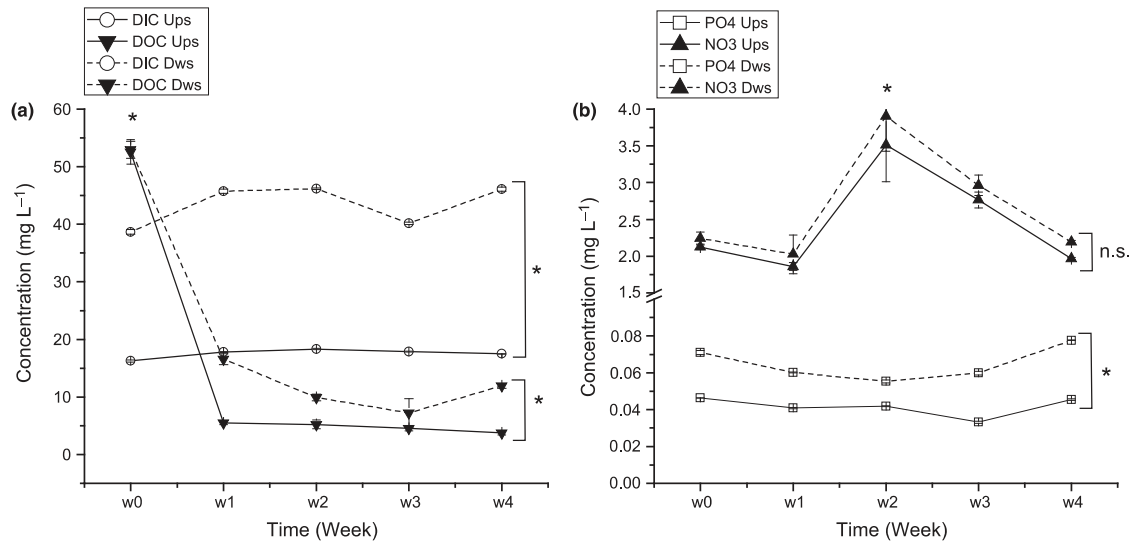
**TABLE 1** Physical and chemical characteristics and pesticide compounds detected (Herbicides [H], Insecticides [I] and Fungicides [F]) in the upstream and downstream waters of the Auzon stream during the laboratory experiment. Values are means and ( $\pm$ ) standard errors of the five sampling times for nutrients ( $n = 15$ ) and four sampling times for pesticides and heavy metals ( $n = 12$ ). Significant differences between sites (ANOVA,  $p < .05$ ) are shown by an asterisk. TU calculation was based on  $EC_{50}$  of *Scenedemus subspicatus*<sup>a</sup>, *Pseudokirchneriella subcapitata*<sup>b</sup>, *Scenedemus quadricauda*<sup>c</sup>, *Scenedemus abundans*<sup>d</sup> and *Chlamydomonas angulosa*<sup>e</sup>.

		Upstream		Downstream	
		Concentration	Mean TU	Concentration	Mean TU
Nutrients	Dissolved organic Carbon (mg/L)	14.32 $\pm$ 9.57		19.71 $\pm$ 8.45*	
	Total Carbon (mg/L)	31.87 $\pm$ 9.25		63.59 $\pm$ 7.28*	
	Phosphate ( $\mu$ g/L)	41.56 $\pm$ 2.34		64.82 $\pm$ 4.13*	
	Conductivity ( $\mu$ S/cm)	204.54 $\pm$ 2.77		552.93 $\pm$ 13.35*	
	pH	7.18 $\pm$ 0.17		7.94 $\pm$ 0.15*	
	Total Nitrogen (mg/L)	1.64 $\pm$ 0.09		1.92 $\pm$ 0.53	
	Nitrate (mg/L)	2.44 $\pm$ 0.19		2.67 $\pm$ 0.21	
Heavy metals	Cadmium ( $\mu$ g/L)	0.024 $\pm$ 0.0		0.03 $\pm$ 0.002*	
	Copper ( $\mu$ g/L)	2.9 $\pm$ 0.4		4.3 $\pm$ 0.2*	
	Manganese ( $\mu$ g/L)	15.75 $\pm$ 3.75		24.53 $\pm$ 1.68*	
	Zinc ( $\mu$ g/L)	54.3 $\pm$ 3.5		77.17 $\pm$ 3.51*	
Xenobiotics	Aminotriazole ( $\mu$ g/L) (H) <sup>a</sup>	<0.005		0.065 $\pm$ 0.0427	2.83 $\times 10^{-5}$
	Biphenyl ( $\mu$ g/L) (F) <sup>e</sup>	<0.005		0.01 $\pm$ 0.01*	7.69 $\times 10^{-6}$
	AMPA ( $\mu$ g/L) (H) <sup>a</sup>	0.705 $\pm$ 0.241	1.10 $\times 10^{-3}$	0.476 $\pm$ 0.157*	7.45 $\times 10^{-4}$
	Glyphosate ( $\mu$ g/L) (H) <sup>c</sup>	0.18 $\pm$ 0.068	4.09 $\times 10^{-5}$	0.332 $\pm$ 0.101*	7.55 $\times 10^{-5}$
	Oxadiazon ( $\mu$ g/L) (H) <sup>a</sup>	<0.005		0.007 $\pm$ 0.001	1.81 $\times 10^{-3}$
	Dinoterb ( $\mu$ g/L) (H) <sup>a</sup>	<0.030		0.02 $\pm$ 0.02	2.70 $\times 10^{-6}$
	Dichloprop ( $\mu$ g/L) (H) <sup>b</sup>	<0.020		0.022 $\pm$ 0.008	3.25 $\times 10^{-7}$
	MCPA ( $\mu$ g/L) (H) <sup>b</sup>	<0.005		0.033 $\pm$ 0.014	4.10 $\times 10^{-7}$
	Mecoprop ( $\mu$ g/L) (H) <sup>b</sup>	<0.005		0.033 $\pm$ 0.012	1.39 $\times 10^{-7}$
	Prosulfocarb ( $\mu$ g/L) (H) <sup>b</sup>	<0.005		0.002 $\pm$ 0.002	4.08 $\times 10^{-5}$
	Lindane ( $\mu$ g/L) (I) <sup>d</sup>	0.0017 $\pm$ 0.0008	7.00 $\times 10^{-7}$	0.004 $\pm$ 0.0003	1.45 $\times 10^{-6}$
	Tributyl phosphate ( $\mu$ g/L) (H) <sup>a</sup>	<0.005		0.01 $\pm$ 0.007	7.69 $\times 10^{-3}$
	Imidacloprid ( $\mu$ g/L) (I) <sup>a</sup>	<0.005		0.027 $\pm$ 0.004	2.65 $\times 10^{-6}$
	Terbumeton ( $\mu$ g/L) (H) <sup>a</sup>	<0.005		0.008 $\pm$ 0.008	8.33 $\times 10^{-4}$
	Terbutylazine ( $\mu$ g/L) (H) <sup>b</sup>	<0.005		0.013 $\pm$ 0.013	1.04 $\times 10^{-3}$
	Terbutryne ( $\mu$ g/L) (H) <sup>b</sup>	<0.005		0.008 $\pm$ 0.002	3.13 $\times 10^{-3}$
	Diuron ( $\mu$ g/L) (H) <sup>c</sup>	<0.005		0.056 $\pm$ 0.004	2.06 $\times 10^{-2}$
	$\Sigma$	0.887	1.14 $\times 10^{-3}$	1.124	3.60 $\times 10^{-2}$ *

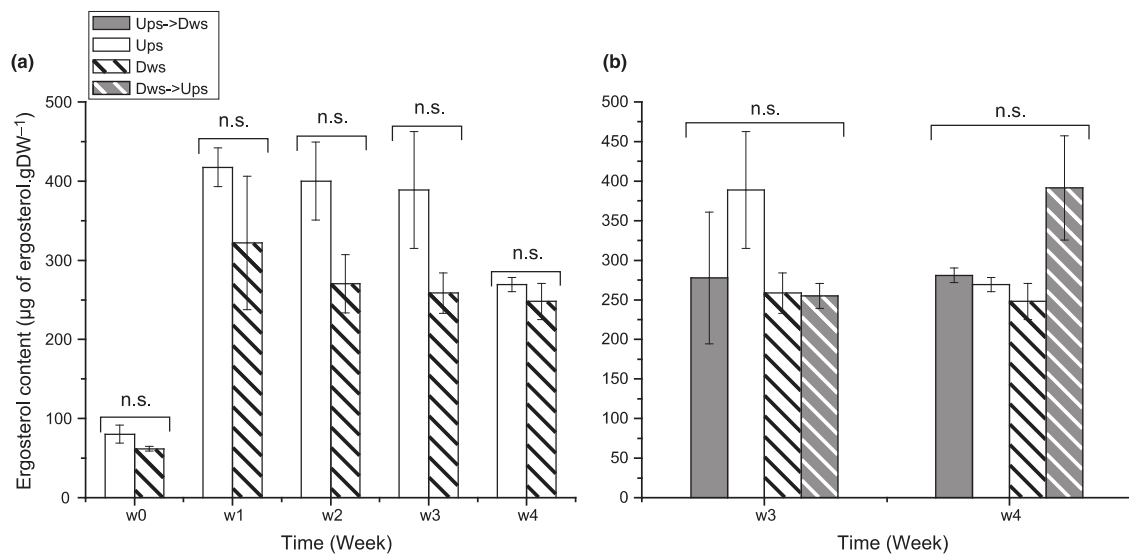
downstream compared to upstream (ANOVA site effect,  $p < .05$ ). Conductivity, pH, dissolved carbon (total and organic), and phosphate concentrations were also greater at the downstream site (ANOVA, site effect,  $p < .001$  for each parameter; Table 1 and Figure 1a, b). Conversely, no differences were observed for total nitrogen and nitrate concentrations between sites (ANOVA, site effect,  $p = .1210$  and  $p = .1197$  respectively). More precisely, average nitrate concentrations were  $0.51 \pm 0.04$  mg/L and  $0.53 \pm 0.04$  mg/L in upstream and downstream channels, respectively (Figure 1b). At week 2,  $N-NO_3^-$  concentration peaked up to  $0.70 \pm 0.10$  mg/L upstream and  $0.78 \pm 0.09$  mg/L downstream (ANOVA time effect,  $p < .0001$ ).

### 3.2 | Litter decomposition rates and lignin content

Decay rates of *Alnus* leaves calculated between weeks 0 and 4 were similar between downstream (0.0162/day) and upstream (0.0101/day) sites, as well as after translocation experiments, although no statistical analysis was performed. Besides, lignin content in *Alnus* leaves (data not shown) decreased from  $10.45 \pm 1.39\%$  DW (week 0) to  $4.30 \pm 0.52\%$  DW (week 4) upstream, representing a loss of 58%. Downstream, lignin loss from leaves appeared lower than upstream (29%) with values ranging from  $8.20 \pm 0.79\%$  DW (week 0) to  $5.82 \pm 1.63\%$  DW (week 4). Specifically, the major decrease in lignin content from leaves was observed at week 2 in upstream and



**FIGURE 1** Dissolved inorganic (DIC) and organic (DOC) concentrations (a) as well as nitrate (NO<sub>3</sub>) and phosphate (PO<sub>4</sub>) concentrations (b) 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 shown by brackets with asterisks for conditions and asterisks only for time (n.s. when not significant, Tukey's test,  $p < .05$ )



**FIGURE 2** Ergosterol concentration on *Alnus* leaves in upstream and downstream samples for the whole experiment (a) and in all samples after translocation (b). Values are means ( $n = 3$ ) and standard errors for the four experimental conditions (upstream samples [Ups], downstream samples [Dws], samples translocated from control to polluted site [Ups->Dws] 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 significant, Tukey's test,  $p < .05$ )

downstream samples (ANOVA, time effect,  $p < .0001$ ). However, no effects of translocation were observed in lignin loss from leaves.

### 3.3 | Biomass and structure of fungal communities

Ergosterol concentration in *Alnus* leaves showed values ranging from  $80.25 \pm 11.45 \mu\text{g/g DM}$  to  $417.37 \pm 24.56 \mu\text{g/g DM}$  upstream and

$61.84 \pm 2.79 \mu\text{g/g DM}$  to  $321.87 \pm 84.24 \mu\text{g/g DM}$  downstream (Figure 2a). The major shifts in ergosterol concentrations were between week 0 and week 1 (Tukey's test,  $p < .0001$ ), evidencing the colonisation phase of *Alnus* leaves by fungi. No significant differences were observed in ergosterol concentration between upstream and downstream sites during the 4 weeks of study (Tukey's test,  $p > .05$ ), but concentrations tended to be slightly higher upstream.

Translocation did not have any effects on ergosterol concentration (Figure 2b).

Fungal band richness tended to increase from weeks 0 to week 4 (ANOVA,  $p < .05$ ) in both upstream (from  $21 \pm 1$  to  $44 \pm 1$  band) and downstream (from  $36 \pm 1$  to  $49 \pm 1$  band) samples, evidencing a fungal species succession on leaves. However, fungal band richness was on average (weeks 0–4) higher in downstream samples ( $S = 41 \pm 1$ ) compared to upstream samples ( $S = 34 \pm 3$ , Tukey test, Site effect,  $p < .05$ ). Moreover, fungal band richness responded to both translocations (Dws  $\rightarrow$  Ups and Ups  $\rightarrow$  Dws), displaying samples with intermediary values (Tukey's test,  $p > .05$ ; Table 2).

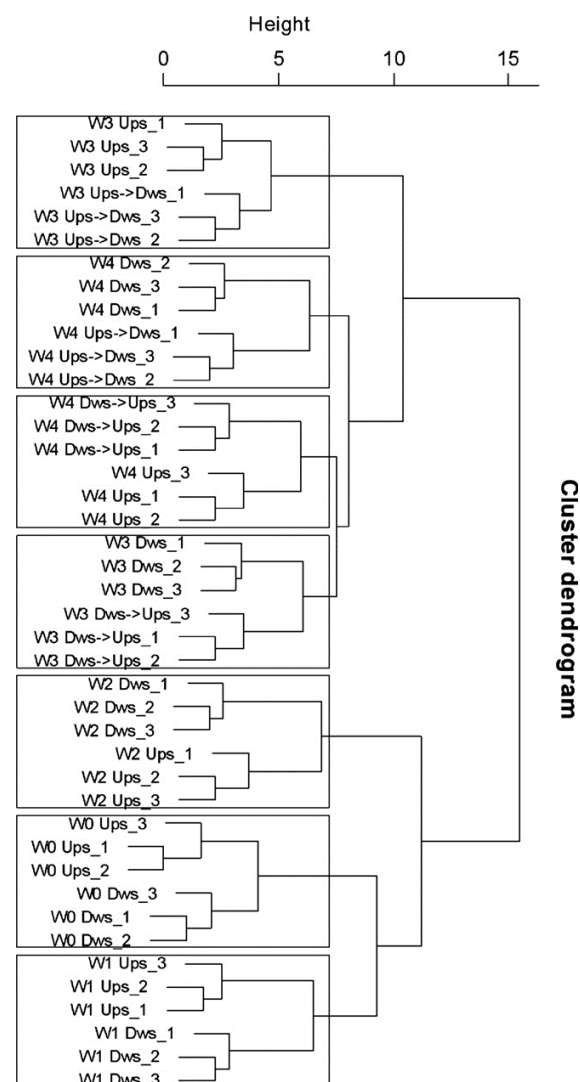
Cluster analysis, performed on fungal community presence/absence matrix, showed a sample ordination, first by time and second by site (Figure 3). Effects of translocation on fungal community structure were perceptible at week 3, and characterised by the splitting of translocated samples from their origin,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

### 3.4 | Enzymatic activities

Peroxidase and phenol oxidase activities measured in *Alnus* leaves were not different between upstream and downstream samples (Tukey test,  $p > .05$ ) (Figure 4a1, b1), though a clear time effect was observed in both activities (ANOVA, time effect,  $p < .0001$ ). Time effects were characterised 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,  $p < .0001$ ), with higher average values (weeks 0–4) in upstream fungal communities ( $1.84 \pm 0.29$  U/g

$\text{DW}^{-1} \text{ hr}^{-1}$ ) comparing to those from downstream ( $0.39 \pm 0.12$  U/g  $\text{DW}^{-1} \text{ hr}^{-1}$ , Figure 4c1). Laccase activity also showed significant differences over time, especially at week 2, where a 3–5 times drop of activity in upstream samples was observed (ANOVA, time effect,  $p < .0001$ ). Concerning translocation experiments, after stress exposure (Ups  $\rightarrow$  Dws translocation, week 4) samples displayed an intermediate state, characterised by a decrease in 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,  $p < .05$ , Figure 4c2). Similarly, after stress removal (Dws

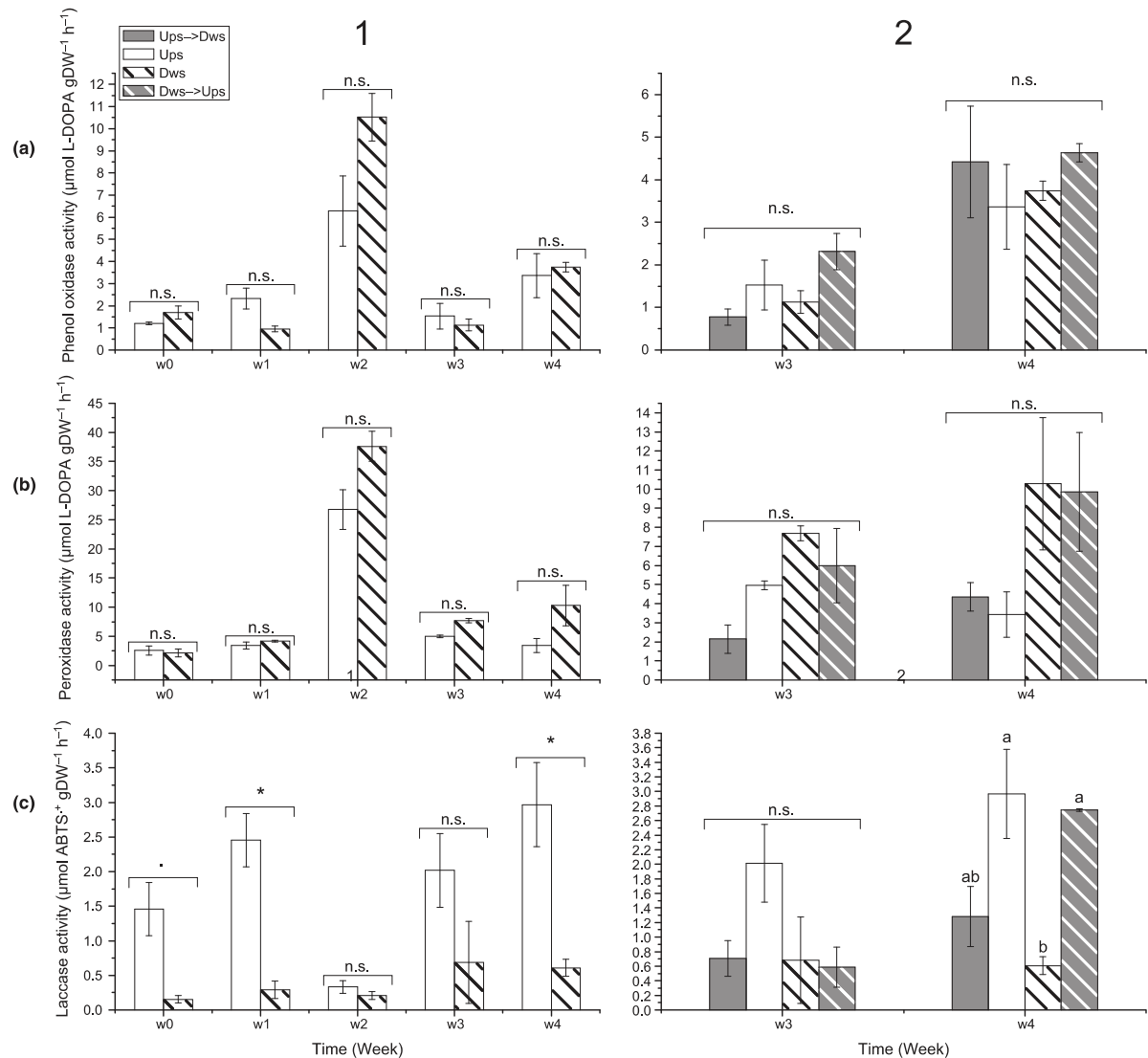


**FIGURE 3** Clustering analysis on the structure of the *Alnus*-associated fungal communities, representing the upstream samples (Ups), downstream samples (Dws), samples translocated from control to polluted site (Ups  $\rightarrow$  Dws) and samples translocated from polluted to control site (Dws  $\rightarrow$  Ups) during the 4 weeks of experiment. Clustering was obtained using the Ward method (Ward dissimilarity index) on fungal DGGE absence/presence matrix

**TABLE 2** 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)  $\pm$  standard errors for each experimental condition. Significant differences between conditions for each week are shown (a  $\neq$  b, Tukey's test,  $p < .05$ )

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





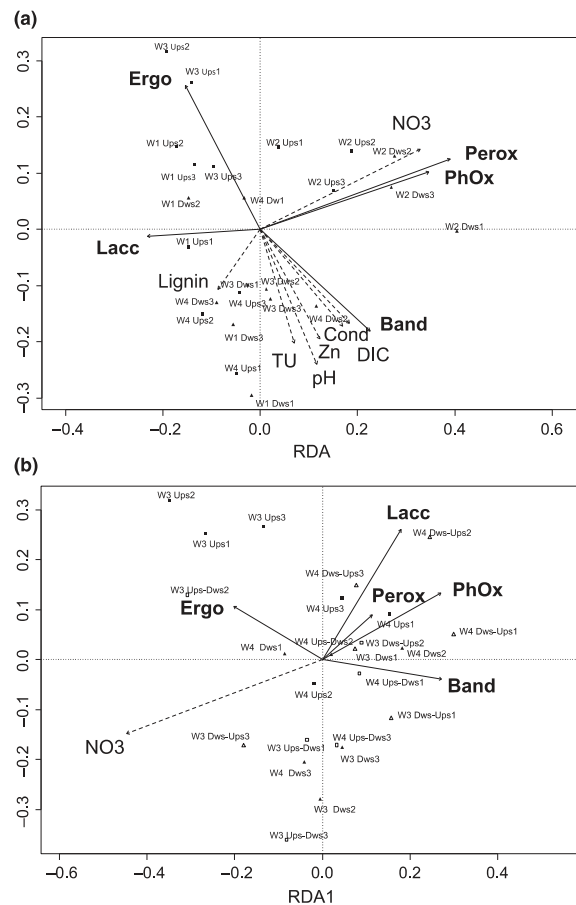
**FIGURE 4** Values of the peroxidase (a), phenol oxidase (b) and laccase (c) activities measured in *Alnus* leaves in upstream and downstream samples for the whole experiment (1) and in all samples after translocation (2). Enzymatic activities are expressed as the amount of 2,3-dihydroindole-5,6-quinone-2-carboxylate (DIQC for peroxidase and phenol oxidase) and oxidised 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS for laccase) released per unit of leaf DM and time (hr). Values are means and standard errors ( $n = 3$ ), and significant differences between conditions for each week are shown by brackets with asterisks and superscript (n.s. when not significant,  $a \neq b$ , Tukey's test,  $p < .05$ )

→ Ups translocation, week 4) translocated samples displayed an increase in laccase activity, reaching values similar to those from the upstream samples (Tukey's test,  $p < .05$ ).

### 3.5 | Relationship between water chemistry and fungal community parameters

RDA<sub>UpDw</sub>, performed with upstream and downstream samples between weeks 0 to 4 (Figure 5a), 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 ( $r = .76$ ) characteristic of samples at week 2 located in the right up corner of the RDA<sub>UpDw</sub> (peak of nitrate concentration), and coincided with high phenol oxidase and peroxidase activities, but low laccase activity. The second axis (RDA 2 explaining 15.59% of the variance) appeared mostly linked with heavy metals concentration (Zn,  $r = -.45$ ) and pesticides toxicity (TU,  $r = -.46$ ), characteristic of samples from the downstream site showing higher values of fungal band richness and lower ergosterol concentrations. In contrast to the



**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). 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 fungal communities to physical and chemical parameters are shown (Permutation test,  $p < .05$ ). Biological variables are composed of phenol oxidase (PhOx), peroxidase (Perox) and laccase (Lacc) activities, ergosterol concentration (Ergo) and band richness (Band). Environmental variables are composed of conductivity (Cond), pH, lignin percentage (Lignin), pesticide toxicity (TU), nutrient (Nitrate [NO<sub>3</sub>], Phosphate [PO<sub>4</sub>], Inorganic carbon [DIC]) and heavy metals concentrations (zinc [Zn])

$RDA_{UpDw}$ , the  $RDA_{Transloc}$  displayed two axes representing a much lower percent of the total variance (46.46%). Here, only nitrate concentration (NO<sub>3</sub>) was retained by the permutation test as explicative of the variance in biological descriptors (Figure 5b). Again, the first axis (RDA1 explaining 38.88% of the variance) was mainly correlated with nitrate concentration ( $r = -.59$ ), but here coinciding with low rates of the three ligninolytic activities (laccase, phenol oxidase and peroxidase). Specifically, samples 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

ligninolytic activities. In addition, fungal band richness appeared also negatively related to the nitrate concentration in water.

## 4 | 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) and were in the same range of that found in field experiments ( $k = 0.0054/\text{day}$ ) Chauvet, 1987; ( $k = 0.0161/\text{day}$ ) Canhoto & Graça, 1996; ( $k = 0.035/\text{day}$ ) 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.

As expected, the Auzon appeared as a contaminated stream (Phyt'eauvergne 2014, Water agency, 2003). While DOC concentrations categorised its upstream and downstream sites in two different class of quality according to the water agency classification system (respectively poor and bad), nutrients (NO<sub>3</sub>, PO<sub>4</sub>) and pesticides categorised the two sites into the same "good" class of quality (Table 1). Despite such small differences in terms of water quality 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 differences in fungal biomass (ergosterol concentration) between communities were observed. The influence of stream water chemistry on the microbial community descriptors was also supported by the translocation of leaves from upstream to downstream waters (stress exposure) which induced a sharp decrease in laccase activity concomitant with changes in the structure of the fungal community, but again without influence on fungal biomass. These results seem to support the "stress gradient" hypothesis proposed by Niyogi, Lewis, and McKnight (2002), 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 appeared to be very sensitive to water chemical stress.

Ligninolytic activities from fungal communities colonising *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 (Baldrian, 2006; Gallo, Amonette, Lauber, Sinsabaugh, & Zak, 2004; 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 peroxidase activities, as observed in the second RDA ( $RDA_{Transloc}$ ) of our study. However,

our study showed also that after a twofold increase of nitrate concentrations probably resulting from the rainfall event occurring at week 2, laccase activity tended to be inhibited (Pearson,  $r = -.497$ ,  $p < .05$ ), whereas peroxidase and phenol oxidase activities tended to be stimulated (Pearson,  $r = .807$  and  $r = .549$  respectively,  $p < .05$ ,  $RDA_{UpsDws}$ , Figure 5b). The fact 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 oxidases (Baldrian, 2006; Sinsabaugh, 2010). Generally, phenol oxidases and peroxidase activities are assayed using L-DOPA as substrate, the latter activity being calculated as the incremental response relative to phenol oxidases (Sinsabaugh, 2010; Sinsabaugh et al., 1994), whereas laccase activities are assayed using a more specific substrate: the 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 two different substrates for activity assays (Bach et al., 2013). Besides, the antagonistic results 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 downstream samples over the 4 weeks of the experiment, whereas  $RDA_{Transloc}$  was performed on week 3 and 4 only. Consequently, the greater ligninolytic activities of translocated samples at week 4 (right up corner of the  $RDA_{Transloc}$ ) may reflect a transient 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 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 between sites. These observations suggest that other factors may be involved in laccase activity regulation. We observed a negative correlation between dissolved organic carbon (DOC) and laccase activity (Pearson,  $r = -.45$ ,  $p < .05$ ) as well as between Toxic Units (TU) and laccase activity (Pearson,  $r = -.50$ ,  $p < .05$ ). As suggested in others studies, we hypothesised that both DOC quantity and quality (Elisashvili, Penninckx, Kachlishvili, Asatiani, & Kvesitadze, 2006; Mikiashvili, Wasser, Nevo, & Elisashvili, 2006; Romani, Artigas, & Ylla, 2012) or the presence of some pesticide compounds could be important in laccase activity regulation (Oliveira et al., 2013; Zapp et al., 2011). Alternatively, shifts in fungal communities structure, highlighted by changes in fungal band richness, can partially explain the differences in laccase activity observed between sites and after translocation (Frey, Knorr, Parrent, & Simpson, 2004; Osono, 2007; Treton, Chauvet, & Charcosset, 2004). When looking at the fungal band absence/presence matrix, fungal species specific of the upstream 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-Raheem and Shearer (2002) and Abdel-Raheem and Ali (2004) in culture experiments, not all aquatic fungal species have shown the

ability to produce all types of ligninolytic enzymes. Besides, since laccases have an indirect role in carbon uptake by fungi from leaf litter (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, more competitive than some high-laccase producers upstream. Our study reveals that stress by 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, suggesting a certain functional redundancy in these communities (Pascoal, Cássio & Marvanová 2005) probably explained by compensation mechanisms in the organic matter degradation by extracellular enzymes.

When stress by pollution was removed through the translocation from the polluted site (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 fungal community structure tended to get closer to that of fungal communities from upstream within 2 weeks. Such observations are consistent with other translocation studies, which showed that aquatic hyphomycetes communities were able to recover in terms of structure, decomposing activity (decay rates), and sporulation rates when translocated from highly metal-exposed sites to cleaner sites in about 2–3 weeks (Sridhar et al., 2005 in the field; Duarte, Pascoal, & Cássio, 2009a in microcosms). Regarding at the fungal band presence/absence 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 communities are very sensitive to nutrient variations in streams (Bärlocher & Corkum, 2003; Suberkropp & Chauvet, 1995), the combined effects of low nutrient concentrations in the upstream channels and the osmotic stress resulting from a sudden change of water chemistry (conductivity values were 2.7 times higher downstream than upstream) may have affected the dominance of certain fungal species from downstream communities.

Overall our study indicates that variations in stream water chemistry, resulting from anthropogenic activities in the watershed, induce changes in the structure and enzymatic pathway of leaf-associated fungal communities. However, recovery assessment allowed us to evidence an almost full recovery in these communities which encourages river restoration programs. But linking fungal community responses to stream water chemistry remains 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 the literature, which decrease the reliability of pesticide risk assessment in these communities (Maltby et al., 2009). Thus, future researches should take into account aquatic fungi as species sensitive to the chemical quality of stream waters.

## ACKNOWLEDGMENTS

This study was funded by the Fédération des Recherches en Environnement (UBP/CNRS FR 3467/INRA). We also thank Fanny Perrière for her help in the water chemical analyses. Florent Rossi was

funded by a PhD grant from the French Ministry of Higher Education and Research.

## REFERENCES

- Abdel-Raheem, A. M., & Ali, E. H. (2004). Lignocellulolytic enzyme production by aquatic hyphomycetes species isolated from the Nile's delta region. *Mycopathologia*, 157, 277–286.
- Abdel-Raheem, A., & Shearer, C. A. (2002). Extracellular enzyme production by freshwater ascomycetes. 11, 1–19.
- Abdullah, S. K., & Taj-Aldeen, S. J. (1989). Extracellular enzymatic activity of aquatic and aero-aquatic conidial fungi. *Hydrobiologia*, 174, 217–223.
- Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., & Pesce, S. (2012). Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. *Aquatic Toxicology*, 122, 197–205.
- Artigas, J., Romani, A. M., & Sabater, S. (2004). Organic matter decomposition by fungi in a Mediterranean forested stream: contribution of streambed substrata. *Annales de Limnologie-International Journal of Limnology*, 40, 269–277.
- Artigas, J., Romani, A. M., & Sabater, S. (2008). Effect of nutrients on the sporulation and diversity of aquatic hyphomycetes on submerged substrata in a Mediterranean stream. *Aquatic Botany*, 88, 32–38.
- Artigas, J., Pascault, N., Bouchez, A., Chastain, J., Debroas, D., Humbert, J. F., ... Pesce, S. (2014). Comparative sensitivity to the fungicide tebuconazole of biofilm and plankton microbial communities in freshwater ecosystems. *Science of The Total Environment*, 468–469, 326–336.
- Bach, C. E., Warnock, D. D., Van Horn, D. J., Weintraub, M. N., Sinsabaugh, R. L., Allison, S. D., & German, D. P. (2013). Measuring phenol oxidase and peroxidase activities with pyrogallol, L-DOPA, and ABTS: Effect of assay conditions and soil type. *Soil Biology and Biochemistry*, 67, 183–191.
- Baldrian, P. (2006). Fungal laccases—occurrence and properties. *FEMS Microbiology Reviews*, 30, 215–242.
- Baldy, V., Chauvet, E., Charcosset, J.-Y., & Gessner, M. O. (2002). Microbial dynamics associated with leaves decomposing in the mainstem and floodplain pond of a large river. *Aquatic Microbial Ecology*, 28, 25–36.
- Baldy, V., Gobert, V., Guerold, F., Chauvet, E., Lambrigt, D., & Charcosset, J.-Y. (2007). Leaf litter breakdown budgets in streams of various trophic status: Effects of dissolved inorganic nutrients on microorganisms and invertebrates. *Freshwater Biology*, 52, 1322–1335.
- Bärlocher, F., & Corkum, M. (2003). Nutrient enrichment overwhelms diversity effects in leaf decomposition by stream fungi. *Oikos*, 101, 247–252.
- Benda, L. E. E., Poff, N. L., Miller, D., Dunne, T., Reeves, G., Pess, G., & Pollock, M. (2004). The network dynamics hypothesis: How channel networks structure riverine habitats. *BioScience*, 54, 413–427.
- Box, G. E., & Cox, D. R. (1964). An analysis of transformations. *Journal of the Royal Statistical Society. Series B (Methodological)*, 26, 211–252.
- Canhoto, C., Gonçalves, A. L., & Bärlocher, F. (2016). Biology and ecological functions of aquatic hyphomycetes in a warming climate. *Fungal Ecology*, 19, 201–218.
- Canhoto, C., & Graça, M. A. (1996). Decomposition of *Eucalyptus globulus* leaves and three native leaf species (*Alnus glutinosa*, *Castanea sativa* and *Quercus faginea*) in a Portuguese low order stream. *Hydrobiologia*, 333, 79–85.
- Chandrashekar, K. R., & Kaveriappa, K. M. (1991). Production of extracellular cellulase by *Lunulospora curvula* and *Flagellospora penicillioides*. *Folia Microbiologica*, 36, 249–255.
- Chauvet, E. (1987). Changes in the chemical composition of alder, poplar and willow leaves during decomposition in a river. *Hydrobiologia*, 148, 35–44.
- Cummins, K. W. (1974). Structure and function of stream ecosystems. *BioScience*, 24, 631–641.
- Duarte, S., Pascoal, C., Alves, A., Correia, A., & Cássio, F. (2008). Copper and zinc mixtures induce shifts in microbial communities and reduce leaf litter decomposition in streams. *Freshwater Biology*, 53, 91–101.
- Duarte, S., Pascoal, C., & Cássio, F. (2009a). Functional stability of stream-dwelling microbial decomposers exposed to copper and zinc stress. *Freshwater Biology*, 54, 1683–1691.
- Duarte, S., Pascoal, C., Garabétian, F., Cássio, F., & Charcosset, J.-Y. (2009b). Microbial decomposer communities are mainly structured by trophic status in circumneutral and alkaline streams. *Applied and Environmental Microbiology*, 75, 6211–6221.
- Elisashvili, V., Penninckx, M., Kachlishvili, E., Asatiani, M., & Kvesitadze, G. (2006). Use of *Pleurotus dryinus* for lignocellulolytic enzymes production in submerged fermentation of mandarin peels and tree leaves. *Enzyme and Microbial Technology*, 38, 998–1004.
- Fernández, D., Voss, K., Bundschuh, M., Zubrod, J. P., & Schäfer, R. B. (2015). Effects of fungicides on decomposer communities and litter decomposition in vineyard streams. *Science of The Total Environment*, 533, 40–48.
- Frey, S. D., Knorr, M., Parrent, J. L., & Simpson, R. T. (2004). Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management*, 196, 159–171.
- Gallo, M., Amonette, R., Lauber, C., Sinsabaugh, R. L., & Zak, D. R. (2004). Microbial community structure and oxidative enzyme activity in nitrogen-amended north temperate forest soils. *Microbial Ecology*, 48, 218–229.
- Gaudes, A., Artigas, J., Romani, A. M., Sabater, S., & Muñoz, I. (2009). Contribution of microbial and invertebrate communities to leaf litter colonization in a Mediterranean stream. *Journal of the North American Benthological Society*, 28, 34–43.
- Gessner, M. O., & Chauvet, E. (1993). Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. *Applied and Environmental Microbiology*, 59, 502–507.
- Gessner, M. O., & Chauvet, E. (1994). Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology*, 75, 1807–1817.
- Gessner, M. O., Chauvet, E., & Dobson, M. (1999). A perspective on leaf litter breakdown in streams. *Oikos*, 377–384.
- Gessner, M. O., & Schmitt, A. L. (1996). Use of solid-phase extraction to determine ergosterol concentrations in plant tissue colonized by fungi. *Applied and Environmental Microbiology*, 62, 415–419.
- Gholz, H. L., Wedin, D. A., Smitherman, S. M., Harmon, M. E., & Parton, W. J. (2000). Long-term dynamics of pine and hardwood litter in contrasting environments: Toward a global model of decomposition. *Global Change Biology*, 6, 751–765.
- Hagen, E. M., Webster, J. R., & Benfield, E. F. (2006). Are leaf breakdown rates a useful measure of stream integrity along an agricultural land-use gradient? *Journal of the North American Benthological Society*, 25, 330–343.
- Hieber, M., & Gessner, M. O. (2002). Contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates. *Ecology*, 83, 1026–1038.
- Iiyama, K., & Wallis, A. F. A. (1988). An improved acetyl bromide procedure for determining lignin in woods and wood pulps. *Wood Science and Technology*, 22, 271–280.
- Ivorra, N., Hettelaar, J., Tubbing, G. M. J., Kraak, M. H. S., Sabater, S., & Admiraal, W. (1999). Translocation of microbenthic algal assemblages used for in situ analysis of metal pollution in rivers. *Archives of Environmental Contamination and Toxicology*, 37, 19–28.
- Johannes, C., & Majcherzyk, A. (2000). Laccase activity tests and laccase inhibitors. *Journal of Biotechnology*, 78, 193–199.
- Junghanns, C., Pecyna, M. J., Böhm, D., Jehmlich, N., Martin, C., Von Bergen, M., ... Schlosser, D. (2009). Biochemical and molecular genetic characterization of a novel laccase produced by the aquatic ascomycete *Phoma* sp. UHH 5-1-03. *Applied Microbiology and Biotechnology*, 84, 1095–1105.



- Krauss, G., Barlocher, F., Schreck, P., Wennrich, R., Glasser, W., & Krauss, G.-J. (2001). Aquatic hyphomycetes occur in hyperpolluted waters in Central Germany. *Nova Hedwigia*, 72, 419–428.
- Krauss, G.-J., Solé, M., Krauss, G., Schlosser, D., Wesenberg, D., & Barlocher, F. (2011). Fungi in freshwaters: Ecology, physiology and biochemical potential. *FEMS Microbiology Reviews*, 35, 620–651.
- Krauss, G., Sridhar, K. R., Jung, K., Wennrich, R., Ehrman, J., & Barlocher, F. (2003). Aquatic hyphomycetes in polluted groundwater habitats of Central Germany. *Microbial Ecology*, 45, 329–339.
- Kristensen, P., & Globevník, L. (2014). European small water bodies: Importance, Threats and Knowledge Gaps. *Biology and Environment: Proceedings of the Royal Irish Academy*, 114B, 281–287.
- Legendre, P., & Gallagher, E. (2001). Ecologically meaningful transformations for ordination of species data. *Oecologia*, 129, 271–280.
- Magbanua, F. S., Townsend, C. R., Blackwell, G. L., Phillips, N., & Matthaei, C. D. (2010). Responses of stream macroinvertebrates and ecosystem function to conventional, integrated and organic farming. *Journal of Applied Ecology*, 47, 1014–1025.
- Maltby, L., Brock, T. C., & van den Brink, P. J. (2009). Fungicide risk assessment for aquatic ecosystems: Importance of interspecific variation, toxic mode of action, and exposure regime. *Environmental Science & Technology*, 43, 7556–7563.
- Mann, K. I. I. (1975). Detritus formation from eelgrass (*Zostera marina* L.): The relative effects of fragmentation, leaching, and decay. *Limnology and Oceanography*, 20, 924–934.
- Mikashvili, N., Wasser, S. P., Nevo, E., & Elisashvili, V. (2006). Effects of carbon and nitrogen sources on *Pleurotus ostreatus* ligninolytic enzyme activity. *World Journal of Microbiology and Biotechnology*, 22, 999–1002.
- Mninishall, G. W. (1967). Role of allochthonous detritus in the trophic structure of a woodland springbrook community. *Ecology*, 139–149.
- Morin, S., Lambert, A.-S., Artigas, J., Coquery, M., & Pesce, S. (2012). Diatom immigration drives biofilm recovery after chronic copper exposure: Role of diatom immigration in recovery. *Freshwater Biology*, 57, 1658–1666.
- Murphy, J., & Riley, J. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, 27, 31–36.
- Niyogi, D. K., Lewis, W. M. Jr, & McKnight, D. M. (2002). Effects of stress from mine drainage on diversity, biomass, and function of primary producers in mountain streams. *Ecosystems*, 5, 554–567.
- Oliveira, T. M., Barroso, M. F., Morais, S., De Lima-Neto, P., Correia, A. N., Oliveira, M. B., & Delerue-Matos, M. (2013). Biosensor based on multi-walled carbon nanotubes paste electrode modified with laccase for pirimicarb pesticide quantification. *Talanta*, 106, 137–143.
- Osono, T. (2007). Ecology of ligninolytic fungi associated with leaf litter decomposition. *Ecological Research*, 22, 955–974.
- Pascoal, C., Cássio, F., & Marvanová, L. (2005). Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Archiv für Hydrobiologie*, 162, 481–496.
- Petersen, R. C., & Cummins, K. W. (1974). Leaf processing in a woodland stream. *Freshwater Biology*, 4, 343–368.
- Phyt'Auvergne (2014). Eau et produits phytosanitaires – Qualité des eaux en Auvergne 2004 – 2012, (Phyt'Auvergne – June 2014).
- Piscart, C., Navel, S., Maazouzi, C., Montuelle, B., Cornut, J., Mermillod-Blondin, F., ... Marmonier, P. (2011). Leaf litter recycling in benthic and hyporheic layers in agricultural streams with different types of land use. *Science of the Total Environment*, 409, 4373–4380.
- Proia, L., Osorio, V., Soley, S., Köck-Schulmeyer, M., Pérez, S., Barceló, D., ... Sabater, S. (2013). Effects of pesticides and pharmaceuticals on biofilms in a highly impacted river. *Environmental Pollution*, 178, 220–228.
- Rasmussen, J. J., Wiberg-Larsen, P., Baattrup-Pedersen, A., Monberg, R. J., & Kronvang, B. (2012). Impacts of pesticides and natural stressors on leaf litter decomposition in agricultural streams. *Science of The Total Environment*, 416, 148–155.
- Romaní, A. M., Artigas, J., & Ylla, I. (2012). Extracellular enzymes in aquatic biofilms: Microbial interactions versus water quality effects in the use of organic matter. In Gavin Lear and Gillian Lewis (Ed.), *Microbial biofilms: Current research and applications* (pp. 153–174). Norfolk: Caister Academic Press.
- Sinsabaugh, R. L. (2010). Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biology and Biochemistry*, 42, 391–404.
- Sinsabaugh, R. L., Osgood, M. P., & Findlay, S. (1994). Enzymatic models for estimating decomposition rates of particulate detritus. *Journal of the North American Benthological Society*, 160–169.
- Sprague, J. B. (1971). Measurement of pollutant toxicity to fish—III: Sublethal effects and “safe” concentrations. *Water Research*, 5, 245–266.
- Sridhar, K. R., Barlocher, F., Krauss, G.-J., & Krauss, G. (2005). Response of aquatic hyphomycete communities to changes in heavy metal exposure. *International Review of Hydrobiology*, 90, 21–32.
- Suberkropp, K., & Chauvet, E. (1995). Regulation of leaf breakdown by fungi in streams: Influences of water chemistry. *Ecology*, 76, 1433–1445.
- Suberkropp, K., & Klug, M. J. (1976). Fungi and bacteria associated with leaves during processing in a woodland stream. *Ecology*, 707–719.
- Treton, C., Chauvet, E., & Charcosset, J.-Y. (2004). Competitive interaction between two aquatic hyphomycete species and increase in leaf litter breakdown. *Microbial Ecology*, 48, 439–446.
- Triska, F. J. (1970). Seasonal distribution of aquatic hyphomycetes in relation to the disappearance of leaf litter from a small woodland stream. Ph.D. thesis, University of Pittsburgh, Pittsburgh, PA. 189 p.
- Water agency (2003). Système d'évaluation de la qualité de l'eau des cours d'eau (SEQ-Eau), grille d'évaluation version 2 - MEDD & Agences de l'eau, 40 p.
- Wallace, J. B., & Webster, J. R. (1996). The role of macroinvertebrates in stream ecosystem function. *Annual Review of Entomology*, 41, 115–139.
- Webster, J. R., & Meyer, J. L. (1997). Organic matter budgets for streams: A synthesis. *Journal of the North American Benthological Society*, 16, 141–161.
- White, T. J., Bruns, T., Lee, S., Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, 18, 315–322.
- Zapp, E., Brondani, D., Vieira, I. C., Scheeren, C. W., Dupont, J., Barbosa, A. M., & Ferreira, V. S. (2011). Biomonitoring of methomyl pesticide by laccase inhibition on sensor containing platinum nanoparticles in ionic liquid phase supported in montmorillonite. *Sensors and Actuators B: Chemical*, 155, 331–339.
- Zubrod, J. P., Feckler, A., Englert, D., Koksharova, N., Rosenfeldt, R. R., Seitz, F., ... Bundschuh, M. (2015). Inorganic fungicides as routinely applied in organic and conventional agriculture can increase palatability but reduce microbial decomposition of leaf litter. *Journal of Applied Ecology*, 52, 310–322.

## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Rossi F, Artigas J, Mallet C. Structural and functional responses of leaf-associated fungal communities to chemical pollution in streams. *Freshwater Biol.* 2017;00:1–13. <https://doi.org/10.1111/fwb.12937>

## 5. Chapter 3: Supplementary Material

Supplementary material: Scored presence/absence band matrix used for fungal community clustering. Samples are represented as columns and band positions are represented as rows. Absence of bands are represented as null, while presence of bands are represented by a 1.

[illegible]

### Main conclusions of the study

We showed that fungal communities associated with alder leaves were sensitive to chemical contamination. Specifically, laccase activity was higher in microbial communities exposed to low chemical stress than in those exposed to high chemical stress. Such differences in the activity response might be explained by differences in the structure of fungal communities.

Leaf-associated microbial communities were capable to recover after stress was removed; both in terms of community function (laccase activity) and structure (fungal community).

The functional descriptor laccase activity showed a strong negative relationship with nitrate concentrations in stream water.



## **Chapter 4: Regulation of microbial extracellular laccase activity subjected to chemical contamination**

Results obtained in our field experiment (Chapter 1) revealed no clear trend in laccase activity regulation in natural communities, although this activity appeared slightly lower in urbanized and agricultural streams as well as in the downstream sections. In the translocation experiment (Chapter 3), laccase activity was lower in microbial communities from downstream comparing to upstream, which confirms the results observed in Chapter 1. Moreover, laccase activity studied in the translocation experiment (Chapter 3) was clearly inhibited by nitrates while stimulated in presence of the fungicide tebuconazole in the artificial channel experiment (Chapter 2). However, in a laboratory experiment (dose-response tests) Artigas *et al*, (2017b) observed no variations of laccase activity in communities exposed to increasing tebuconazole concentrations and concluded that such activity was a poor indicator of fungicide contamination, at least for the tebuconazole exposure assessment in microbial communities (Annex 1). Despite the lack of consensus between experiments, microbial laccase activity regulation seems to be tightly linked to shifts in water chemical contamination.

Given the observed results in both field and microcosm experiments in this thesis work, we wanted to go further in the understanding of the regulation of laccase activity in leaf-associated microbial communities. This chapter investigated the specific interaction between nutrients and pesticides on microbial laccase activity regulation.

# **Regulation of microbial extracellular laccase activity subjected to chemical contamination (in prep)**

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***Running head:*** Nitrogen stimulate laccase activity in leaf-associated microbes

***Keywords:*** Laccase activity, nitrogen, tebuconazole, microbial communities, toxicity test.

## Abstract

The decomposition of leaf litter by microorganisms is mediated by the action of several extracellular ligninolytic enzymes, including laccase. Though it is generally accepted that laccase activity varies according to lignin content in leaves, the effects of stream water chemistry on such enzymatic activity is still unclear. This study aims to improve knowledge on the specific effect of nitrates and a fungicide (tebuconazole, TBZ) on laccase activity from microbial communities associated with *Alnus glutinosa* leaf litter. Here, we performed a dose-response experiment using microbial communities from a natural well preserved stream exposed to increasing concentrations of nitrate and TBZ. Results showed that laccase activity was not sensitive to TBZ concentration increase regardless of nitrate concentrations. However, a clear activity stimulation was observed when increasing nitrate concentrations. The Effective Concentration inducing a variation of 50% in laccase activity ( $EC_{50}$ ) for nitrates was comprised between  $18.61 \pm 4.55 \text{ mg L}^{-1}$  of N- $\text{NO}_3$  for the highest TBZ concentration ( $20 \text{ mg L}^{-1}$  of TBZ) and  $36.45 \pm 6.34 \text{ mg L}^{-1}$  of N- $\text{NO}_3$  for the control (without TBZ). The observed response of laccase activity was probably associated with the osmotic stress generated by high nitrate concentrations, and this stress was still greater for the treatment with the highest TBZ concentration. This can be explained by a specific stress response of the microbial community to extremely high nitrate and TBZ concentrations resulting from the acute exposure conditions (36h). However, laccase was not sensitive to lower nitrate concentrations variations (ranging between 0 to  $1 \text{ mg N-NO}_3/\text{L}$ ) in this experiment which contrasts with observations from field and microcosm experiments. In the field, laccase activity in microbial communities from well preserved streams was more sensitive to low nitrate variations, whereas this activity appeared less sensitive in more contaminated sites. Here such laccase activity regulation can be explained by N repression, as laccase activity is involved in N acquisition by fungi. Moreover, the present study highlights the gap between standard ecotoxicological laboratory tests and field observations when assessing microbial laccase activity regulation in leaf-associated microbial communities.

## Introduction

The functioning of headwater forested streams is largely based on heterotrophic processes and thus deeply rely on allochthonous organic matter inputs from the riparian forest (Minshall, 1967). In these ecosystems, leaves usually represent the major part of these inputs, ranging from 41 to 98% of the total organic matter in terms of biomass (Minshall, 1967; Abelho, 2001b). The chemical composition of leaves involves several types of complex polymers including cellulose, hemicellulose, pectin and lignin, embedding a large quantity of nutrients (Sarkar *et al.*, 2009). While the first three compounds are generally considered as labile, lignin, a highly branched aromatic polymer conferring rigidity and resistance to plant tissues, is much more recalcitrant (Vanholme *et al.*, 2010). In order to release the nutrients imprisoned in leaves (i. e. nitrogen, phosphorus and other essential micro-nutrients such as potassium, manganese ...), microorganisms must breakdown lignin. The degradation of lignin involves the action of a large panel of ligninolytic enzymes, including phenol oxidases, peroxidases and laccases (Abdullah & others, 1989; Chandrashekar & Kaveriappa, 1991; Abdel-Raheem & Ali, 2004). Among them, laccase enzymes have drawn considerable attention over the last century (Durán *et al.*, 2002). Belonging to the group of blue oxidases, laccases (EC 1.10.3.2) are copper-containing oxidase enzymes that catalyze the oxidation of many phenolic compounds as well as some inorganic ions, accompanied by the reduction of molecular oxygen to water (Wong, 2009). Due to its low substrate specificity, this enzyme has been also shown to transform many phenol-like pollutants and thus make a very good candidate in many waste treatment applications (Durán & Esposito, 2000). In stream ecosystems, several organisms are able to synthesize laccase enzymes synthesis, including higher plants and some bacteria, but the main producers of extracellular laccases in leaf-associated microbial communities are aquatic fungi (mostly ascomycetes, (Baldrian, 2006)). In the context of the global contamination of streams and given the involvement of fungal laccase enzymes in carbon cycling, using this enzymatic activity as descriptor could give valuable information on the effects of water chemical contamination on leaf-litter decomposition processes in streams.

Rivers and streams are often subjected to chemical contamination, including nutrient inputs. Among the pollution sources, agriculture remains the main contributor of nitrogen and phosphorus discharge into the environment. While contaminations by phosphorus have greatly decreased over the last two decades (-42% in phosphate concentration in European streams between 1992 and 2012), nitrogen inputs from agriculture (mostly issued from fertilizer spreading) remain constant and a major driver of stream eutrophication (WISE WFD database).

Based on annual averages, more than 35% of European surface waters show nitrate concentrations above  $10 \text{ mg L}^{-1}$ , with huge variations among countries. For example, in France, nitrate concentrations above  $25 \text{ mg L}^{-1}$  are detected in more than 50% of the monitored surface waters. Currently, the effects of nitrogen on leaf-associated microbial communities and their extracellular laccase activity remains unanswered. Generally, nitrogen enrichment is associated with increases in fungal biomass and diversity which often leads to an increase in enzymatic activity, including laccases (Artigas, Romaní & Sabater, 2004c; Hagen *et al.*, 2006; Magbanua *et al.*, 2010). However, high nitrate and ammonium concentrations in eutrophic streams have also been shown to decrease fungal diversity and biomass, as well as their potential for decomposing leaf litter (Baldy *et al.*, 2002; Duarte *et al.*, 2009). In soil ecosystems, where laccases are the most abundant enzymes, ligninolytic activities were found to be highly sensitive to nitrogen inputs (Baldrian, 2006). In most cases, a sharp decrease in ligninolytic activity was observed after a nitrogen amendment, probably explained by the concomitant decrease in fungal biomass (Litvintseva & Henson, 2002; Matocha, Haszler & Grove, 2004; Zeglin *et al.*, 2007). However, based on the abundance and diversity of laccase genes, several authors also observed a decrease in laccase activity explained by a reduced gene expression with no variation in terms of microbial community structure and abundance (Blackwood *et al.*, 2007; Hofmockel, Zak & Blackwood, 2007; Hassett *et al.*, 2009; Sinsabaugh, 2010). This suggests a link between laccase activity regulation and the availability of nitrogen. In contrast with studies in soils, very few studies reported nitrogen effects on laccase activity in streams. Rossi, Artigas & Mallet, (2017) showed a negative relationship between laccase activity and nitrate concentration, and laccase variations were not explained by shifts in the amount of fungal biomass. Studies working on single fungal strain showed that the response of laccase activity appears tightly linked to the type of nitrogen source. For example, in glucose-containing culture media, (Mikiashvili *et al.*, 2006) showed that nitrogen inorganic forms tended to inhibit laccase activity in *Pleurotus ostreatus*, while organic forms tended to stimulate it. However, (Elisashvili *et al.*, 2006) also showed that nitrogen addition in both organic or inorganic forms had no effect on laccase activity from *Pleurotus dryinus* IBB 903 strain when lignocellulosic substrates were already present (mandarin peels and tree leaves). These results suggest that the effects of nitrogen on laccase activity also depend on the considered strain as well as the nature and amount of the carbon substrate supplied.

In addition to nutrients, rivers and streams in urbanized and agricultural areas are often subjected to pesticide loads. Pesticides constitute the other major threat posed to stream

ecosystem functioning, possibly also affecting aquatic microbial communities and their extracellular enzymatic potential. Pesticides include herbicides, fungicides, insecticides, nematicides, plant growth regulators, among other molecules. Among fungicides, tebuconazole (TBZ) is one of the most studied molecules and is frequently detected in surface waters from Europe (e.g. 11% of French rivers, NAIADES database, EauFrance, 2010-2015). TBZ ( $C_{16}H_{22}ClN_3O$ ) is a triazole fungicide that inhibits the sterol C-14  $\alpha$ -demethylation of 24-methylenedihydrolanosterol (which is a precursor of ergosterol, a key component of the fungal cell membrane) thus limiting the development of fungal biomass (Copping & Hewitt, 2007). To our knowledge, only Artigas et al. (2017b) have attempted to assess the acute exposure effect of tebuconazole on extracellular laccase activity from leaf-associated microbial communities. In their study, increasing concentrations of TBZ enhanced laccase activity at the fungal population level (*Alatospora acuminata* species) but decreased ergosterol concentration as well as the amount of 18S RNA gene copies. The authors suggested that the enhanced extracellular laccase activity was probably linked to a detoxification mechanism used by the fungus to reduce TBZ toxicity. A recent laboratory experiment, showed also a stimulation of microbial laccase activity by chronic inputs of TBZ (Rossi *et al.*, 2018). Such results seem to support the potential involvement of laccase activity in microbial stress responses.

Since both stressors (nutrients and pesticides) are likely to co-occur in contaminated streams (Pesce *et al.*, 2008), it appears essential to assess their interaction on the regulation of extracellular laccase activity produced by leaf-associated microbial communities. If nitrates modify laccase regulation in response to TBZ, the former could potentially impair the ability of leaf-associated microbial communities to display proper stress response. Hence, the present study aim to assess the acute exposure effects of the interaction between nitrates and the fungicide TBZ on extracellular laccase activity from alder leaves-associated microbial communities. Acute toxicity tests were performed at increasing concentrations of both TBZ (0, 0.01, 0.1, 1, 10, 20 mg/L of TBZ) and nitrogen (0, 0.1, 0.5, 1, 5, 10, 50 and 100 mg/L of N- $NO_3$ ) for a duration of 36h. We hypothesized that increasing TBZ concentrations would increase laccase activity by triggering detoxification mechanisms, but that high nitrate concentrations would impair the stress response of microbial communities by inhibiting laccase activity. To which extent dose-response toxicity tests results can be transposable to microcosms and/or field observations will be further discussed, using data from microcosm (chapter 3 (Rossi *et al.*, 2017) and field experiments (chapter 1(Rossi *et al.*, 2019) derived from this thesis.



## Material and methods

### Microbial inoculum

Natural microbial communities associated with *Alnus glutinosa* (L.) Gaertn leaves were obtained from the upstream of the Artière River, a well preserved mesotrophic third-order forested stream section in the Puy-de-Dôme region (Centre France). Freshly fallen *Alnus* leaves were harvested in October 2017, transported to the laboratory and dried at room temperature for 72h. Dried leaves were placed into 9 litter bags (10-12 leaves per bag) of 0.5 mm mesh size (l x w = 15 x 15 cm) and immersed in the same stream section (described above) to allow microbial colonization. After 2 weeks of colonization in stream, litter bags were retrieved and leaves were cleaned with filtered stream water (0.2 µm) and cut into small circles (0.8 cm in diameter) that were used for the subsequent toxicity tests.

### Toxicity tests

Acute toxicity tests were used to assess the effects of nitrates and TBZ separately, as well as their interaction on microbial laccase activity. Six concentrations of TBZ were set up (0, 0.01, 0.1, 1, 10, 20 mg/L of TBZ) and for each TBZ concentration, a dose-response curve with increasing nitrogen concentrations was performed (0, 0.1, 0.5, 1, 5, 10, 50 and 100 mg/L of N-NO<sub>3</sub>). Nine replicates were performed per treatment (6 TBZ x 8 N x 9 replicates = 432 samples) to better assess enzymatic activity variability. For each sample preparation, TBZ and nitrogen solutions (issued from stock solutions at 30 mg/L for TBZ and 300 mg/L for N-NO<sub>3</sub>) were dissolved into Ringer's solution (800 µL final volume) transferred into 2 mL Eppendorf tubes containing two colonized leaf disks. Laccase activity was then measured after 36 hours of incubation in the corresponding treatment (TBZ, N-NO<sub>3</sub> or both) in a thermo-regulated chamber (20°C) under agitation (80 rpm) in the dark.

### Laccase activity measurements

Potential laccase activity of microbial communities associated with *Alnus* leaves was measured according to the protocol described in Johannes & Majcherczyk (2000). In each Eppendorf tube, 800 µl of 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS, 3 mM final

concentration) in acetate buffer (pH 4.65, Sigma-Aldrich, St. Louis, Missouri, USA) were added (1600 µl final volume). After 2 hours of incubation, laccase activity was measured spectrophotometrically (415 nm) using an Ultrospec 2000 (Pharmacia Biotech, Trowbridge, UK). All laccase activity measurements were conducted under saturating conditions of substrate (Artigas *et al.*, 2017). The enzymatic activity was expressed as 1 U = 1 µmol ABTS oxidized per g of leaf Dry Mass<sup>-1</sup> h<sup>-1</sup> ( $\epsilon_{420} = 36 \text{ M}^{-1} \text{ cm}^{-1}$ , Johannes and Majcherczyk, 2000).

### **Comparison with microcosms and field experiments**

Data from two published studies (Rossi *et al.*, 2017, 2019a) were used to assess the sensitivity of laccase activity to nitrates and pesticides (including the TBZ) in situations of greater ecological relevance. The dataset from Rossi *et al.* (2017) consisted in an artificial stream channels experiment surveying microbial laccase activity during *Alnus* leaves decomposition using water and microbial communities from the upstream (less contaminated) and downstream (more contaminated) sections of the Auzon stream (Centre France). This dataset contains 24 observations (2 sites x 4 weeks x 3 replicates). For each of these observations, laccase activity and biomass from microbial communities as well as nitrate concentrations and pesticides concentrations in water were measured. The dataset from Rossi *et al.* (2019) consisted in the monitoring of *Alnus* leaves decomposition and its related laccase activity in 6 streams (each including one upstream and one downstream section) from the Puy-de-Dôme region (Centre France) over 4 seasons (spring, summer, winter, autumn). Each stream belongs to a different watershed. According to their land use, watersheds were grouped into 3 categories: forested, urbanized and agricultural (see Rossi *et al.* (2019b)). The dataset contains 144 observations (6 streams x 2 sections x 4 seasons x 3 replicates). As described above, laccase activity, microbial biomass, fungal diversity, nitrate and pesticide concentrations were measured.

## Statistical analyses

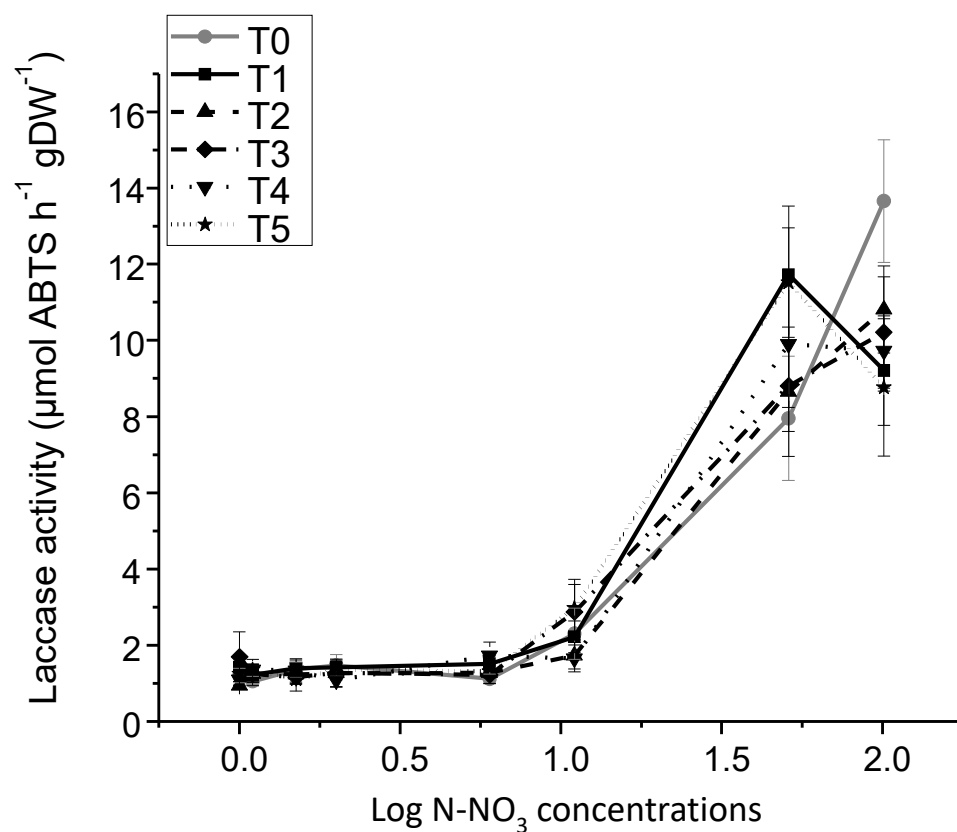
Laccase activity data obtained from the dose-response curve were fitted to a four-parameter logistic curve model to assess the sensitivity to nitrogen at different TBZ concentrations using the following equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(\text{center} - x)}}$$

Where  $y$  is the value of the measured laccase activity,  $x$  is the concentration of nitrogen from the dose response,  $\min$  is the minimum laccase activity value (or baseline),  $\max$  is the maximum laccase activity value (plateau of the curve) and center is the point of inflection of the curve. The Effective Concentration inhibiting 50% of laccase activity ( $EC_{50}$ ) after exposure to nitrogen and TBZ was obtained using the following calculation:  $10^{\text{center}}$ . Fittings were performed using the dosResp non-linear curve fit from the Origin 2016 software. Differences between  $EC_{50}$  and max values among treatments were assessed by permutation test (999 permutations) followed by pairwise comparison tests using Tukey contrast ( $P < 0.05$ ).

Concerning microcosm and field experiments data, relationship between laccase activity and nitrogen concentrations using all dataset ( $n = 165$ ) was assessed by exponential regression using the following equation:  $y = y_0 + Ae^{R_0x}$ . Another set of regressions was performed to assess the relationship between laccase activity and nitrogen concentrations for each section (upstream or downstream) of each type of watershed. Either exponential or linear fitting were used regarding the best fitting based on  $R^2$ . Given the Auzon watershed land use (i.e. mostly forests, less than 17% of agriculture in terms of surface area), data from the microcosm experiment were grouped with the forested watersheds in the field experiment.

## Results



**Figure 1:** Response of laccase activity to increasing nitrogen (N-NO<sub>3</sub>, log scale) concentrations for the different TBZ concentrations (T0 = 0, T1 = 0.01, T2 = 0.1, T3 = 1, T4 = 10 and T5 = 20 mg L<sup>-1</sup>).

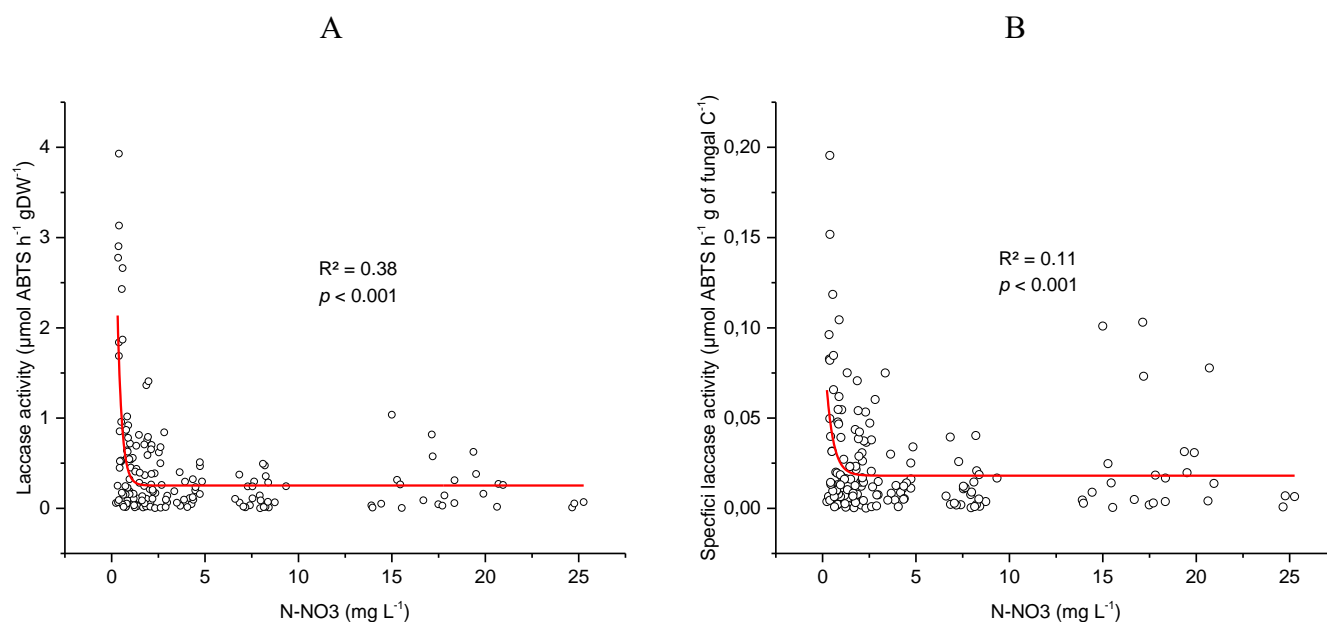
**Table 1:** Parameters estimated from the four-parameter logistic curve fitting regarding the response of laccase activity to increasing nitrogen concentrations at different TBZ concentrations (T0 = 0, T1 = 0.01, T2 = 0.1, T3 = 1, T4 = 10 and T5 = 20 mg L<sup>-1</sup>). Asterisks represent significant difference with the control ( $P < 0.05$ , permutation test, 999 permutations)

[TBZ]	max	slope	EC50	R <sup>2</sup>
T0	13.72 ± 1.56	25.21 ± 4.68	36.45 ± 6.34	0.99
T1	10.78 ± 1.48	20.87 ± 8.79	22.06 ± 4.42	0.92
T2	11.62 ± 1.15	28.58 ± 10.70	36.53 ± 5.91	0.97
T3	10.48 ± 1.37	14.09 ± 4.89	25.21 ± 4.84	0.95
T4	11.17 ± 1.03	23.27 ± 8.73	37.07 ± 6.05	0.94
T5	10.32 ± 1.46	25.20 ± 7.42	18.61 ± 4.55 *	0.86

### Interaction between TBZ and Nitrogen on laccase activity: toxicity test approach

Laccase activity appeared very sensitive to nitrogen concentration increase ( $> 0.5$  mg N-NO<sub>3</sub> L<sup>-1</sup>) in our study, but not to the increase in TBZ concentrations (Fig.1). On average, laccase activity was 818 times higher at the highest nitrogen concentration (100 mg N-NO<sub>3</sub> L<sup>-1</sup>) compared to the control (0 mg N-NO<sub>3</sub> L<sup>-1</sup> of nitrate). When plotting laccase activity as a function of TBZ concentration, non-significant activity variations were observed (data not shown). At the highest nitrogen concentrations, no differences were observed in terms of maximal laccase activity between TBZ treatments, although maximal activity value for the control treatment (absence of TBZ) appeared higher than for the TBZ contaminated ones (ANOVA  $P = 0.51$ , Table 1).

Nitrogen concentration EC<sub>50</sub> coefficients (concentration increasing 50% of laccase activity) were different between TBZ treatments (ANOVA  $P < 0.05$ ). While nitrogen EC<sub>50</sub> concentrations for most of treatments ranged between 22 and 37 mg N-NO<sub>3</sub>L<sup>-1</sup>, the EC<sub>50</sub> at TBZ treatment (20 mg L<sup>-1</sup>) was significantly lower comparing to the control TBZ (36.45 and 18.61 mg NO<sub>3</sub>/L respectively, Tukey test  $P < 0.05$ ).



**Figure 2:** **A** Relationship between laccase activity and N-NO<sub>3</sub> concentrations, **B** relationship between specific laccase activity (corrected by fungal biomass) and increasing N-NO<sub>3</sub> concentrations. Dataset include data observed from both field and microcosm experiment (n = 165). Red solid line represent fitting to an exponential model (see material and methods) with its associated p value and R<sup>2</sup>.

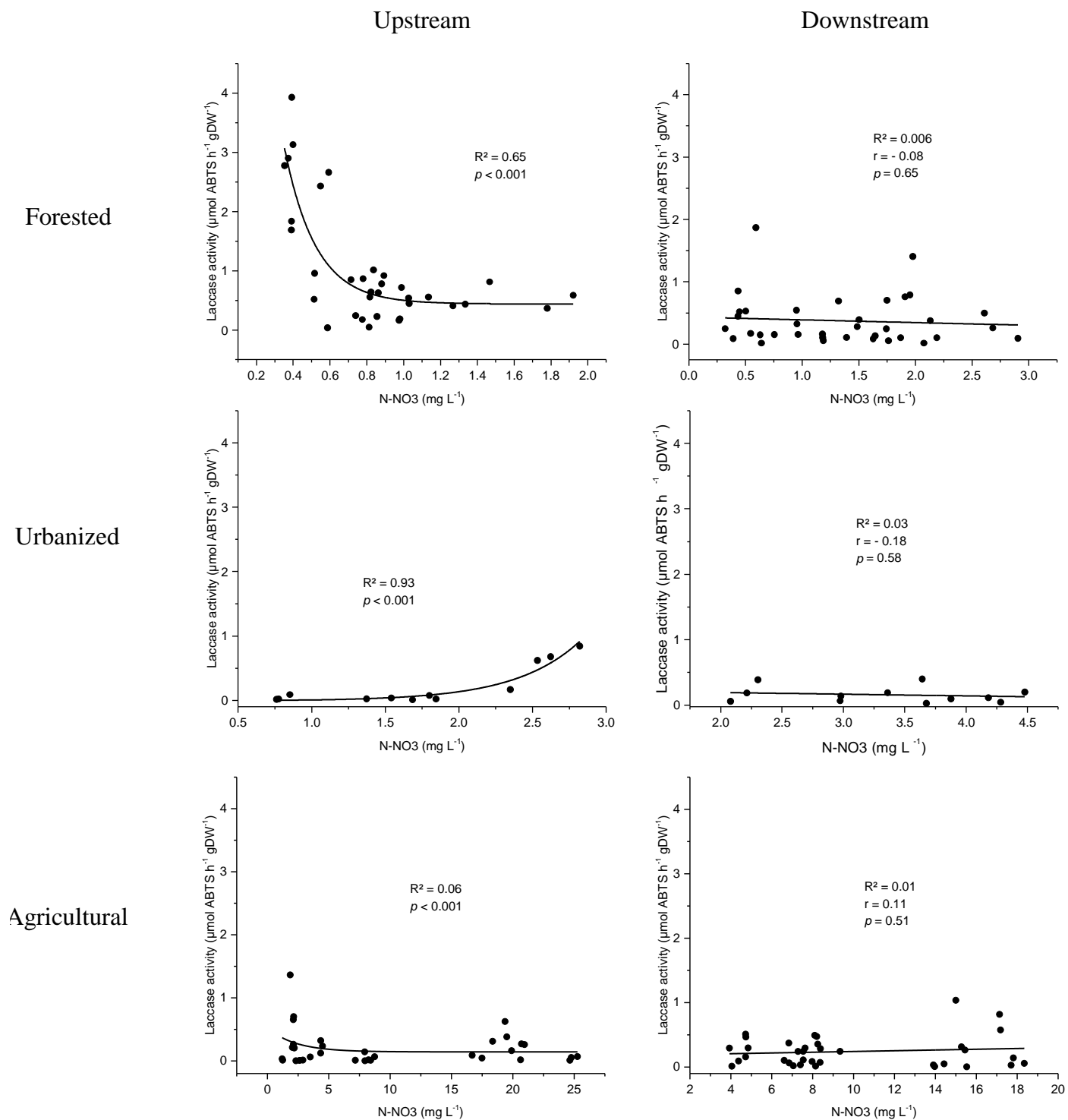
### Relationships between laccase activity, N-NO<sub>3</sub> and xenobiotics: field and microcosm approaches

Nitrate concentrations (in N-NO<sub>3</sub>) observed from both field and microcosm experiments ranged between 0.24 mg L<sup>-1</sup> at the least contaminated stream (Couze upstream) to 25.27 mg L<sup>-1</sup> at the most contaminated one (Charlet upstream). Significant negative correlation between laccase activity and nitrogen concentrations were obtained ( $P < 0.001$ ,  $R^2 = 0.38$ , Fig.2 A). Here, laccase activity decreased exponentially when increasing nitrogen concentration. A similar trend was observed in specific laccase activity (corrected by fungal biomass) but with a weaker fitting ( $P < 0.001$ ,  $R^2 = 0.11$ , Fig.2 B).

When regression analyses between laccase and N-NO<sub>3</sub> were performed separately for each section (upstream and downstream) of each type of watershed (forested, urbanized and

agricultural), different trends were observed (Fig. 3). Overall, laccase activity in upstream sections in all type of watersheds appeared sensitive to N-NO<sub>3</sub> increase, although different relationships were observed. For instance, laccase activity in the upstream sections of the forested watersheds clearly decreased exponentially with N-NO<sub>3</sub> concentration increase ( $P < 0.001$ ,  $R^2 = 0.65$ ). Similarly, laccase activity in the upstream sections of the agricultural watersheds decreased exponentially with N-NO<sub>3</sub> increase, although the fitting of the data to the exponential model appeared much weaker ( $P < 0.001$ ,  $R^2 = 0.06$ , Fig.2 B). In contrast, laccase activity in the upstream section of the urbanized watershed showed a positive exponential relationship with N-NO<sub>3</sub>. ( $P < 0.001$ ,  $R^2 = 0.93$ ). However, amplitudes for laccase activity in the upstream sections of both agricultural and urbanized watersheds were rather small (1.36  $\mu\text{mol ABTS}^{-1} \text{ gDW}^{-1}$  and 0.83  $\mu\text{mol ABTS}^{-1} \text{ gDW}^{-1}$  respectively) compared to the upstream sections of the forested watersheds (3.91  $\mu\text{mol ABTS}^{-1} \text{ gDW}^{-1}$ ). Concerning the downstream sections of all watershed types, no significant correlation between laccase activity and N-NO<sub>3</sub> were observed. Similar trends were observed in the relationship between specific laccase activity and N-NO<sub>3</sub>, regardless of the watershed type or the section.





**Figure 3:** Relationship between laccase activity and N-NO<sub>3</sub> concentrations for each type of watersheds (forested, urbanized and agricultural) at each section (upstream or downstream)

obtained from both field and microcosm experiment. Solid line represent fitting to either an exponential or linear model with its associated p value and R<sup>2</sup>.

## Discussion

In the present study, we investigated the regulation of extracellular laccase activity from microbial communities associated to decomposing *Alnus* leaves exposed to increasing nutrient and fungicide concentrations. Our results confirm those of Artigas *et al.* (2017a) in which laccase activity proved a poor indicator for TBZ acute toxicity assessment of leaf-associated microbial communities. A first explanation for this lack of response to the fungicide could be related to the complexity and interactions within the microbial assemblage colonizing leaves. While Artigas *et al.* (2017a) observed no effect of the TBZ at the community level (including bacteria and fungi) they observed a stimulation in laccase activity in the single fungal strain treatment. They associated this difference to a number of biotic (ex. species diversity and interactions) and abiotic factors (community architecture, molecule exchanges, biofilm structure), limiting the effects of the fungicide at the community level (Lagadic, Caquet & Ramade, 1994; Forbes, Palmqvist & Bach, 2006; Moons, Michiels & Aertsen, 2009; Lear, 2016). A second explanation for the lack of response could be the delay between the exposure to the fungicide and the microbial response (Walker & White, 2017b). Perhaps a 36 hours exposure of communities to the fungicide was not enough to observe a response in terms of extracellular laccase activity in our experiment. A third explanation could be linked to the diversity of microbial enzymatic detoxification mechanisms employed by the analyzed microbial community. Microbial oxidative enzymes other than laccase, such as phenol oxidases, may have been used by the analyzed microbial community to reduce TBZ toxicity. There is evidence in the literature about the ability of certain phenol oxidases to degrade phenolic-like pollutants such as the TBZ (Fragoeiro & Magan, 2005; Mukherjee & Roy, 2013; Mukherjee *et al.*, 2013; Sharma, Dangi & Shukla, 2018a). Further measurements of TBZ metabolites during microbial communities' exposure could permit us to draw conclusions on this question.

The main result of this study revealed a strong response of microbial laccase activity to the increase in nitrogen concentrations, and this response was regardless of the presence of the fungicide. This result is contradictory to what has been suggested in the literature (Saiya-Cork, Sinsabaugh & Zak, 2002; Sinsabaugh, 2010; Rossi *et al.*, 2017), and thus contradicts our initial

hypothesis of laccase activity inhibition by nitrogen. From our results, nitrogen  $EC_{50}$  values were high, ranging between 18 to 36 mg N- $NO_3$  L<sup>-1</sup>. Given the ability of fungi to utilize nitrate for their growth (Walker & White, 2017b), and considering that they were oversupplied in nitrogen in our study, the drastic stimulation of laccase activity probably reflects an osmotic stress suffered by leaf-associated fungal communities. Although we did not measure conductivity in our experiment, conductivity can be expected to be much higher in high N- $NO_3$  treatments in comparison with controls. Besides, fungi are capable of living in high osmotic media by increasing their internal osmotic potential through the synthesis of molecules such as glycerol, trehalose, arabitol, and mannitol, (Walker & White, 2017b). Hence the increase in laccase activity could reflect an increase in decomposition activity in order to compensate the energy costs set up by fungi to cope with the unfavorable conditions. The presence of TBZ in the media also contributes to increase osmotic stress, which might explain the nitrogen  $EC_{50}$  decrease at the highest TBZ concentration treatment (20 mg L<sup>-1</sup>) comparing to the control (without TBZ).

Contrary to the dose-response experiment, the relationship between laccase activity and nitrogen concentrations in stream water (using data from microbial communities in field and microcosms experiment) was negative and this despite the high nitrate concentrations values recorded (i. e. 25.27 mg N- $NO_3$  L<sup>-1</sup> in the Charlet stream) which were close to the  $EC_{50}$  values obtained in the dose-response experiment. However, we suspect different mechanisms to be involved in such differences. In the field and microcosms, relationships between laccase and nitrogen appeared mostly watershed and section-specific. Specifically, extracellular laccase activity in microbial communities from well preserved forested streams was more sensitive to low nitrate variations, whereas this activity appeared less sensitive in the more contaminated sites. Given the involvement of laccase in nitrogen uptake for fungi (Sinsabaugh, 2010), the regulation of such activity in microbial communities in the field can be explained by nitrogen substrate availability in water. The low nitrogen availability in water at the upstream-well preserved sections of the forested watersheds forced the fungi to produce extracellular laccase in order to get nitrogen from the leaves. However, a slight increase in nitrogen concentration in these sections (above 1 mg L<sup>-1</sup> of N- $NO_3$  in water) was sufficient to greatly repressed laccase activity. In contrast, the low sensitivity in laccase activity at the more contaminated downstream sections suggest that other parameters are involved in laccase regulation when nitrogen availability is sufficient. Besides, previous studies have shown that fungal community structure was rather different between watershed types and upstream and downstream sections of the

streams (Rossi *et al.*, 2019b). Considering that laccase activity has been shown to vary considerably from one specie to another for the same substrate (Elisashvili *et al.*, 2006; Mikiashvili *et al.*, 2006; Elisashvili & Kachlishvili, 2009), the observed differences in extracellular laccase sensitivity to nitrogen availability could be mediated by changes in fungal community structure due to the presence of N-sensitive species. However, it has to be noted that physical and chemical parameters as well as laccase activity measurements performed in the field experiment are only punctual samplings. Physical and chemical analyzes of stream water were performed twice per season and per site (at the laying of bags and at their recovery) whereas laccase activity measurements were only performed once (at the recovery of bags). Hence, nitrogen values reported from the field may not be representative of exposure history during the residence time of the bags containing alder leaves and there might be a delay between nitrogen variation and the associated microbial enzymatic response. This is especially the case in the downstream sections where nitrogen inputs can be highly variable due to increased anthropogenic activity in comparison to the upstream ones. The potential gap between the actual nitrogen exposure and pre-exposure history to nitrogen in microbial communities coupled with the different temporal scales (i.e. punctual nitrogen samplings and more integrative potential laccase activity) may probably explain the low  $R^2$  obtained between laccase activity and nitrate in field data, and especially for the downstream contaminated sections.

Given the differences observed between toxicity tests in the laboratory and field and microcosms experiments on extracellular laccase activity regulation by nutrients and fungicides, this study highlights some of the limits of relevance of the toxicological tests in aquatic ecotoxicology (Carpenter, 1996; Gerhardt, 2007). Acute toxicity tests are simple tests that can be useful to determine the potential toxicity of a compound in living organisms and their short term response, but they do not consider the environmental complexity, and hence, their representability to actual stream ecosystems is questionable. Another important issue involves the short term exposure at very high toxicant concentrations provoking an additional osmotic stress to microbes that is rarely observed in the field (Gerhardt, 2007). Although alternatives exist, such as the pollution-induced concept (PICT) which has proven itself quite efficient in many ecotoxicological risk assessment situations (Blanck *et al.*, 1988; Tlili *et al.*, 2016), the reliability of ecotoxicology tests in assessing contamination effects in microbial enzymatic activities can be questionable.

In conclusion, laccase activity from leaf-associated microbial communities appeared rather sensitive to nitrate variations but not to TBZ, and especially in communities issued from stream

sections displaying low anthropogenic pressure. These results further underline the sensitivity of headwater streams to nutrient chemical contamination regarding microbial recycling activities. In addition, we observed a clear gap between the results obtained from the toxicological tests and the in situ/microcosm observations, mostly because of the lack of environmental complexity and the short term exposure at very high concentrations generally used in the toxicological tests. Overall, these findings highlight that great caution should be taken when extrapolating results from toxicological tests to actual stream ecology, and especially regarding microbial communities. Indeed, while such tests might be useful for screening purposes, they need to be combined with ecological or ecotoxicological approaches.

### Main conclusions of the study

We showed that extremely high nitrogen concentrations stimulated microbial laccase activity, and this was probably explained by microbial stress response mechanisms to cope with high osmotic pressure provoked by high nitrate concentration. Conversely, the fungicide tebuconazole did not affect laccase activity.

Relationships between nitrogen and laccase activity followed different trends between laboratory and field and microcosm experiments. In the laboratory, nitrogen stimulated laccase activity while in the field we observed a negative correlation between laccase and nitrates. We found that laccase activity in microbial communities from well preserved stream sections was more sensitive to nitrogen than in communities from contaminated stream sections.

Our study highlight the gap between standard ecotoxicological tests and field observations when trying to perform environmental risk assessment.

## **Conclusions, general discussion and perspectives**



## 6. Conclusions and general discussion

Industry, urbanization and agriculture are the main factors contributing to the massive inputs of chemical compounds into the biosphere over the last century, and streams are among the most exposed ecosystems. From this context raises the question about the effects of multi-chemical contamination stress on stream ecosystem services and functions. In this thesis we assessed the effects of multi-chemical contamination, including that of pesticides, pharmaceuticals and nutrients, on the decomposition activity of heterotrophic microbial communities associated with submerged leaf-litter. Multi-chemical contamination effects were assessed on the structure, biomass and enzymatic activities of microbial communities degrading *alnus glutinosa*'s leaves which is considered a reference species in the majority of leaf-litter decomposition studies. Due to the pivotal role of microbial decomposition on stream carbon cycling, results from this thesis give new insights on the consequences of the global change process potentially affecting whole stream functioning.

### **Effects of multi-chemical contamination on the decomposition activity of heterotrophic micro and macroorganisms associated with submerged leaf-litter**

One of the main results from this thesis revealed that chemical contamination as observed in urbanized and agricultural watersheds (especially by nitrates and pesticides) slightly enhanced microbial decomposition of alder leaves in the field. There are several explanations to such differences in microbial decomposition rates between more and less contaminated watersheds in Chapter 1. One explanation is that high dissolved nutrient availability observed in both urbanized and agricultural watersheds probably masked the negative effects of xenobiotics and even enhanced the microbial decomposition activity. This result agrees with the stimulatory effect of nutrients (both nitrogen and phosphorus) on fungal biomass and thus on microbial litter decomposition (Artigas *et al.*, 2004b; Hagen *et al.*, 2006; Magbanua *et al.*, 2010). Besides, the observed compensatory mechanism between nutrients and xenobiotics has already been described in literature (Fernández *et al.*, 2016; Gardeström *et al.*, 2016). However, in our field experiment (Chapter 1), the slightly higher decay rates of alder leaves in urbanized and agricultural watersheds was accompanied by a lower fungal biomass accumulation in comparison with forested watersheds. In the artificial channel experiment (Chapter 2), pesticide effects (by the herbicide S-metolachlor and the fungicide tebuconazole, alone or in mixture) on

alder decay rates were observed in eutrophic channels but not in mesotrophic channels. These two examples show that high nutrient availability does not always compensate the toxicity of xenobiotics, and that this probably depends on the exposure history of microbial communities to stream water chemical contamination (Feckler *et al.*, 2017). For instance, microbial communities never or less exposed to xenobiotics and/or high nutrient concentrations (i.e. the inoculum used in Chapter 2) were expected to display a greater sensitivity to water contamination than communities long adapted to nutrients and xenobiotics contamination (i.e. communities from urbanized and agricultural watersheds in the field experiment, Chapter 1), mostly because of pollution-induced community tolerance (Blanck, Wängberg & Molander, 1988; Tlili *et al.*, 2016). To some extent, the chronic exposure to xenobiotics and/or high nutrients levels in stream waters from polluted sites can open new ecological niches for microorganisms (Lewis, 2009). Overall, leaf-associated microbial communities have been shown to adapt to stream's water chemical contamination and maintain pivotal ecosystem functions.

In contrast with the microbial decomposition rates in leaves, fungal biomass appeared very sensitive to water chemical contamination in the field experiment (Chapter 1). Here, fungal biomass in leaves showed the lowest values in agricultural and urbanized watersheds and the highest values in those from the forested watersheds. These results joins those from the literature in which fungal biomass is commonly reduced in the presence of xenobiotics (Artigas *et al.*, 2012; Dimitrov *et al.*, 2014; Fernández *et al.*, 2015). The fact that microbial communities from the contaminated watersheds exhibited the lowest fungal biomass but the highest microbial decomposition rates is surprising. This might suggest a higher efficiency in microbial communities subjected to a stress for leaf-litter decomposition, probably explained by fungal community adaptation (Pascoal, Cássio & Marvanová, 2005b; Feckler *et al.*, 2017) and a more efficient resource partitioning (Gessner *et al.*, 2010; Lawrence & Barraclough, 2016) between microbial species. However, the interpretation and representativeness of biomass response to contamination and its link with decomposition rates can be questionable. While leaf decomposition rates measurements are highly integrative of the whole microbial decomposition process, fungal biomass accumulation during leaf decomposition can vary considerably. Unfortunately, we performed just one fungal biomass measurement per season (at the recovery of bags) which complicate the integration of the biomass responsible of the leaf mass loss between the deployment and recovery of bags. To overcome this limitation, an alternative could be to integrate the total amount of fungal biomass accumulated in leaves during the

decomposition experiment. However, this alternative approach would become expensive and highly time consuming.

Indirect effects of stream water chemical contamination on the macroinvertebrate *Gammarus fossarum* feeding activity was not observed in Chapter 2. Since aquatic invertebrates are highly dependent on leaf conditioning by microbes, increasing leaf palatability and nutritional quality, this lack of response can mostly be explained by the similar fungal biomass accumulation in leaves between treatments (i.e. control or treated by pesticides). However, the exposed gammarids ingested slightly less fungal carbon in the mixture of pesticides treatment (tebuconazole + S-metolachlor). This response was not explained by a lower fungal biomass accumulation on leaves, but perhaps by a shift the fungal community structure, that could influence gammarids feeding activity.

Although aquatic invertebrate feeding activity was not assessed in the field experiment (Chapter 1), the lower fungal biomass accumulation in leaves observed in agricultural and urbanized watersheds raises the question of the indirect effects of water contamination on higher trophic level organisms within the stream. Extrapolating the indirect effects of chemical contamination on leaf palatability for macroinvertebrates at the stream ecosystem scale remains a challenge. By combining the results of this thesis and those from the literature, two main hypotheses can be emitted.

- i) The lower fungal biomass observed in agricultural and urbanized watersheds coupled with the presence of complex mixture of pesticides and pharmaceuticals in these sites drastically decreases the nutritional quality of leaves for macroinvertebrates. According to the concept of compensatory feeding (Bärlocher, 1985), we might thus expect macroinvertebrates to consume more leaf material, greatly accelerating the processes of organic matter decomposition in streams.
- ii) The presence of pesticides (TBZ and S-Met, alone or in mixture) deeply affected the structure of microbial communities associated with leaf-litter. As observed by Bundschuh *et al.* (2011), unpalatable fungal species for aquatic invertebrates tend to dominate on leaves exposed to contamination. Hence, an alternative hypothesis could be that be the presence of fungal species with poor attractiveness for macroinvertebrates might deter the latters to feed on leaves, decreasing organic matter decomposition rates in streams.

## Extracellular laccase activity regulation in contaminated streams

Fungi and bacteria use extracellular enzymatic activities to convert polymeric compounds such as cellulose, hemicellulose, and lignin into smaller molecules that can be easily assimilated (Chróst, 1991; Romaní *et al.*, 2006b). The degradation of these compounds involves a multi-enzyme system allowing the simultaneous transformation of lignin and carbohydrates into carbonates (Leonowicz *et al.*, 2001). Hence, these enzymes directly participate in leaf mass loss, and various examples in the literature showed a good correlation between these enzymatic activities and leaf litter decomposition (Artigas *et al.*, 2004b; Sinsabaugh, 2010). However, extracellular enzymatic activities measured in the field experiment (Chapter 1) revealed quite hard to interpret. Both cellulolytic and ligninolytic enzyme activities showed high variability. This is an issue that has also been frequently reported in literature (Sinsabaugh, Carreiro & Rebert, 2002; Artigas *et al.*, 2004b; Rossi *et al.*, 2017). Hence, enzymatic activities were integrated among the whole sampling times in the artificial channel experiment (Chapter 2). Although this assumes to draw enzymatic kinetics (and thus to multiply the sampling effort), this might provide a more relevant picture of microbial cumulative activity during leaf decomposition. Specifically, this activity calculation allowed us to compare the influence of the TBZ and S-Met treatments to control for laccase activity in Chapter 2.

In the different experiments of this thesis, the microbial extracellular laccase activity has shown sensitivity to water chemical contamination including nutrients, fungicide and herbicide. Interestingly, we were able to highlight a positive relationship between such activity and high nitrogen concentrations rather than pesticide toxicity, especially in stream contaminated sections (Chapters 1 and 3). However, stimulations of extracellular laccase activity in presence of tebuconazole was also observed in eutrophic conditions (Chapter 2) or with increasing nitrate concentrations (dose-response experiment, Chapter 4). These results agrees with those of Artigas *et al.* (2017a) who suggested that this activity could be involved in detoxification or stress response mechanisms. Based on our results and those from the literature, we can better define the role and regulation of extracellular laccases in leaf-associated microbial communities in streams:

- Lignin decomposition and mass loss: the main goal of extracellular laccases is to degrade lignin from leaves. Although their relative contribution to the overall leaf mass loss remains unknown, laccases are known to be involved in nutrient uptake (especially Nitrogen). By degrading recalcitrant lignin from the outer plant cell walls, they allow the fungi to access the inner part of the cells, thus releasing nutrients and especially

nitrogen in the media (Osono, 2007; Baldrian & Valášková, 2008b; Horn *et al.*, 2012b; Walker & White, 2017b). This may partially explain the negative relationship between stream water nitrate and microbial laccase activity in leaves observed in Chapters 1 and 3. More globally, lignin degradation by extracellular laccases participates to carbon catabolism and carbon cycling in streams (Walker & White, 2017b). Although we did not observe any correlation between laccase activity and decomposition rates in our experiment, many studies already showed that laccase activity is often correlated with leaf mass loss (Fioretto *et al.*, 2000).

- Detoxification/stress defense mechanism: microbial laccase activity can modify the structure of certain phenolic compounds (including pesticides such as the tebuconazole) and reduce their toxicity. This mechanism was suggested in the study of (Artigas *et al.* (2017a) and could therefore explain the stimulation of both laccase and phenol oxidase activities exposed to the fungicide tebuconazole in Chapter 2. However, laccase could also be involved directly or indirectly in osmotic stress response as shown by the stimulation under high nitrate concentrations in Chapter 4. However, we must note that exposure times to xenobiotics were different between studies. The fact that laccase and phenol oxidase stimulation occurred only 32 days after exposure to the fungicide in Chapter 2 against 36h and 96h, respectively in Chapter 4 and in the study of Artigas *et al.* (2017a) might highlight the time needed for the compound to display toxicity toward fungal cells when fungicide concentrations are low. Alternatively, this might also suggest that the stress response mechanism occurs rapidly after stress exposure but that such rapid response was not observed in Chapter 2 because of the sampling scheme (samplings occurred every weeks).

In addition, the stimulation observed for the extracellular laccase activity in presence of the fungicide was not observed in presence of the mixture of both the fungicide and the herbicide. We hypothesized that such difference might come from an antagonistic-type interaction between the fungicide and the herbicide on this enzymatic activity. We thus concluded that a mixture of pesticide compounds, even at low concentrations and even if they present low threat to the considered organisms, may potentially impair the ability of microbial communities to display proper stress response (Dawoud *et al.*, 2017). However, studying the literature dealing with the effects of xenobiotics on leaf-litter decomposition, only few studies tested the

interaction between eutrophication and different pesticides compounds, and to our knowledge, none of them tested the effect of an herbicide (Flores *et al.*, 2014; Dawoud *et al.*, 2017). Overall, these results highlight the need to include herbicides when assessing pesticide mixtures effects. This would be particularly relevant considering that herbicides currently represent the most frequently detected compounds in rivers and streams worldwide, and that their effect on microbial decomposers might be largely underestimated.

### **Strengths and weaknesses of experimental approaches:**

During this thesis, I studied leaf-litter decomposition processes using different scales analysis: *in vitro* dose-response tests, microcosm experiments and field study. A fundamental question that can be asked in aquatic ecotoxicology is how *in vitro* toxicological tests and microcosms approaches can be ecologically relevant and extrapolated to the field? Dose-responses and microcosm experiments exhibit a large array of advantages: control of many parameters, easy to set-up, less expensive and generally gives fast and easily interpretable results (Carpenter, 1996). Although in some cases simplified models give results similar to what has been observed in field experiments (Larsen *et al.*, 1986), laboratory experiments are generally hard to extrapolate to the field. This is typically what we observed in Chapter 4 when trying to compare results from the dose-response experiment with those from the field study. Here, we argued that such differences were mostly due to the lack of environmental complexity and the conditions of exposure (i.e. high concentrations of stressors and short-time exposure).

Another major issue when trying to simulate stream ecosystems is their upstream to downstream continuum, whereas laboratory experiments only simulate a disconnected portion. As observed in the translocation experiment (Chapter 3), microbial communities can adapt fast to their changing environment (Findlay & Sinsabaugh, 2006; Besemer, 2015). Specifically, we identified that the resilience ability of communities in downstream contaminated sites was mediated by species immigration from communities of the upstream non-contaminated sites (Chapter 3). Such species immigration phenomenon could help to recover microbial communities from acute toxicity episodes and therefore mask xenobiotic effects in long-term surveys in the field.

Another major drawback of laboratory experiments concerns the exposure to mixtures of xenobiotic molecules. While most studies assess the effects of few compounds and their interactions (up to 3 generally), stream water exhibits a much higher number of xenobiotic

compounds, most of them at very low concentrations (see Chapter 1 and 3). For example, Papadakis *et al.* (2015) monitored 302 different pesticide compounds in the basin of Lake Vistonis (Greece) over a 2 year period. Such a mixture of pesticide compounds can lead to complex interactions and therefore complex modelisation of effects on microbial communities. Despite limitations intrinsic to laboratory approaches, researchers agreed that simplified models give valuable information for supportive or heuristic purposes (Carpenter, 1996).

However, despite all these drawbacks, scientists must be able to change scales for a good understanding of ecological processes. While laboratory experiments may not be relevant for the field and hardly extrapolable, they can give information on a potential effect that needs to be then checked in the field.

### **Microbial leaf-litter decomposition as bio-assessment tool for stream waters contamination**

Leaf-litter decomposition has been well documented as a bio-assessment tool for detecting anthropogenic disturbances in the literature (Webster & Benfield, 1986; Gessner & Chauvet, 2002; Sandin & Solimini, 2009). Evidence showed that this tool is quite sensitive, integrative (several weeks to several months), easy to use and relatively cheap (Gessner & Chauvet, 1994; Gessner *et al.*, 1999). However, the use of this tool still undergoes several limitations, such as the lack of standardization (i. e. intra- and inter-species effect, amount of leaves, sampling frequency, leaf species ...) and its relevance in streams and rivers where autotrophic processes dominates. Within leaf-litter decomposition studies, several revealed the greater sensitivity of macroinvertebrate communities to xenobiotic contamination, while reports on the sensitivity of leaf-associated microbial communities to xenobiotics are more contrasted (Piscart *et al.*, 2009). Given the results obtained in terms of fungal biomass for the urbanized and agricultural watersheds in Chapter 1 (i.e. decrease in comparison to forested watersheds), fungal biomass could be a useful complementary metric for bio-assessment purposes, especially in determining stream water contamination effects on the microbial compartment (Gessner & Chauvet, 2002). However, considering that fungal biomass can be highly variable during the incubation time, this suggests to draw kinetics, which implies multiple samplings.

**Table 3:** Framework of the breakdown rate module for assessing functional stream integrity by means of litter breakdown assays; this module can be complemented by a variety of additional modules.

Method	Assessment parameter	Criterion	Score
Comparison with reference	Ratio of breakdown coefficients at impacted ( $k_i$ ) and reference ( $k_r$ ) site	$k_i:k_r = 0.75-1.33$	2
		$k_i:k_r = 0.5-0.75$ or $1.33-2.0$	1
		$k_i:k_r < 0.5$ or $>2.0$	0
Absolute value	Breakdown coefficient at impacted site ( $k_i$ )	$k_i = 0.01-0.03/d$	2
		$k_i = 0.005-0.01/d$ or $0.03-0.05/d$	1
		$k_i < 0.005/d$ or $>0.05/d$	0
Absolute value of ratio	Ratio of breakdown coefficients in coarse ( $k_c$ ) and fine ( $k_f$ ) mesh bags <sup>†</sup>	$k_c:k_f = 1.2-1.5$	2
		$k_c:k_f = 1.5-2.0$ or $<1.2$	1
		$k_c:k_f > 2.0$	0

<sup>†</sup> If sizable numbers of shredders are predicted to occur in the stream.

In contrast with our initial hypothesis, microbial decomposition was not repressed by stream water chemical contamination (including nutrients, pesticides, and pharmaceuticals), but instead appeared slightly enhanced. Gessner & Chauvet, (2002), proposed a framework for assessing stream integrity based on leaf breakdown rates variation. The assessment consisted in the calculation of  $k_{\text{impacted}}/k_{\text{reference}}$  ratios arguing that a ratio  $< 0.5$  or  $> 2$  may be indicative of severely compromised stream functioning (Table 3). Further, they suggested that decomposition rate coefficients varying between 0.75 to 1.25 reveal no clear evidence of impact on ecosystem functioning. In our field experiment (Chapter 1), the ratio  $k_{\text{impacted}}/k_{\text{reference}}$  for the urbanized and agricultural watersheds (each corrected by decomposition rates obtained for the forested watersheds as  $K_{\text{reference}}$ ) were 1.06 and 1.20, respectively. According to these results, the agricultural and urbanized watersheds in our study did not show any impairment in microbial litter decomposition regarding the Gessner and Chauvet (2002) criterion. Yet, results of the field experiment (Chapter 1) clearly highlighted a negative effect of the water chemical contamination from agricultural and urbanized watersheds on fungal biomass, mostly because of pesticides' toxicity. This suggests that the proposed assessment tool is not adapted for assessing microbial decomposition only, and can only be used when assessing overall leaf breakdown rates (including both the microbial and macroinvertebrates contribution to leaf mass loss). Perhaps, complementary analyses would be necessary to adapt this assessment tool to the specific response of microbial decomposer communities. Further, considering that the section where the stream shifts from a heterotrophic-dominated to an autotrophic-dominated metabolism is commonly fixed at Stralher order 3 (Minshall, 1978). Then, assessing microbial



leaf-litter decomposition in streams displaying Stralher order > 3 can be debated, and the adjustments of decay rates should be considered.

**Table 4:** Coefficients of variation in decay rates (K) calculated in the field experiment for each type of watershed including upstream and downstream sections.

	Urbanized		Agricultural		Forested	
	Upstream	Downstream	Upstream	Downstream	Upstream	Downstream
Coefficient of variation (%)	19,8 ± 4.5		13,2 ± 1.8		15,5 ± 3.3	

Other weaknesses regarding microbial leaf litter decomposition process as bio-assessment tool involve reproducibility. Here, we calculated the coefficients of variation (CV) in microbial decomposition rates of leaves using data from our field experiment (Table 4). Results show that 13 to 20% of leaf decomposition rates variability in this experiment can be found between inter-replicate variability of the same experimental condition. This is almost the variation reported by Gessner & Chauvet (2002) between the seven preserved sites that served as basis for their proposed framework (Table 3). These huge differences in terms of leaf-decomposition between replicates can be explained by the localization of the bags in the stream reaches coupled with heterogeneous microbial colonization. Although leaf bags were disposed close to each other and in a very limited space during the field experiment, there might be variations in water velocity sufficient enough to provoke differences on leaf decomposition (Bastias Álamo, 2018). In addition, leaves placed into the litter bags and subjected to water velocity tend to overlap and stack together. Hence, leaves in the middle of the pack may be less colonized than leaves from the outside, possibly leading to variation in leaf decomposition, especially if the number and the size of leaves varied between litter bags (Joly et al., submitted). Overall, these results highlight that further research should be made to provide more standardization to the leaf litter decomposition tool, especially when assessing microbial communities associated to leaf litter.

Beyond the above cited aspects, microbial communities also display high resistance and resilience capacities to stream water chemical contamination. As observed in Chapter 3, fungal communities displayed rather low resistance (one week) but high resilience (two weeks)

capacities after translocation between more contaminated and less contaminated sites. Even if leaf litter decomposition is a highly integrative tool, small contamination events in streams, may be compensated by microbial communities' plasticity and therefore not detected. In conclusion, the results of this thesis show that water chemical contamination (including pesticides and pharmaceuticals) only have a limited effect on microbial decomposition of leaf-litter in stream ecosystems. While overall leaf-litter decomposition (including that of macroinvertebrates) has been proved an efficient descriptor of water chemical contamination and stream functioning impairment, its use for microbial communities' assessment can be questionable unless other microbial descriptors such as fungal biomass are taken into account.

## 7. Perspectives

In the context of global change, human activity has produced and released a huge quantity of chemical compounds into streams and rivers and their effects on microbial communities colonizing leaf-litter are still hard to predict. While microcosm experiments could give a good understanding on the impact of certain chemical compounds on microbes, to the detriment of ecological relevance, testing all the compounds and their potential interactions appears impossible (Schäfer & Bundschuh, 2018). Hence in a river bio-assessment purpose, much more efforts should be allowed to perform field studies in order to assess the effects of chemical contamination, even if it implies more complexity and less understanding. All the data obtained from field experiments may be used for modelisation purpose. Conceptual models have already been described in literature to predict litter decomposition in streams (Graça *et al.*, 2015) or even to predict the effects of climate change and contamination on stream macroinvertebrates (Kattwinkel *et al.*, 2016); (Li *et al.*, 2018). Adapting such models to aquatic microbial communities would provide a powerful tool for stream managers in the context of the Water Framework Directive in environmental risk assessment application.

One major problem of field approaches is how to evaluate the potential toxicity of complex mixtures of xenobiotics to aquatic microorganisms associated with leaves. The first major issue concerns the sampling methods. Beyond the purely technical aspects relative to xenobiotics identification and detection in stream water, and that can only be fixed by improvement of knowledge on xenobiotic compounds and their metabolites coupled with new analytic methods,

samplings often lack representativeness. Actual sampling methods mostly consist in monthly punctual samplings that often miss peak exposures, and thus, underestimate xenobiotic concentrations in streams (Stehle, Knäbel & Schulz, 2013). To overcome this issue, passive samplers (i. e. Polar Organic Chemical Integrative Sampler (POCIS) or Semipermeable membrane devices (SPMDs) among others) have been developed. However, these passive samplers often target specific families of compounds (Vrana *et al.*, 2005) underrepresenting the real diversity of chemical molecules. Moreover, these samplers can saturate rapidly, especially in heavily contaminated streams. Rasmussen *et al.* (2012) used event triggered samplers that can trap water when stream water level increase during rainfall events and further analyzed their content using gas-chromatography mass-spectrometry (GC–MS) or liquid-chromatography mass-spectrometry (LC–MS). Although this allowed to characterize pesticides released in water during the rainfall event, this approach may probably underestimate pesticide concentrations in the case of long rainfall events.

While there are clearly no perfect techniques to evaluate stream contamination by xenobiotics, a good alternative would be to combine all the existing technics. For example, punctual samplings could give information on the basal contamination of the stream whereas passive ones could inform on peak exposures. However, given the cost of chemical analyze and passive samplers, assessing as precisely as possible xenobiotic compounds in stream water will still probably remain a challenge. Hence, focusing on fewer compounds that are more likely to impact the considered organism can remain a good option, even if it implies a loss of information.

The second major concern is to integrate xenobiotic contamination for aquatic microbial heterotrophic communities. The default model to assess the potential toxicity of mixtures of xenobiotics, and the one we used for the field experiment (Chapter 1), is the concentration addition model (CA) using the toxic units concept (TU, i.e. that rely on EC<sub>50</sub> for standard test organisms). Although this model can suffer from serious limitations, especially when dealing with acute effects, it is still recognized as largely protective for the risk assessment of xenobiotics mixtures (Schell *et al.*, 2018). Many reports in literature showed that CA model was the best model to describe the toxicity of a mixture of xenobiotics, especially in the case of very complex ones (Tian *et al.*, 2012; Rodea-Palomares *et al.*, 2015; Schell *et al.*, 2018). Despite this, the predictions of the CA model using TU are highly dependent on the considered organisms. Yet, standard test organisms for freshwater ecosystems include bacteria (such as *Vibrio fischeri*), various microalgae (such as *Scenedemus subspicatus* or *Scenedesmus*

*quadricauda*) and microcrustaceans (such *Daphnia magna* or *Daphnia Pulex*) but lack fungal representatives. Until now, studies assessing the potential toxicity of contaminated-waters to microbial communities colonizing leaf-litter mostly used microalgae as surrogates for TU calculations (Rasmussen *et al.*, 2012; Bundschuh, Goedkoop & Kreuger, 2014; Rossi *et al.*, 2019b) which raises the question of the accuracy of potential toxicity assessment. It becomes obvious that more EC<sub>50</sub> data should be available concerning the effects of xenobiotic compounds on heterotrophic microbes such as aquatic hyphomycetes, which represent the most important contributor to leaf decomposition in streams. Hence, and as already proposed by (Maltby *et al.*, 2009), we propose that aquatic hyphomycetes should be included as standard organisms in the regulatory documentation of the xenobiotic compounds. However, the question of the reliability of a model based on EC<sub>50</sub> on a single strain and its projection at the community scale is still debatable. Hence, an alternative would be to directly assess the EC<sub>50</sub> of each xenobiotic compound on the considered microbial community. Although testing all xenobiotics for all the studied communities appears impossible, testing at least the main detected compounds might give more accurate toxicity informations than estimating an overall toxicity using the TU model.

In addition to xenobiotics, nutrient contamination (especially nitrogen) appears as another major factor affecting microbial decomposition of submerged leaves. While nutrients are commonly associated with positive effects on leaf-associated microbial communities, their interactions with xenobiotic compounds are still unclear. Many literature reports showed that nutrients can compensate xenobiotic's toxicity though we observed that they also can exacerbate their effect. Hence, it would be interesting to assess which nutrients (i.e. inorganic or organic forms of nutrients) and their thresholds (i.e. concentrations of both P and N) are able to compensate or exacerbate pesticide effects on microbes. According to the study of Fernandes *et al.* (2009) assessing the interaction between a range of zinc (0.03 mg L<sup>-1</sup>, 0.98 mg L<sup>-1</sup>, and 9.8 mg L<sup>-1</sup>) and phosphate (0.05 mg L<sup>-1</sup>, 0.2 mg L<sup>-1</sup>, and 0.5 mg L<sup>-1</sup>) concentrations, the strongest inhibition of *Alnus Glutinosa* decomposition was observed in leaves exposed to both zinc and high phosphate concentrations. The authors suggested that such absence of compensation could be explained by the stimulation of some fungal species by nutrients which were at the same time the most sensitive to zinc exposure. To validate the hypothesis of the microbial structure-related compensation of nutrients over xenobiotics, and based on the experiment performed in Chapter 2, we could test the effects of various concentrations of nutrients in microbial communities exposed to a fungicide (tebuconazole) at environmentally

relevant concentrations. The classical descriptors (microbial biomass and activity) would be coupled with next generation sequencing techniques in order to assess precisely the effects nutrient concentrations and the presence of the fungicide on the structure of microbial communities.

The results obtained in Chapter 2 suggest that aquatic fungi associated to leaves might be involved in the transformation of pesticide compounds thanks to their ligninolytic activities (i.e. laccases and phenol oxidases). Many studies already highlighted the involvement of ligninolytic enzymes in phenolic compounds degradation (Fragoeiro & Magan, 2005; Mukherjee *et al.*, 2013; Sharma, Dangi & Shukla, 2018b). However, their potential role in xenobiotic's detoxification processes is still unclear. For example, Carles *et al.* (2017) (Annex 2), reported that microbial communities colonizing submerged leaf-litter were able to bio-transform the herbicide nicosulfuron (used in maize crops), but did not survey the evolution of enzymatic activities. The same authors further isolated from these microbial communities an ascomycete fungal strain, (*Plectosphaerella cucumerina* AR1), which was able to co-metabolize nicosulfuron in the presence of glucose (Carles *et al.* *submitted*). In their study, laccase activity was reduced in the presence of the herbicide nicosulfuron, which contrasts with the detoxification hypothesis presented above. It would thus be interesting to investigate whether laccases and/or other types of phenol oxidases can actually be mobilized by fungi for detoxification/protection purposes or if the observed stimulation in Chapter 2 was attributable to something else. Using the inoculum from chapter 2 experiment (coming from the upstream site of the Couze watershed) and in the presence of decaying alder leaves, we could perform degradation tests at environmental concentrations of TBZ and S-Met (alone or in mixture). Dissipation of both TBZ and S-Met molecules would be assessed over time and accompanied by metabolite production monitoring. Several enzymatic activities such as laccase, phenol oxidase and peroxidase would be monitored, and accompanied by microbial diversity analyses using sequencing techniques in order to highlight pesticide resistant/sensitive species. These measurements could be complemented by RNA sequencing in order to evaluate the implication of specific genes in these detoxification processes and eventually coupled with marked pesticide compounds followed by Stable Isotope Probing (SIP) techniques in order to evaluate whether the compounds were completely mineralized (used as carbon source) or just degraded.

While most of this thesis deals with the effect of chemical contamination on microbial communities and their organic matter decomposition activity, indirect effects of contamination were also investigated on higher trophic-level organisms within the stream food web (i. e. macroinvertebrates). As described in the introduction section, the interface microbes/macroinvertebrates is crucial in stream functioning (i.e. organic matter decomposition and food web support), and microbes play an important role in macroinvertebrate diet and feeding behavior. In Chapter 2, we focused on how different combinations of pesticide compounds (a fungicide and herbicide alone or in mixture) may affect leaf palatability for macroinvertebrates and concluded that environmentally relevant concentrations of these molecules have a negligible impact. However, it has to be noted that studies assessing indirect effects on macroinvertebrates (including ours) are generally short-term exposure experiments (from 12h to several days). In contrast, the effect of prolonged exposure of macroinvertebrates (above 20 days) to food previously contaminated are less studied. Baudy *et al.* (2017) tested the effects of a 56 days exposure of the macroinvertebrate *Hyaella Azteca* to water containing a mixture of fungicides at environmentally realistic concentrations (i.e. 5 and 25  $\mu\text{g L}^{-1}$ ). They showed that such exposure impaired the organism energy budget and reduced their reproductive performance. Considering that many macroinvertebrates have been shown to bio-accumulate pesticides (Katagi and Tanaka, 2016; Lazorchak *et al.*, 2015; Ruhí *et al.*, 2016) and that a part of these compounds are transferred via the food supply (i.e. compounds accumulated on microbial biofilms and leaf material), a longer exposition of macroinvertebrates to contaminated food supply appears relevant. To which extent indirect pesticide bioaccumulation via the trophic chain impairs higher trophic level organisms (biomass, reproduction and feeding activity) and its relative importance compared to direct bioaccumulation (i.e. from water) would be interesting to determine. Based on previous results from Baudy *et al.* (2017), we could test the effects of a long-term exposure (56 days) to water containing i) a mixture of pesticides (including herbicides and fungicides) with clean leaves previously colonized by microorganisms ii) clean water but leaves previously colonized by microorganisms exposed to the same mixture of pesticides, and iii) water containing the mixture of pesticides with leaves previously colonized by microorganisms exposed to the same mixture of pesticides.

Finally, according to the United Nations, the world human population is expected to reach about 9.6 billion in 2050 years and 10.9 billion in 2100 (Bradshaw & Brook, 2014) whereas urbanization level is expected to grow accordingly and reach more than 80% worldwide

(Grimm *et al.*, 2008). To fit such human expansion, global food demand is also expected to increase rapidly, and predictions on global crop demand in 2050 have been estimated to 100 to 110% increase (Tilman *et al.*, 2011), probably accompanied by an augmentation of fertilizers and pesticides use despite some willingness to diminish them. Obviously, such human expansion will have serious repercussions on freshwater resources, especially considering that water used for irrigation accounts for 80% of the total freshwater consumption (Rosegrant, Cai & Cline, 2002). Hence, xenobiotic and nutrient concentrations in stream ecosystems can be expected to drastically increase within the next 50 years. In addition to these findings, the predicted increase in stream water temperature due to climate change is expected to increase by 1.0–2.2°C by 2100 (van Vliet *et al.*, 2013), whereas the frequency and magnitude of runoff events will be reduced, potentially leading to higher occurrence of drought events. Up to now, only few studies already tested the interaction between drought event and a fungicide or between nutrients and mixtures of fungicides (Pesce *et al.*, 2016; Fernández *et al.*, 2016), but none included both nutrients, drought and pesticides. Considering that context, testing the triple interaction between nutrient increase, drought events and the presence of pesticides in mixture on organic matter decomposition processes in streams appears relevant.

Besides, temperature variations might also affect the fate and effects of xenobiotics in stream ecosystems. Indeed, temperature increase may influence sorption of xenobiotics (Gavrilescu, 2005) in soils and in streams. Another consequence of water temperature increase involve the decrease in dissolved oxygen, in turn leading to a decrease in water pH, further affecting the sorption properties and the toxicity of xenobiotic compounds in the water (Delpla *et al.*, 2009). To which extent stream water temperature increases might affect the fate and effects of xenobiotics in streams, further affecting leaf-associated microbial communities? Addressing that question by testing the sorption on stream sediments and leaves of a pesticide compound under different temperature scenarios and evaluating the resulting effects toward microbial communities as well as their responses could give valuable informations for the future ecotoxicological risk assessment in stream ecosystems.

## **Extended French summary**



La grande majorité de la surface de la Terre (>70%) est recouverte d'eau. Alors qu'une partie importante de cette eau se retrouve dans les océans et les mers sous forme d'eau salée (>96%), une infime partie seulement (0.01%) se trouve dans les glaciers, les nappes phréatiques, les lacs et les rivières à l'état d'eau douce (Shiklomanov, 1993b). Pourtant, ces écosystèmes d'eau douce revêtent d'une importance capitale, à la fois en terme de valeur écologique (plus de 100 000 espèces biologiques retrouvées dans ces écosystèmes, soit 6% des espèces actuellement connues) mais aussi en terme de valeur socio-économique (Dudgeon *et al.*, 2006). Le bien être des hommes et le fonctionnement des sociétés modernes reposent en grande partie sur les apports en eau douce, que ce soit pour satisfaire les demandes en eau potable comme celles pour l'agriculture, l'industrie, la production d'énergie, le transport ou encore les activités récréatives (pêche, baignade, ...). Malheureusement, cette surexploitation de la ressource en eau, couplée à la modernisation des techniques d'agriculture (utilisation de pesticides entre autre) et leur intensification a conduit à l'introduction massive de nutriments et de xénobiotiques dans un grand nombre d'écosystèmes aquatiques.

A l'heure actuelle, les rivières et les cours d'eau font certainement partie des écosystèmes aquatiques les plus impactés de la planète. C'est plus particulièrement le cas des cours d'eau de tête de bassin versant, dont la très petite surface du lit de la rivière les rend très sensibles aux intrants de composés chimiques. Pourtant, malgré leur petite taille et les faibles débits généralement observés dans ce type d'écosystèmes, ils revêtent d'une importance capitale. Outre le fait qu'ils représentent la grande majorité des réseaux hydrographiques en termes de linéaire (entre 60% et 70% du linéaire total), les cours d'eau de tête de bassins versant constituent une réserve importante d'espèces et jouent donc un rôle primordial dans le maintien de la diversité et de la fonction des écosystèmes lotiques, notamment dans leur partie aval.

En l'absence de perturbations anthropiques, les cours d'eau en tête de bassin versant se caractérisent généralement par la présence d'une végétation riveraine. Cette dernière a pour effet non seulement d'apporter de l'ombre au niveau du lit du cours d'eau mais également de fournir ce dernier en matière organique, telle que des litières de feuilles, des branchages ou bien encore toute autre forme de matière organique d'origine végétale (fruits, spores, etc...). En conséquence, il est admis que le fonctionnement des cours d'eau de tête de bassin versant est très largement basé sur l'hétérotrophie. Les processus de décomposition de la matière organique ont donc un rôle primordial dans le fonctionnement de ces écosystèmes, permettant non seulement de faire le lien avec les écosystèmes terrestre, mais permettant également le transfert

d'énergie et de nutriment au travers du réseau trophique au sein de la rivière (Cummins, 1974; Gessner & Chauvet, 1994; Webster & Meyer, 1997).

La matière organique d'origine allochtone (c.-à-d. depuis la ripisylve) arrivant au niveau du lit du cours d'eau peut être répartie dans deux catégories : les apports verticaux (directement depuis les arbres) et les apports latéraux (depuis les sols avoisinants). Dans les deux cas, la grande majorité de ces apports est essentiellement constituée de litière de feuilles. Suivant le type d'espèces végétales constituant la ripisylve, l'ordre de Strahler ou encore le degré de perturbation anthropique du cours d'eau (végétation riveraine altérée ou non), la contribution de la litière de feuilles varie entre 41% et 98% de l'apport en matière organique allochtone totale. D'autres facteurs tels que la saison interviennent dans les apports en matières organiques. Par exemple, la majorité des apports en litières ont lieu en automne dans les cours d'eau forestiers tempérés, tandis que pour les cours d'eau de régions plus arides (p. ex. Australie), ceux-ci ont lieu majoritairement au printemps ou en été (Abelho & Graça, 1996b, 1998b; Grigg & Mulligan, 1999b).

Dans les cours d'eau de tête de bassin versant, on distingue deux facteurs majoritairement impliqués dans la décomposition des litières de feuilles : les facteurs abiotiques (lessivage et fragmentation physique essentiellement, Petersen and Cummins, 1974; Benfield, 1996; Gessner *et al.*, 1999) et les facteurs biotiques (intervention d'organismes). Parmi les facteurs biotiques, les communautés microbiennes hétérotrophes ont un rôle primordial. Composées principalement de champignons aquatiques et de bactéries organisés sous forme de biofilms à la surface des feuilles, ces communautés sont capables de coloniser ces dernières quelques heures à peine après leur entrée dans le cours d'eau. La contribution des communautés microbiennes dans la décomposition des feuilles est estimée variant entre 25 et 45% de la perte total de masse (Bärlocher & Kendrick, 1975; Hieber & Gessner, 2002; Baldy *et al.*, 2007b). Les champignons, et notamment le groupe des hyphomycètes aquatiques sont souvent considérés comme étant les plus fortement impliqués dans ces processus (Duarte *et al.*, 2010; Fischer *et al.*, 2006; Pascoal and Cássio, 2004), bien que certains rapports fassent état de contribution similaires entre bactéries et champignons aquatiques (Hieber & Gessner, 2002). L'activité de décomposition chez les micro-organismes est possible notamment grâce à l'utilisation d'un large panel d'enzymes extracellulaires, telles que les enzymes cellulolytiques (qui dégradent la cellulose, par exemple les  $\beta$ -glucosidases et les cellobiohydrolases, (Romaní *et al.*, 2006a)) et les enzymes lignolytiques (qui dégradent la lignine, par exemple les laccases, les phénols

oxydases et les peroxydases, Abdullah et al. 1989; Chandrashekar & Kaveriappa 1991; Abdel-Raheem & Ali 2004).

Outre leur contribution directe dans la perte de masse des feuilles, les communautés microbiennes hétérotrophes ont également un rôle important dans le conditionnement du substrat feuille et son appétence pour des organismes de niveau trophiques supérieurs tels que les macro-invertébrés déchiqueteurs (Mann, 1975b; Suberkropp *et al.*, 1976; Gessner *et al.*, 1999; Gaudes *et al.*, 2009). En effet, bien que la contribution de ces derniers dans la perte de masse des feuilles soit très largement supérieure à celles des micro-organismes, la présence de micro-organismes à la surface des feuilles fournit aux macro-invertébrés des micronutriments généralement présents en faibles quantités dans les tissus végétaux. De plus, le fait que les feuilles aient été partiellement dégradées par les micro-organismes les rend plus facilement assimilables par les macro-invertébrés. L'activité des communautés microbiennes hétérotrophes associées aux litières végétales est donc déterminante dans la décomposition de la matière organique, et donc par extension, dans le maintien des réseaux trophiques de cours d'eau de tête de bassin versant.

Cependant, à l'heure du développement des sociétés humaines et de la mondialisation, les activités anthropogéniques telles que l'agriculture, l'urbanisation, l'industrialisation ou encore les activités de transport ont un effet néfaste sur les écosystèmes de rivière. On peut citer par exemple des modifications du lit du cours d'eau pour faciliter le passage des bateaux, la suppression des ripisylves en bord d'activité agricole ou encore la contamination chimique des cours d'eau par des molécules xénobiotiques (pesticides, pharmaceutiques) ou des nutriments (provenant de fertilisants). Toutes ces modifications ont malheureusement des effets importants sur la biodiversité des cours d'eau ainsi que sur les services écosystémiques qu'ils assurent (Harding *et al.*, 1998; Sala *et al.*, 2000).

En Europe, et en France notamment, l'une des principales causes de l'altération des écosystèmes lotiques est l'eutrophisation. Bien que l'eutrophisation soit un phénomène naturel, il est très largement reconnu que l'activité humaine a contribué très fortement à l'apport massif de nutriments (phosphore et nitrate notamment) dans les cours d'eau au cours des dernières décennies. On peut distinguer des causes directes et indirectes. Les causes directes correspondent à l'introduction directe de nutriment dans l'eau (fertilisateurs en agriculture, rejets de station d'épuration ou encore rejets domestiques) tandis que les causes indirectes résultent

de modifications physiques du cours d'eau et de ses alentours (déforestation), augmentant les apports en nutriments par lessivage des sols.

L'Europe fait partie des régions les plus touchées par l'eutrophisation des cours d'eau (European Environment Agency, 2010, Leaf, 2017). Tandis qu'une nette amélioration peut être observée en termes de phosphates (diminution d'un facteur de 3 au cours des 20 dernières années), les concentrations en nitrates tendent à rester stables, avoisinant les 2 mg L<sup>-1</sup> (Water Information System for Europe, WISE WFD Database, Malaj *et al.* (2014)). Ces fortes concentrations ne sont pas sans conséquences sur les communautés aquatiques et les fonctions écosystémiques qu'elles assurent (Vitousek *et al.*, 1997a b; Nijboer *et al.*, 2004). De manière générale, il est admis que l'augmentation des concentrations en nutriments provoque une augmentation des processus de décomposition microbiens des litières de feuilles (Ferreira *et al.*, 2015). Cette augmentation d'activité s'explique en grande partie par une augmentation de la biomasse fongique qui s'accompagne d'une stimulation des activités enzymatiques extracellulaires associées (Artigas *et al.*, 2004b; Hagen *et al.*, 2006; Magbanua *et al.*, 2010). Bien qu'il soit difficile de prédire les effets de l'eutrophisation à long terme, cette augmentation de l'activité microbienne pourrait profondément altérer le fonctionnement des cours d'eau, notamment en accélérant les processus de décomposition et de la minéralisation de la matière organique de l'écosystème.

Outre l'eutrophisation, la contamination chimique des cours d'eau par des pesticides constitue la seconde majeure source de perturbation. Ces composés xénobiotiques peuvent être classés en fonction de leur mode d'action, et comptent notamment les herbicides, les fongicides, les insecticides ou encore les nématicides. Leur utilisation concerne un grand nombre de domaines, allant de l'agriculture à l'entretien des espaces verts. Cependant, le changement des pratiques agricoles dans le but d'augmenter les rendements (c.-à-d. utilisation d'un grand nombre de molécules différentes à faible grammage et en mélange pour augmenter leur efficacité) constitue la principale cause de contamination globale des cours d'eau. En 2015, on compte une consommation globale en pesticides d'environ 250 000 tonnes de molécules actives avec une tendance stable depuis 2010. En Europe, plus de la moitié des cours d'eau sous surveillance présentent une contamination par au moins une molécule de pesticide (WISE WFD Database, Malaj *et al.* (2014)). Leurs effets sur la décomposition de la litière de feuilles dans les cours d'eau ont fait l'objet de nombreuses études au cours de la dernière décennie, et notamment les fongicides dont le mode d'action les rend susceptibles d'impacter les champignons aquatiques. De manière générale, l'exposition aux fongicides entraîne une diminution des taux de

décomposition microbiens qui peut s'expliquer notamment par une diminution de la biomasse fongique associée à des modifications de la structure génétique de la communauté (Zubrod *et al.*, 2011a, 2015b a; Bundschuh *et al.*, 2011a; Artigas *et al.*, 2012). Cette diminution en termes de biomasse a également été démontrée comme pouvant affecter la qualité nutritionnelle de la feuille, diminuant alors les taux de décomposition associés aux macro-invertébrés (Zubrod *et al.*, 2011a; Bundschuh *et al.*, 2011a). En comparaison, il existe très peu de littérature concernant l'effet des herbicides et insecticides sur les communautés microbiennes associées aux feuilles. Sachant que les herbicides peuvent avoir un impact direct sur les organismes autotrophes (Villeneuve, Larroudé & Humbert, 2011b; Pesce, Bouchez & Montuelle, 2012) et sachant que ces derniers ont un effet positif sur la décomposition de la litière de feuilles, il est possible que la présence d'herbicide puisse diminuer de manière indirecte la décomposition microbienne.

Une autre cause de contamination chimique des cours d'eau concerne les composés pharmaceutiques, dont la présence dans les cours d'eau a suscité un intérêt relativement récent en comparaison des pesticides. Il existe environ 4 000 molécules pharmaceutiques réparties dans différentes familles, incluant les antibiotiques, les bêta-bloquants, les anxiolytiques, les antihypertenseurs, les anti-inflammatoires, les analgésiques ou encore les stimulants (Monteiro & Boxall, 2010). La plupart de ces composés ont été développés pour des utilisations humaines ou vétérinaires, mais une partie d'entre eux (notamment les antibiotiques) sont également utilisés en agriculture pour lutter contre certaines maladies (Rosi-Marshall & Royer, 2012). L'occurrence des produits pharmaceutiques dans les cours d'eau est relativement variable, en fonction des régions ou encore des saisons. Cependant, certains antibiotiques tels que le triméthoprim, la ciprofloxacine ou encore le sulfaméthoxazole sont fréquemment détectés à des concentrations comprises entre 10 et 2000 ng/L. Les quelques études ayant tenté d'évaluer les effets de produits pharmaceutiques, tels que les antibiotiques, sur les processus de décomposition n'ont montré que des effets négligeables à concentrations environnementales (Bundschuh *et al.*, 2009, 2017; Jonsson *et al.*, 2015). Seules de fortes concentrations (>100 µg/L) semblent avoir des effets significatifs sur les communautés microbiennes (Maul *et al.*, 2006). Cependant, étant donné la diversité des molécules pharmaceutiques et leur occurrence dans les cours d'eau, des effets significatifs de ces composés, dus notamment à des interactions entre eux, sur les communautés microbiennes et leur activité de décomposition de la matière organique ne peuvent pas totalement être exclus.

La contamination chimique des cours d'eau en Europe, et dans le monde d'une manière générale, est devenue particulièrement préoccupante, d'autant que les effets sur les organismes

aquatiques, sur l'intégrité des écosystèmes voir sur la santé humaine, sont encore mal connus. Devant ce constat alarmant, le 23 octobre 2000, le parlement européen décide d'adopter la Directive Cadre sur l'Eau (DCE) ou Directive 2000/60/EC. Cette directive, adoptée par l'ensemble des pays de l'Union Européenne, consiste à établir un plan d'action pour le retour au bon état chimique et écologique des cours d'eau en Europe. Le bon état chimique est défini comme la conformité des cours d'eau par rapport aux normes de qualités établies au niveau européen en termes de substances chimiques. Il est établi sur la base de seuils pour de nombreuses substances (y compris les nutriments, les métaux lourds et les xénobiotiques) et une liste de 45 substances prioritaires présentant un risque important pour les environnements aquatiques. Le bon état écologique est défini comme l'état de l'écosystème en l'absence de perturbation et désigne principalement l'intégrité des communautés biologiques ou leur déviation par rapport à un système sans perturbations (système de référence). Cette évaluation repose sur l'intégrité du réseau trophique, allant des producteurs primaires (micro algues, telles que les diatomées) jusqu'aux consommateurs que sont les macro-invertébrés et les poissons. Il intègre également des notions telles que l'hydro-morphologie du cours d'eau (c.-à-d. l'absence de modification du lit du cours d'eau) et la physico-chimie de l'eau qui sont des paramètres importants dans le maintien des espèces dans les écosystèmes lotiques.

Bien que la qualité des cours d'eau en Europe et en France se soit grandement améliorée au cours des cinquante dernières années, les objectifs de la Directive Cadre sur l'Eau n'ont toujours pas été atteints. Prévu initialement pour 2015, ces derniers ont par la suite été reportés pour 2027. Par ailleurs, selon les rapports de l'Agence Européenne pour l'Environnement, plus de 57% des cours d'eau en Europe présenterait un statut écologique inférieur au statut « bon » de référence. De plus, les critères d'évaluations de la qualité chimique et écologique peuvent largement être remis en cause. En effet, il subsiste un manque de connaissances probant sur le nombre et la nature des composés chimiques (tels que les pesticides ou les pharmaceutiques) présents dans les cours d'eau. Les récents progrès en chimie analytique permettent régulièrement de mettre en évidence de nouveaux composés xénobiotiques. Il faudrait également prendre en compte les métabolites (produits de dégradation), dont la toxicité peut largement excéder celle des molécules mère (Carles, 2016).

Devant ce constat, il devient évident que les indicateurs utilisés par la DCE sont insuffisants. Les indices actuels de la qualité biologique des eaux de surface reposent essentiellement sur la densité et la présence / l'absence d'espèces sensibles telles que les diatomées, les macro-invertébrés ou encore les poissons. Cependant, ces indicateurs n'intègrent pas les aspects

fonctionnels de l'écosystème. De plus, les communautés microbiennes hétérotrophes, bien que à la base du réseau trophique et jouant un rôle important dans le recyclage de la matière organique, ne sont pas prises en compte. Dans ce contexte, l'utilisation de la litière de feuilles comme outils afin d'évaluer l'intégrité fonctionnelle des écosystèmes lotiques apparaît donc pertinent (Gessner & Chauvet, 2002; Young *et al.*, 2008). En effet, il s'agit d'un outil sensible et intégrateur. Cette thèse a donc pour objectif d'évaluer les effets de contaminations chimiques complexes, dans un contexte de changement global résultant de l'activité humaine, sur les processus microbiens de décomposition de la matière organique par l'intermédiaire de la litière de feuilles comme descripteur.

### Structure et objectifs de la thèse :

A l'heure actuelle, de nombreux travaux scientifiques ont tenté de mesurer les effets de composés xénobiotiques et/ou des nutriments sur la décomposition de la litière de feuilles. En revanche, la grande majorité de ces travaux sont des expériences de laboratoire, utilisant souvent des concentrations en xénobiotiques (pesticides ou autres) bien au-delà de celles généralement retrouvées dans l'environnement (Zubrod *et al.*, 2011b). Bien que les expériences de laboratoire, telles que les approches en microcosmes, possèdent un grand nombre d'avantages (paramètres contrôlables, faciles à mettre en œuvre et peu coûteuses) et peuvent fournir des réponses rapides à des questions très spécifiques, leur nature simplifiée pose la question de leur représentabilité par rapport aux réponses dans les écosystèmes naturels. Notamment se pose la question de la difficulté d'extrapoler ces résultats au milieu naturel. Il semble donc nécessaire d'effectuer d'avantage de mesures *in situ* des effets de contaminations chimiques sur la décomposition de la litière de feuilles.

Dans les cours d'eau exposés aux contaminants, les xénobiotiques sont systématiquement présents sous forme de mélanges avec d'autres composés, tels que les nutriments. Pourtant, la plupart des études n'ont testé l'influence que des nutriments ou des pesticides seuls (Gulis & Suberkropp, 2003; Dimitrov *et al.*, 2014). Au regard des interactions potentielles nutriments/pesticides ou pesticides/pesticides qui peuvent avoir lieu dans l'environnement, il apparaît donc nécessaire d'évaluer de telles interactions. Quelques études ont néanmoins étudié les interactions entre nutriments et fongicides ou bien entre fongicides et insecticides (Flores *et al.*, 2014; Dawoud *et al.*, 2017). Mais les interactions entre pesticides en mélange et nutriments n'ont encore jamais été évaluées. Etant donné que les herbicides sont les composés les plus

fréquemment détectés dans les cours d'eau, il serait intéressant de voir comment ils influent, directement ou via leurs interactions avec les nutriments et/ou un fongicide, sur la décomposition de la litière de feuilles.

Un grand nombre d'étude a déjà mis en évidence des effets de la contamination chimique sur la structure des communautés microbiennes et leur activité de décomposition de la litière de feuilles (Rasmussen *et al.*, 2012). En revanche, leur capacité à résister ou à récupérer de ce type de stress a moins été étudiée. Dans le contexte de la DCE qui vise à supprimer les intrants de xénobiotiques et de nutriments dans les cours d'eau, il semble donc pertinent de savoir si les communautés microbiennes ayant subies une exposition prolongée à des stress chimiques peuvent retourner à un état pré-contamination.

Bien que la transformation et la minéralisation de la matière organique dans les cours d'eau par les activités enzymatiques microbiennes extracellulaires aient bien été étudiées, les effets de contaminations chimiques sur ces processus enzymatiques restent mal compris. La littérature récente suggère que l'activité laccase, une enzyme lignolytiques extracellulaire, pourrait être impliquée dans les mécanismes de réponse au stress et de détoxification chez les champignons aquatiques. Cette enzyme pourrait donc être utilisée comme outil de bio-évaluation de contamination chimique des cours d'eau.

Compte tenu de ces lacunes, les objectifs de cette thèse sont donc:

i) de comparer l'activité de décomposition microbienne de la litière de feuille *in situ* dans des bassins versants présentant différentes utilisations du sol (agriculture, urbanisation, forêts) et gradients de contamination (sites en amont peu contaminés et en aval plus contaminés) et de discriminer qui des paramètres physico-chimiques (nutriments, conductivité, oxygène, pH, entre autres) ou des xénobiotiques (produits pharmaceutiques et pesticides) influencent le plus ce processus (chapitre 1).

ii) d'évaluer *in vitro* (approche en microcosmes) les effets des interactions entre l'eutrophisation (conditions mésotrophe ou eutrophe) et la contamination par des pesticides (fongicide, herbicide et mélange des deux) des cours d'eau sur l'activité de décomposition microbienne. Nous considérerons également les effets de cette contamination multiple sur l'appétence des feuilles pour les macro-invertébrés (chapitre 2).

iii) d'évaluer, par une expérience de translocation (transfert des communautés d'un site pollué vers un site moins pollué et inversement) en conditions contrôlées (microcosmes), la



capacité de résistance et de récupération des communautés fongiques et de leur activité de décomposition de la litière de feuilles, à une contamination chimique multiple (chapitre 3).

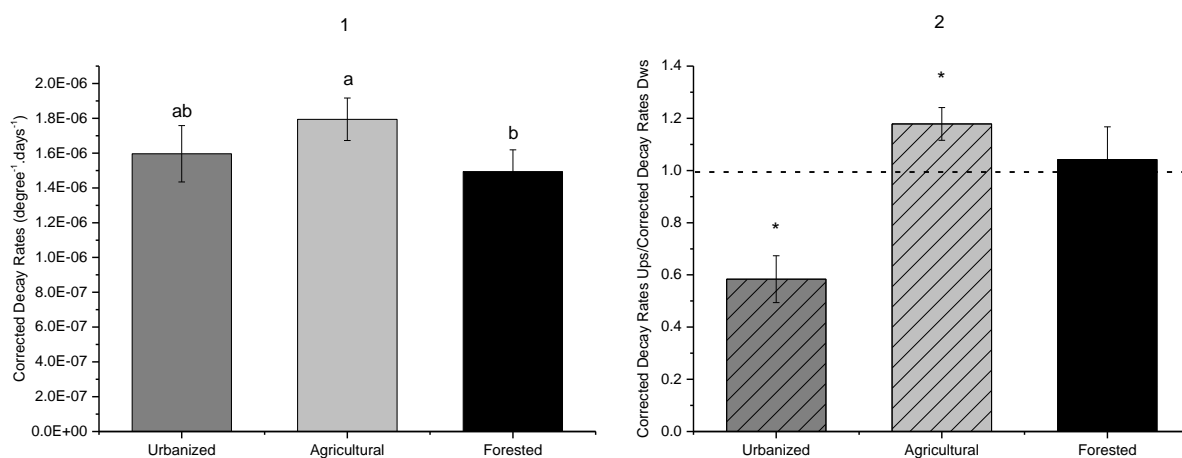
iv) d'étudier *in vitro* (tests doses-réponses) la réponse de l'activité laccase extracellulaire chez les communautés microbiennes exposée à des concentrations croissantes d'un fongicide et de nitrates (chapitre 4). Les résultats de cette étude seront ensuite comparés avec ceux obtenus au cours des chapitres 1 et 3 afin de mieux comprendre la régulation de l'activité laccase extracellulaire soumise à une contamination chimique.

### Chapitre 1: Stimulation ou inhibition : décomposition microbienne des feuilles dans des cours d'eau soumis à une contamination chimique complexe

Dans un premier temps, l'objectif était de définir un état des lieux des processus de décomposition microbiens dans les cours d'eau soumis à différents types de contaminations (non impacté ou forestier, agricole et urbain). Pour ce faire, six cours d'eau appartenant au bassin versant de l'Allier ont été sélectionnés dans le département du Puy-de-Dôme (Centre France). Le choix de ces bassins versants s'est effectué en collaboration avec le groupe Eaux et Produits Phytosanitaires en Auvergne-Rhône-Alpes, ex Phyt'Eauvergne (<http://www.eauetphyto-aura.fr>), chargé de la surveillance de la qualité chimique (pesticides) des cours d'eau en Auvergne, et l'Agence de l'Eau Loire-Bretagne, à partir de données de surveillance de pesticides dans l'eau sur une période de 5 ans ainsi que des pourcentages d'occupation des sols. Pour chaque cours d'eau, un site amont (proche des sources) et un site aval (à la confluence avec l'Allier) ont été sélectionnés afin d'évaluer les effets du gradient de contamination sur les processus de décomposition microbiens.

Afin d'évaluer les effets de l'occupation des sols et du gradient de contamination (amont/aval) sur l'activité de décomposition microbienne des litières de feuilles, des sacs à mailles fines (0.5 mm de diamètre) contenant de la litière de feuilles d'aulne (*Alnus glutinosa*) ont été disposés en triplicat sur chaque site (amont et aval de chaque cours d'eau, n = 36). La variabilité temporelle a également été étudiée en répétant l'expérience à chaque saison entre juin 2016 et mars 2017 (printemps, été, automne et hiver, n = 144 au total). Les taux de décomposition microbiens et les activités enzymatiques associées (laccases, phénol oxydase, peroxydase, cellobiohydrolase et  $\beta$ -glucosidase) ainsi que la structure et la biomasse des communautés

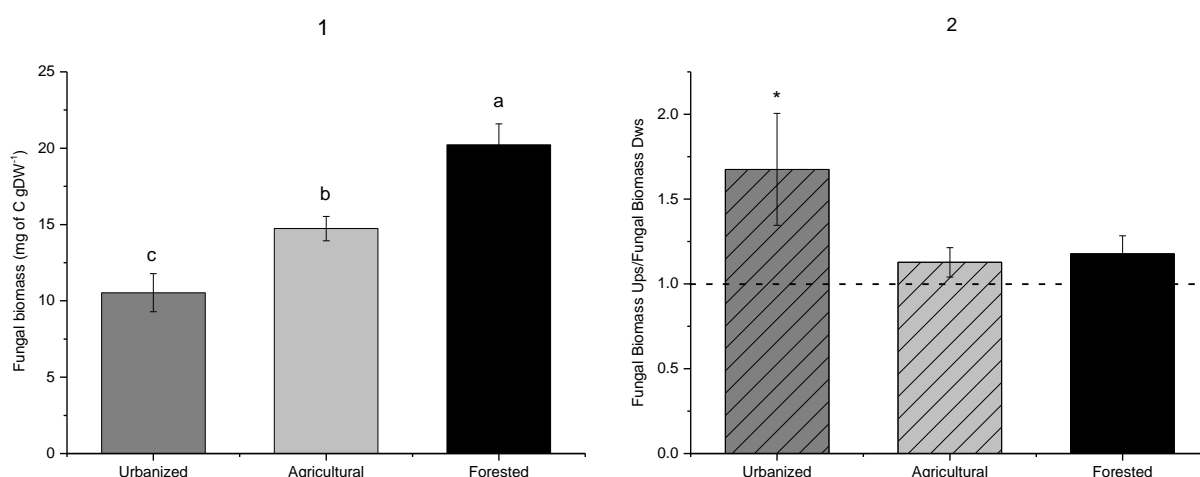
microbiennes associées aux litières de feuilles (champignons et bactéries) ont été utilisés comme descripteurs. Afin de limiter l'effet de la température dans notre étude (notamment due aux variations entre saisons), les taux de décomposition obtenus ont été corrigés par la température cumulée lors de la durée de l'incubation des feuilles à chaque site. En parallèle, des mesures physico-chimiques de l'eau ont été réalisées (phosphate, nitrate, carbone organique et inorganique, pH, conductivité, oxygène dissous) et couplées à des analyses de résidus de pesticides et de produits pharmaceutiques.



**Figure 1: 1** Taux de décomposition microbiens en degrés jours pour chaque type de bassin versant (moyenne des amonts et avals pour les 4 saisons +/- erreur standard,  $n = 24$  pour les bassins versants urbanisés,  $n = 72$  pour les bassins versants agricoles et  $n = 48$  pour les bassins versants forestiers). **2** Ratios amont/aval pour les taux de décomposition en degrés jours pour chaque type de bassin versant ( $n = 12$  pour les bassins versants urbanisés,  $n = 36$  pour les bassins versants agricoles et  $n = 24$  pour les bassins versants forestiers). Les astérisques indiquent des différences significatives avec la ligne pointillée, qui représente un ratio de 1 (T test,  $p < 0.05$ ).

Les résultats des analyses physico-chimiques de l'eau ont permis de mettre en évidence des concentrations en nutriments et une toxicité potentielle par des pesticides et pharmaceutiques (exprimés en unités toxiques, TU) supérieures pour les bassins versant urbanisés et agricoles par rapport aux bassins versants forestiers (ou control). De manière surprenante, une tendance similaire a été observé sur les taux de décomposition microbiens (corrigés par la température), avec des valeurs légèrement plus élevées pour les bassins versants urbanisés et agricoles (Fig. 1.1) par rapport aux bassins versants forestiers. Cette observation est encore plus flagrante lorsque l'on compare l'amont du bassin versant urbanisé par rapport à son aval (Fig. 1.2). Ces

résultats suggèrent donc que l'effet toxique des pesticides et des composés pharmaceutiques a probablement été compensé par la présence des nutriments en forte concentrations. En revanche, le fait que la biomasse fongique soit réduite d'un facteur de 2 et 1.4 pour les bassins versants urbanisés et agricoles respectivement par rapport aux bassins versants forestiers semble démontrer que l'effet compensatoire des nutriments n'est que partiel (Fig. 2). De plus, les résultats d'une analyse multi-variée ont montré que la diminution de la biomasse fongique associée aux litières feuilles au niveau des bassins versants anthropisés (c.-à-d. urbanisés et agricoles) peut être majoritairement expliquée par la toxicité des pesticides. Aucune tendance nette n'a cependant pu être observée sur les activités enzymatiques généralement associées à la décomposition de la litière de feuilles. Ces résultats suggèrent que la régulation des activités enzymatiques extracellulaires chez les communautés microbiennes est d'avantage influencée par plusieurs facteurs, tels que la composition chimique du substrat, de l'eau ou encore la structure de la communauté.



*Figure 2: 1 Biomasse fongique pour chaque type de bassin versant (moyenne des amonts et avals pour les 4 saisons +/- erreur standard, n = 24 pour les bassins versants urbanisés, n = 72 pour les bassins versants agricoles et n = 48 pour les bassins versants forestiers). 2 Ratios amont/aval de la biomasse fongique pour chaque type de bassins versant (n = 12 pour les bassins versants urbanisés, n = 36 pour les bassins versants agricoles et n = 24 pour les bassins versants forestiers). Les astérisques indiquent des différences significatives avec la ligne pointillée, qui représente un ratio de 1 (T test, p < 0.05).*

Les résultats de cette étude de terrain montrent donc que la contamination chimique des cours d'eau liée à l'agriculture et à l'urbanisation tend à stimuler les processus de décomposition microbiens des feuilles d'aulne et que cette stimulation peut s'expliquer par des effets

compensatoires des nutriments (principalement l'azote). Cependant, la diminution de la biomasse fongique en présence de contamination chimique souligne tout de même une forte sensibilité chez les communautés microbiennes, même à de faibles concentrations en xénobiotiques. Globalement, ces résultats tendent à montrer que les communautés microbiennes sont plus efficaces en termes de décomposition de la litière de feuilles lorsqu'elles sont exposées à la contamination des cours d'eau, ce qui peut s'expliquer par des mécanismes d'adaptation accompagnés de modifications de la structure des communautés microbiennes associées aux feuilles. L'accroissement de l'urbanisation couplé aux nouvelles pratiques agricoles résultant de l'activité humaine suggère que les cours d'eau seront de plus en plus pollués par des nutriments et des micropolluants. Un tel mélange, même à de faibles concentrations, pourrait ainsi accélérer la décomposition microbienne de la litière de feuilles, entraînant un rétrécissement de la fenêtre temporelle de décomposition pour des organismes de niveaux trophiques supérieurs (c.-à-d. macro-invertébrés et poissons). Ces résultats soulignent donc l'importance de considérer que la biomasse microbienne et l'activité de décomposition dans l'évaluation des risques écologiques et écotoxicologiques au niveau des écosystèmes de cours d'eau.

## Chapitre 2: Effets interactifs des pesticides et des nutriments sur les communautés microbiennes responsables de la décomposition de la litière dans les cours d'eau

Comme nous avons pu l'observer dans le chapitre précédent, la contamination chimique de l'eau peut avoir un effet stimulant sur l'activité de décomposition microbienne. Nous avons donc émis l'hypothèse que cette stimulation était essentiellement due à la présence de fortes concentrations en nutriments, contrebalançant les fortes toxicités potentielles en pesticides et pharmaceutiques observées aux bassins versant contaminés (c.-à-d. urbanisés et agricoles). Cependant, il reste difficile de déterminer si cette stimulation peut effectivement être attribuée aux nutriments. D'une part parce que nutriments et xénobiotiques co-occurrent généralement dans les cours d'eau contaminés et d'autre part parce que les résultats de notre analyse multi-variée ont révélés une forte corrélation positive entre les taux de décomposition et la toxicité des pesticides. D'une manière générale, il est toujours très compliqué d'observer des tendances

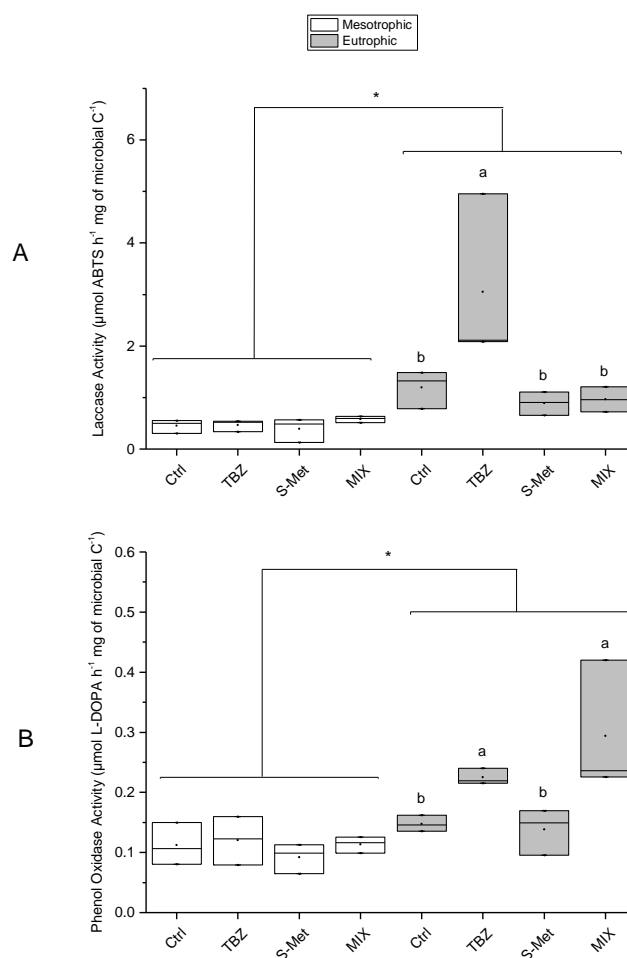
nettes sur le terrain, premièrement à cause des variables confondantes mais également à cause de la forte variabilité entre sites d'études et entre saisons (Liess and Ohe, 2005).

Dans ce second chapitre, nous avons donc voulu essayer de déterminer qui des nutriments ou des pesticides influencent le plus l'activité de décomposition microbienne ainsi que la structure des communautés fongiques. Afin de répondre à cet objectif, nous avons mis en place une étude en conditions contrôlées (microcosme de 24 canaux de rivières artificielles). L'expérience s'est déroulée sur une durée de 40 jours, en utilisant 2 conditions nutritives différentes (mésotrophe et eutrophe) ainsi que 4 traitements (control sans contamination, fongicide et herbicide seuls ou en mélange). Les conditions nutritives ont été établies à partir des données obtenues sur le terrain (Chapitre 1) et de la littérature (classification de Dodds & Smith, 2016, mésotrophe =  $0.8 \text{ mg L}^{-1} \text{ N-NO}_3$  et  $0.05 \text{ mg L}^{-1} \text{ P-PO}_4$  ; eutrophe =  $8 \text{ mg L}^{-1} \text{ N-NO}_3$  et  $0.5 \text{ mg L}^{-1} \text{ P-PO}_4$ ). Nous avons choisi comme fongicide le tébuconazole (TBZ) et comme herbicide le S-métolachlore (S-met). Le premier (TBZ) a été choisi en fonction de son mode d'action, qui suppose des effets sur les champignons aquatiques tandis que le second (S-met) a été sélectionné pour son occurrence dans les cours d'eau en France (24% des cours d'eau, NAIADES, EauFrance, 2010-2015). L'impact de ces différentes conditions a été testé sur des communautés microbiennes obtenues à partir d'un inoculum provenant d'un site peu contaminée (Couze d'Ardes amont). Afin de simuler des piques d'expositions, les différents traitements ont été renouvelés chaque semaine par des changements d'eau. Les taux de décomposition microbiens et les activités enzymatiques associées (laccases, phénol oxydase, leucine-aminopetidase, phosphatase et  $\beta$ -glucosidase) ainsi que la structure et la biomasse des communautés microbiennes associées aux litières de feuilles (champignons et bactéries) ont été utilisés comme descripteurs à partir de prélèvements réalisés chaque semaine. Les effets indirect de l'exposition aux nutriments et aux pesticides sur l'appétence des feuilles précédemment exposées aux différents traitements (pendant 40 jours) pour le macro-invertébrée *Gammarus fossarum* ont également été mesurés.

*Table 1 : Taux de décomposition microbiens des feuilles d'aulnes (K) en jours<sup>-1</sup> dans les canaux exposés au fongicide (TBZ), à l'herbicide (S-Met), aux deux pesticides en mélange (MIX) ou non exposés (Ctrl) en condition eutrophe ou mésotrophe. Les valeurs ont été obtenues après ajustement des pertes de masse en % à un modèle exponentiel décroissant. La qualité de l'ajustement est représentée par le R<sup>2</sup>. Les valeurs en gras indiquent des différences significatives par rapport au contrôle, et l'astérisque des différences entre conditions nutritives (Test de Kruskal-Wallis, P < 0.05).*

	Ctrl		+ TBZ		+ S-Met		+ MIX		
	K	R <sup>2</sup>	K	R <sup>2</sup>	K	R <sup>2</sup>	K	R <sup>2</sup>	
<b>Eutrophic</b>	13.34 x 10 <sup>-3</sup> ± 1.52 x 10 <sup>-3</sup>	0.94	<b>11.19 x 10<sup>-3</sup></b> ± 1.23 x 10 <sup>-3</sup>	0.95	<b>11.39 x 10<sup>-3</sup></b> ± 1.34 x 10 <sup>-3</sup>	0.94	<b>10.48 x 10<sup>-3</sup></b> ± 1.27 x 10 <sup>-3</sup>	0.93	*
<b>Mesotrophic</b>	8.81 x 10 <sup>-3</sup> ± 1.91 x 10 <sup>-3</sup>	0.81	8.69 x 10 <sup>-3</sup> ± 1.79 x 10 <sup>-3</sup>	0.83	7.49 x 10 <sup>-3</sup> ± 1.52 x 10 <sup>-3</sup>	0.83	7.38 x 10 <sup>-3</sup> ± 1.39 x 10 <sup>-3</sup>	0.85	

Les résultats de cette étude nous ont permis de mettre en évidence une interaction entre nutriments et pesticides sur l'activité microbienne, uniquement en présence de fortes concentrations en nutriments (phosphore et azote). Plus spécifiquement, une diminution des taux de décomposition microbiens a été observée dans les canaux exposés au TBZ et au S-met en comparaison avec les canaux contrôles (sans pesticides) en conditions eutrophes (Table 1). Ces résultats suggèrent que les nutriments pourraient exacerber les effets des pesticides sur les taux de décomposition microbiens. Ces résultats pourraient aussi majoritairement s'expliquer par des différences de structure des communautés (fongiques et bactériennes) entre traitements. Aucune interaction n'a cependant été observée entre les deux pesticides (TBZ et S-met) sur les taux de décomposition. De plus, une stimulation des activités lignolytiques (laccase et phénol oxydase) a été observée en présence du fongicide (Fig. 3). Ces résultats pourraient suggérer la mise en place de mécanismes de détoxification utilisés par les champignons afin de pallier à la toxicité du TBZ. Cependant, aucune stimulation de l'activité laccase en présence de TBZ et de S-met en mélange n'a pas été observée. Ceci suggère une interaction antagoniste entre ces deux pesticides sur l'activité laccase. Concernant l'impact indirect sur les organismes de niveau trophique supérieurs, aucune influence des pesticides n'a été observée sur la palatabilité des feuilles pour le macro-invertébré.



*Figure 3 : Activités spécifiques laccase (A) et phénol oxydase (B) mesurées dans les canaux exposés au fongicide (TBZ), à l'herbicide (S-Met), aux deux pesticides en mélange (MIX) ou non exposés (Ctrl) en condition eutrophe (gris) ou mésotrophe (blanc). Les valeurs ont été obtenues après intégration des activités enzymatiques pour chaque condition pour la durée de l'expérience (40 jours). Les différences entre conditions nutritives sont indiquées par un astérisque (Kruskal-Wallis,  $P < 0.05$ ) tandis que les différences entre traitements sont indiquées par des lettres ( $a > b$ ).*

En conclusion, les résultats de cette étude nous ont permis de mettre en évidence que les nutriments constituent le paramètre principal influençant la structure et le fonctionnement des communautés microbiennes associées aux litière de feuilles d'aulne. Cependant, nos résultats ont également montré que des concentrations réalistes en fongicide (TBZ) et en herbicide (S-met), seuls ou en mélange, suffisent à induire des changements au niveau de la structure des communautés, conduisant à une légère diminution de l'activité microbienne de décomposition associée. Cependant, aucun effet indirect de l'exposition aux pesticides n'a été observé sur l'appétence des feuilles pour des organismes de niveau trophiques supérieurs (macro-

invertébrés). De manière surprenante, les effets des pesticides dans cette étude n'ont été observés qu'en présence de fortes concentrations en nutriments (condition eutrophe), suggérant des interactions de type antagonistes entre les nutriments et les xénobiotiques sur la décomposition microbienne.

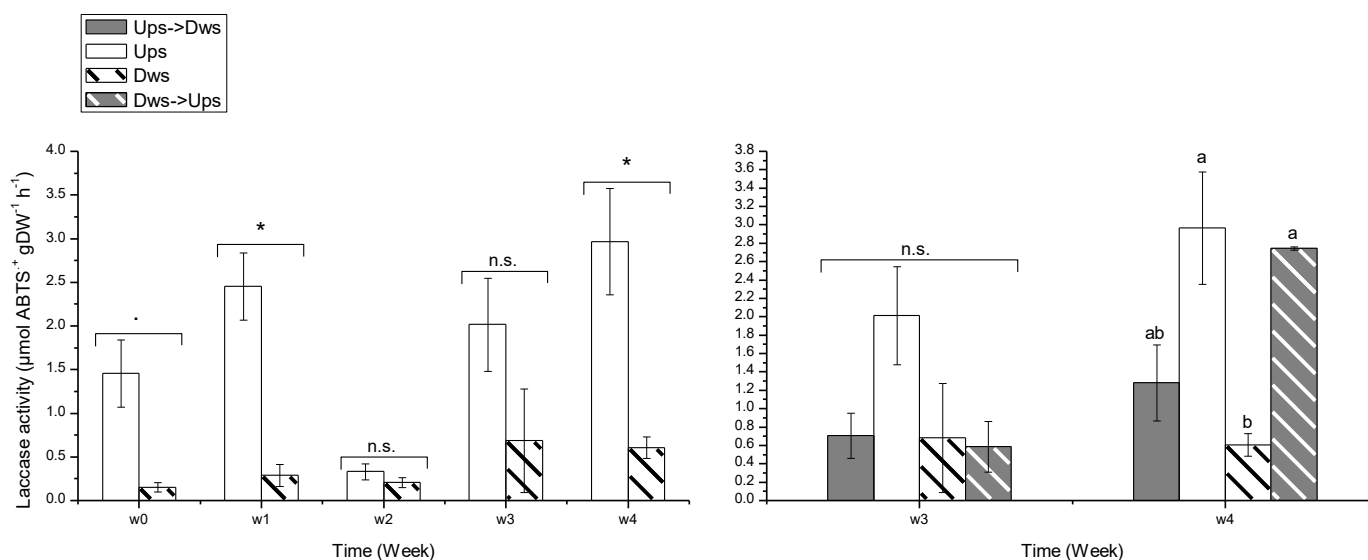
### Chapitre 3: Réponses structurelles et fonctionnelles des communautés fongiques de litières de feuilles soumises à une pollution chimique d'un cours d'eau

Comme nous avons pu le constater dans les chapitres 1 et 2, la contamination chimique de l'eau peut impacter les communautés microbiennes associées aux litières de feuilles. Alors que les effets d'une telle contamination sur la décomposition microbienne sont contrastés, la biomasse et la structure de ces communautés se révèlent plus sensibles, et apparaissent souvent altérées. Dans un contexte réglementaire de préservation des masses d'eau ou la Directive Cadre sur l'Eau vise à réduire drastiquement les intrants de nutriments et pesticides dans les cours d'eau, une question reste en suspens : les communautés microbiennes ayant été exposées chroniquement à une contamination chimique peuvent-elles revenir à un état pré-perturbation une fois la contamination éliminée.

Dans le but de répondre à cette question, nous avons mis en place une expérience en canaux de rivières artificiels afin de tester la résistance et la résilience des communautés microbiennes associées aux litières de feuilles à une contamination chimique complexe réaliste. Pour ce faire, 6 canaux ont été mis en place, divisés en deux conditions, contrôle et contaminée, alimentés par de l'eau du site amont ou du site aval de la rivière Auzon (Puy-de-Dôme). Les communautés microbiennes (champignons et bactéries) ont été obtenues à partir d'inocula provenant de l'amont et de l'aval de cette même rivière. Les taux de décomposition microbiens et les activités enzymatiques lignolytiques associées (laccases, phénol oxydase, peroxydase) ainsi que la structure et la biomasse des communautés fongiques associées aux litières de feuilles ont été utilisés comme descripteurs à partir de prélèvements réalisés chaque semaine. Après deux semaines de suivi, les capacités de résistance et de résilience des communautés fongiques ont été évaluées en effectuant une translocation depuis l'amont vers l'aval (évaluation de la résistance des communautés) ou de l'aval vers l'amont (évaluation de la résilience des communautés). En parallèle, des mesures physico-chimiques (phosphate, nitrate, carbone organique et inorganique, pH, conductivité, oxygène dissous) et des analyses multi-résidus



pesticides de l'eau des canaux ont été réalisées afin de caractériser précisément la composition physico-chimique de l'eau de chaque condition.



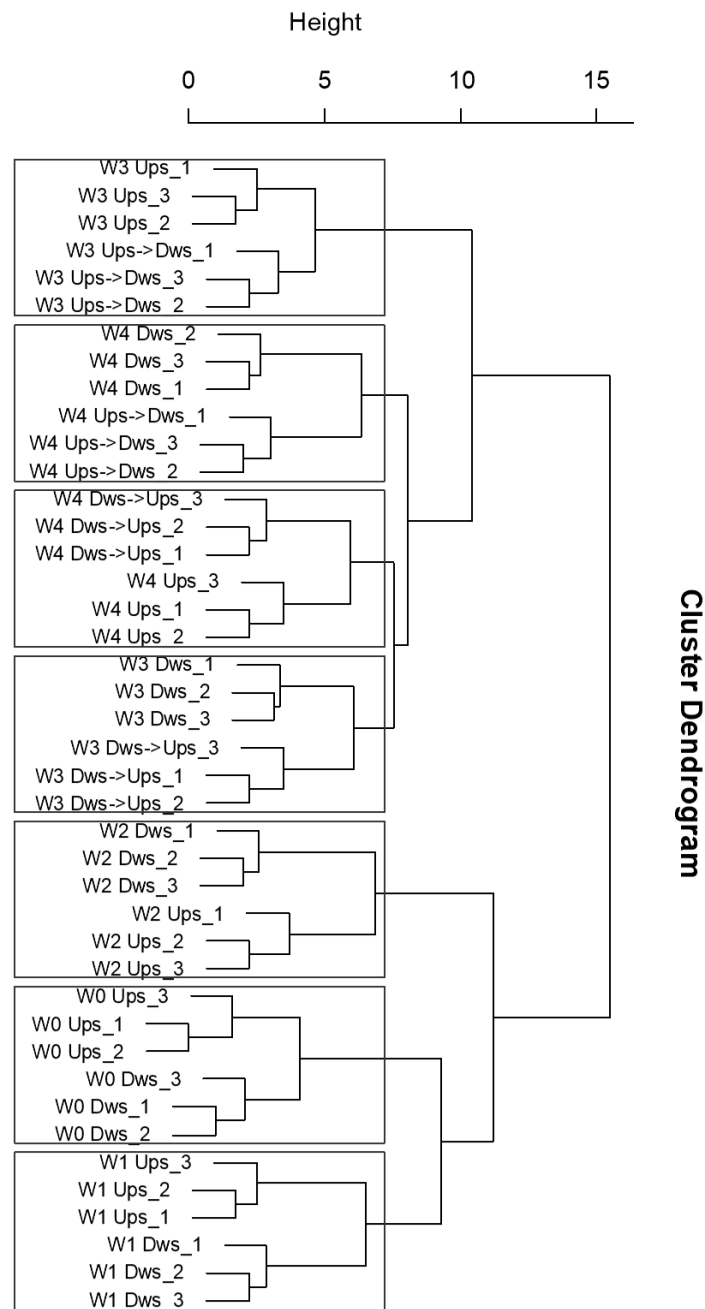
*Figure 4 : Activité laccase mesurée dans les canaux amont et aval au cours de l'expérience (moyenne et standard erreur, n = 3). Les différences significatives entre conditions sont exprimées par des astérisques et des lettres (a ≠ b, test de Tukey's, P < 0.05).*

Les résultats des analyses physico-chimiques de l'eau ont permis de mettre en évidence une toxicité potentielle liée aux pesticides (TU) et des concentrations en nutriments supérieures dans l'eau de l'aval de la rivière Auzon par rapport à l'eau de l'amont. Parallèlement à ces résultats, des valeurs d'activité laccase plus faibles ont été obtenues pour les communautés exposées à l'eau de l'aval par rapport à celles exposées à l'eau de l'amont (Fig. 4). Ces différences peuvent s'expliquer en partie par des différences en termes de structure des communautés fongiques (Fig. 5), malgré une richesse d'Unités Taxonomiques Opérationnelles (OTUs) fongiques supérieures pour les communautés exposées à l'eau de l'aval. De plus, à la suite d'un pique de concentration en nitrate, nous avons pu mettre en évidence une corrélation négative entre les nitrates et l'activité laccase, quel que soit le site.

Les résultats de cette expérience de translocation nous ont permis de montrer une diminution rapide (1 semaine) de l'activité laccase accompagnée de changements au niveau de la structure des communautés fongiques pour les communautés déplacées depuis les canaux amont vers les

canaux aval. Ainsi, les communautés déplacées de l'amont vers l'aval (Ups -> Dws) montrent une structure différentes de celles de l'amont (Ups) mais proche de celles l'aval (Dws) à la semaine 4 (Fig. 5). Ces résultats suggèrent une faible résistance chez les communautés microbiennes associées aux litières de feuilles d'aulnes. De même, une forte capacité de récupération (2 semaines) fonctionnelle (activité laccase) et structurale (structure des communautés fongiques) ont pu être observées pour les communautés déplacées depuis les canaux aval vers les canaux amont. Ici encore, les communautés déplacées de l'aval vers l'amont (Dws -> Ups) montrent une structure différentes de celles de l'aval (Dws) mais proche de celles l'amont (Ups) à la semaine 4 (Fig. 5). Ces résultats suggèrent donc une forte capacité de récupération chez les communautés microbiennes associées aux litières de feuilles. Dans les deux cas, résistance ou résilience, les changements observés au niveau de la structure des communautés fongiques peuvent s'expliquer par des transferts d'espèces depuis les feuilles restées dans les canaux vers celles qui ont été déplacées au moment de l'expérience de translocation.

Les résultats de cette étude ont donc permis de mettre en évidence que l'activité laccase et la structure des communautés fongiques associées aux litières de feuilles étaient sensibles et peu résistantes aux variations en termes de physico-chimie de l'eau. Ceci souligne donc l'importance de préserver ces écosystèmes. Cependant, ces communautés ont montré une forte capacité de résilience une fois le stress éliminé. Les cours d'eau de tête de bassin versant s'avèrent importants en termes de réservoir d'espèces lors des programmes de restauration des cours d'eau. Les résultats de cette étude ont également permis de mettre en évidence l'utilisation potentielle de l'activité laccase en tant que bio-marqueur de contamination chimique des cours d'eau.

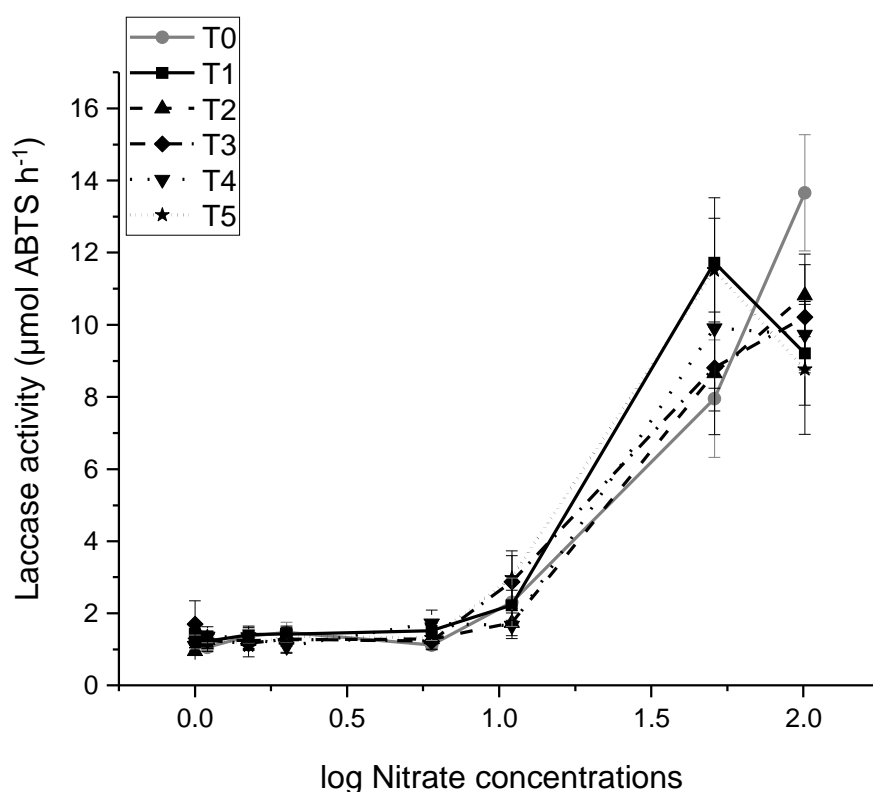


*Figure 5 : Dendrogramme représentant la structure des communautés fongiques associées aux litières de feuilles d'aulnes pour chaque semaine (semaine 1 (W1), semaine 2 (W2), semaine 3 (W3) et semaine 4(W4)) dans les canaux amont (Ups), aval (Dws), déplacées depuis l'amont vers l'aval (Ups -> Dws) et déplacées depuis l'aval vers l'amont (Dws -> Ups). Le dendrogramme a été obtenu en utilisant la méthode de Ward (indice de dissimilarité de Ward) à partir d'une matrice présence/absence obtenue en DGGE.*

#### Chapitre 4: Régulation de l'activité laccase extracellulaire microbienne soumise à une contamination chimique

Comme nous avons pu l'observer en chapitre 1, aucune tendance nette n'a été observée concernant l'effet d'une contamination chimique sur l'activité laccase, malgré des valeurs légèrement plus faible pour les bassins versant exposés à une contamination urbaine et agricole ainsi qu'aux sites aval. Lors de l'expérience de translocation du chapitre 3, une activité laccase plus faible a été obtenue pour les communautés exposées à de l'eau du site contaminé (riche en nutriments et xénobiotiques) par rapport à celles du site moins contaminé. Les résultats de ces deux chapitres semblent aller dans le même sens. En revanche, alors que les nitrates ont clairement inhibé l'activité laccase en chapitre 3, une stimulation de cette même activité enzymatique a été observée en présence de fortes concentrations en nitrate et de tébuconazole en chapitre 2. Ces résultats semblent montrer que l'activité laccase est sensible à la contamination chimique des cours d'eau et que la régulation de cette activité chez les communautés microbiennes associées aux litières de feuilles semble fortement dépendante des caractéristiques chimiques de l'eau.

Afin de comprendre plus précisément l'influence des nitrates et des pesticides sur cette activité enzymatique, nous avons donc mis en place une expérience de doses-réponses permettant d'évaluer les effets de concentrations croissantes en nitrates (0, 0.1, 0.5, 1, 5, 10, 50 et 100 mg/L de  $\text{N-NO}_3$ ) et en tébuconazole (0, 0.01, 0.1, 1, 10, 20 mg/L de TBZ) seuls ou en mélange, sur l'activité laccase microbienne associée à la litière de feuilles d'aulnes. Dans cette étude, des feuilles pré-colonisées pendant une période de deux semaines à l'amont de la rivière Artière (site de référence) ont été utilisées, et l'activité laccase a été mesurée après 36 heures d'exposition aux nitrates et/ou au TBZ. La concentration effective 50 (EC50), qui évalue la concentration en TBZ ou nitrate nécessaire pour provoquer une variation de 50% par rapport à la valeur de référence (sans TBZ ni nitrate) sur l'activité laccase, a été utilisée.



*Figure 6 : Réponse en termes d'activité laccase exposée à des concentrations croissantes en azote (N-NO<sub>3</sub>, échelle logarithmique) pour chaque concentration en TBZ (T0 = 0, T1 = 0.01, T2 = 0.1, T3 = 1, T4 = 10 and T5 = 20 mg L<sup>-1</sup>).*

Comme précédemment démontré par Artigas *et al.*, (2017b), les résultats de cette étude ont montré que l'activité laccase issue de communautés microbiennes complexes était peu sensible au TBZ. Cependant, une forte stimulation de cette même activité a été observée en présence de concentrations croissantes en nitrates, et ce, quelle que soit la concentration en TBZ (Fig. 6). Néanmoins, cette stimulation n'a pu être observée qu'à de très fortes concentrations en nitrates (EC50 comprises entre  $18.61 \pm 4.55$  mg L<sup>-1</sup> de N-NO<sub>3</sub> pour la plus forte concentration en TBZ et  $36.45 \pm 6.34$  mg L<sup>-1</sup> de N-NO<sub>3</sub> en absence de TBZ), très supérieures à celles observées sur le terrain à l'exception du Charlet (site agricole, 25.27 mg de N-NO<sub>3</sub> L<sup>-1</sup>).

Globalement, ces résultats diffèrent de ce qui a précédemment obtenu lors de l'étude de terrain (chapitre 1) et de l'expérience de translocation (chapitre 3), où une relation négative entre l'activité laccase et les concentrations en azote (nitrate) ont été observés. Sachant que ces

stimulations sont observées uniquement à de très fortes concentrations en nitrate, nous avons émis l'hypothèse que l'augmentation d'activité pouvait être due à une réponse au stress provoqué par un choc osmotique. Par ailleurs, il reste très compliqué de comparer des études de terrain avec des tests d'éco-toxicologie, peu représentatifs de l'environnement. Ces résultats soulèvent donc la pertinence des tests standards éco-toxicologiques pour l'analyse des effets d'un toxique chez les communautés microbiennes.

En analysant plus en détails les données obtenues lors de l'étude de terrain et de l'expérience de translocation (chapitres 1 et 3), il apparaît que l'activité laccase semble plus sensible à la contamination chimique de l'eau chez les communautés issues des sites amont (moins contaminés) par rapport à celles issues des sites aval (plus contaminés). Il est donc probable que la réponse de communautés microbiennes à une contamination chimique soit fortement liée à la structure des communautés. L'absence de réponse d'activité laccase au nitrate chez les communautés issues du site plus contaminé peut donc s'expliquer par des mécanismes de d'acquisition de tolérance ou de résistance à la pollution, probablement expliqués par l'exclusion d'espèces sensibles et maintien des espèces tolérantes à la contamination.

## **Discussion et conclusions**

L'industrie, l'urbanisation et l'agriculture sont les principaux facteurs ayant largement contribué aux apports massifs de composés chimiques dans la biosphère au cours du siècle dernier, et les écosystèmes de rivières comptent certainement parmi les plus exposés. De ce constat se pose la question des effets d'un tel stress chimique sur les services écosystémiques et les fonctions dispensés par les écosystèmes de cours d'eau. Dans cette thèse, nous avons évalué les effets d'une contamination chimique complexe, incluant celle des pesticides, des produits pharmaceutiques et des nutriments, sur l'activité de décomposition des communautés microbiennes associées à la litière de feuilles. Les réponses de ces communautés en termes de structure de communauté, de biomasse et d'activités enzymatiques associées ont également été évaluées. Ces travaux s'inscrivent dans le contexte de l'évaluation des risques écotoxicologiques liés à la contamination chimique des cours d'eau et contribuent à améliorer les connaissances sur les conséquences du changement global résultant de l'activité humaine sur le fonctionnement des cours d'eau de tête de bassin versant.

Parmi les principaux résultats de ces travaux de thèse, nous avons pu observer que la contamination chimique liée à une utilisation agricole ou urbaine des sols a provoqué à une

stimulation des taux de décomposition observé sur le terrain. Nous avons émis l'hypothèse que cette stimulation résultait en grande partie de la présence de fortes concentrations en nutriments généralement observées dans les cours d'eau impactés par l'activité humaine. En effet, l'augmentation des concentrations en nutriments est généralement associée à une augmentation des taux de décomposition microbiens associés à une augmentation de la biomasse microbienne (Artigas *et al.*, 2004b; Hagen *et al.*, 2006; Magbanua *et al.*, 2010). Par ailleurs, la compensation des nutriments sur les xénobiotiques est un phénomène qui a déjà été décrit dans la littérature scientifique (Fernández *et al.*, 2016; Gardeström *et al.*, 2016). Cependant, le fait que la biomasse fongique soit fortement impactée pour les bassins versant agricoles et urbanisés semble montrer que ces mécanismes ne sont que partiel. Ces observations sont renforcées par les résultats obtenus lors du chapitre 2, où les effets des pesticides n'ont été observés qu'en présence de fortes concentrations en nutriment simulant un phénomène d'eutrophisation. Dans les deux cas, ces modifications en termes d'activité de décomposition peuvent s'expliquer par des changements au niveau de la structure des communautés microbiennes (bactéries et champignons). Dans l'ensemble, ces résultats montrent que malgré la contamination chimique des cours d'eau et son impact potentiel sur les communautés microbiennes, ces dernières sont capables de s'adapter afin de maintenir leur fonction de décomposition de la matière organique à l'échelle de l'écosystème et que les effets indirects de cette contamination sur les macro-invertébrés semblent négligeables.

L'autre résultat marquant de ces travaux de thèse concerne les activités enzymatiques extracellulaires associées à la décomposition des litières de feuilles. Parmi ces dernières, les enzymes lignolytiques sont bien connues pour leur implication dans la dégradation de molécules récalcitrantes de haut poids moléculaire, telle que la lignine, permettant l'accès aux compartiments internes des cellules végétales, riches en micronutriments pour les micro-organismes (Chróst, 1991; Romaní *et al.*, 2006b). Cependant, bien qu'une corrélation entre ces enzymes et la décomposition de la litière de feuilles a fréquemment été observée dans la littérature (Artigas *et al.*, 2004b; Sinsabaugh, 2010), aucune tendance nette n'a été observée au cours de ces travaux de thèse. En revanche, l'activité laccase extracellulaire s'est montrée relativement sensible à la contamination chimique. D'une manière générale, nous avons pu observer une activité laccase plus faible aux sites les plus contaminés en lien avec la présence d'azote. Ces résultats nous ont d'abord conduits à penser que l'activité laccase pourrait servir de bio-marqueur de contamination chimique. Cependant une stimulation de cette activité chez les communautés microbiennes a également été observée en présence du fongicide

tébuconazole dans l'étude d'Artigas *et al.* (2017a) ainsi que lors de l'expérience en microcosme du chapitre 3, très probablement explicable par des mécanismes de détoxification/défense contre le stress mis en place par les champignons aquatiques (Baldrian, 2006; Sinsabaugh, 2010; Karas *et al.*, 2011). De manière surprenante, les résultats obtenus lors des tests doses-réponses du chapitre 4 ont montré une tendance inverse, caractérisée par une stimulation de l'activité laccase en présence de nitrate mais aucun effet du fongicide. Cependant, différents mécanismes semblent être impliqués entre ces différentes études. La stimulation de l'activité laccase observée lors des tests dose-réponse peut être expliquée par une réponse au stress probablement causée par le stress osmotique résultant des concentrations extrêmes en azote. A l'inverse, et sachant que l'activité laccase extracellulaire est impliquée dans l'acquisition d'azote, l'inhibition de cette activité sur le terrain peut refléter une répression par le substrat, probablement dû au fait que les communautés microbiennes répondaient déjà à leurs besoins nutritionnels en azote. Des plus, les différences de structure des communautés entre sites suggèrent que la composition de la communauté microbienne pourrait également jouer un rôle dans le potentiel d'activité de la laccase extracellulaire (Sinsabaugh & Linkins, 1987; Songulashvili *et al.*, 2007). Dans l'ensemble, ces résultats tendent à montrer la forte implication de la chimie de l'eau dans la régulation de l'activité laccase extracellulaire probablement par l'intermédiaire de changements au niveau de la structure des communautés microbiennes.





## References

- Abdel-Raheem A.M. & Ali E.H. (2004) Lignocellulolytic enzyme production by aquatic hyphomycetes species isolated from the Nile's delta region. *Mycopathologia* **157**, 277–286.
- Abdel-Raheem A. & Shearer C.A. (2002) Extracellular enzyme production by freshwater ascomycetes.
- Abdullah S.K. & others (1989) Extracellular enzymatic activity of aquatic and aero-aquatic conidial fungi. *Hydrobiologia* **174**, 217–223.
- Abelho M. (2001a) From Litterfall to Breakdown in Streams: A Review. *The Scientific World JOURNAL* **1**, 656–680.
- Abelho M. (2001b) From Litterfall to Breakdown in Streams: A Review. *The Scientific World JOURNAL* **1**, 656–680.
- Abelho M. & Graça M.A.S. (1996a) Effects of eucalyptus afforestation on leaf litter dynamics and macroinvertebrate community structure of streams in Central Portugal. *Hydrobiologia* **324**, 195–204.
- Abelho M. & Graça M.A.S. (1996b) Effects of eucalyptus afforestation on leaf litter dynamics and macroinvertebrate community structure of streams in Central Portugal. *Hydrobiologia* **324**, 195–204.
- Abelho M. & Graça M.A.S. (1998a) Litter in a first-order stream of a temperate deciduous forest (Margaraça Forest, central Portugal). *Hydrobiologia* **386**, 147–152.
- Abelho M. & Graça M.A.S. (1998b) Litter in a first-order stream of a temperate deciduous forest (Margaraça Forest, central Portugal). *Hydrobiologia* **386**, 147–152.
- Allison S.D. & Martiny J.B.H. (2008) Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences* **105**, 11512–11519.
- Allison S.D. & Vitousek P.M. (2005) Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biology and Biochemistry* **37**, 937–944.
- Arheimer B., Andersson L. & Lepistö A. (1996) Variation of nitrogen concentration in forest streams—influences of flow, seasonality and catchment characteristics. *Journal of Hydrology* **179**, 281–304.
- Arsuffi T.L. & Suberkropp K. (1989) Selective feeding by shredders on leaf-colonizing stream fungi: comparison of macroinvertebrate taxa. *Oecologia* **79**, 30–37.
- Artigas J., Majerholc J., Foulquier A., Margoum C., Volat B., Neyra M., *et al.* (2012) Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. *Aquatic Toxicology* **122**, 197–205.

- Artigas J., Romaní A.M. & Sabater S. (2004a) Organic matter decomposition by fungi in a Mediterranean forested stream: contribution of streambed substrata. In: *Annales de Limnologie-International Journal of Limnology*. pp. 269–277. Cambridge Univ Press.
- Artigas J., Romaní A.M. & Sabater S. (2004b) Organic matter decomposition by fungi in a Mediterranean forested stream : contribution of streambed substrata. *Annales de Limnologie - International Journal of Limnology* **40**, 269–277.
- Artigas J., Romaní A.M. & Sabater S. (2004c) Organic matter decomposition by fungi in a Mediterranean forested stream: contribution of streambed substrata. In: *Annales de Limnologie-International Journal of Limnology*. pp. 269–277. Cambridge Univ Press.
- Artigas J., Rossi F., Gerphagnon M. & Mallet C. (2017) Sensitivity of laccase activity to the fungicide tebuconazole in decomposing litter. *Science of The Total Environment* **584**, 1084–1092.
- Baldrian P. (2006) Fungal laccases—occurrence and properties. *FEMS microbiology reviews* **30**, 215–242.
- Baldrian P. & Valášková V. (2008a) Degradation of cellulose by basidiomycetous fungi. *FEMS microbiology reviews* **32**, 501–521.
- Baldrian P. & Valášková V. (2008b) Degradation of cellulose by basidiomycetous fungi. *FEMS microbiology reviews* **32**, 501–521.
- Baldy V., Chauvet E., Charcosset J.-Y. & Gessner M.O. (2002) Microbial dynamics associated with leaves decomposing in the mainstem and floodplain pond of a large river. *Aquatic Microbial Ecology* **28**, 25–36.
- Baldy V., Gessner M.O. & Chauvet E. (1995) Bacteria, fungi and the breakdown of leaf litter in a large river. *Oikos*, 93–102.
- Baldy V., Gobert V., Guerold F., Chauvet E., Lambrigot D. & CHARCOSSET J.-Y. (2007a) Leaf litter breakdown budgets in streams of various trophic status: effects of dissolved inorganic nutrients on microorganisms and invertebrates. *Freshwater Biology* **52**, 1322–1335.
- Baldy V., Gobert V., Guerold F., Chauvet E., Lambrigot D. & Charcosset J.-Y. (2007b) Leaf litter breakdown budgets in streams of various trophic status: effects of dissolved inorganic nutrients on microorganisms and invertebrates. *Freshwater Biology* **52**, 1322–1335.
- Bandoni R.J. (1972) Terrestrial occurrence of some aquatic hyphomycetes. *Canadian Journal of Botany* **50**, 2283–2288.

- Barbash J.E., Thelin G.P., Kolpin D.W. & Gilliom R.J. (2001) Major herbicides in ground water. *Journal of Environmental Quality* **30**, 831–845.
- Bärlocher F. (1985) The role of fungi in the nutrition of stream invertebrates. *Botanical journal of the Linnean Society* **91**, 83–94.
- Bärlocher F. & Kendrick B. (1975) Leaf-conditioning by microorganisms. *Oecologia* **20**, 359–362.
- Barnett T., Malone R., Pennell W., Stammer D., Semtner B. & Washington W. (2004) The effects of climate change on water resources in the west: introduction and overview. *Climatic Change* **62**, 1–11.
- Barnett T.P., Pierce D.W. & Schnur R. (2001) Detection of anthropogenic climate change in the world's oceans. *Science* **292**, 270–274.
- Baschien C., Tsui C.K.-M., Gulis V., Szewzyk U. & Marvanová L. (2013) The molecular phylogeny of aquatic hyphomycetes with affinity to the Leotiomycetes. *Fungal Biology* **117**, 660–672.
- Bastias Álamo E. (2018) *The role of leaf litter quality and streambed hydro-morphology on in-stream leaf litter dynamics= El rol de la qualitat de la fullaraca i de la hidro-morfologia del tram fluvial en les dinàmiques de la fullaraca als rierols*. Universitat de Barcelona.
- Baudy P., Zubrod J.P., Kenschak M., Weil M., Schulz R. & Bundschuh M. (2017) Does long-term fungicide exposure affect the reproductive performance of leaf-shredders? A partial life-cycle study using *Hyalella azteca*. *Environmental Pollution* **222**, 458–464.
- Beketov M.A., Kefford B.J., Schäfer R.B. & Liess M. (2013) Pesticides reduce regional biodiversity of stream invertebrates. *Proceedings of the National Academy of Sciences* **110**, 11039–11043.
- Benda L.E.E., Poff N.L., Miller D., Dunne T., Reeves G., Pess G., *et al.* (2004) The network dynamics hypothesis: how channel networks structure riverine habitats. *BioScience* **54**, 413–427.
- Benda L., Hassan M.A., Church M. & May C.L. (2005) Geomorphology of steep-land headwaters: the transition from hillslopes to channels 1. *JAWRA Journal of the American Water Resources Association* **41**, 835–851.
- Benfield E.F. (1997) Comparison of litterfall input to streams. *Journal of the North American Benthological Society* **16**, 104–108.
- Benfield E.F. (1996) Leaf breakdown in stream ecosystems. *Methods in stream ecology*, 579–590.

- Bereswill R., Golla B., Streloke M. & Schulz R. (2012) Entry and toxicity of organic pesticides and copper in vineyard streams: erosion rills jeopardise the efficiency of riparian buffer strips. *Agriculture, Ecosystems & Environment* **146**, 81–92.
- Berg B. (1986) Nutrient release from litter and humus in coniferous forest soils—a mini review. *Scandinavian journal of forest research* **1**, 359–369.
- Bernhardt E.S., Palmer M.A., Allan J.D., Alexander G., Barnas K., Brooks S., *et al.* (2005) *Synthesizing US river restoration efforts*. American Association for the Advancement of Science.
- Berninger U.-G., Finlay B.J. & Kuuppo-Leinikki P. (1991) Protozoan control of bacterial abundances in freshwater. *Limnology and oceanography* **36**, 139–147.
- Besemer K. (2015) Biodiversity, community structure and function of biofilms in stream ecosystems. *Research in Microbiology* **166**, 774–781.
- Blackwood C.B., Waldrop M.P., Zak D.R. & Sinsabaugh R.L. (2007) Molecular analysis of fungal communities and laccase genes in decomposing litter reveals differences among forest types but no impact of nitrogen deposition. *Environmental Microbiology* **9**, 1306–1316.
- Blanck H., Wängberg S.-\AA & Molander S. (1988) Pollution-induced community tolerance—a new ecotoxicological tool. In: *Functional testing of aquatic biota for estimating hazards of chemicals*. ASTM International.
- Bradshaw C.J. & Brook B.W. (2014) Human population reduction is not a quick fix for environmental problems. *Proceedings of the National Academy of Sciences* **111**, 16610–16615.
- Bravard J.P. & Petit F. (2009) Geomorphology of Streams and Rivers. In: *Encyclopedia of Inland Waters*. pp. 387–395. Elsevier.
- Van den Brink P.J., Maltby L., Wendt-Rasch L., Heimbach F. & Peeters F. (2007) New improvements in the aquatic ecological risk assesment of fungicidal pesticides and biocides.
- Briones M.J.I. & Ineson P. (1996) Decomposition of eucalyptus leaves in litter mixtures. *Soil Biology and Biochemistry* **28**, 1381–1388.
- Brown L.R. & Kane H. (1995) *Full house: Reassessing the earth's population carrying capacity*. Earthscan.
- Brown T.C. (2000) Projecting US freshwater withdrawals. *Water Resources Research* **36**, 769–780.

- Bundschuh M., Goedkoop W. & Kreuger J. (2014) Evaluation of pesticide monitoring strategies in agricultural streams based on the toxic-unit concept—experiences from long-term measurements. *Science of the Total Environment* **484**, 84–91.
- Bundschuh M., Hahn T., Gessner M.O. & Schulz R. (2017) Antibiotic mixture effects on growth of the leaf-shredding stream detritivore *Gammarus fossarum*. *Ecotoxicology* **26**, 547–554.
- Bundschuh M., Hahn T., Gessner M.O. & Schulz R. (2009) ANTIBIOTICS AS A CHEMICAL STRESSOR AFFECTING AN AQUATIC DECOMPOSER–DETRITIVORE SYSTEM. *Environmental Toxicology and Chemistry* **28**, 197.
- Bundschuh M., Zubrod J.P., Kosol S., Maltby L., Stang C., Duester L., *et al.* (2011a) Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. *Aquatic Toxicology* **104**, 32–37.
- Bundschuh M., Zubrod J.P., Kosol S., Maltby L., Stang C., Duester L., *et al.* (2011b) Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. *Aquatic toxicology* **104**, 32–37.
- Caldwell B.A. (2005) Enzyme activities as a component of soil biodiversity: a review. *Pedobiologia* **49**, 637–644.
- Canhoto C. & Graça M.A. (1996) Decomposition of Eucalyptus globulus leaves and three native leaf species (*Alnus glutinosa*, *Castanea sativa* and *Quercus faginea*) in a Portuguese low order stream. *Hydrobiologia* **333**, 79–85.
- Canhoto C. & Graça M.A.S. (1995) Food value of introduced eucalypt leaves for a Mediterranean stream detritivore: *Tipula lateralis*. *Freshwater Biology* **34**, 209–214.
- Cargill A.S., Cummins K.W., Hanson B.J. & Lowry R.R. (1985) The role of lipids as feeding stimulants for shredding aquatic insects. *Freshwater Biology* **15**, 455–464.
- Carles L. (2016) *Devenir de mélanges de pesticides: étude des voies de biodégradation et développement d'une méthode préventive de bioremédiation*. Université Blaise Pascal-Clermont-Ferrand II.
- Carles L., Rossi F., Joly M., Besse-Hoggan P., Batisson I. & Artigas J. (2017) Biotransformation of herbicides by aquatic microbial communities associated to submerged leaves. *Environmental Science and Pollution Research* **24**, 3664–3674.
- Carpenter S.R. (1996) Microcosm experiments have limited relevance for community and ecosystem ecology. *Ecology* **77**, 677–680.

- Carpita N.C. & Gibeaut D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **3**, 1–30.
- Carrias J.-F. & Sime-Ngando T. (2009) Bacteria, Attached to Surfaces. In: *Encyclopedia of Inland Waters*. pp. 182–192. Elsevier.
- Chandrashekar K.R. & Kaveriappa K.M. (1991) Production of extracellular cellulase by *Lunulospora curvula* and *Flagellospora penicillioides*. *Folia microbiologica* **36**, 249–255.
- Chauvet E. (1987) Changes in the chemical composition of alder, poplar and willow leaves during decomposition in a river. *Hydrobiologia* **148**, 35–44.
- Chróst R.J. (1991) Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In: *Microbial enzymes in aquatic environments*. pp. 29–59. Springer.
- Connors M.E. & Naiman R.J. (1984) Particulate allochthonous inputs: relationships with stream size in an undisturbed watershed. *Canadian Journal of Fisheries and Aquatic Sciences* **41**, 1473–1484.
- Copping L.G. & Hewitt H.G. (2007) *Chemistry and mode of action of crop protection agents*. Royal Society of Chemistry.
- Cummins K.W. (1974) Structure and function of stream ecosystems. *BioScience* **24**, 631–641.
- Danger M., Cornut J., Chauvet E., Chavez P., Elger A. & Lecerf A. (2013) Benthic algae stimulate leaf litter decomposition in detritus-based headwater streams: a case of aquatic priming effect? *Ecology* **94**, 1604–1613.
- Das M., Royer T.V. & Leff L.G. (2007) Diversity of Fungi, Bacteria, and Actinomycetes on Leaves Decomposing in a Stream. *Applied and Environmental Microbiology* **73**, 756–767.
- Dawoud M., Bundschuh M., Goedkoop W. & McKie B.G. (2017) Interactive effects of an insecticide and a fungicide on different organism groups and ecosystem functioning in a stream detrital food web. *Aquatic Toxicology* **186**, 215–221.
- Dawson J.O. & Funk D.T. (1981) Seasonal change in foliar nitrogen concentration of *Alnus glutinosa*. *Forest Science* **27**, 239–243.
- Delpla I., Jung A.-V., Baures E., Clement M. & Thomas O. (2009) Impacts of climate change on surface water quality in relation to drinking water production. *Environment international* **35**, 1225–1233.
- Dighton J. (1983) Phosphatase production by mycorrhizal fungi. *Plant and soil* **71**, 455–462.



- Dimitrov M.R., Kosol S., Smidt H., Buijse L., Van den Brink P.J., Van Wijngaarden R.P., *et al.* (2014) Assessing effects of the fungicide tebuconazole to heterotrophic microbes in aquatic microcosms. *Science of The Total Environment* **490**, 1002–1011.
- Dodds W.K. (2007) Trophic state, eutrophication and nutrient criteria in streams. *Trends in ecology & evolution* **22**, 669–676.
- Dodds W.K. & Cole J.J. (2007) Expanding the concept of trophic state in aquatic ecosystems: it's not just the autotrophs. *Aquatic Sciences* **69**, 427–439.
- Dodds W.K. & Smith V.H. (2016) Nitrogen, phosphorus, and eutrophication in streams. *Inland Waters* **6**, 155–164.
- Downing J.A., Cole J.J., Duarte C.M., Middelburg J.J., Melack J.M., Prairie Y.T., *et al.* (2012) Global abundance and size distribution of streams and rivers. *Inland waters* **2**, 229–236.
- Duarte S., Pascoal C., Alves A., Correia A. & Cássio F. (2010) Assessing the dynamic of microbial communities during leaf decomposition in a low-order stream by microscopic and molecular techniques. *Microbiological research* **165**, 351–362.
- Duarte S., Pascoal C., Garabétian F., Cássio F. & Charcosset J.-Y. (2009) Microbial decomposer communities are mainly structured by trophic status in circumneutral and alkaline streams. *Applied and environmental microbiology* **75**, 6211–6221.
- Durán N. & Esposito E. (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied Catalysis B: Environmental* **28**, 83–99.
- Durán N., Rosa M.A., D'Annibale A. & Gianfreda L. (2002) Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. *Enzyme and microbial technology* **31**, 907–931.
- Elisashvili V. & Kachlishvili E. (2009) Physiological regulation of laccase and manganese peroxidase production by white-rot Basidiomycetes. *Journal of Biotechnology* **144**, 37–42.
- Elisashvili V., Penninckx M., Kachlishvili E., Asatiani M. & Kvesitadze G. (2006) Use of *Pleurotus dryinus* for lignocellulolytic enzymes production in submerged fermentation of mandarin peels and tree leaves. *Enzyme and Microbial Technology* **38**, 998–1004.
- Elosegi A. & Pozo J. (2005) Litter input. In: *Methods to Study Litter Decomposition*. pp. 3–11. Springer.

- Enríquez S., Duarte C.M. & Sand-Jensen K.A.J. (1993) Patterns in decomposition rates among photosynthetic organisms: the importance of detritus C: N: P content. *Oecologia* **94**, 457–471.
- Eskin N.M. & Shahidi F. (2012) *Biochemistry of foods*. Academic Press.
- Evert R.F. (2006) *Esau's plant anatomy: meristems, cells, and tissues of the plant body: their structure, function, and development*. John Wiley & Sons.
- Feckler A., Goedkoop W., Konschak M., Bundschuh R., Kenngott K.G.J., Schulz R., *et al.* (2017) History matters: Heterotrophic microbial community structure and function adapt to multiple stressors. *Global Change Biology*.
- Fenchel T. (1986) The ecology of heterotrophic microflagellates. In: *Advances in microbial ecology*. pp. 57–97. Springer.
- Fernandes I., Duarte S., Cássio F. & Pascoal C. (2009) Mixtures of zinc and phosphate affect leaf litter decomposition by aquatic fungi in streams. *Science of The Total Environment* **407**, 4283–4288.
- Fernández D., Tummala M., Schreiner V.C., Duarte S., Pascoal C., Winkelmann C., *et al.* (2016) Does nutrient enrichment compensate fungicide effects on litter decomposition and decomposer communities in streams? *Aquatic Toxicology* **174**, 169–178.
- Fernández D., Voss K., Bundschuh M., Zubrod J.P. & Schäfer R.B. (2015) Effects of fungicides on decomposer communities and litter decomposition in vineyard streams. *Science of The Total Environment* **533**, 40–48.
- Ferreira V., Castagneyrol B., Koricheva J., Gulis V., Chauvet E. & Graça M.A.S. (2015) A meta-analysis of the effects of nutrient enrichment on litter decomposition in streams: Nutrient enrichment and litter decomposition. *Biological Reviews* **90**, 669–688.
- Ferreira V. & Chauvet E. (2011) Synergistic effects of water temperature and dissolved nutrients on litter decomposition and associated fungi. *Global Change Biology* **17**, 551–564.
- Ferreira V., Encalada A.C. & Graça M.A. (2012) Effects of litter diversity on decomposition and biological colonization of submerged litter in temperate and tropical streams. *Freshwater Science* **31**, 945–962.
- Ferreira V., Graça M.A., de Lima J.L. & Gomes R. (2006) Role of physical fragmentation and invertebrate activity in the breakdown rate of leaves. *Archiv für Hydrobiologie* **165**, 493–513.
- Ferreira V., Raposeiro P.M., Pereira A., Cruz A.M., Costa A.C., Graça M.A., *et al.* (2016) Leaf litter decomposition in remote oceanic island streams is driven by microbes and

- depends on litter quality and environmental conditions. *Freshwater Biology* **61**, 783–799.
- Findlay S. & Sinsabaugh R.L. (2006) Large-scale variation in subsurface stream biofilms: a cross-regional comparison of metabolic function and community similarity. *Microbial ecology* **52**, 491–500.
- Finlay B.J. & Esteban G.F. (1998) Freshwater protozoa: biodiversity and ecological function. *Biodiversity & Conservation* **7**, 1163–1186.
- Fioretto A., Papa S., Curcio E., Sorrentino G. & Fuggi A. (2000) Enzyme dynamics on decomposing leaf litter of *Cistus incanus* and *Myrtus communis* in a Mediterranean ecosystem. *Soil Biology and Biochemistry* **32**, 1847–1855.
- Fisher S.G. & Likens G.E. (1973) Energy flow in Bear Brook, New Hampshire: an integrative approach to stream ecosystem metabolism. *Ecological monographs* **43**, 421–439.
- Fisher S.G. & Sponseller R.A. (2009) Streams and Rivers as Ecosystems. In: *Encyclopedia of Inland Waters*. pp. 491–498. Elsevier.
- Flores L., Banjac Z., Farré M., Larrañaga A., Mas-Martí E., Muñoz I., *et al.* (2014) Effects of a fungicide (imazalil) and an insecticide (diazinon) on stream fungi and invertebrates associated with litter breakdown. *Science of The Total Environment* **476-477**, 532–541.
- Forbes V.E., Palmqvist A. & Bach L. (2006) The use and misuse of biomarkers in ecotoxicology. *Environmental Toxicology and Chemistry* **25**, 272–280.
- Fragoeiro S. & Magan N. (2005) Enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *Phanerochaete chrysosporium* and *Trametes versicolor*. *Environmental Microbiology* **7**, 348–355.
- Gardeström J., Ermold M., Goedkoop W. & McKie B.G. (2016) Disturbance history influences stressor impacts: effects of a fungicide and nutrients on microbial diversity and litter decomposition. *Freshwater Biology* **61**, 2171–2184.
- Gaudes A., Artigas J., Romaní A.M., Sabater S. & Muñoz I. (2009) Contribution of microbial and invertebrate communities to leaf litter colonization in a Mediterranean stream. *Journal of the North American Benthological Society* **28**, 34–43.
- Gavrilescu M. (2005) Fate of pesticides in the environment and its bioremediation. *Engineering in life sciences* **5**, 497–526.
- Gessner M.O. (1991) Differences in processing dynamics of fresh and dried leaf litter in a stream ecosystem. *Freshwater Biology* **26**, 387–398.

- Gessner M.O. (2005) Proximate lignin and cellulose. In: *Methods to study litter decomposition*. pp. 115–120. Springer.
- Gessner M.O. & Chauvet E. (2002) A case for using litter breakdown to assess functional stream integrity. *Ecological applications* **12**, 498–510.
- Gessner M.O. & Chauvet E. (1994) Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology* **75**, 1807–1817.
- Gessner M.O., Chauvet E. & Dobson M. (1999) A perspective on leaf litter breakdown in streams. *Oikos*, 377–384.
- Gessner M.O. & Schwoerbel J. (1989) Leaching kinetics of fresh leaf-litter with implications for the current concept of leaf-processing in streams. *Archiv für Hydrobiologie* **115**, 81–90.
- Gessner M.O., Swan C.M., Dang C.K., McKie B.G., Bardgett R.D., Wall D.H., *et al.* (2010) Diversity meets decomposition. *Trends in ecology & evolution* **25**, 372–380.
- Gomi T., Sidle R.C. & Richardson J.S. (2002) Understanding processes and downstream linkages of headwater systems: headwaters differ from downstream reaches by their close coupling to hillslope processes, more temporal and spatial variation, and their need for different means of protection from land use. *AIBS Bulletin* **52**, 905–916.
- Götz T. & Böger P. (2004) The Very-Long-Chain Fatty Acid Synthase Is Inhibited by Chloroacetamides. *Zeitschrift für Naturforschung C* **59**.
- Graça M.A., Bärlocher F. & Gessner M.O. (2005) *Methods to study litter decomposition: a practical guide*. Springer Science & Business Media.
- Graça M.A.S. (2001a) The Role of Invertebrates on Leaf Litter Decomposition in Streams - a Review. *International Review of Hydrobiology* **86**, 383–393.
- Graça M.A.S. (2001b) The Role of Invertebrates on Leaf Litter Decomposition in Streams - a Review. *International Review of Hydrobiology* **86**, 383–393.
- Graça M.A.S., Ferreira V., Canhoto C., Encalada A.C., Guerrero-Bolaño F., Wantzen K.M., *et al.* (2015) A conceptual model of litter breakdown in low order streams: Litter breakdown in low order streams. *International Review of Hydrobiology* **100**, 1–12.
- Graça M.A.S., Maltby L. & Calow P. (1993) Importance of fungi in the diet of *Gammarus pulex* and *Asellus aquaticus* I: feeding strategies. *Oecologia* **93**, 139–144.
- Grigg A.H. & Mulligan D.R. (1999a) Litterfall from two eucalypt woodlands in central Queensland. *Australian journal of Ecology* **24**, 662–664.
- Grigg A.H. & Mulligan D.R. (1999b) Litterfall from two eucalypt woodlands in central Queensland. *Australian journal of Ecology* **24**, 662–664.

- Grimm N.B., Faeth S.H., Golubiewski N.E., Redman C.L., Wu J., Bai X., *et al.* (2008) Global change and the ecology of cities. *science* **319**, 756–760.
- Guenet B., Danger M., Abbadie L. & Lacroix G. (2010) Priming effect: bridging the gap between terrestrial and aquatic ecology. *Ecology* **91**, 2850–2861.
- Gulis V., Kuehn K.A. & Suberkropp K. (2009a) Fungi. In: *Encyclopedia of Inland Waters*. pp. 233–243. Elsevier.
- Gulis V., Kuehn K.A. & Suberkropp K. (2009b) Fungi. In: *Encyclopedia of Inland Waters*. pp. 233–243. Elsevier.
- Gulis V. & Suberkropp K. (2003) Effect of Inorganic Nutrients on Relative Contributions of Fungi and Bacteria to Carbon Flow from Submerged Decomposing Leaf Litter. *Microbial Ecology* **45**, 11–19.
- Hagen E.M., Webster J.R. & Benfield E.F. (2006) Are leaf breakdown rates a useful measure of stream integrity along an agricultural landuse gradient? *Journal of the North American Benthological Society* **25**, 330–343.
- Hansen W.F. (2001) Identifying stream types and management implications. *Forest Ecology and Management* **143**, 39–46.
- Harding J.S., Benfield E.F., Bolstad P.V., Helfman G.S. & Jones E.B.D. (1998) Stream biodiversity: the ghost of land use past. *Proceedings of the national academy of sciences* **95**, 14843–14847.
- Haritash A.K. & Kaushik C.P. (2009) Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *Journal of hazardous materials* **169**, 1–15.
- Hassett J.E., Zak D.R., Blackwood C.B. & Pregitzer K.S. (2009) Are basidiomycete laccase gene abundance and composition related to reduced lignolytic activity under elevated atmospheric NO<sub>3</sub>- deposition in a northern hardwood forest? *Microbial ecology* **57**, 728–739.
- Heino J. (2005) Functional biodiversity of macroinvertebrate assemblages along major ecological gradients of boreal headwater streams. *Freshwater Biology* **50**, 1578–1587.
- Heino J., Tolkkinen M., Pirttilä A.M., Aisala H. & Mykrä H. (2014) Microbial diversity and community-environment relationships in boreal streams. *Journal of Biogeography* **41**, 2234–2244.
- Hieber M. & Gessner M.O. (2002) Contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates. *Ecology* **83**, 1026–1038.
- Hodkinson I.D. (1975) Energy flow and organic matter decomposition in an abandoned beaver pond ecosystem. *Oecologia* **21**, 131–139.

- Hofmockel K.S., Zak D.R. & Blackwood C.B. (2007) Does atmospheric NO<sub>3</sub>- deposition alter the abundance and activity of ligninolytic fungi in forest soils? *Ecosystems* **10**, 1278–1286.
- Holloway J.M. & Dahlgren R.A. (2001) Seasonal and event-scale variations in solute chemistry for four Sierra Nevada catchments. *Journal of Hydrology* **250**, 106–121.
- Horn S.J., Vaaje-Kolstad G., Westereng B. & Eijsink V. (2012a) Novel enzymes for the degradation of cellulose. *Biotechnology for biofuels* **5**, 45.
- Horn S.J., Vaaje-Kolstad G., Westereng B. & Eijsink V. (2012b) Novel enzymes for the degradation of cellulose. *Biotechnology for biofuels* **5**, 45.
- Hughes S.R., Kay P. & Brown L.E. (2016) Impact of anti-inflammatories, beta-blockers and antibiotics on leaf litter breakdown in freshwaters. *Environmental Science and Pollution Research* **23**, 3956–3962.
- Jeffree C.E. (1986) The cuticle, epicuticular waxes and trichomes of plants, with reference to their structure, functions and evolution. *Insects on the plant surface*, 23–64.
- Jensen V. (1974) Decomposition of angiosperm tree leaf litter. *Biology of plant litter decomposition* **1**, 69–104.
- Johannes C. & Majcherczyk A. (2000) Laccase activity tests and laccase inhibitors. *Journal of Biotechnology* **78**, 193–199.
- Jonsson M., Ershammar E., Fick J., Brodin T. & Klaminder J. (2015) Effects of an antihistamine on carbon and nutrient recycling in streams. *Science of The Total Environment* **538**, 240–245.
- Karas P.A., Perruchon C., Exarhou K., Ehaliotis C. & Karpouzas D.G. (2011) Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. *Biodegradation* **22**, 215–228.
- Katagi T. & Tanaka H. (2016) Metabolism, bioaccumulation, and toxicity of pesticides in aquatic insect larvae. *Journal of Pesticide Science* **41**, 25–37.
- Kattwinkel M., Reichert P., Rüegg J., Liess M. & Schuwirth N. (2016) Modeling Macroinvertebrate Community Dynamics in Stream Mesocosms Contaminated with a Pesticide. *Environmental Science & Technology* **50**, 3165–3173.
- Kiran U. (1996) Fungi and food preferences of aquatic invertebrates. *National Academy Science Letters-India* **19**, 188–190.
- Klemm D., Heublein B., Fink H.-P. & Bohn A. (2005) Cellulose: fascinating biopolymer and sustainable raw material. *Angewandte Chemie International Edition* **44**, 3358–3393.

- Kolpin D.W., Furlong E.T., Meyer M.T., Thurman E.M., Zaugg S.D., Barber L.B., *et al.* (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999- 2000: A national reconnaissance. *Environmental science & technology* **36**, 1202–1211.
- Kolpin D.W., Thurman E.M. & Linhart S.M. (2001) Occurrence of cyanazine compounds in groundwater: degradates more prevalent than the parent compound. *Environmental science & technology* **35**, 1217–1222.
- Kominoski J.S., Hoellein T.J., Kelly J.J. & Pringle C.M. (2009) Does mixing litter of different qualities alter stream microbial diversity and functioning on individual litter species? *Oikos* **118**, 457–463.
- Kominoski J.S., Marczak L.B. & Richardson J.S. (2011) Riparian forest composition affects stream litter decomposition despite similar microbial and invertebrate communities. *Ecology* **92**, 151–159.
- Kominoski J.S., Rosemond A.D., Benstead J.P., Gulis V., Maerz J.C. & Manning D.W. (2015) Low-to-moderate nitrogen and phosphorus concentrations accelerate microbially driven litter breakdown rates. *Ecological Applications* **25**, 856–865.
- Krogh K.A., Halling-Sørensen B., Mogensen B.B. & Vejrup K.V. (2003) Environmental properties and effects of nonionic surfactant adjuvants in pesticides: a review. *Chemosphere* **50**, 871–901.
- Kuehn K.A., Francoeur S.N., Findlay R.H. & Neely R.K. (2014) Priming in the microbial landscape: periphytic algal stimulation of litter-associated microbial decomposers. *Ecology* **95**, 749–762.
- Kumar V. & Chandra R. (2018) Characterisation of manganese peroxidase and laccase producing bacteria capable for degradation of sucrose glutamic acid-Maillard reaction products at different nutritional and environmental conditions. *World Journal of Microbiology and Biotechnology* **34**, 32.
- Kuzyakov Y. (2010) Priming effects: interactions between living and dead organic matter. *Soil Biology and Biochemistry* **42**, 1363–1371.
- Lagadic L., Caquet T. & Ramade F. (1994) The role of biomarkers in environmental assessment (5). Invertebrate populations and communities. *Ecotoxicology* **3**, 193–208.
- Larrañaga S., Díez J.R., Elozegi A. & Pozo J. (2003) Leaf retention in streams of the Agüera basin (northern Spain). *Aquatic Sciences* **65**, 158–166.

- Larsen D.P., Stay F., Shiroyama T. & DeNoyelles Jr F. (1986) Comparisons of single-species, microcosm and experimental pond responses to atrazine exposure. *Environmental Toxicology and Chemistry: An International Journal* **5**, 179–190.
- Lawrence D. & Barraclough T.G. (2016) Evolution of resource use along a gradient of stress leads to increased facilitation. *Oikos* **125**, 1284–1295.
- Lazorchak J.M., Griffith M.B., Mills M., Schubauer-Berigan J., McCormick F., Brenner R., *et al.* (2015) Proof of concept for the use of macroinvertebrates as indicators of polychlorinated biphenyls (PCB) contamination in Lake Hartwell: Use of macroinvertebrates as indicators of PCB contamination. *Environmental Toxicology and Chemistry* **34**, 1277–1282.
- Lear G. (2016) *Biofilms in Bioremediation*. Caister Academic Press.
- Lecerf A., Usseglio-Polatera P., Charcosset J.-Y., Lambrigt D., Bracht B. & Chauvet E. (2006) Assessment of functional integrity of eutrophic streams using litter breakdown and benthic macroinvertebrates. *Archiv für Hydrobiologie* **165**, 105–126.
- Leonowicz A., Cho N., Luterek J., Wilkolazka A., Wojtas-Wasilewska M., Matuszewska A., *et al.* (2001) Fungal laccase: properties and activity on lignin. *Journal of Basic Microbiology* **41**, 185–227.
- Leopold L.B., Wolman M.G. & Miller J.P. (2012) *Fluvial processes in geomorphology*. Courier Corporation.
- Leu C., Singer H., Stamm C., Müller S.R. & Schwarzenbach R.P. (2004) Simultaneous assessment of sources, processes, and factors influencing herbicide losses to surface waters in a small agricultural catchment. *Environmental Science & Technology* **38**, 3827–3834.
- Lewis W.M. (2009) The Ecological Niche in Aquatic Ecosystems. In: *Encyclopedia of Inland Waters*. pp. 411–415. Elsevier.
- Limburg K.E. (2009) Aquatic Ecosystem Services. In: *Encyclopedia of Inland Waters*. pp. 25–30. Elsevier.
- Litvintseva A.P. & Henson J.M. (2002) Cloning, characterization, and transcription of three laccase genes from *Gaeumannomyces graminis* var. *tritici*, the take-all fungus. *Applied and environmental microbiology* **68**, 1305–1311.
- Li X., Zhang Y., Guo F., Gao X. & Wang Y. (2018) Predicting the effect of land use and climate change on stream macroinvertebrates based on the linkage between structural equation modeling and bayesian network. *Ecological Indicators* **85**, 820–831.



- Logue J.B., Bürgmann H. & Robinson C.T. (2008) Progress in the Ecological Genetics and Biodiversity of Freshwater Bacteria. *BioScience* **58**, 103–113.
- López-Mondéjar R., Zühlke D., Becher D., Riedel K. & Baldrian P. (2016) Cellulose and hemicellulose decomposition by forest soil bacteria proceeds by the action of structurally variable enzymatic systems. *Scientific Reports* **6**.
- Lowe W.H. & Likens G.E. (2005) Moving headwater streams to the head of the class. *AIBS Bulletin* **55**, 196–197.
- Magbanua F.S., Townsend C.R., Blackwell G.L., Phillips N. & Matthaei C.D. (2010) Responses of stream macroinvertebrates and ecosystem function to conventional, integrated and organic farming. *Journal of Applied Ecology* **47**, 1014–1025.
- Malaj E., Peter C., Grote M., Kühne R., Mondy C.P., Usseglio-Polatera P., *et al.* (2014) Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. *Proceedings of the National Academy of Sciences* **111**, 9549–9554.
- Malavoi J.-R. & Souchon Y. (2002) Description standardisée des principaux faciès d'écoulement observables en rivières: clé de détermination qualitative et mesure physiques. *Bulletin Français de la Pêche et de la Pisciculture*, 357–372.
- Maltby L., Brock T.C. & van den Brink P.J. (2009) Fungicide risk assessment for aquatic ecosystems: importance of interspecific variation, toxic mode of action, and exposure regime. *Environmental science & technology* **43**, 7556–7563.
- Mann K.I.I. (1975a) Detritus formation from eelgrass (*Zostera marina* L.): The relative effects of fragmentation, leaching, and decay. *Limnol. Oceanogr* **20**, 924–934.
- Mann K.I.I. (1975b) Detritus formation from eelgrass (*Zostera marina* L.): The relative effects of fragmentation, leaching, and decay. *Limnol. Oceanogr* **20**, 924–934.
- Mann M.E., Bradley R.S. & Hughes M.K. (1998) Global-scale temperature patterns and climate forcing over the past six centuries. *Nature* **392**, 779.
- Margalef O., Sardans J., Fernández-Martínez M., Molowny-Horas R., Janssens I.A., Ciais P., *et al.* (2017) Global patterns of phosphatase activity in natural soils. *Scientific reports* **7**, 1337.
- Matocha C.J., Haszler G.R. & Grove J.H. (2004) Nitrogen fertilization suppresses soil phenol oxidase enzyme activity in no-tillage systems. *Soil science* **169**, 708–714.
- Maul J.D., Schuler L.J., Belden J.B., Whiles M.R. & Lydy M.J. (2006) EFFECTS OF THE ANTIBIOTIC CIPROFLOXACIN ON STREAM MICROBIAL COMMUNITIES AND DETRITIVOROUS MACROINVERTEBRATES. *Environmental Toxicology and Chemistry* **25**, 1598.

- McCann M.C. (1991) Architecture of the primary cell wall. *The cytoskeletal basis of plant growth and form*, 109–129.
- Méthé B.A., Hiorns W.D. & Zehr J.P. (1998) Contrasts between marine and freshwater bacterial community composition: Analyses of communities in Lake George and six other Adirondack lakes. *Limnology and Oceanography* **43**, 368–374.
- Meyer J.L., Strayer D.L., Wallace J.B., Eggert S.L., Helfman G.S. & Leonard N.E. (2007) The contribution of headwater streams to biodiversity in river networks1. *JAWRA Journal of the American Water Resources Association* **43**, 86–103.
- Meyer J.L. & Wallace J.B. (2001) Lost linkages and lotic ecology: rediscovering small streams. *Ecology: Achievement and challenge* **29** 5317.
- Mikiashvili N., Wasser S.P., Nevo E. & Elisashvili V. (2006) Effects of carbon and nitrogen sources on *Pleurotus ostreatus* ligninolytic enzyme activity. *World Journal of Microbiology and Biotechnology* **22**, 999–1002.
- Minguez L., Pedelucq J., Farcy E., Ballandonne C., Budzinski H. & Halm-Lemeille M.-P. (2016) Toxicities of 48 pharmaceuticals and their freshwater and marine environmental assessment in northwestern France. *Environmental Science and Pollution Research* **23**, 4992–5001.
- Minshall G.W. (1978) Autotrophy in stream ecosystems. *BioScience* **28**, 767–771.
- Minshall G.W. (1967) Role of allochthonous detritus in the trophic structure of a woodland springbrook community. *Ecology*, 139–149.
- Molénat J., Gascuel-Oudou C., Ruiz L. & Gruau G. (2008) Role of water table dynamics on stream nitrate export and concentration in agricultural headwater catchment (France). *Journal of Hydrology* **348**, 363–378.
- Monteiro S.C. & Boxall A.B. (2010) Occurrence and fate of human pharmaceuticals in the environment. In: *Reviews of environmental contamination and toxicology*. pp. 53–154. Springer.
- Montgomery D.R. (1999) PROCESS DOMAINS AND THE RIVER CONTINUUM 1. *JAWRA Journal of the American Water Resources Association* **35**, 397–410.
- Moons P., Michiels C.W. & Aertsen A. (2009) Bacterial interactions in biofilms. *Critical reviews in microbiology* **35**, 157–168.
- Mukherjee P. & Roy P. (2013) Cloning, sequencing and expression of novel trichloroethylene degradation genes from *Stenotrophomonas maltophilia* PM102: a case of gene duplication. *J Bioremed Biodeg* **4**, 10–4172.

- Mukherjee S., Basak B., Bhunia B., Dey A. & Mondal B. (2013) Potential use of polyphenol oxidases (PPO) in the bioremediation of phenolic contaminants containing industrial wastewater. *Reviews in Environmental Science and Bio/Technology* **12**, 61–73.
- Mulholland P.J., Hall Jr R.O., Sobota D.J., Dodds W.K., Findlay S.E., Grimm N.B., *et al.* (2009) Nitrate removal in stream ecosystems measured by <sup>15</sup>N addition experiments: denitrification. *Limnology and Oceanography* **54**, 666–680.
- Mulholland P.J., Helton A.M., Poole G.C., Hall R.O., Hamilton S.K., Peterson B.J., *et al.* (2008) Stream denitrification across biomes and its response to anthropogenic nitrate loading. *Nature* **452**, 202.
- Mykrä H., Tolkkinen M. & Heino J. (2017) Environmental degradation results in contrasting changes in the assembly processes of stream bacterial and fungal communities. *Oikos* **126**, 1291–1298.
- Nijboer R.C., Verdonchot P.F.M., Johnson R.K., Sommerhäuser M. & Buffagni A. (2004) Establishing reference conditions for European streams. In: *Integrated Assessment of Running Waters in Europe*. pp. 91–105. Springer.
- Nikolaou A., Meric S. & Fatta D. (2007) Occurrence patterns of pharmaceuticals in water and wastewater environments. *Analytical and bioanalytical chemistry* **387**, 1225–1234.
- Nikolcheva L.G., Bourque T. & Bärlocher F. (2005) Fungal diversity during initial stages of leaf decomposition in a stream. *Mycological Research* **109**, 246–253.
- Ocampo C.J., Sivapalan M. & Oldham C. (2006) Hydrological connectivity of upland-riparian zones in agricultural catchments: Implications for runoff generation and nitrate transport. *Journal of Hydrology* **331**, 643–658.
- O'Neill M.A. & York W.S. (2003) The composition and structure of plant primary cell walls. *The plant cell wall*, 1–54.
- Osono T. (2007) Ecology of ligninolytic fungi associated with leaf litter decomposition. *Ecological Research* **22**, 955–974.
- Papadakis E.-N., Tsaboula A., Kotopoulou A., Kintzikoglou K., Vryzas Z. & Papadopoulou-Mourkidou E. (2015) Pesticides in the surface waters of Lake Vistonis Basin, Greece: Occurrence and environmental risk assessment. *Science of The Total Environment* **536**, 793–802.
- Pascoal C. & Cássio F. (2004) Contribution of fungi and bacteria to leaf litter decomposition in a polluted river. *Applied and environmental microbiology* **70**, 5266–5273.

- Pascoal C., Cássio F., Marcotegui A., Sanz B. & Gomes P. (2005a) Role of fungi, bacteria, and invertebrates in leaf litter breakdown in a polluted river. *Journal of the North American Benthological Society* **24**, 784–797.
- Pascoal C., Cássio F. & Marvanová L. (2005b) Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Archiv für Hydrobiologie* **162**, 481–496.
- Paul S., Gable K., Beaudoin F., Cahoon E., Jaworski J., Napier J.A., *et al.* (2006) Members of the Arabidopsis FAE1-like 3-Ketoacyl-CoA Synthase Gene Family Substitute for the Elop Proteins of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **281**, 9018–9029.
- Pesce S., Bouchez A. & Montuelle B. (2012a) Effects of organic herbicides on phototrophic microbial communities in freshwater ecosystems. In: *Reviews of environmental contamination and toxicology*. pp. 87–124. Springer.
- Pesce S., Bouchez A. & Montuelle B. (2012b) Effects of organic herbicides on phototrophic microbial communities in freshwater ecosystems. In: *Reviews of environmental contamination and toxicology*. pp. 87–124. Springer.
- Pesce S., Fajon C., Bardot C., Bonnemoy F., Portelli C. & Bohatier J. (2008) Longitudinal changes in microbial planktonic communities of a French river in relation to pesticide and nutrient inputs. *Aquatic Toxicology* **86**, 352–360.
- Pesce S., Zoghlami O., Margoum C., Artigas J., Chaumot A. & Foulquier A. (2016) Combined effects of drought and the fungicide tebuconazole on aquatic leaf litter decomposition. *Aquatic Toxicology* **173**, 120–131.
- PETERSEN R.C. & CUMMINS K.W. (1974) Leaf processing in a woodland stream. *Freshwater biology* **4**, 343–368.
- Peterson B.J., Wollheim W.M., Mulholland P.J., Webster J.R., Meyer J.L., Tank J.L., *et al.* (2001) Control of nitrogen export from watersheds by headwater streams. *Science* **292**, 86–90.
- Piontek K., Antorini M. & Choinowski T. (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *Journal of Biological Chemistry* **277**, 37663–37669.
- Piscart C., Genoel R., Doledec S., Chauvet E. & Marmonier P. (2009) Effects of intense agricultural practices on heterotrophic processes in streams. *Environmental Pollution* **157**, 1011–1018.

- Poff N.L. (1997) Landscape filters and species traits: towards mechanistic understanding and prediction in stream ecology. *Journal of the north american Benthological society* **16**, 391–409.
- Poole G.C. (2002) Fluvial landscape ecology: addressing uniqueness within the river discontinuum. *Freshwater Biology* **47**, 641–660.
- Postel S.L., Daily G.C. & Ehrlich P.R. (1996) Human appropriation of renewable fresh water. *Science* **271**, 785–788.
- Rabiet M., Margoum C., Gouy V., Carlier N. & Coquery M. (2010) Assessing pesticide concentrations and fluxes in the stream of a small vineyard catchment—effect of sampling frequency. *Environmental Pollution* **158**, 737–748.
- Rasmussen J.J., Wiberg-Larsen P., Baattrup-Pedersen A., Monberg R.J. & Kronvang B. (2012) Impacts of pesticides and natural stressors on leaf litter decomposition in agricultural streams. *Science of The Total Environment* **416**, 148–155.
- Reeves G.H., Benda L.E., Burnett K.M., Bisson P.A. & Sedell J.R. (1995) A disturbance-based ecosystem approach to maintaining and restoring freshwater habitats of evolutionarily significant units of anadromous salmonids in the Pacific Northwest. In: *American Fisheries Society Symposium*. 17: 334–349. .
- Ribblett S.G., Palmer M.A. & Wayne Coats D. (2005) The importance of bacterivorous protists in the decomposition of stream leaf litter. *Freshwater Biology* **50**, 516–526.
- Rice S.P., Greenwood M.T. & Joyce C.B. (2001) Tributaries, sediment sources, and the longitudinal organisation of macroinvertebrate fauna along river systems. *Canadian Journal of Fisheries and Aquatic Sciences* **58**, 824–840.
- Richardson J.S. & Danehy R.J. (2007) A synthesis of the ecology of headwater streams and their riparian zones in temperate forests. *Forest Science* **53**, 131–147.
- Richardson K. & Jørgensen B.B. (1996) Eutrophication: definition, history and effects. *Eutrophication in coastal marine ecosystems*, 1–19.
- Río Andrade J.C. del, Rencoret J., Gutiérrez Suárez A., Lan W., Kim H. & Ralph J. (2017) Lignin monomers from outside the canonical monolignol biosynthetic pathway.
- Rodea-Palomares I., Makowski M., Gonzalo S., González-Pleiter M., Leganés F. & Fernández-Piñas F. (2015) Effect of PFOA/PFOS pre-exposure on the toxicity of the herbicides 2, 4-D, Atrazine, Diuron and Paraquat to a model aquatic photosynthetic microorganism. *Chemosphere* **139**, 65–72.

- Romaní A.M., Amalfitano S., Artigas J., Fazi S., Sabater S., Timoner X., *et al.* (2013) Microbial biofilm structure and organic matter use in mediterranean streams. *Hydrobiologia* **719**, 43–58.
- Romaní A.M., Artigas J. & Ylla I. (2012) Extracellular enzymes in aquatic biofilms: microbial interactions versus water quality effects in the use of organic matter. *Microbial Biofilms: Current Research and Applications*.(Eds G. Lear & G. Lewis), 153–174.
- Romaní A.M., Fischer H., Mille-Lindblom C. & Tranvik L.J. (2006a) Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. *Ecology* **87**, 2559–2569.
- Romaní A.M., Fischer H., Mille-Lindblom C. & Tranvik L.J. (2006b) Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. *Ecology* **87**, 2559–2569.
- Rong Q., Sridhar K.R. & Bärlocher F. (1995) Food selection in three leaf-shredding stream invertebrates. *Hydrobiologia* **316**, 173–181.
- Rosegrant M.W., Cai X. & Cline S.A. (2002) *World water and food to 2025: dealing with scarcity*. Intl Food Policy Res Inst.
- Rosi-Marshall E.J. & Royer T.V. (2012) Pharmaceutical compounds and ecosystem function: an emerging research challenge for aquatic ecologists. *Ecosystems* **15**, 867–880.
- Rossi F., Artigas J. & Mallet C. (2017) Structural and functional responses of leaf-associated fungal communities to chemical pollution in streams. *Freshwater Biology* **62**, 1207–1219.
- Rossi F., Mallet C., Portelli C., Donnadieu F., Bonnemoy F. & Artigas J. (2019a) Stimulation or inhibition: Leaf microbial decomposition in streams subjected to complex chemical contamination. *Science of The Total Environment* **648**, 1371–1383.
- Rossi F., Mallet C., Portelli C., Donnadieu F., Bonnemoy F. & Artigas J. (2019b) Stimulation or inhibition: Leaf microbial decomposition in streams subjected to complex chemical contamination. *Science of The Total Environment* **648**, 1371–1383.
- Rossi F., Pesce S., Mallet C., Margoum C., Chaumot A., Masson M., *et al.* (2018) Interactive Effects of Pesticides and Nutrients on Microbial Communities Responsible of Litter Decomposition in Streams. *Frontiers in Microbiology* **9**.
- Roy S.B., Chen L., Girvetz E.H., Maurer E.P., Mills W.B. & Grieb T.M. (2012) Projecting water withdrawal and supply for future decades in the US under climate change scenarios. *Environmental science & technology* **46**, 2545–2556.

- Ruhí A., Acuña V., Barceló D., Huerta B., Mor J.-R., Rodríguez-Mozaz S., *et al.* (2016) Bioaccumulation and trophic magnification of pharmaceuticals and endocrine disruptors in a Mediterranean river food web. *Science of The Total Environment* **540**, 250–259.
- Sabater S., Artigas J., Gaudes A., Muñoz I., Urrea G. & Romaní A.M. (2011) Long-term moderate nutrient inputs enhance autotrophy in a forested Mediterranean stream: Long-term nutrient input in a forested Mediterranean stream. *Freshwater Biology* **56**, 1266–1280.
- Sabater S., Guasch H., Muñoz I. & Romaní A. (2006) Hydrology, light and the use of organic and inorganic materials as structuring factors of biological communities in Mediterranean streams. *Limnetica* **25**, 335–348.
- Saiya-Cork K.R., Sinsabaugh R.L. & Zak D.R. (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology and Biochemistry* **34**, 1309–1315.
- Sala O.E., Chapin F.S., Armesto J.J., Berlow E., Bloomfield J., Dirzo R., *et al.* (2000) Global biodiversity scenarios for the year 2100. *science* **287**, 1770–1774.
- Sanders R.W. (2009) Protists. In: *Encyclopedia of Inland Waters*. pp. 252–260. Elsevier.
- Sandin L. & Solimini A.G. (2009) Freshwater ecosystem structure–function relationships: from theory to application. *Freshwater Biology* **54**, 2017–2024.
- Sarkar P., Bosneaga E. & Auer M. (2009) Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *Journal of Experimental Botany* **60**, 3615–3635.
- Schäfer R.B. & Bundschuh M. (2018) Ecotoxicology. In: *Riverine Ecosystem Management*. pp. 225–239. Springer.
- Schäfer R.B., Caquet T., Siimes K., Mueller R., Lagadic L. & Liess M. (2007) Effects of pesticides on community structure and ecosystem functions in agricultural streams of three biogeographical regions in Europe. *Science of the Total Environment* **382**, 272–285.
- Scheller H.V. & Ulvskov P. (2010) Hemicelluloses. *Annual review of plant biology* **61**.
- Schell T., Goedkoop W., Zubrod J.P., Feckler A., Lüderwald S., Schulz R., *et al.* (2018) Assessing the effects of field-relevant pesticide mixtures for their compliance with the concentration addition model—An experimental approach with *Daphnia magna*. *Science of The Total Environment* **644**, 342–349.

- Schulze D.J. & Walker K.F. (1997) Riparian eucalypts and willows and their significance for aquatic invertebrates in the River Murray, South Australia. *Regulated Rivers: Research & Management: An International Journal Devoted to River Research and Management* **13**, 557–577.
- Schulz R. (2004) Field studies on exposure, effects, and risk mitigation of aquatic nonpoint-source insecticide pollution. *Journal of Environmental Quality* **33**, 419–448.
- Sharma B., Dangi A.K. & Shukla P. (2018a) Contemporary enzyme based technologies for bioremediation: a review. *Journal of environmental management* **210**, 10–22.
- Sharma B., Dangi A.K. & Shukla P. (2018b) Contemporary enzyme based technologies for bioremediation: A review. *Journal of Environmental Management* **210**, 10–22.
- Shearer C.A., Descals E., Kohlmeyer B., Kohlmeyer J., Marvanová L., Padgett D., *et al.* (2007) Fungal biodiversity in aquatic habitats. *Biodiversity and Conservation* **16**, 49–67.
- Sherr E.B. & Sherr B.F. (1994) Bacterivory and herbivory: key roles of phagotrophic protists in pelagic food webs. *Microbial Ecology* **28**, 223–235.
- Shiklomanov I.A. (1993a) *World Fresh Water Resource In: Gleick, PH (Ed.) Water crisis: A guide to world fresh water resources*. Oxford University Press.
- Shiklomanov I.A. (1993b) *World Fresh Water Resource In: Gleick, PH (Ed.) Water crisis: A guide to world fresh water resources*. Oxford University Press.
- Singh nee' Nigam P. & Pandey A. eds (2009) *Biotechnology for Agro-Industrial Residues Utilisation*. Springer Netherlands, Dordrecht.
- Sinsabaugh R.L. (2010) Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biology and Biochemistry* **42**, 391–404.
- Sinsabaugh R.L., Carreiro M.M. & Repert D.A. (2002) Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. *Biogeochemistry* **60**, 1–24.
- Sinsabaugh R.L., Hill B.H. & Shah J.J.F. (2009) Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. *Nature* **462**, 795.
- Sinsabaugh R.L. & Linkins A.E. (1987) Inhibition of the *Trichoderma viride* cellulase complex by leaf litter extracts. *Soil Biology and Biochemistry* **19**, 719–725.
- Slater J.H. & Lovatt D. (1984) Biodegradation and the significance of microbial communities. *Microbiology Series[MICROBIOL. SER.]. 1984*.



- Somerville C., Bauer S., Brininstool G., Facette M., Hamann T., Milne J., *et al.* (2004) Toward a systems approach to understanding plant cell walls. *Science* **306**, 2206–2211.
- Songulashvili G., Elisashvili V., Wasser S.P., Nevo E. & Hadar Y. (2007) Basidiomycetes laccase and manganese peroxidase activity in submerged fermentation of food industry wastes. *Enzyme and Microbial Technology* **41**, 57–61.
- Souza P.M. de, Bittencourt M.L. de A., Caprara C.C., Freitas M. de, Almeida R.P.C. de, Silveira D., *et al.* (2015) A biotechnology perspective of fungal proteases. *Brazilian Journal of Microbiology* **46**, 337–346.
- Stehle S., Knäbel A. & Schulz R. (2013) Probabilistic risk assessment of insecticide concentrations in agricultural surface waters: a critical appraisal. *Environmental monitoring and assessment* **185**, 6295–6310.
- Strahler A.N. (1957) Quantitative analysis of watershed geomorphology. *Transactions, American Geophysical Union* **38**, 913.
- Stuart V., Lucas M.I. & Newell R.C. (1981) Heterotrophic Utilization Of Particulate Matter From The Kelp Laminaria-Pallida. *Marine Ecology Progress Series* **4**, 337–348.
- Suberkropp K., Godshalk G.L. & Klug M.J. (1976) Changes in the chemical composition of leaves during processing in a woodland stream. *Ecology*, 720–727.
- Suberkropp K. & Klug M.J. (1976) Fungi and bacteria associated with leaves during processing in a woodland stream. *Ecology*, 707–719.
- Suberkropp K. & Wallace J.B. (1992) Aquatic hyphomycetes in insecticide-treated and untreated streams. *Journal of the North American Benthological Society* **11**, 165–171.
- Sukhodolov A.N., Kozerski H.-P. & Rhoads B.L. (2009) Currents in Rivers.
- Swan C.M. & Palmer M.A. (2004) Leaf diversity alters litter breakdown in a Piedmont stream. *Journal of the North American Benthological Society* **23**, 15–28.
- Tachet H., Bournaud M. & Richoux P. (1991) Introduction à l'étude des macroinvertébrés des eaux douces (systématique élémentaire et aperçu écologique).
- Tian D., Lin Z., Yu J. & Yin D. (2012) Influence factors of multicomponent mixtures containing reactive chemicals and their joint effects. *Chemosphere* **88**, 994–1000.
- Tilman D., Balzer C., Hill J. & Belfort B.L. (2011) Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences* **108**, 20260–20264.

- Tlili A., Berard A., Blanck H., Bouchez A., Cássio F., Eriksson K.M., *et al.* (2016) Pollution-induced community tolerance (PICT): towards an ecologically relevant risk assessment of chemicals in aquatic systems. *Freshwater Biology* **61**, 2141–2151.
- Tobor-Kaplon M.A., Bloem J., Römken P.F. & Ruiter P. de (2005) Functional stability of microbial communities in contaminated soils. *Oikos* **111**, 119–129.
- Trewavas A. (2002) Malthus foiled again and again. *Nature* **418**, 668.
- Tukey Jr H.B. (1970) The leaching of substances from plants. *Annual review of plant physiology* **21**, 305–324.
- Vanholme R., Demedts B., Morreel K., Ralph J. & Boerjan W. (2010) Lignin biosynthesis and structure. *Plant Physiology* **153**, 895–905.
- Vannote R.L., Minshall G.W., Cummins K.W., Sedell J.R. & Cushing C.E. (1980) The river continuum concept. *Canadian journal of fisheries and aquatic sciences* **37**, 130–137.
- Villeneuve A., Larroudé S. & Humbert J.-F. (2011a) *Herbicide contamination of freshwater ecosystems: impact on microbial communities*. InTech Open.
- Villeneuve A., Larroudé S. & Humbert J.-F. (2011b) *Herbicide contamination of freshwater ecosystems: impact on microbial communities*. InTech Open.
- Vitousek P.M., Aber J.D., Howarth R.W., Likens G.E., Matson P.A., Schindler D.W., *et al.* (1997a) Human alteration of the global nitrogen cycle: sources and consequences. *Ecological applications* **7**, 737–750.
- Vitousek P.M., Mooney H.A., Lubchenco J. & Melillo J.M. (1997b) Human domination of Earth's ecosystems. *Science* **277**, 494–499.
- Van Vliet M.T.H., Franssen W.H.P., Yearsley J.R., Ludwig F., Haddeland I., Lettenmaier D.P., *et al.* (2013) Global river discharge and water temperature under climate change. *Global Environmental Change* **23**, 450–464.
- Vörösmarty C.J., McIntyre P.B., Gessner M.O., Dudgeon D., Prusevich A., Green P., *et al.* (2010) Global threats to human water security and river biodiversity. *Nature* **467**, 555–561.
- Vrana B., Allan I.J., Greenwood R., Mills G.A., Dominiak E., Svensson K., *et al.* (2005) Passive sampling techniques for monitoring pollutants in water. *TrAC Trends in Analytical Chemistry* **24**, 845–868.
- Walker G.M. & White N.A. (2017a) Introduction to fungal physiology. *Fungi: biology and applications*, 1–35.
- Walker G.M. & White N.A. (2017b) Introduction to fungal physiology. *Fungi: biology and applications*, 1–35.

- Wallace J.B. & Eggert S.L. (2009) Benthic Invertebrate Fauna, Small Streams. In: *Encyclopedia of Inland Waters*. pp. 173–190. Elsevier.
- Webster J. (1992) Anamorph-teleomorph relationships. In: *The ecology of aquatic hyphomycetes*. pp. 99–117. Springer.
- Webster J.R. (2007) Spiraling down the river continuum: stream ecology and the U-shaped curve. *Journal of the North American Benthological Society* **26**, 375–389.
- Webster J.R. & Benfield E.F. (1986) Vascular plant breakdown in freshwater ecosystems. *Annual review of ecology and systematics* **17**, 567–594.
- Webster J.R., Covich A.P., Tank J.L. & Crockett T.V. (1994) Retention of coarse organic particles in streams in the southern Appalachian Mountains. *Journal of the North American Benthological Society* **13**, 140–150.
- Webster J.R. & Meyer J.L. (1997) Organic matter budgets for streams: a synthesis. *Journal of the North American Benthological Society* **16**, 141–161.
- Weigelhofer G. & Waringer J.A. (1994) Allochthonous input of coarse particulate organic matter (CPOM) in a first to fourth order Austrian forest stream. *Internationale Revue der Gesamten Hydrobiologie und Hydrographie* **79**, 461–471.
- Winkelmann C., Worischka S., Koop J.H.E. & Benndorf J. (2007) Predation effects of benthivorous fish on grazing and shredding macroinvertebrates in a detritus-based stream food web. *Limnologia - Ecology and Management of Inland Waters* **37**, 121–128.
- Wipfli M.S., Richardson J.S. & Naiman R.J. (2007) Ecological Linkages Between Headwaters and Downstream Ecosystems: Transport of Organic Matter, Invertebrates, and Wood Down Headwater Channels 1. *JAWRA Journal of the American Water Resources Association* **43**, 72–85.
- Witkamp M. & Frank M.L. (1969) Loss of weight, cobalt-60, and cesium-137 from tree litter in three subsystems of a watershed. *Environmental Science & Technology* **3**, 1195–1198.
- Wong D.W. (2009) Structure and action mechanism of ligninolytic enzymes. *Applied biochemistry and biotechnology* **157**, 174–209.
- Wright I.J., Reich P.B. & Westoby M. (2001) Strategy shifts in leaf physiology, structure and nutrient content between species of high-and low-rainfall and high-and low-nutrient habitats. *Functional Ecology* **15**, 423–434.
- Yannarell A.C. & Kent A.D. (2009) Bacteria, Distribution and Community Structure. In: *Encyclopedia of Inland Waters*. pp. 201–210. Elsevier.

- Young R.G., Matthaei C.D. & Townsend C.R. (2008) Organic matter breakdown and ecosystem metabolism: functional indicators for assessing river ecosystem health. *Journal of the North American Benthological Society* **27**, 605–625.
- Zeglin L.H., Stursova M., Sinsabaugh R.L. & Collins S.L. (2007) Microbial responses to nitrogen addition in three contrasting grassland ecosystems. *Oecologia* **154**, 349–359.
- Zubrod J.P., Bundschuh M., Feckler A., Englert D. & Schulz R. (2011a) Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. *Environmental Toxicology and Chemistry* **30**, 2718–2724.
- Zubrod J.P., Bundschuh M., Feckler A., Englert D. & Schulz R. (2011b) Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. *Environmental Toxicology and Chemistry* **30**, 2718–2724.
- Zubrod J.P., Englert D., Feckler A., Koksharova N., Konschak M., Bundschuh R., *et al.* (2015a) Does the Current Fungicide Risk Assessment Provide Sufficient Protection for Key Drivers in Aquatic Ecosystem Functioning? *Environmental Science & Technology* **49**, 1173–1181.
- Zubrod J.P., Feckler A., Englert D., Koksharova N., Rosenfeldt R.R., Seitz F., *et al.* (2015b) Inorganic fungicides as routinely applied in organic and conventional agriculture can increase palatability but reduce microbial decomposition of leaf litter. *Journal of Applied Ecology* **52**, 310–322.



## Annex I



Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: [www.elsevier.com/locate/scitotenv](http://www.elsevier.com/locate/scitotenv)

# Sensitivity of laccase activity to the fungicide tebuconazole in decomposing litter

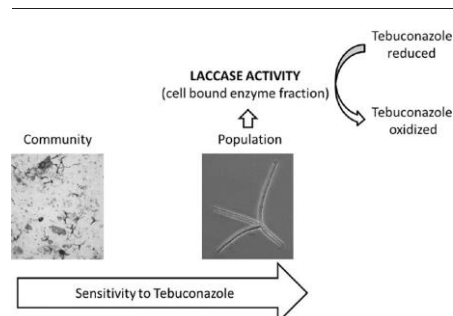
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## HIGHLIGHTS

- Laccase sensitivity to tebuconazole (TBZ) was assessed in decomposing leaves.
- The fungicide enhances laccase activity of *Alatospora acuminata* populations.
- Activity correction by fungal community descriptors improved toxicity assessment.
- Only laccase activity from the cell bound fraction was sensitive to TBZ.
- Fungal laccase activity can be involved in the detoxification of TBZ.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

## Article history:

Received 16 December 2016  
 Received in revised form 24 January 2017  
 Accepted 24 January 2017  
 Available online xxxx

Editor: D. Barcelo

## Keywords:

Microbial interactions  
 Cell bound enzyme  
 Diffusible enzyme  
 Functional descriptor  
 Aquatic hyphomycete  
 Bacteria

## ABSTRACT

The present study investigates the sensitivity of laccase activity to the fungicide tebuconazole (TBZ) in order to seek for new functional toxicity descriptors in aquatic microbial communities associated to decomposing litter. With this aim, we analyzed the sensitivity of laccase from the different microbial components (fungi and bacteria growing separately and in co-existence), as well as that of their corresponding enzyme fractions (cell bound and diffusible), forming microbial communities in *Alnus glutinosa* leaves. Results show that fungi are pivotal for laccase activity in leaves and that their activity is repressed when they co-exist with bacteria. The sensitivity of laccase activity to the TBZ was only detectable in leaves colonized by fungi separately (*Alatospora acuminata* populations), but absent in those colonized by bacteria separately and/or mixed fungi plus bacteria. Specifically, the increase of TBZ concentration enhances laccase activity in *Alatospora acuminata* populations but decreases ergosterol concentration as well as the amount of 18S RNA gene copies. This activity response suggests a detoxification mechanism employed by the fungus in order to reduce TBZ toxicity. Besides, enzyme fractioning showed that laccase activity in the cell bound fraction (76% of the total activity) was sensitive to the fungicide, but not that in the diffusible fraction (24% of total activity). Hence, TBZ would influence laccase activity in the presence of fungal cells but not in enzymes already synthesized in the extracellular space. The present study highlights the importance of the biological complexity level (i. e. population, community, ecosystem) when seeking for appropriate functional ecotoxicity descriptors in aquatic microbial communities.

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## 1. Introduction

Microbial communities contribute to the transformation and mineralization of carbon and nutrients from leaf litter allowing their own nutrient supply (Schimel and Weintraub, 2003). Leaf litter decomposition is possible through the synthesis extracellular enzymes which production can be viewed as a form of foraging strategy where microbial cells demand are aligned with nutrient and energy supplies (Burns et al., 2013). Among leaf-litter constituents, the lignin is one of the most abundant naturally occurring compounds. It can be depolymerized by the action of oxidative enzymes mostly for the supply of carbon to the microorganisms (Hendel et al., 2007). Laccase enzymes (EC 1.10.3.2) belong to the group of phenoloxidases and can be secreted by fungi, higher plants and bacteria (Claus, 2003; Giardina et al., 2010). In fungi and bacteria, laccases catalyze the oxidation of various substrates, typically a p-dihydroxyl phenol or another phenolic compound, coupled to reduction of oxygen to water (Baldrian, 2006). Overall, laccase activity constitutes a good descriptor for organic matter decomposition since it reflects the degradation of phenolic compounds in leaf litter over time (Sinsabaugh et al., 1994). However, its use as functional descriptor is much more frequent in forest litter and soil (i. e. Criquet et al., 1999; Ghosh et al., 2003) than in submerged litter in rivers (Hendel and Marxsen, 2000; Artigas et al., 2004).

Rivers and streams draining agricultural areas in Europe and the US are often contaminated by fungicides (Kreuger, 1998; Kahle et al., 2008; Vogel et al., 2008). The fungicide tebuconazole (TBZ) is frequently detected in vineyard regions (Rabiet et al., 2010; Herrero-Hernandez et al., 2013) but also through municipal wastewater effluents (i. e. Stamatis et al., 2010). The TBZ ( $C_{16}H_{22}ClN_3O$ ) is a broad-spectrum azole fungicide that inhibits the ergosterol biosynthesis required for the development of fungal biomass (Copping and Hewitt, 1998). The impact of TBZ has been widely documented for the structure (Artigas et al., 2014), diversity (Pascual et al., 2014), and activity (Artigas et al., 2012; Donnadiou et al., 2016) of aquatic microbial communities, as well as for the decomposer-detritivore system (Zubrod et al., 2015). Specifically for leaf-associated microbial communities, the TBZ has been shown to reduce fungal biomass, change the diversity of aquatic hyphomycete community (Bundschuh et al., 2011), and modify the kinetics (decrease of the Michaelis constant) of two cellulolytic ( $\beta$ -glucosidase and cellobiohydrolase) and one hemicellulolytic ( $\beta$ -xylosidase) enzyme activities (Artigas et al., 2012). At present, efforts are needed to identify new ecologically-relevant endpoints suitable for fungicide risk assessment in aquatic ecosystems, and especially to target effects in aquatic fungal communities (Maltby et al., 2009). Since laccase enzyme activity is both involved in relevant ecological process (litter decomposition) and reactive to the biodegradation of certain organic pesticides (Solé et al., 2008; Krauss et al., 2011), it could constitute a good candidate.

Typically, extracellular enzyme activity has been considered as weakly specific physiological descriptor to assess the impact of toxicants in aquatic microbial communities (Sabater et al., 2007). Their transient responses following exposure to a chemical compound (Forbes et al., 2006) and/or their maintenance whatever the species diversity present in microbial communities (Pascoal et al., 2005; Findlay and Sinsabaugh, 2006; Frossard et al., 2012), greatly reduces their applicability for routine monitoring of environmental quality. This weak sensitivity of extracellular enzyme activity descriptors is enhanced in acute toxicity testing. Indeed, the variable rates of survival of individual enzymes in the extracellular space and/or the response velocity of new enzyme synthesis can influence the activity response (Jones and Lock, 1989). However, recent investigations have highlighted that the non-specificity of extracellular enzyme activity to toxicants can be just a matter of normalization of the enzyme activity data. For instance, the normalization of enzyme activity per live bacteria cell revealed that phosphatase and peptidase activities in biofilms were sensitive to the herbicide diuron (Ricart et al., 2009; Proia et al., 2011).

The objective of the present study is to assess the sensitivity of laccase activity as descriptor of TBZ toxicity in microbial communities associated to decomposing plant litter. Considering that laccase activity values normalization can be crucial for an appropriate assessment of TBZ toxicity, our study analyzes the sensitivity of laccase expressed by i) fungi and bacteria grown separately and in co-existence, and ii) their corresponding enzyme fractions (cell bound versus diffusible enzyme activity) of each microbial component. Accordingly, acute toxicity tests have been conducted in the laboratory using i) natural communities colonizing *Alnus glutinosa* leaves from upstream and downstream sites of the Artière river (Centre France), ii) a simplified microbial community, iii) fungi separately and iv) bacteria separately. For each microbial condition, fungal (ergosterol concentration, 18S RNA gene copies), bacterial density, and leaf litter (dry mass, lignin content) descriptors were measured, and a new protocol to separate the cell bound from the diffusible enzyme fraction was applied. Our hypothesis expects a progressive decrease of laccase activity when increasing TBZ concentrations, due to the direct effect of the fungicide on fungal biomass development and consequently on their activity. This effect would be more consistent in leaves colonized by fungi separately. Besides, the sensitivity of cell bound fraction is expected to be greater than that of the diffusible fraction, since enzyme synthesis in the former is still possible. An increased sensitivity of the diffusible fraction to the TBZ would suggest a direct effect of the fungicide in laccase enzymes.

## 2. Materials and methods

### 2.1. Preparation of microbial assemblages

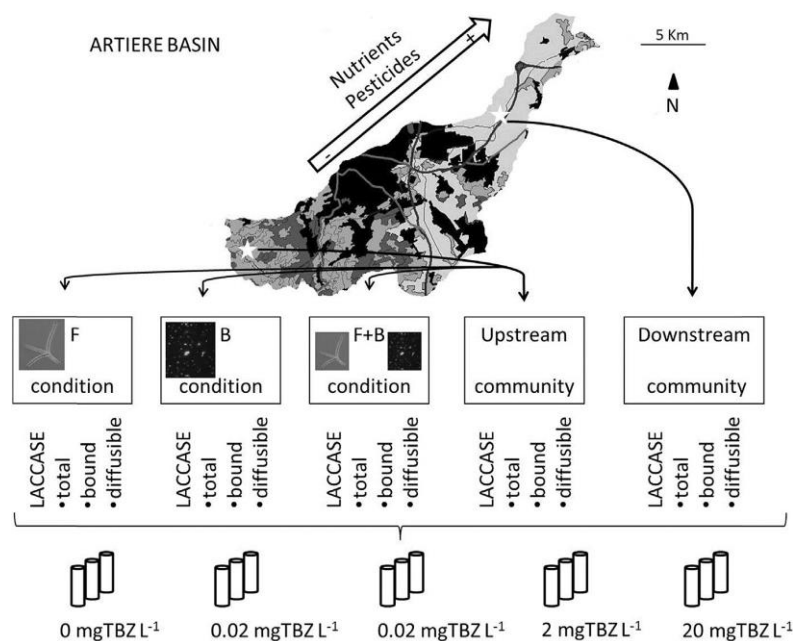
Dose response experiments between laccase activity and tebuconazole (TBZ) in *Alnus glutinosa* (L.) Gaertn. leaf species (*Alnus*) were performed in the laboratory using different microbial assemblages: two natural communities (Upstream and Downstream), a simplified community (F + B), fungi alone (F), and bacteria alone (B) (Fig. 1).

Natural microbial communities were obtained directly from the upstream (Saint-Genès-Champanelle) and downstream (Aulnat) sites of the Artière river (Puy-de-Dôme region, Centre France). These two sites show contrasting contamination levels, in terms of pesticides (i. e. aminomethylphosphonic acid, glyphosate, and diuron; Phyt'eauvergne, 2016) and nutrient concentrations (nitrate, phosphate, and dissolved organic carbon; unpublished data) in water, being the downstream site the most polluted. Triplicate litter bags of 0.5 mm mesh size, each containing ten pre-dried leaves of *Alnus*, were led to colonize at the two sites at the end of spring 2016. The colonization of leaves lasted for four weeks, the time necessary to obtain natural microbial communities representative of the two sites before TBZ toxicity tests. During this period, average TBZ concentration in the Artière's water was  $<0.005 \mu\text{g L}^{-1}$  upstream and  $0.026 \pm 0.010 \mu\text{g L}^{-1}$  downstream (unpublished data).

The simplified microbial community (F + B) was obtained after the isolation of the aquatic hyphomycetes species *Alatospora acuminata* Ingold (*Alatospora*), without axenic conditions, thus in the presence of bacterial community. Isolation was performed from *Alatospora* conidia physically isolated using glass micropipettes under a microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany). A total of  $6666 \pm 154$  spores of *Alatospora* and  $2 \times 10^6 \pm 2 \times 10^2$  bacterial cells were inoculated into triplicate Erlenmeyer flasks containing each ten sterile *Alnus* leaves in 500 mL of sterile Volvic water. Sterilization of leaves and water was performed separately by autoclaving at  $121^\circ\text{C}$  during 20 min. Leaves were led to colonize for two weeks in the dark using a thermo-regulated chamber (Thermo Scientific Max Q6000, Ohio, US) set at  $20^\circ\text{C}$  and 80 rpm agitation before TBZ toxicity tests.

The fungi alone (F) and bacteria alone (B) conditions were obtained from the simplified microbial community (F + B). In the case of F, bacteria were removed by directly adding a mixture of chloramphenicol





**Fig. 1.** Experimental design testing laccase activity sensitivity to the fungicide TBZ in upstream and downstream communities of the Artière river, as well as in the F + B (*Alatospora acuminata* + bacterial community), F (*Alatospora acuminata*), and B (bacterial community) conditions. For each microbial condition, the sensitivity of laccase was assessed for total activity, cell bound and diffusible enzyme fractions.

( $0.5 \text{ g L}^{-1}$ ) and ampicillin ( $0.05 \text{ g L}^{-1}$ ). In the case of B, fungal-free suspensions were obtained after centrifugation ( $1000g$  during  $15 \text{ min}$ ) and filtration ( $0.45 \mu\text{m}$ ) of the inoculum (Romani et al., 2006). As described above, 6666 spores of *Alatospora* (for the F condition) and  $2 \times 10^6$  bacterial cells (for the B condition) were inoculated into triplicate Erlenmeyer flasks containing each ten sterile *Alnus* leaves in  $500 \text{ mL}$  of sterile Volvic water. Leaves were led to colonize for two weeks at the same conditions as described above before TBZ toxicity tests.

## 2.2. Fractioning between cell bound and diffusible enzyme fractions

The cell bound and diffusible enzyme fractions (*sensu* Burns et al., 2013) were separated for the five microbial conditions at the end of their respective colonization of *Alnus* leaves in the field (Upstream and Downstream) and/or in the laboratory (F + B, F, and B; Fig. 1). Previous tests were performed in the laboratory to determine the most appropriate sonication ( $60 \text{ s} + 60 \text{ s}$ ,  $120 \text{ s} + 120 \text{ s}$ ,  $180 \text{ s} + 180 \text{ s}$ ;  $35 \text{ W}$  power and  $50/60 \text{ Hz}$  frequency, Fisher Scientific FB 15048, Germany) and centrifugation ( $6000$ ,  $8000$ , or  $12,000 \text{ rpm}$  during  $20 \text{ min}$ ; Eppendorf 5415R, Germany) conditions to separate the two enzyme fractions. All tested conditions were performed in triplicate samples composed of three leaf disks in  $2 \text{ mL}$  sterile Volvic water. After fractioning,  $1 \text{ mL}$  of supernatant was transferred into a new and sterile tube and considered as the dissolved enzyme fraction. The remaining  $1 \text{ mL}$  (containing the three leaf disks) was discarded and refilled with  $1 \text{ mL}$  of new and sterile Volvic water in order to obtain the cell bound enzyme fraction. Then, laccase activity was measured in the two fractions separately for each of the sonication and centrifugation conditions tested ( $n = 54$ ). Results revealed that the fractioning protocol at  $60 \text{ s} + 60 \text{ s}$  sonication and  $8000 \text{ rpm}$  centrifugation for  $20 \text{ min}$ , provided i) the lowest values of bacterial density in the diffusible fraction ( $1.7 \times 10^2 \pm 8.3 \times 10^1 \text{ cells leaf disk}^{-1}$ ) and ii) the best recovery when comparing the sum of activities in fractions with respect to the total activity in the intact community ( $100.4 \pm 1.5\%$ ; Supplementary material, Table 1). According to these findings, we separated the cell bound and diffusible enzyme fractions

from the Upstream, Downstream, F + B, F, and B conditions before testing TBZ toxicity.

## 2.3. Toxicity tests

Leaves colonized by Upstream and Downstream microbial communities, F + B, F, and B conditions were cut into disks ( $1 \text{ cm}$  in diameter) to run acute toxicity tests between total laccase activity (intact community) and the TBZ. Also, the cell bound and diffusible enzyme fractions obtained for each microbial condition were exposed separately to the fungicide. Laccase activity measurements were all performed in triplicate along a TBZ concentration gradient of  $0$ ,  $0.02$ ,  $0.2$ ,  $2$ , and  $20 \text{ mg L}^{-1}$  (Fig. 1). The TBZ was dissolved in sterile deionized water below its solubility limit ( $36 \text{ mg L}^{-1}$ , Agritox, 2016). Samples for total laccase activity determination consisted of  $20$  leaf disks (from each respective microbial condition) in  $5 \text{ mL}$  sterile Volvic water (each containing its respective TBZ dose) in test tubes incubated for  $96 \text{ h}$  in a thermo-regulated chamber set up at  $20^\circ \text{C}$  temperature and  $80 \text{ rpm}$  agitation in the dark. Samples for the cell bound (three leaf disks in  $1 \text{ mL}$  of sterile Volvic water) and diffusible ( $1 \text{ mL}$  of supernatant) laccase activity determination were exposed to the same TBZ concentration gradient for  $96 \text{ h}$  at the same temperature and agitation conditions as described above. After incubations, laccase activity was measured on the intact microbial communities (total laccase) as well as on the enzyme fractions (cell bound and diffusible).

## 2.4. Laccase activity measurements

Laccase (EC 1.10.3.2) activity was measured in triplicate in leaf samples (intact community and enzyme fractions) using the 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) commercial substrate (Sigma-Aldrich, St. Louis, Missouri, USA). Enzyme activity assays were conducted according to the protocol of Johannes and Majcherzyk (2000) with some modifications. Substrate saturating conditions were fixed at  $3 \text{ mmol ABTS L}^{-1}$  (calculated from substrate

saturation curves in *Alnus*-associated microbial communities from the Artière river) and incubations were for 1 h at 20 °C, under agitation (80 rpm) in the dark. ABTS transformation was measured spectrophotometrically (420 nm) using an Ultrospec 2000 device (Pharmacia Biotech, Trowbridge, UK). The enzymatic activity was expressed as  $1 \text{ U} = 1 \mu\text{mol ABTS oxidized g leaf Dry Mass}^{-1} \text{ h}^{-1}$  ( $\epsilon_{420} = 36 \text{ M}^{-1} \text{ cm}^{-1}$ , Johannes and Majcherzyk, 2000). After laccase activity measurements on intact communities, the 20 leaf disks were kept for the subsequent analyses of fungal biomass (ten disks), bacterial density (one disk), 18S RNA gene copies (five disks), and lignin content (four disks). Dry mass was determined systematically for each leaf disk resulting from activity measurements in enzyme fractions.

## 2.5. Fungal and bacterial descriptors

Fungal descriptors in leaves were estimated from ergosterol concentration and the number of 18S RNA gene loci copies. For ergosterol concentration determination, extractions were performed on ten *Alnus* disks per sample and following the protocol described by Artigas et al. (2012). Besides, the amount of 18S RNA gene loci copies in leaves (five disks per sample) was achieved by quantitative PCR using FR1/FF390 primer set (5'-AICCATTCATCGGTAIT and 5'-CGATAACGAACGAGACCT respectively) (Prévost-Bouré et al., 2011). Amplification reactions were conducted in a 12.5  $\mu\text{L}$  final reaction volume, containing 6.25  $\mu\text{L}$  of MESA green qPCR Master mix Plus for SYBR® assay (Eurogentec), 400 mM of each primer, 250  $\mu\text{g mL}^{-1}$  of BSA, 2.5  $\mu\text{L}$  of DNA template (which correspond to a 100-fold dilution). Runs were performed with a Realplex Mastercycler (Eppendorf) under the following conditions: a 5 min denaturation at 95 °C, followed by 40 cycles consisting of denaturation (45 s at 95 °C), annealing (45 s at 48 °C), extension (70 s at 72 °C). A final step was added to obtain a specific denaturation curve from 70 °C to 95 °C. Standard curves were prepared by doing a ten-fold serial dilutions (ranging from  $10^7$  to  $10^1$  copies  $\mu\text{L}^{-1}$  of plasmid contained insert from fungi). Quantifications were performed in triplicate and expressed as 18S RNA gene copies per g leaf Dry Mass (DM).

The diversity of aquatic hyphomycetes community was assessed after the sporulation of leaves (three disks per sample) from the different microbial conditions (Upstream, Downstream, F + B, and F) before acute toxicity tests. *Alnus* disks were incubated in 10 mL of sterile diluted Volvic water (1:100) during 72 h and 20 °C under agitation in the dark. The water suspensions containing fungal conidia were filtered through 5  $\mu\text{m}$  filters (Whatmann Cellulose Nitrate Membrane filters, Dassel, Germany) and then stained with 0.1% Trypan blue in 60% acid lactic before observation in the microscope (Leica DM IRB, Wetzlar, Germany). Among 200–300 spores at 400 $\times$  were counted per filter and later identified using Gulis et al. (2007). Conidia numbers were expressed in mean percent contribution for each inoculum.

Bacterial density in leaves was estimated by flow cytometric counts (Borrel et al., 2012). Leaf disks (one per sample) were stored in TE buffer (10 mM Tris, 1 mM EDTA) and fixed with paraformaldehyde (2% final concentration) before analysis. Samples were incubated with sodium pyrophosphate (10 mM final concentration) and sonicated 30s + 30s using a sonication bath in order to improve cells disaggregation. The bacterial suspensions were then centrifuged at 800g for 60 s and the supernatants diluted 10-fold and stained with SYBR Green I (Molecular Probes) before counting bacterial cells with a BD FACSCalibur flow cytometer (15 mW at 488 nm, Becton Dickinson, U.S.A.).

## 2.6. Lignin content

Lignin content in *Alnus* leaves (four disks per sample) was measured using the acetyl bromide procedure described by Iyama and Wallis (1990). Leaf disks were digested with 2.5 mL of acetyl bromide solution in acetic acid (25% w/w) at 70 °C for 30 min. 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 (280 nm) and expressed as lignin percentage per leaf Dry Mass (DM) by considering a lignin absorption coefficient of  $20 \text{ g}^{-1} \text{ L cm}^{-1}$ .

## 2.7. Statistical analyses

A two-way ANOVA was used to determine the effect of TBZ concentration (Factor I), microbial condition (Factor II) as well as their interactions, on the total, cell bound, and diffusible laccase activity. The same test was applied on ergosterol concentration, 18S RNA gene copies, bacterial density, and lignin content. All variables were checked for normality and homogeneity of variances prior to ANOVA tests and  $\log(x + 1)$  transformations applied when necessary. A *post hoc* multiple comparison test (Tukey HSD) was used to examine differences between levels of each factor.

Dose response relationships between TBZ and laccase activity (normalized by fungal, bacterial and/or litter descriptors) were expressed as the percentage of the control (namely TBZ concentration at  $0 \text{ mg L}^{-1}$ ). Laccase sensitivity to TBZ was estimated as Effective Concentration 50% ( $\text{EC}_{50}$ ) by fitting the descriptor to the four-parameter logistic curve model as described below:

$$Y = \min + \frac{\max - \min}{1 + \left(\frac{x}{\text{EC}_{50}}\right)^{\text{hillslope}}}$$

where the variable  $x$  corresponds to TBZ concentration of the dose response test and  $y$  is the value of the measured laccase activity. The parameter  $\min$  equals the baseline, and  $\max$  is the plateau of the curve. The parameter  $\text{EC}_{50}$  gives the transition center corresponding to the concentration causing 50% of modification of the measured descriptor. The hillslope is the slope of the curve at the transition center.

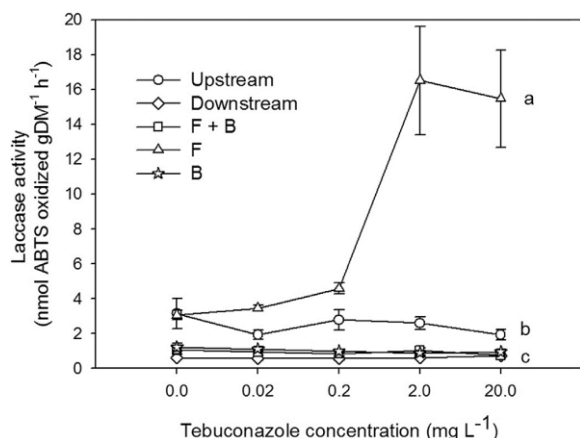
## 3. Results

### 3.1. Laccase activity: total versus fractions

Total laccase activity was significantly different between the microbial conditions tested ( $P < 0.0001$ , Fig. 2). The highest activity values were recorded for the F condition (fungi alone) followed by those in the Upstream community, whereas the lowest values were recorded in the Downstream community, F + B, and B conditions (Tukey's test,  $P < 0.05$ ). The effect of TBZ was condition- and concentration-dependent (condition  $\times$  concentration interaction,  $P < 0.05$ ; Fig. 2). Laccase activity in the F condition was the only that responded to the TBZ concentration increase. Specifically, the activity in F was 3.5 to 4 times increased in the presence of TBZ concentrations ranging 2–20  $\text{mg L}^{-1}$  (Tukey's test,  $P < 0.05$ ). Besides, no effect of TBZ was observed in the rest of microbial conditions tested.

Laccase activity was greater in the cell bound (62–76% of the total activity) than in the diffusible fraction (24–38%), except in the case of the B condition (bacteria alone) in which the activities of both fractions were rather similar (Table 1). Irrespective of the fungicide concentration tested, differences in laccase activity among microbial conditions were also detected after enzyme fractioning (Tukey's test,  $P < 0.05$ , Fig. 3). For the cell bound enzyme fraction, the activity of F was significantly greater than that of the rest of microbial conditions. However, the activity of the diffusible fraction of both F and B was greater than that of F + B, Upstream and Downstream conditions (Tukey's test,  $P < 0.05$ , Fig. 3).

The two enzyme fractions responded in different manner to the TBZ (Fig. 3). With the exception of the cell bound fraction for F condition (condition  $\times$  concentration interaction,  $P < 0.0001$ ) neither diffusible nor cell bound fraction respond to the TBZ concentration increase ( $P = 0.835$ ) whatever the considered condition (Fig. 3). Responses observed in the cell bound fraction of the F condition were similar to that observed for total activity measurements, except by the fact that activity



**Fig. 2.** Effects of TBZ on laccase activity in the five microbial conditions tested (Upstream, Downstream, F + B, F, and B). Values are means ( $n = 3$ ) and standard errors of each condition at each fungicide concentration. Differences between microbial conditions are marked by letters ( $a > b > c$ ; Tukey's test,  $P < 0.05$ ).

in the former was stimulated at lower TBZ concentrations (from 0.2 mg TBZ L<sup>-1</sup>, Tukey's test,  $P < 0.05$ ).

### 3.2. Fungi, bacteria, and lignin content in leaves

The two fungal descriptors were significantly affected by the TBZ ( $P < 0.001$ ) though their responses to the increasing fungicide concentrations were different (Fig. 4). While ergosterol concentration decreased when increasing TBZ concentration, the number of 18S RNA gene copies slightly increased in most of the microbial conditions tested (Tukey test,  $P < 0.05$ ). Regarding the ergosterol concentration, TBZ effects were drastic in F (46.3% ergosterol reduction between 0 and 20 mg TBZ L<sup>-1</sup>), moderate in both F + B and Upstream (27.4% and 21% reduction, respectively), and extremely low in Downstream communities (2.7% reduction). Conversely, the number of 18S RNA gene copies was slightly enhanced in the Upstream, Downstream, and F + B conditions between 0 and 20 mg TBZ L<sup>-1</sup>, except in the case of the F condition (fungi alone) (condition  $\times$  concentration interaction,  $P < 0.0001$ ) where the number of 18S RNA gene copies was drastically reduced from  $16.09 \pm 1.2 \times 10^3$  to  $0.13 \pm 0.033 \times 10^3$  (at 0 and 20 mg TBZ L<sup>-1</sup>, respectively) (Fig. 4). Differences in ergosterol concentrations and 18S RNA gene copies among microbial conditions were also observed ( $P < 0.001$  and  $P < 0.0001$ , respectively). While the highest ergosterol concentrations were measured in the F + B and Downstream conditions, the highest 18S RNA gene values were recorded in the Upstream condition (Tukey's test,  $P < 0.05$ ; Fig. 4).

Bacterial densities varied slightly between microbial conditions ( $P < 0.001$ ) but not between TBZ concentrations ( $P = 0.621$ , Fig. 5).

**Table 1**

Percentage of cell bound and diffusible laccase activity determined in the five microbial conditions. The percentage of total laccase activity was calculated by comparing the sum of activities in cell bound and diffusible fractions versus the total activity in intact leaves. Values are percentage averages and standard errors of activity from the five TBZ concentrations tested ( $n = 15$ ) for each microbial condition.

Inoculum	% bound activity	% diffusible activity	% total activity
Upstream	61.7 $\pm$ 1.5	38.3 $\pm$ 1.0	116.5 $\pm$ 10.9
Downstream	68.0 $\pm$ 1.3	32.0 $\pm$ 1.1	97.0 $\pm$ 11.6
F + B	63.0 $\pm$ 0.6	37.0 $\pm$ 1.5	99.5 $\pm$ 7.2
F	75.8 $\pm$ 3.2	24.2 $\pm$ 3.1	92.4 $\pm$ 9.2
B	43.0 $\pm$ 1.0	57.0 $\pm$ 0.9	110.2 $\pm$ 12.3

The greatest difference was observed in the absence of TBZ (0 mg L<sup>-1</sup>) between the Downstream communities and the rest of microbial conditions (Tukey's test,  $P < 0.05$ ). TBZ had no effect on bacterial densities in most of the microbial conditions tested, except in the Downstream condition where trends were to decrease (80% reduction). Finally, results showed that fungi and bacteria were significantly under-represented in the B (<7  $\mu$ g ergosterol gDM<sup>-1</sup> and/or <22 copies 18S RNA gene gDM<sup>-1</sup>, in average between 0 and 20 mg TBZ L<sup>-1</sup>) and F (<5  $\times 10^2$  bacterial cells gDM<sup>-1</sup>, in average) conditions, respectively, as expected (Tukey's test,  $P < 0.05$ , Figs. 4 and 5).

A total of 12 different aquatic hyphomycete species were identified in the present experiment (Table 2). The greatest species richness was observed in the Upstream communities (11 species), followed by those from Downstream (5 species), and finally the assemblages in F + B and F which were monospecific for the species *Alatospora acuminata*. The dominant species in the Upstream communities were *Alatospora acuminata* and *Flagellospora curvula*, whereas the species *Clavariopsis aquatica* largely dominant in Downstream communities. The species *Alatospora acuminata* was present at significant percent contributions (26–35%) in both Upstream and Downstream communities (Table 2).

Lignin content in leaves was rather different between microbial conditions ( $P < 0.0001$ , Fig. 5). Leaves from Upstream and F + B conditions were richer in lignin content (Tukey's test,  $P < 0.05$ ). Moreover, TBZ concentration effect on lignin content was condition dependent (condition  $\times$  concentration interaction,  $P < 0.01$ ). While lignin content did not vary in most of the microbial conditions exposed to TBZ, a significant decrease was observed in the Downstream condition (80% reduction between 0 and 20 mg TBZ L<sup>-1</sup>).

### 3.3. Relationships between tebuconazole and laccase activity

Dose response curves (expressed as the percentage of the control, namely TBZ concentration at 0 mg L<sup>-1</sup>) were calculated for laccase activity normalized by leaf DM, ergosterol concentration, 18S RNA gene copies, bacterial density, and lignin content. Results showed that fitting of dose response data to logistic model varied depending on the activity normalization applied and the complexity of the microbial condition tested (Table 3). The less complex is the microbial assemblage, the more significant are the effects of TBZ on laccase activity. This is the case of the F condition which experienced stimulation of laccase activity when increasing TBZ concentration. This stimulation of activity was irrespective of changes in bacterial density, leaf DM or lignin content but coincided with decreases of the ergosterol concentration and 18S RNA gene copies. EC<sub>50</sub> values in F ranged from 0.249 to 0.617 mg TBZ L<sup>-1</sup>, the highest effective concentration being obtained when normalizing laccase activity by ergosterol concentration and the lowest being obtained when normalizing activity by leaf DM, 18S RNA gene copies, and lignin content (Table 3). A similar stimulation of laccase activity was observed in Downstream communities when normalizing their activity by bacterial density values (EC<sub>50</sub> = 0.025 mg TBZ L<sup>-1</sup>). Here, the stimulation of laccase was instead explained by the reduction of bacterial densities at high TBZ concentrations. In contrast to the F and Downstream conditions, the B condition (bacteria alone) experienced inhibition of laccase activity in the presence of TBZ. This activity inhibition was specifically observed when normalizing laccase by bacterial density (EC<sub>50</sub> = 0.002 mg L<sup>-1</sup>) and lignin content (EC<sub>50</sub> = 0.024 mg L<sup>-1</sup>) values.

Concerning laccase activity in enzyme fractions, dose response fittings were only possible for activity normalized by the leaf Dry Mass. Overall, the F condition showed lower EC<sub>50</sub> values in the cell bound fraction (0.193 mg TBZ L<sup>-1</sup>;  $P < 0.05$ ) rather than in the total laccase activity measured in the intact community described above. No other statistically significant fittings were observed for the rest of the microbial conditions and enzyme fractions tested.



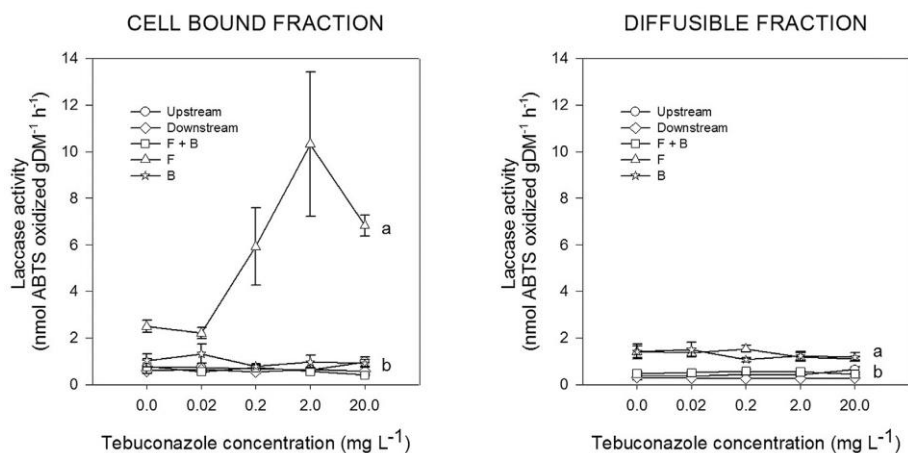


Fig. 3. Effects of TBZ on the cell-bound (left panel) and freely-diffusible (right panel) fractions of laccase activity in the five microbial conditions tested. Values are means ( $n = 3$ ) and standard errors of each condition and fungicide concentration. Differences between microbial conditions are marked by letters ( $a > b > c$ , Tukey's test,  $P < 0.05$ ).

#### 4. Discussion

Laccase activity proves poor indication for the assessment of TBZ acute toxicity in microbial communities associated to decomposing litter in rivers. The activity of both intact microbial community (total) and enzyme fractions (cell bound and diffusible) were not sensitive to the fungicide at the community level, but at the population level. Specifically, leaves colonized by *Alatospora acuminata* (an aquatic hyphomycete species dominant in decomposing litter from the Artière river (this experiment) and more globally in temperate-type streams (Suberkropp and Klug, 1976; Gulis et al., 2007)) experienced a stimulation of laccase activity when increasing TBZ concentration. The absence of laccase response to TBZ in communities with fungi and bacteria growing in co-existence (upstream and downstream of the Artière river, F + B conditions) may be explained by a number of biotic (ex. species diversity and interactions) and abiotic (ex. community architecture, molecule exchanges) factors avoiding the extrapolation of contaminant effects from the population level to the community level (Lagadic et al., 1994; Forbes et al., 2006). From our observations, the absence of laccase response to TBZ in Upstream and Downstream communities, both dominated by *Alatospora acuminata* species, let us suggest an activity

repression due to the presence of other microorganisms in the same community, notably bacteria.

The sensitivity of laccase to TBZ (estimated by the  $EC_{50}$  parameter) varied depending on the normalization applied on enzyme activity values. Lower laccase sensitivity ( $EC_{50}$  ranging 0.249–0.617 mg TBZ L<sup>-1</sup>) was obtained when normalizing activity values by fungal community descriptors (ergosterol concentration, 18S RNA gene copies) and/or leaf litter characteristics (leaf Dry Mass, lignin content), than when normalizing activity by bacterial density ( $EC_{50} = 0.002$  mg TBZ L<sup>-1</sup>). Overall, these  $EC_{50}$  values are significantly lower than those observed for freshwater algae (*Cryptococcus laurentii*,  $EC_{50} = 3.20$  mg TBZ L<sup>-1</sup>, Ochoa-Acuña et al., 2009) invertebrate (*Gammarus pulex*,  $EC_{50} = 1.64$  mg TBZ L<sup>-1</sup>, Adam et al., 2009) or fish species (*Oncorhynchus mykiss*,  $EC_{50} = 5.32$  mg TBZ L<sup>-1</sup>, Knauer et al., 2007), but in the same range to that observed for the aquatic yeast species *Cryptococcus laurentii* ( $EC_{50} = 0.13$  mg TBZ L<sup>-1</sup>, Wiesner, 2014). The choice of the most appropriate activity correction for determining accurate  $EC_{50}$  values remains difficult, since laccase activity is essentially found in fungi (Baldrian, 2006) but also in bacteria (Kellner et al., 2008). On the light of our results, the greater laccase activity was overall recorded in the F condition ( $8.62 \pm 3.02$  nmol ABTS oxidized gDM<sup>-1</sup> h<sup>-1</sup>; average

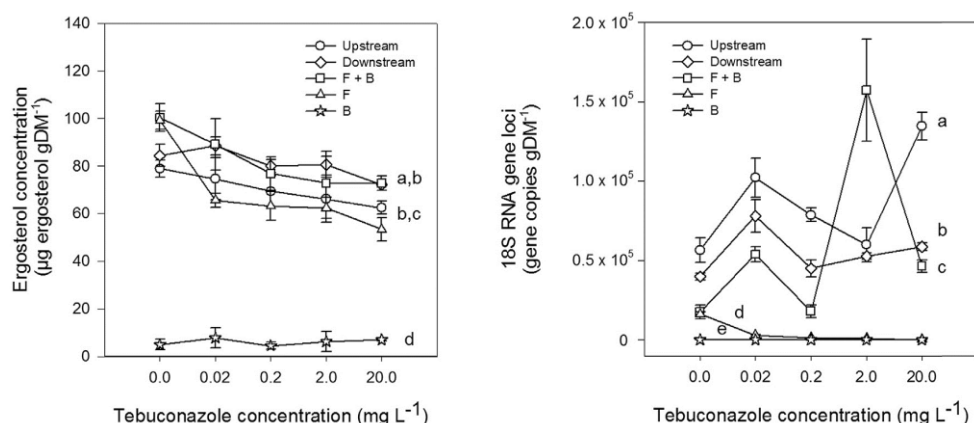
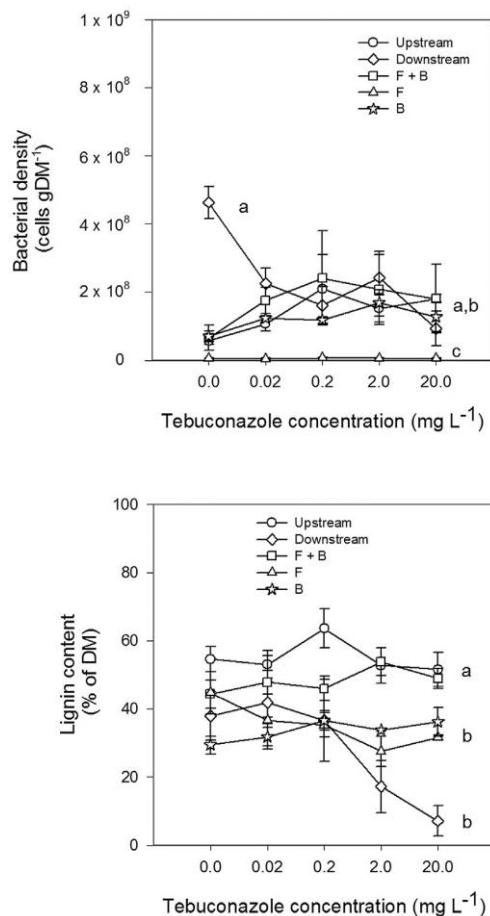


Fig. 4. Response of fungal descriptors (ergosterol concentration (left panel) and 18S RNA gene copies (right panel)) to the different TBZ concentrations in the five microbial conditions tested. Values are means ( $n = 3$ ) and standard errors per TBZ concentration and differences between microbial conditions are marked by letters ( $a > b > c > d$ , Tukey's test,  $P < 0.05$ ).

Please cite this article as: Artigas, J., et al., Sensitivity of laccase activity to the fungicide tebuconazole in decomposing litter, Sci Total Environ (2017), <http://dx.doi.org/10.1016/j.scitotenv.2017.01.167>



**Fig. 5.** Bacterial density (upper panel) and lignin content (lower panel) in leaves colonized by the five microbial inocula and exposed to TBZ. Values are means ( $n = 3$ ) and standard errors for the number of bacterial cells and/or the percent of lignin in leaf DM. Differences between microbial conditions are marked by letters ( $a > b > c$ , Tukey's test,  $P < 0.05$ ).

of the five TBZ concentrations,  $n = 15$ ) comparing to the B condition ( $1.04 \pm 0.06$  nmol ABTS oxidized  $\text{gDM}^{-1} \text{h}^{-1}$ ,  $n = 15$ ), which permits us to state the major role of fungi in laccase activity from *Alnus glutinosa*

**Table 2**

Aquatic hyphomycete conidia from leaves colonized by the different microbial inocula. Values are means ( $n = 3$ ) of percent contributions for each species.

Species	Upstream	Downstream	F + B	F
<i>Alatospora acuminata</i> Ingold	34.6	26.4	100	100
<i>Articulospora tetracladia</i> Ingold		13.2		
<i>Clavariopsis aquatica</i> De Wild.	1.9	49.1		
<i>Culicidospora</i> sp.	1.9			
<i>Flagellospora curvula</i> Ingold	22.6			
<i>Fontanospora eccentric</i> (R.H. Petersen)	5.7			
Dyko				
<i>Heliscella stellata</i> (Ingold & V.J.Cox)	7.5			
Marvanová				
<i>Heliscus lugdunensis</i> Sacc. & Thérny	9.4	3.8		
<i>Lemonniera aquatica</i> De Wild.	10.1			
<i>Lemonniera terrestris</i> Tubaký	2.5			
<i>Stenocladia neglecta</i> Marvanová & Descals	1.9			
<i>Tetracladium marchalianum</i> De Wild.	1.9	7.5		

**Table 3**

Four-parameter logistic curve model on laccase activity of the five microbial inocula. Dose-response curves data on laccase activity corrected by dry mass, ergosterol concentration, 18S RNA gene density, bacterial density, and/or lignin content were tested for each inoculum. The coefficients of regression ( $R^2$ ), effective concentration ( $EC_{50}$ ), the hillslope, as well as the average of maximum and minimum percentage values of activity response are shown.

		$R^2$	$EC_{50}$ ( $\text{mg L}^{-1}$ )	Hillslope	Max (%)	Min (%)
Dry mass-specific	Upstream	ns	ns	ns	ns	ns
	Downstream	ns	ns	ns	ns	ns
	F + B	ns	ns	ns	ns	ns
	F	0.99*	0.249***	−9.74	521.04	106.46
	B	ns	ns	ns	ns	ns
Ergosterol-specific	Upstream	ns	ns	ns	ns	ns
	Downstream	ns	ns	ns	ns	ns
	F + B	ns	ns	ns	ns	ns
	F	0.99*	0.617*	−1.67	964.90	133.48
	B	−	−	−	−	−
18S gene-specific	Upstream	ns	ns	ns	ns	ns
	Downstream	ns	ns	ns	ns	ns
	F + B	ns	ns	ns	ns	ns
	F	0.99*	0.249*	−9.74	521.04	106.46
	B	−	−	−	−	−
Bacterial density-specific	Upstream	ns	ns	ns	ns	ns
	Downstream	0.94*	0.025*	−7.89	300.42	100.00
	F + B	ns	ns	ns	ns	ns
	F	−	−	−	−	−
	B	0.93*	0.002*	0.82	99.99	39.33
Lignin-specific	Upstream	ns	ns	ns	ns	ns
	Downstream	ns	ns	ns	ns	ns
	F + B	ns	ns	ns	ns	ns
	F	0.96*	0.249**	−9.78	795.23	116.72
	B	0.99*	0.024*	1.90	99.99	31.90

(\*\*\*)  $P < 0.0005$ , (\*\*)  $P < 0.005$ , (\*)  $P < 0.05$ , and (ns) not significant show statistical differences.

(−) Fittings were not calculated for bacteria in the F inoculum and for fungi in the B inoculum.

decomposing litter. Moreover, the lack of bacterial density response to TBZ questions the validity of  $EC_{50}$  values obtained after laccase normalization by bacterial densities. Accordingly, activity normalization by fungal descriptors ( $EC_{50}$  values in the same range of concentration; see above) seems to be the most appropriate way to assess TBZ toxicity for laccase activity in decomposing leaves. Nevertheless, the similar  $EC_{50}$  values obtained when correcting laccase by litter characteristics (i. e. leaf Dry Mass) could represent a less expensive and less time consuming option for TBZ toxicity assessment.

The sensitivity of laccase between the cell bound and diffusible enzyme fractions to TBZ were different. Such differences were observed in the F condition (*Alatospora acuminata* populations) where the sensitivity of the activity in the cell bound fraction was similar to that observed for the intact microbial community. But, this was not the case for the activity in the diffusible fraction (freely-diffusible enzymes) that remained insensitive to the increase of TBZ concentration. These results suggest two different explanations. First, the TBZ may not have a direct effect (inhibitory and/or stimulatory) on laccase enzymes already synthesized, but probably induce their biosynthesis in the presence of microbial cells. And second, the low amount of enzymes recovered in the diffusible fraction and/or their variable rates of survival in the extracellular space (Jones and Lock, 1989) could have masked any toxic effects of TBZ. According to our results, the second explanation can be partly rejected since activity values in the diffusible fraction ( $1.42 \pm 0.25$  nmol ABTS oxidized  $\text{gDM}^{-1} \text{h}^{-1}$ ,  $n = 3$ ) were in the same range to those measured in the cell bound fraction for the F condition at  $0 \text{ mg TBZ L}^{-1}$ . Therefore, the first explanation sounds the most likely. Besides, it is interesting to remark that laccase activity in the diffusible fraction of the F and B conditions was significantly greater than that measured for the conditions when fungi and bacteria grew in co-existence (F + B, Upstream, and Downstream). This result highlights a certain antagonism between fungi and bacteria when growing in co-

existence, as observed in the literature (i. e. Mille-Lindblom and Tranvik, 2003). Such antagonism is observed again when comparing laccase activity between the F + B and F conditions for the cell bound fraction.

The hypothesis suggesting that TBZ would inhibit microbial laccase activity has been rejected. Instead, we observed that high TBZ concentrations enhance drastically laccase activity in the F condition. Laccase activity stimulation can reflect a stress response of *Alatospora acuminata* populations when exposed to high TBZ concentration (Baldrian, 2006; Jaszek et al., 2006). This stress response was concomitant with the lowest fungal biomass and density values (estimated from ergosterol concentration and the amount of 18S RNA gene copies, respectively) recorded in *Alatospora acuminata* populations evidencing the impact of TBZ. Regarding the literature, many authors have associated the role of oxidative cleavage as a prelude to the detoxification of many pesticides (Kelley et al., 1993; Kanaly and Harayama, 2000). In the case of TBZ, oxygenase-type enzymatic reactions have been described in the initial oxidation of the molecule (cleavage of the triazole ring) by soil and/or wood-decaying associated microorganisms (Strickland et al., 2004; Obanda and Shupe, 2009). This reaction could explain the observed increase in laccase activity when increasing TBZ concentration in the present experiment. Moreover, Johannes et al. (1996) observed that in the presence of a mediator (i. e. an appropriate low-molecular-mass compound) laccase is able to enhance the oxidation of a wide range of aromatic compounds including the TBZ and other azole fungicides (i. e. imazalil, Maruyama et al., 2007). Among these mediators, the 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was shown to be necessary for the oxidation of anthracene (Johannes et al., 1996) and/or veratryl alcohol (Bourbonnais and Paice, 1990). Considering that the ABTS was used for laccase activity assays in the present experiment, the enhanced ABTS turnover at TBZ concentrations  $> 0.2 \text{ mg L}^{-1}$  suggest a greater potential oxidation of TBZ by the laccase activity of *Alatospora acuminata* populations. According to these observations, TBZ and TBZ-metabolites concentration measurements are needed to be included in further experiments to understand how fungal laccases contribute to TBZ detoxification in leaf-associated microbial communities.

## 5. Conclusions

The present study reveals that laccase activity from *Alatospora acuminata* populations in decomposing leaves can react to TBZ and reduce its toxicity. This activity response was only observed in the cell bound enzyme fraction of the fungal population suggesting that the fungicide influence laccase activity in the presence of fungal cells but not in enzymes already synthesized in the extracellular space. According to these findings, the need for searching new functional descriptors, other than laccase activity, for TBZ toxicity assessment in microbial communities associated to decomposing litter is still required. However, it cannot be ruled out that microbial laccase activity can be sensitive to other types of pesticides. When selecting new functional descriptors to assess pesticide toxicity, it is important to remark that responses observed at the population level can be diluted when increasing biological complexity (i. e. population, community, ecosystem). Further tests are needed to confirm our observations by eventually enlarging to other leaf species, and/or microbial communities in the river ecosystem (i. e. sediments) where bacteria would be dominant over fungi in terms of enzyme activity.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2017.01.167>.

## Acknowledgments

This study has been funded by the project EcoBioPest (Agence de l'Eau Loire-Bretagne). Florent Rossi was funded by a PhD grant from the French Ministry of Higher Education and Research. Finally, we thank Hermine Billard for her help in flow cytometry analyses.

## References

- Adam, O., Badot, P.M., Degiorgi, F., Crini, G., 2009. Mixture toxicity assessment of wood preservative pesticides in the freshwater amphipod *Gammarus pulex* (L.). *Ecotoxicol. Environ. Saf.* 72, 441–449.
- Agritox, 2016. <http://www.agritox.anses.fr>.
- Artigas, J., Romani, A.M., Sabater, S., 2004. Organic matter decomposition by fungi in a Mediterranean forested stream: contribution of streambed substrata. *Ann. Limnol.* 40, 269–277.
- Artigas, J., Majerholc, J., Foulquier, A., Margoum, C., Volat, B., Neyra, M., Pesce, S., 2012. Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. *Aquat. Toxicol.* 122–123, 197–205.
- Artigas, J., Pascual, N., Bouchez, A., Chastain, J., Debroas, D., Humbert, J.F., Leloup, J., Tadonleké, R.D., ter Halle, A., Pesce, S., 2014. Comparative sensitivity to the fungicide tebuconazole of biofilm and plankton microbial communities in freshwater ecosystems. *Sci. Total Environ.* 468–469, 326–336.
- Baldrian, P., 2006. Fungal laccases-occurrence and properties. *FEMS Microbiol. Rev.* 30, 215–242.
- Borrel, G., Colombet, J., Robin, A., Lehours, A.C., Prangishvili, D., Sime-Ngando, T., 2012. Unexpected and novel putative viruses in the sediments of a deep-dark permanently anoxic freshwater habitat. *ISME J* 6, 2119–2127.
- Bourbonnais, R., Paice, M.G., 1990. Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. *FEBS Lett.* 267, 99–102.
- Bundschuh, M., Zubrod, J.P., Kosol, S., Maltby, L., Stang, C., Duyster, L., Schulz, R., 2011. Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. *Aquat. Toxicol.* 104, 32–37.
- Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A.M., 2013. Soil enzymes in a changing environment: current knowledge and future directions. *Soil Biol. Biochem.* 58, 216–234.
- Claus, H., 2003. Laccases and their occurrence in prokaryotes. *Arch. Microbiol.* 179, 145–150.
- Copping, L.G., Hewitt, H.G., 1998. Chemistry and mode of action of crop protection agents. The Royal Society of Chemistry, Cambridge, UK, p. 160.
- Criquet, S., Tagger, S., Vogt, G., Iacazio, G., Le Petit, J., 1999. Laccase activity of forest litter. *Soil Biol. Biochem.* 31, 1239–1244.
- Donnadieu, F., Besse-Hoggar, P., Forestier, C., Artigas, J., 2016. Influence of streambed substratum composition on stream microbial communities exposed to the fungicide tebuconazole. *Freshw. Biol.* 61, 2026–2036.
- Findlay, S., Sinsabaugh, R.L., 2006. Large-scale variation in subsurface stream biofilms: a cross-regional comparison of metabolic function and community similarity. *Microb. Ecol.* 52, 491–500.
- Forbes, V.E., Palmqvist, A., Bach, L., 2006. The use and misuse of biomarkers in ecotoxicology. *Environ. Toxicol. Chem.* 25, 272–280.
- Frossard, A., Gerull, L., Mutz, M., Gessner, M.O., 2012. Disconnect of microbial structure and function: enzyme activities and bacterial communities in nascent stream corridors. *ISME J* 6, 680–691.
- Ghosh, A., Frankland, J.C., Thurston, C.F., Robinson, C.H., 2003. Enzyme production by *Mycena galopus* mycelium in artificial media and in *Picea sitchensis* F-1 horizon needle litter. *Mycol. Res.* 107, 996–1008.
- Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., Sanna, G., 2010. Laccases: a never-ending story. *Cell. Mol. Life Sci.* 67, 369–385.
- Gulis, V., Marvanová, L., Descals, E., 2007. An illustrated key to the common temperate species of aquatic hyphomycetes. In: Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.), *Methods to Study Litter Decomposition: A Practical Guide*. Springer, Dordrecht, Netherlands, pp. 153–168.
- Hendel, B., Sinsabaugh, R.L., Marxsen, J., 2007. Lignin-degrading enzymes: phenoloxidase an peroxidase. In: MAS, Graça, Bärlocher, F., Gessner, M.O. (Eds.), *Methods to Study Litter Decomposition: A Practical Guide*. Springer, Dordrecht, The Netherlands, pp. 273–278.
- Hendel, B., Marxsen, J., 2000. Extracellular enzyme activity associated with degradation of beech wood in a Central European stream. *Int. Rev. Hydrobiol.* 85, 95–105.
- Herrero-Hernandez, E., Andrades, M.S., Alvarez-Martin, E., Pose-Juan, E., Rodriguez-Cruz, M.S., Sanchez-Martin, M.J., 2013. Occurrence of pesticides and some of their degradation products in waters in a Spanish wine region. *J. Hydrol.* 486, 234–245.
- Iyama, K., Wallis, A.F.A., 1990. Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. *J. Sci. Food Agric.* 51, 145–151.
- Jaszek, M., Grzywnowicz, K., Malarczky, E., Leonowicz, A., 2006. Enhanced extracellular laccase activity as a part of the response system of white rot fungi: *Trametes versicolor* and *Abortiporus biennis* to paraquat-caused oxidative stress conditions. *Pestic. Biochem. Physiol.* 85, 147–154.
- Johannes, C., Majcherzyk, A., Huttermann, A., 1996. Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. *Appl. Microbiol. Biotechnol.* 46, 313–317.
- Johannes, C., Majcherzyk, A., 2000. Laccase activity tests and laccase inhibitors. *J. Biotechnol.* 78, 193–199.
- Jones, S.E., Lock, M.A., 1989. Hydrolytic extracellular enzyme activity in heterotrophic biofilms from two contrasting streams. *Freshw. Biol.* 22, 289–296.
- Kanly, R.A., Harayama, S., 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol.* 182, 2059–2067.
- Kahle, M., Buerge, I.J., Hauser, A., Müller, M.D., Poiger, T., 2008. Azole fungicides: occurrence and fate in wastewater and surface waters. *Environ. Sci. Technol.* 42, 7193–7200.
- Kellner, H., Luis, P., Zimdars, B., Kiesel, B., Busco, F., 2008. Diversity of bacterial laccase-like multicopper oxidase genes in forest and grassland Cambisol soil samples. *Soil Biol. Biochem.* 40, 638–648.

- Kelley, I., Freeman, J.P., Evans, F.E., Ceringlia, C.E., 1993. Identification of metabolites from the degradation of fluoranthene by *Mycobacterium* sp. strain PYR-1. *Appl. Environ. Microbiol.* 59, 800–806.
- Knauer, K., Lampert, C., Gonzalez-Valero, J., 2007. Comparison of in vitro and in vivo acute fish toxicity in relation to toxicant mode of action. *Chemosphere* 68, 1435–1441.
- Krauss, G.J., Solé, M., Krauss, G., Schlosser, D., Wesenberg, D., Bärlocher, F., 2011. Fungi in freshwaters: ecology, physiology and biochemical potential. *FEMS Microbiol. Rev.* 35, 620–651.
- Kreuger, J., 1998. Pesticides in stream water within an agricultural catchment in southern Sweden, 1990–1996. *Sci. Total Environ.* 216, 227–251.
- Lagadic, L., Caquet, T., Ramade, F., 1994. The role of biomarkers in environmental assessment (5). Invertebrate populations and communities. *Ecotoxicology* 3, 193–208.
- Maltby, L., Brock, T.C., van den Brink, P.J., 2009. Fungicide risk assessment for aquatic ecosystems: importance of interspecific variation, toxic mode of action, and exposure regime. *Environ. Sci. Technol.* 43, 7556–7563.
- Maruyama, T., Komatsu, C., Michizoe, J., Sakai, S., Goto, M., 2007. Laccase-mediated degradation and reduction of toxicity of the postharvest fungicide imazalil. *Process Biochem.* 42, 459–461.
- Mille-Lindblom, C., Tranvik, L.J., 2003. Antagonism between bacteria and fungi on decomposing aquatic plant litter. *Microb. Ecol.* 45, 173–182.
- Obanda, D.N., Shupe, T.F., 2009. Biotransformation of tebuconazole by microorganisms: evidence of a common mechanism. *Wood Fiber Sci.* 41, 157–167.
- Ochoa-Acuña, H.G., Bialkowski, W., Yale, G., Hahn, L., 2009. Toxicity of soybean rust fungicides to freshwater algae and *Daphnia magna*. *Ecotoxicology* 18, 440–446.
- Pascual, N., Roux, S., Artigas, J., Pesce, S., Leloup, J., Taddonleke, R.D., Debroas, D., Bouchez, A., Humbert, J.F., 2014. A high-throughput sequencing ecotoxicology study of freshwater bacterial communities and their responses to tebuconazole. *FEMS Microbiol. Ecol.* 90, 563–574.
- Pascoal, C., Cassio, F., Marvanova, L., 2005. Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low-order stream. *Arch. Hydrobiol.* 162, 481–496.
- Phyt'eauvergne, 2016. Eau et produits phytosanitaires-Qualité des eaux en Auvergne-2004-2012. *Phyt'Eauvergne* (82p).
- Prévost-Bouré, N.C., Christen, R., Dequiedt, S., Mougé, C., Lelièvre, M., Jolivet, C., Shabbazkia, H.R., Guillou, L., Arrouays, D., Ranjard, L., 2011. Validation and application of a PCR primer set to quantify fungal communities in the soil environment by Real-Time Quantitative PCR. *PLoS One* <http://dx.doi.org/10.1371/journal.pone.0024166>.
- Proia, L., Morin, S., Peipoch, M., Romani, A.M., Sabater, S., 2011. Resistance and recovery of river biofilms receiving short pulses of triclosan and diuron. *Sci. Total Environ.* 409, 3129–3137.
- Rabiet, M., Margoum, C., Gouy, V., Carlier, N., Coquery, M., 2010. Assessing pesticide concentrations and fluxes in the stream of a small vineyard catchment-effect of sampling frequency. *Environ. Pollut.* 158, 737–748.
- Ricart, M., Barceló, D., Geiszinger, A., Guasch, H., Lopez de Alda, M., Romani, A.M., Vidal, G., Villagrassa, M., Sabater, S., 2009. Effects of low concentrations of the phenylurea herbicide diuron on biofilm algae and bacteria. *Chemosphere* 76, 1392–1401.
- Romani, A.M., Fischer, H., Mille-Lindblom, C., Tranvik, L.J., 2006. Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. *Ecology* 87, 2559–2569.
- Sabater, S., Guasch, H., Ricart, M., Romani, A.M., Vidal, G., Klünder, C., Schmitt-Jansen, M., 2007. Monitoring the effect of chemicals on biological communities. The biofilm as an interface. *Anal. Bioanal. Chem.* 387, 1425–1434.
- Schimel, J.P., Weintraub, M.N., 2003. The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. *Soil Biol. Biochem.* 35, 549–563.
- Sinsabaugh, R.L., Osgood, M.P., Findlay, S., 1994. Enzymatic models for estimating decomposition rates of particulate detritus. *J. N. Am. Benthol. Soc.* 13, 160–169.
- Solé, M., Kellner, H., Brock, S., Buscot, F., Schlosser, D., 2008. Extracellular laccase activity and transcript levels of putative laccase genes during removal of the xenoestrogen technical nonylphenol by the aquatic hyphomycete *Clavariopsis aquatica*. *FEMS Microbiol. Lett.* 288, 47–54.
- Stamatis, N., Hela, D., Konstantinou, I., 2010. Occurrence and removal of fungicides in municipal sewage treatment plant. *J. Hazard. Mater.* 175, 829–835.
- Strickland, T.C., Potter, T.L., Joo, H., 2004. Tebuconazole dissipation and metabolism in Tifton loamy sand during laboratory incubation. *Pest Manag. Sci.* 60, 703–709.
- Suberkropp, K., Klug, M.J., 1976. Fungi and bacteria associated with leaves during processing in a woodland stream. *Ecology* 57, 707–719.
- Vogel, J.R., Majewski, M.S., Capel, P.D., 2008. Pesticides in Rain in Four Agricultural Watersheds in the United States (USGS Staff — Published Research. Paper 3).
- Wiesner, L., 2014. Aquatic Risk Assessment for Triazole Fungicides: The Impact of Epoxiconazole and Tebuconazole on Aquatic Fungi. M. Sc. Swiss Centre for Applied Ecotoxicology, Eawag, Dübendorf (70pp).
- Zubrod, J.P., Englert, D., Feckler, A., Koksharova, N., Konschak, M., Bundschuh, R., Schnetzer, N., Englert, K., Schilz, R., Bundschuh, M., 2015. Does the current fungicide risk assessment provide sufficient protection for key drivers in aquatic ecosystem functioning? *Environ. Sci. Technol.* 49, 1173–1181.

## **Annex II**



RESEARCH ARTICLE

# Biotransformation of herbicides by aquatic microbial communities associated to submerged leaves

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Received: 30 June 2016 / Accepted: 1 November 2016  
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**Abstract** Leaf microbial communities possess a large panel of enzymes permitting the breakdown of leaf polymers as well as the transformation of organic xenobiotic compounds present in stream waters. This study aims to assess the potential of leaf microbial communities, exhibiting different exposure histories to pesticides (upstream versus downstream), to biotransform three maize herbicides (mesotrione, S-metolachlor, and nicosulfuron) in single and cocktail molecule exposures. The results showed a high dissipation of nicosulfuron (sulfonylurea herbicide) (from  $29.1 \pm 10.8\%$  to  $66 \pm 16.2\%$ , day 40) in both single and cocktail exposures, respectively, but not of mesotrione and S-metolachlor. The formation of nicosulfuron metabolites such as ASDM (2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide) and ADMP (2-amino-4,6-dimethoxypyrimidine) and the weak sorption ( $<0.4\%$ ) on the leaf matrix confirmed the

transformation of this molecule by leaf microorganisms. In addition, the downstream communities showed a greater ability to transform nicosulfuron than the upstream communities suggesting that the exposure history to pesticides is an important parameter and can enhance the biotransformation potential of leaf microorganisms. After 40-day single exposure to nicosulfuron, the downstream communities were also those experiencing the greatest shifts in fungal and bacterial community diversity suggesting a potential adaptation of microorganisms to this herbicide. Our study emphasizes the importance of leaf microbial communities for herbicide biotransformation in polluted stream ecosystems where fungi could play a crucial role.

**Keywords** Leaf-associated microorganisms · Biodegradation · Mesotrione · Nicosulfuron · S-metolachlor · Herbicide mixture

an Artigas contributed equally to the work

Responsible Editor: Philippe Garrigues

**Electronic supplementary material** The online version of this article (doi:10.1007/s11356-016-8035-9) contains supplementary material, which is available to authorized users.

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## Introduction

Microbial heterotrophic communities in forested streams play a central role in leaf litter decomposition (Kaushik and Hynes 1971) and its conditioning for further consumption by macroinvertebrate shredders (Gessner et al. 2007). Leaf-associated microbial communities are essentially dominated by aquatic hyphomycetes (98.4 to 99.8% of leaf microbial biomass), and to a lesser extent by bacteria (Gulis and Suberkropp 2003), both having the ability to produce extracellular enzymes capable to degrade complex leaf polymers (i.e., cellulose, hemicellulose, lignin) into readily absorbable monomers (Artigas et al. 2011). Fungal extracellular lac

Apart from extracellular enzymatic oxidation, organic xenobiotics can also be transformed by fungal intracellular enzymes into hydroxylated, methylated, and conjugated degradation products or be mineralized to CO<sub>2</sub> (Junghanns et al. 2005; Augustin et al. 2006; Prenafeta-Boldu et al. 2006; Martin et al. 2009). Up to date, few studies have assessed the ability of aquatic hyphomycetes to metabolize organic xenobiotics (e.g., pesticides) in stream ecosystems (Krauss et al. 2011).

Our study focused on the herbicide cocktail composed by S-metolachlor (chloroacetanilide), mesotrione ( $\beta$ -triketone), and nicosulfuron (sulfonylurea) often used together to ensure maize, alfalfa, and soybean crop yields (Whaley et al. 2006; Joly et al. 2013) and susceptible to reach stream ecosystems and their inhabiting aquatic microbial communities. Indeed, S-metolachlor, mesotrione and nicosulfuron are relatively mobile and bioavailable in soil, as attested by their water solubilities (488, 160, and 7500 mg L<sup>-1</sup>, respectively) and sorption coefficients (1.08 to 9.32 L kg<sup>-1</sup>, 0.13 to 5.0 L kg<sup>-1</sup>, and 0.14 to 2.15 L kg<sup>-1</sup> according to the soil characteristics, respectively) (Oliveira et al. 2001; Dyson et al. 2002; Chaabane et al. 2008; Zemolin et al. 2014; Agritox 2016). The presence of these three herbicides has been detected in French surface waters (SOeS 2013). In 2014, annual pesticide surveys in 84 river sites from the Auvergne's region (Centre France) also revealed that the detection of S-metolachlor (0.9–1.5  $\mu$ g L<sup>-1</sup> in April–May), used as pre-emergence herbicide, tends to be earlier than mesotrione and nicosulfuron (0.03–0.05  $\mu$ g L<sup>-1</sup> in May–June), both used as post-emergence herbicides (Phyt'eauvergne 2015).

Some studies have reported the impact of mesotrione (Joly et al. 2012), nicosulfuron (Šantrić et al. 2004; de Oliveira et al. 2009; Karpouzas et al. 2014a, b; Petric et al. 2016), S-metolachlor (Joly et al. 2014) and of the cocktail of these three herbicides (Joly et al. 2015) on the activity, structure, and diversity of soil microbial communities. Concerning the aquatic compartment, the impact of herbicides has been shown toward fishes (Saglio et al. 2003; Kreutz et al. 2008), amphibians (Fort et al. 1999), crayfishes (Cook and Moore 2008), *Daphnia* (Neves et al. 2015), and *Lemna minor* (Aliferis et al. 2009). Some other studies also revealed an impact of mesotrione (Valiente Moro et al. 2012; Ni et al. 2014), nicosulfuron (Seguin et al. 2001; Nélieu et al. 2005) and S-metolachlor (Debenest et al. 2009; Elias and Bernot 2014) on aquatic microbial communities, but no data are available concerning the toxicity of these three herbicides in cocktail toward aquatic microorganisms.

Several microorganisms have shown the potential to biotransform these three compounds separately. Some mesotrione (e.g., *Bacillus* sp. and *Pantoea* sp.) (Durand et al. 2006; Batisson et al. 2009; Pileggi et al. 2012; Youness 2013; Romdhane et al. 2015; Liu et al. 2015), S-metolachlor (e.g., *Klebsiella* sp.) (Martins et al. 2007) and nicosulfuron-

degrading strains (e.g. *Serratia* sp., *Talaromyces* sp. and *Alcaligenes* sp.) (Zhang et al. 2012; Lu et al. 2012; Song et al. 2013; Zhao et al. 2015) have already been isolated from aquatic and terrestrial environments. As a sulfonylurea herbicide, nicosulfuron is removed from soil mainly via microbial activity and chemical hydrolysis, the second way becoming preponderant in acidic soils (Berger and Wolfe 1996; Sarmah and Sabadie 2002). The microbial degradation of pesticides has been mostly associated to the capacity of microorganisms for acquiring alternative nutrient sources (Alexander 1994), such as nitrogen in the case of nicosulfuron (Zhang et al. 2012; Song et al. 2013), but also carbon and/or phosphorus in the case of organophosphorus compounds (e.g., glyphosate) (Singh and Walker 2006). Although nutrient acquisition activities (extracellular enzymes) in leaf-associated microbial communities have been mostly associated to the utilization of leaf polymers (Artigas et al. 2008), it cannot be ruled out that pesticides available in water and/or adsorbed onto the leaf matrix are used by these microorganisms.

The repeated exposure of microbial communities to pesticides has been observed to enhance their pesticide-degradation potential because of microbial acclimation (Topp et al. 1996; Arthur et al. 1997). Shorter acclimation times will be observed when appropriate degradative enzymes are already present in the microbial community, while longer acclimation is expected when development of new traits permitting biodegradation are required (i.e., evolution of catabolic genes by either a relaxation of substrate specificity or inducer specificity of existing enzymes) (van der Meer 1994). Apart from the appropriate enzymatic potential for an effective pesticide biodegradation, other factors such as (i) sub-optimal environmental conditions, (ii) co-metabolism (fortuitous degradation of a pesticide by enzymes generated for other substrate degradation, the pesticide being not used as carbon and energy source for microbial growth), (iii) limited availability of contaminants, (iv) low concentrations of contaminants in the environment, and (v) protozoan predation of degrading microorganisms, can limit biodegradation (Providenti et al. 1993; Liu et al. 2000). However, there is no report of the impact of pesticides (alone or in cocktail) on the biodegradation abilities of microbial communities associated to submerged leaves. In streams, microbial adaptations to pesticide degradation would be naturally expected in downstream sections (close to the watershed outlet) because of the increase of concentration and diversity of herbicide molecules coming from the entire watershed compared to upstream sections (headwaters), as observed in various studies (e.g., Pesce et al. (2010)). The intensity and frequency in which these herbicides are found in stream waters is expected to favor microbial acclimation and enhance their degradation potential.

The present study aimed to assess the capacity of leaf-associated microbial communities to transform and/or degrade three maize herbicides (mesotrione, S-metolachlor, and

nicosulfuron) often found in streams draining agricultural areas of the Auvergne's region (Phyt'auvergne 2015). The impact of these molecules on microbial community structure was also investigated. Considering that the three herbicides can be found simultaneously and/or separately in stream waters, our study was designed to compare single versus cocktail molecule exposures. The importance of exposure history to contaminants will also be assessed in the impact (microbial community structure) and biotransformation capacity of upstream versus downstream communities (less and more exposed to contaminants, respectively). Our hypothesis is that the leaf-associated microbial communities with greater exposure history to contaminants, belonging to the downstream site in our study, would present a more resistant microbial structure and greater capacity to biotransform the three maize herbicides.

## Materials and methods

### Chemicals and media

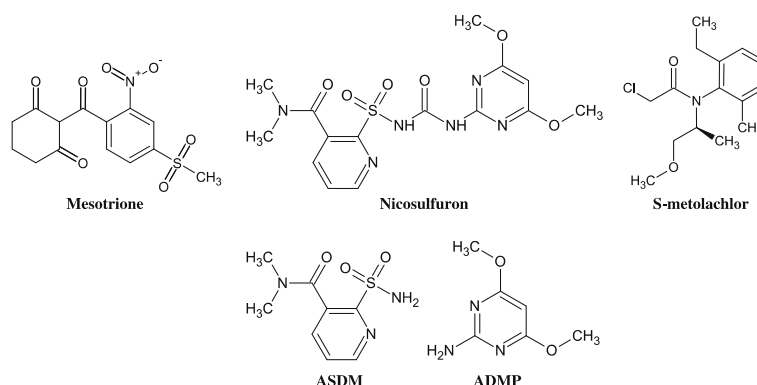
Nicosulfuron (Pestanal, purity 99.6%), mesotrione (Pestanal, purity 99.9%), S-metolachlor (Pestanal, purity 98.4%), and ADMP (2-amino-4,6-dimethoxypyrimidine, purity 98.0%) were purchased from Sigma Aldrich (France), and ASDM (2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide, purity 98%) was purchased from J and K Scientific (Germany). The structure of these compounds is described in the Fig. 1. Mineral salt medium (MSM), pH 6.5, was composed of ( $L^{-1}$ ) 1 g  $(NH_4)_2HPO_4$ , 1 g  $KH_2PO_4$ , 1 g  $KNO_3$ , 0.2 g  $MgSO_4$ , 0.1 g  $NaCl$ , 20 mg  $CaCl_2$ , 5 mg  $FeSO_4 \cdot 7H_2O$ , 1 mL of a salt stock solution, and 1 mL of a vitamin stock solution. The salt stock solution contained ( $L^{-1}$ ) 20 g boric acid, 18 g  $MnSO_4 \cdot H_2O$ , 2 g  $ZnSO_4$ , 1 g  $CuSO_4$ , 2.5 g  $Na_2MoO_4$ , and 10 mg  $Co(NO_3)_2$ . The vitamin stock solution contained ( $L^{-1}$ ) 2 mg biotin and 5 mg thiamine-HCl.

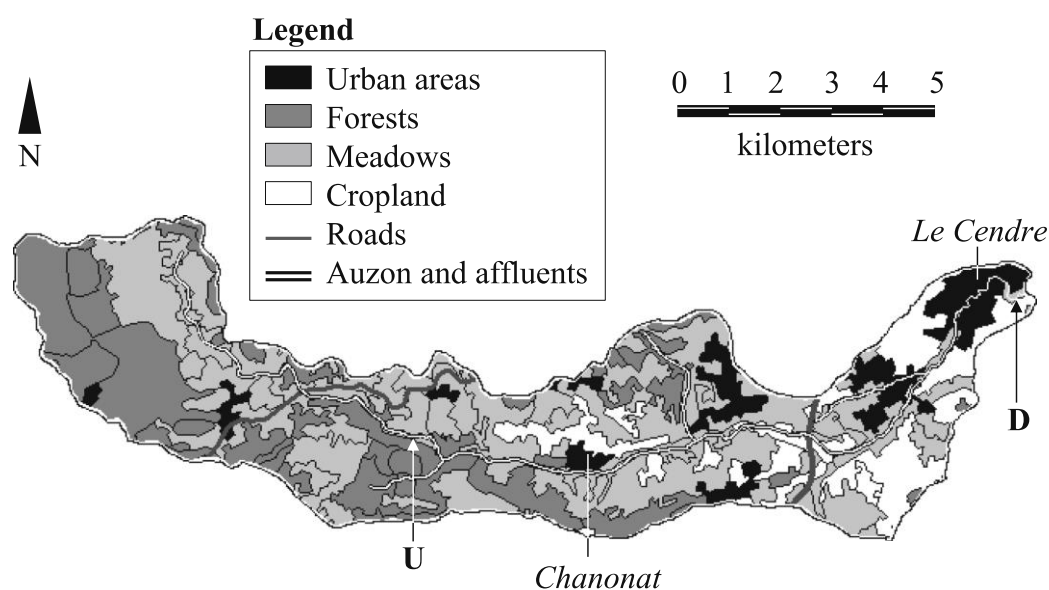
### Microbial communities

Natural microbial communities colonizing *Alnus glutinosa* (L.) Gaertn. leaf species were obtained from the Auzon stream located in the Auvergne's region (Centre France). Leaves of *A. glutinosa* tree species were selected in our experiment since they are a major component of riparian vegetation in the site studied. The Auzon is a fourth-order temperate stream of 18.7 Km in length with a basin surface area of 60.7  $Km^2$ . Two sites separated by 14.6 Km were selected for *A. glutinosa* leaves colonization during October 2014 (4 weeks), an upstream site (first order) in Chanonat and a downstream in Le Cendre (fourth order) (Fig. 2). In parallel, water samples were taken at weeks 1 and 4 in triplicate from upstream and downstream sites to determine water chemical characteristics (including nutrients, dissolved organic carbon, and pesticide concentrations).

Both sites presented well-developed riparian vegetation composed by alder, ash and hazel tree species and streambeds composed by gravel and cobbles in the most upstream section and sand in the most downstream section. Average discharge, temperature, pH, conductivity, dissolved organic carbon and phosphate concentrations were higher in the downstream site than in the upstream site during microbial community colonization (Table 1, MANOVA,  $P < 0.05$ ). Concerning the pesticide concentrations (Table 1), the herbicide glyphosate and its main degradation product (aminomethylphosphonic acid—AMPA) were present in both sites. However, a large number of herbicide and insecticide molecules were only detected downstream at concentrations ranging  $0.13\text{--}0.02 \mu g L^{-1}$ . During our study, the concentrations of mesotrione, S-metolachlor and nicosulfuron were below quantification limits ( $0.005 \mu g L^{-1}$  S-metolachlor,  $0.01 \mu g L^{-1}$  nicosulfuron, and mesotrione) of multi residue analyses (Laboratoire de Touraine, France). Nevertheless, historical data on pesticide monitoring in the Auzon stream (period 2012–2013) revealed greater pesticide contamination in the downstream site comparing to the upstream site (SMVVA 2014). Specifically, S-

**Fig. 1.** Structure of the three herbicides studied and the two nicosulfuron metabolites detected (ADMP (2-amino-4,6-dimethoxypyrimidine) and ASDM (2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide))





**Fig. 2.** Map of the Auzon watershed. Upstream (U) and downstream (D) sampling sites are indicated with arrows

metolachlor and nicosulfuron were detected in spring 2014 in the downstream site of the Auzon at 1.3 and 0.05  $\mu\text{g L}^{-1}$ , respectively (Phyt'auvergne 2015). Differences in water chemistry between sites converged with strong variations in land use along the stream basin, the upstream section being mostly surrounded by forests and prairies (47% forests, 48% prairies and 5% urban of the basin surface area), and a downstream section essentially devoted to agriculture and urbanization (68% croplands, 17% prairies and 15% urban) (Phyt'auvergne 2015). Recently fallen *A. glutinosa* leaves from the Auzon's riparian forest were collected and dried at room temperature for 48 h. Six nylon mesh bags (mesh diameter of 0.5 mm) were each filled with 5 grams of dried leaves and immersed to the upstream (three bags corresponding to three replicates) and downstream (three bags) sites. After 4-week colonization, bags containing leaves were retrieved and transported to the laboratory in zip bags at 4 °C.

### Herbicide exposure experiments

We assessed the influence of the (i) study site (upstream versus downstream) and (ii) herbicide (mesotrione, S-metolachlor and nicosulfuron, alone or as a ternary cocktail) on leaves microbial communities structure and their pesticide degradation potential. Each exposure condition was tested in triplicate (24 flasks in total). Flasks were each filled with 25 mL of MSM supplemented (separated or in cocktail) with nominal concentrations of 25  $\text{mg L}^{-1}$  nicosulfuron, 25  $\text{mg L}^{-1}$  mesotrione and/or 35  $\text{mg L}^{-1}$  S-metolachlor, and two leaf disks of 2 cm in diameter directly cut from leaf bags (described above). Herbicide concentrations in our study are

relatively high comparing to those found in streams and rivers, though they were far below  $\text{IC}_{50}$  concentrations ( $\text{mg L}^{-1}$ )

**Table 1.** Physical and chemical water characteristics during the colonization of upstream and downstream leaf-associated microbial communities. The pesticide concentrations (in  $\mu\text{g L}^{-1}$ ) during the colonization are also represented in the lower part of the table. Values are means ( $n = 12$ ) and standard errors (in parenthesis) of the different descriptors during the 4-week colonization in the Auzon stream

Physicochemical characteristics	Upstream	Downstream
Discharge ( $\text{L s}^{-1}$ )	180 (20.5)	250 (33.6)*
Temperature ( $^{\circ}\text{C}$ )	6.5 (1.2)	7.9 (0.5)*
pH	7.18 (0.17)	7.94 (0.15)*
Conductivity ( $\mu\text{S cm}^{-1}$ )	204.55 (2.77)	552.93 (13.36)*
DOC ( $\text{mg L}^{-1}$ )	14.32 (9.57)	19.71 (8.45)*
Nitrate ( $\text{mg L}^{-1}$ )	2.45 (0.31)	2.67 (0.35)
Phosphate ( $\text{mg L}^{-1}$ )	0.041 (0.002)	0.065 (0.004)*
Pesticide concentration ( $\mu\text{g L}^{-1}$ )		
AMPA (H)	0.64 (0.56)	0.82 (0.33)
Glyphosate (H)	0.32 (0.07)	0.25 (0.09)
Aminotriazole (H)	—	0.13 (0.03)
Dinoterb (H)	—	0.08 (0.002)
Diuron (H)	—	0.07 (0.01)
Terbutylazine (H)	—	0.05 (0.01)
Mecoprop (H)	—	0.04 (0.003)
Dichloprop (H)	—	0.04 (0.001)
MCPA (H)	—	0.04 (0.01)
Terbumeton (H)	—	0.03 (0.01)
Fipronil (I)	—	0.02 (0.003)
Imidacloprid (I)	—	0.03 (0.005)
Terbutryn (H)	—	0.001 (0.004)

H herbicide, I insecticide

\*Significant differences between sites (MANOVA,  $P < 0.05$ )



described in the literature: S-metolachlor,  $178.4 \pm 22.8$ ; mesotrione,  $43.6 \pm 2.4$ ; and nicosulfuron,  $167.8 \pm 21.8$  (Joly et al. 2013). Three additional flasks per herbicide exposure condition without leaf disks served as abiotic controls.

The flasks were incubated at 28 °C on an orbital shaker at 150 rpm in the darkness to avoid photolysis. The culture media were sampled at days 0, 7, 14, 30 and 40 to determine herbicide degradation (dissipation and/or metabolite production) by HPLC analyses. At the end of the experiment, only nicosulfuron showing significant dissipation was analyzed for adsorption on leaves and for effects on fungal and bacterial community structure.

### Herbicide concentration determination

The quantification of the herbicides in the culture media (dissipation) and adsorbed on leaves (after overnight extraction of one leaf disk in 1 mL of MeOH) was performed by HPLC on an Agilent Series 1100 chromatograph (Courtaboeuf, France). HPLC was equipped with a DAD detector and a reverse phase column (Zorbax Eclipse XDB-C18, 3.5  $\mu\text{m}$ , 75 mm  $\times$  4.6 mm). The mobile phase was composed of acetonitrile (Solvent A) and acidified water ( $\text{H}_3\text{PO}_4$ , 0.01% v/v; pH 2.9) (Solvent B) with a flow rate of 1 mL min<sup>-1</sup>, injection volume: 5  $\mu\text{L}$ ,  $\lambda = 220$  and 254 nm. Each sample was analyzed twice. *Mesotrione*: 0–1 min 2%B, 1–8 min 2–70%B, 8–9 min 70–100%B, 9–10 min 100–0%B, 10–11 min 0%B; *Nicosulfuron and metabolites*: 0–2 min 0%B, 2–10 min 0–70%B, 10–11 min 70–100%B, 11–12 min 100–0%B, 12–12.5 min 0%B; *S-Metolachlor*: 0–1 min 2%B, 1–17 min 2–70%B, 17–19 min 70–100%B, 19–21 min 100–2%B, 21–21.5 2%B; *Mixture*: 0–5 min 5%B, 5–30 min 5–70%B, 30–32 min 70–100%B, 32–33,100–5%B; 33–34 min 5%B. The detection limits were 25  $\mu\text{g L}^{-1}$  for mesotrione, 45  $\mu\text{g L}^{-1}$  for nicosulfuron, and 30  $\mu\text{g L}^{-1}$  for S-metolachlor. The herbicide dissipation percentages in each biotic condition were calculated by subtracting the dissipation percentage measured in the corresponding abiotic control.

### Analysis of fungal and bacterial communities structure

Genomic DNA from fungal and bacterial communities was extracted from upstream and downstream *A. glutinosa* leaves at the beginning (day 0) and at the end (day 40) of exposure to nicosulfuron in single and cocktail conditions. DNA extraction was performed from one leaf disk per replicate using the Fast DNA SPIN Kit for soil (MP Biomedicals, USA) following the manufacturer's instructions. Extracted DNA was then amplified by targeting the fungal internal transcribed region (ITS 3 and 4 (White et al. 1990)) and the bacterial 16S rDNA gene (regions V6-V8, (Heuer et al. 1999)) using the primer pairs ITS 3-GC (5'- CGC CCG CCG CGC CCC GCG CCC

GGC CCG CCG CCC CCG CCC CGC ATC GAT GAA GAA - 3') and ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC 3') for fungi and 968f-GC (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GG G GCA CGG GGG GAA CGC GAA GAA CCT TAC - 3') and 1401r (5'- CGG TGT GTA CAA GGC CC - 3') for bacteria. Polymerase chain reactions (PCR) conditions were: 5 min at 95 °C, followed by 35 steps [30 s at 95 °C, 45 s at 55 °C and 1 min at 72 °C] for fungal communities, and 5 min at 95 °C, followed by 35 steps [45 s at 95 °C, 45 s at 58 °C and 45 s at 72 °C] for bacterial communities. A final elongation step at 72 °C for 7 min for both conditions was performed. The quality of PCR amplicons was checked on 1% (w/v) agarose gel and quantified by spectrophotometry ( $\lambda_{\text{excitation}} = 360$  nm and  $\lambda_{\text{emission}} = 460$  nm) using the DNA quantitation kit, fluorescence assay (Sigma-Aldrich, Steinheim, Germany).

Denaturing Gradient Gel Electrophoresis (DGGE) on fungal and bacterial PCR products were used to assess the structure of microbial communities. A fixed quantity of PCR product (500 ng) was loaded onto 12% (w/v) acrylamide gels containing urea/formamide gradient of 25–48% for fungi and 45–65% for bacteria. Gels were run in 1 $\times$  TAE buffer (pH 8) for 16 h at 59 °C, with a voltage of 90 V (fungi) or 140 V (bacteria), using TTGEK 2401–220 equipment (CBS Scientific, USA). After migration, gels were stained in a bath containing 0.5  $\mu\text{g mL}^{-1}$  Gel Star (Lonza, USA) for 45 min in the dark and digitized using a BioSpectrum® Imaging system™ (UVP, USA). Numerical analysis of digitized gels was performed using Gel ComparII software (Ver. 4.6, Applied Maths, Kortrijk, Belgium) and based on the relative intensity of DNA bands considered as operational taxonomic units (OTUs). The diversity of fungal and bacterial communities was assessed by calculating OTUs richness and diversity (Shannon–Weaver,  $H'$ ) using the Evenness software (MFC Applications, 2007).

### Statistical analyses

The differences in mesotrione, S-metolachlor and nicosulfuron dissipation percentages (single versus cocktail exposures) from upstream and downstream leaf-associated microbial communities were assessed using three-way ANOVA. The sources of variation were estimated for single compound (mesotrione, S-metolachlor, nicosulfuron), mixture of compounds (single versus cocktail) and site (upstream versus downstream) factors, separately, as well as for their interactions. The differences in water physico-chemical characteristics between upstream and downstream sites were assessed using one-way MANOVA. The values were log-transformed prior to ANOVA and MANOVA tests in order to meet assumptions of normality and homogeneity of variances.

Separate post hoc comparisons (Tukey's test,  $P < 0.05$ ) were applied to determine differences in remaining herbicide concentrations between control, upstream and downstream samples, and between sampling times. The statistical analyses were performed using SPSS for Windows Ver 17.0.

Principal component analyses on fungal and bacterial OTUs matrices (square root transformed) were used to reveal ordination trends between microbial communities at the beginning of the experiment (day 0) and after 40 days of exposure to nicosulfuron (single exposure) and to herbicide cocktail (mesotrione + S-metolachlor + nicosulfuron). Post hoc multiple comparisons (Tukey's test,  $P < 0.05$ ) were tested on log-transformed diversity indices (species richness and Shannon-Weaver's diversity) from fungal and bacterial communities, separately, in order to assess for differences between sites and modes of exposure.

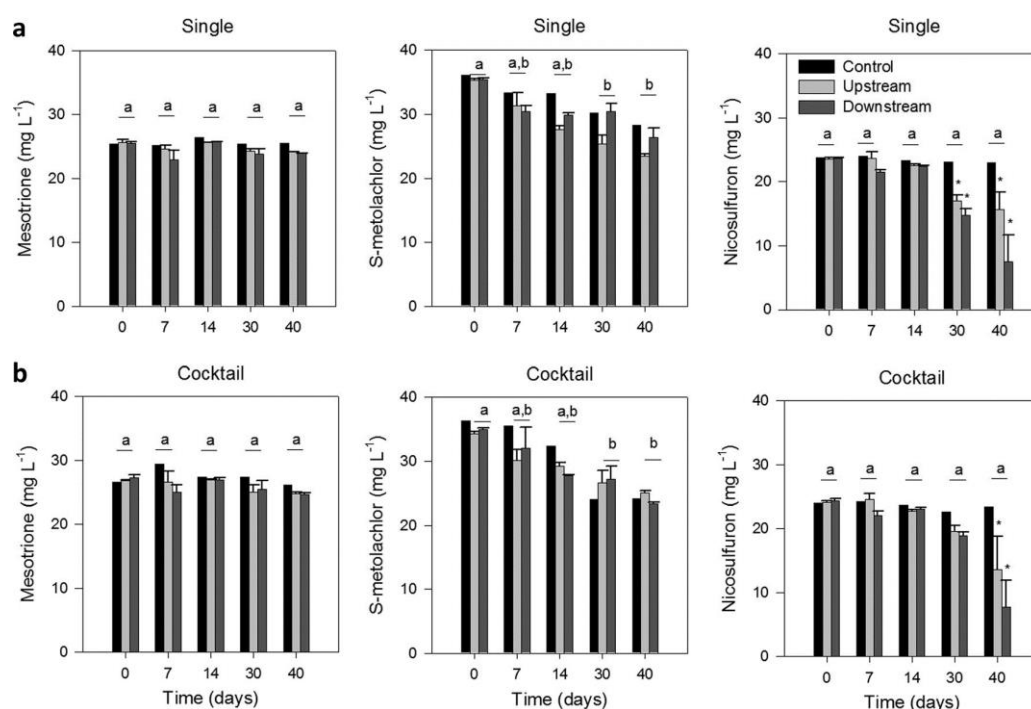
## Results

### Dissipation of herbicides

#### (i) Single herbicide exposure

Leaf-associated microbial communities from upstream and downstream sites, as well as abiotic controls, were exposed separately to mesotrione, S-metolachlor and nicosulfuron. Figure 3a shows the evolution of the herbicide concentration during the incubation of single pesticide. In the abiotic controls, the herbicide concentrations do not change during the 40 days except in the case of S-metolachlor for which a dissipation of  $21.8 \pm 0.9\%$  was observed. This dissipation is probably due to volatilization (Zemolin et al. 2014) since this molecule possesses a relatively high Henry's constant ( $2.2 \cdot 10^{-3} \text{ Pa m}^{-3} \text{ mol}^{-1}$ ). Moreover, a similar dissipation was observed in the presence of microorganisms. In the presence of microbial communities, the dissipation percentages presented in Table 2 are previously corrected by the dissipation measured in abiotic controls.

No significant dissipation of the three molecules was observed after 14 days of exposure (Tukey's test,  $P < 0.05$ ). After a longer time of exposure (30 to 40 days), a significant dissipation of nicosulfuron (ranging from  $29.1 \pm 10.8\%$  (upstream) to  $64.4 \pm 17.4\%$  (downstream) at day 40) was observed in the culture medium containing leaves (Molecule effect, ANOVA,  $P < 0.0001$ ; Tukey's test,  $P < 0.05$ ). No dissipation was observed for mesotrione and as explained above for abiotic controls, the concentration of S-metolachlor tends to decrease



**Fig. 3.** Concentrations of mesotrione, S-metolachlor and nicosulfuron (in  $\text{mg L}^{-1}$ ) in abiotic controls (without leaves), in the culture media of upstream and downstream leaf communities (a single and b cocktail) during the 40-day experiment. The values are means  $\pm$  standard errors

( $n = 3$ ) of herbicide concentration per condition at days 0, 7, 14, 30 and 40. Significant differences between conditions (in asterisks) and sampling times (in letters  $a > b$ ) are also represented (Tukey's test,  $P < 0.05$ )

**Table 2.** Dissipation percentages (average) and standard errors (in parenthesis) for the three maize herbicides exposed alone or in cocktail in culture medium containing leaf-associated microbial communities from the upstream and downstream sites of the Auzon stream ( $n = 3$ )

Site	Mesotrione		S-metolachlor		Nicosulfuron	
	Single	Cocktail	Single	Cocktail	Single	Cocktail
Upstream	6.1 (1.5)	5.9 (1.2)	11.8 (0.9)	0.6 (0.4)	29.1 (10.8)	40.7 (21.3)
Downstream	6.2 (0.7)	7.7 (0.5)	4.8 (2.4)	0.0 (0.0)	64.4 (17.4)	66 (16.2)

over time irrespectively to the conditions tested (Fig. 3, Table 2), showing no involvement of microbial communities in its dissipation (Molecule effect, ANOVA,  $P < 0.0001$ ; Tukey's test,  $P < 0.05$ ). Moreover, no new peaks on the HPLC chromatograms of samples from mesotrione and S-metolachlor conditions were observed during the incubation, suggesting the absence of metabolite production from these compounds. Conversely, the dissipation of nicosulfuron was significantly different between sites (Site  $\times$  Molecule effect, ANOVA,  $P < 0.05$ ) at day 40, the highest rates being observed in the downstream communities ( $64.4 \pm 17.4\%$  degradation) (Table 2).

#### (ii) Cocktail herbicide exposure

The exposure of leaf microbial communities to the herbicide cocktail composed of mesotrione, S-metolachlor and nicosulfuron did not modify greatly the dissipation percentages of each molecule (Cocktail effect, ANOVA,  $P = 0.968$ ). Nicosulfuron was the only herbicide exhibiting significant dissipation after 40 days of exposure in cocktails (Fig. 3b, Table 2). Moreover, the nicosulfuron dissipation percentage was again greater in downstream ( $66.0 \pm 16.2\%$ ) than in upstream communities ( $40.7 \pm 21.3\%$ ) though the differences in dissipation between sites are less pronounced for the cocktail (Site  $\times$  Molecule effect, ANOVA,  $P < 0.05$ ).

The nicosulfuron dissipation observed can be due to biodegradation but also to a sorption mechanism on leaves. The proportion of this last process was assessed by extracting nicosulfuron from leaves with methanol at the end of the experiment, after 40-day exposure. No difference was observed between nicosulfuron sorption percentages in single ( $0.36 \pm 0.04\%$  upstream,  $0.24 \pm 0.02\%$  downstream) and cocktail ( $0.18 \pm 0.02\%$  upstream,  $0.25 \pm 0.05\%$  downstream) exposures.

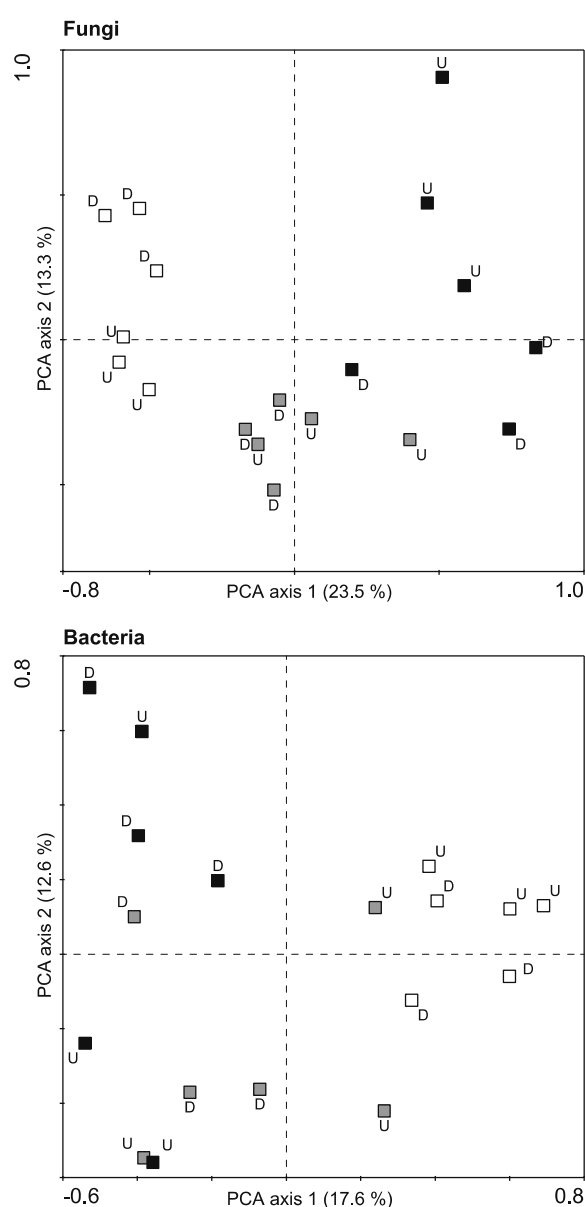
#### Degradation of nicosulfuron

The HPLC chromatograms of samples from both single and cocktail exposures of the leaf-associated microbial communities to nicosulfuron showed the appearance of new peaks during the incubation that could correspond to the production of three metabolites. These peaks are not present in the abiotic

controls. Two of them were assigned as ADMP and ASDM (same retention times and same UV spectra as those obtained with standards), known metabolites coming from hydrolysis of nicosulfuron (Fig. 1). The concentration of ADMP reached  $1.1 \pm 0.6 \text{ mg L}^{-1}$  (upstream),  $3.4 \pm 1.1 \text{ mg L}^{-1}$  (downstream), and  $2.2 \pm 1.3 \text{ mg L}^{-1}$  (upstream),  $3.9 \pm 1.3 \text{ mg L}^{-1}$  (downstream) after 40 days of exposure to nicosulfuron alone and in cocktail, respectively. At the same exposure time, the concentration of ASDM was  $0.3 \pm 0.1 \text{ mg L}^{-1}$  (upstream),  $0.6 \pm 0.2 \text{ mg L}^{-1}$  (downstream), and  $0.7 \pm 0.3 \text{ mg L}^{-1}$  (upstream),  $1.2 \pm 0.4 \text{ mg L}^{-1}$  (downstream) after exposure to nicosulfuron alone and in cocktail, respectively. Overall, the average concentrations of metabolites are greater downstream than upstream, confirming the greater nicosulfuron transformation in the former. The dissipation rate of nicosulfuron and production of ADMP and ASDM were positively correlated as attested by the Pearson correlation coefficients  $r = 0.99$ ,  $P < 0.001$  and  $r = 0.99$ ,  $P < 0.0001$ , respectively (single exposure),  $r = 0.99$ ,  $P < 0.0001$  and  $r = 0.97$ ,  $P < 0.01$ , respectively (cocktail exposure).

#### Changes in fungal and bacterial communities' structure

The effects of nicosulfuron and the herbicide cocktail on leaf-associated microbial communities were investigated. The structure of bacterial communities in *A. glutinosa* leaves was rather similar between upstream and downstream sites at the beginning of the experiment while the fungal ones were different (Fig. 4 and S1). After 40-day exposure to the herbicides, the shifts in microbial community structure were moderate in the case of the single exposure to nicosulfuron and strong in the case of the herbicide cocktail exposure (PC1 Axis explaining 23.5 and 17.6% of the variance in fungal and bacterial communities, respectively; Fig. 4). These shifts were also observed in terms of diversity indices, although the response of fungal and bacterial communities was rather different. While species richness and diversity of fungal communities tend to increase after single and cocktail exposures, the opposite trend was observed in bacterial communities (Tukey's test,  $P < 0.05$ , Table 3). Comparing to microbial communities at day 0, fungal communities increase 1–25 OTUs richness, whereas bacterial communities loss 8–12



**Fig. 4.** Principal component analysis on relative band intensities obtained from fungal (*upper plot*) and bacterial (*lower plot*) communities observed on PCR-DGGE analysis before (day 0, *white square*) and after nicosulfuron-single (*gray square*) and cocktail (*black square*) 40-day exposure. Upstream (U) and downstream (D) communities are also indicated in the plots

OTUs richness after both single and cocktail exposures to the herbicides.

The site effects were only observed in the structure of fungal communities, and mostly in herbicide cocktail exposure (PC2 Axis explaining 13.3 and 12.6% of the variance in fungal and bacterial communities, respectively; Fig. 4). This site effect was especially evident in the structure of downstream

fungal communities exposed to the herbicide cocktail (Fig. 4). The greatest effects of nicosulfuron (single exposure) were also observed in downstream communities where the highest values of fungal species richness ( $38 \pm 8$  OTUs) and diversity ( $H' = 3.2 \pm 0.1$ ) and the lowest values of bacterial species richness ( $20 \pm 1$  OTUs) and diversity ( $H' = 2.6 \pm 0.1$ ) were recorded (Tukey's test,  $P < 0.05$ , Table 3).

## Discussion

It has been shown for the first time that aquatic microbial communities associated to submerged leaves have the potential to biotransform nicosulfuron (at a very high concentration and in a short time), a herbicide frequently detected in surface (Battaglin et al. 2009; de Lafontaine et al. 2014; Cessna et al. 2015; SOeS 2015) and groundwater (ADES 2016). This is not the case for mesotrione and S-metolachlor. The absence of mesotrione or S-metolachlor biodegradation could be explained by many factors such as (i) the absence of degrading strains, (ii) the sensitivity of degrading strains to the herbicides at the high doses tested, (iii) lag phases  $> 40$  days, or (iv) sub-optimal incubation conditions.

The nicosulfuron dissipation has been attributed to biotransformation processes since adsorption and abiotic degradation were negligible. Moreover, the production of metabolites, such as ADMP and ASDM, coming from hydrolytic reaction, was only observed in the presence of microbial communities, and can be correlated to the disappearance of nicosulfuron. Only 14-day acclimation was required for microbial communities to transform nicosulfuron that corresponds to a rather fast adaptation comparing to some other biological degradation of pesticides described by natural microflora of river water which was characterized by an extended lag phase ranging from 45 to 60 days (El-Dib and Abou-Waly 1998). In the literature, several fungal and bacterial strains, isolated from agricultural and wastewater treatment systems, have been described as able to transform nicosulfuron (Zhang et al. 2012; Lu et al. 2012; Song et al. 2013; Zhao et al. 2015). Although the estimation of the specific contribution of fungi and/or bacteria in nicosulfuron transformation potential is difficult on leaves substratum, the observed increase in fungal species richness and Shannon's diversity and decrease in bacterial community diversity in presence of nicosulfuron would suggest a better adaptation of the former. Indeed, some fungal strains have already been described as nicosulfuron-degraders in waste water treatment plant systems (Lu et al. 2012; Song et al. 2013). Moreover, fungi constitute  $> 90\%$  of microbial biomass in submerged leaves (Hieber and Gessner 2002; Artigas et al. 2012) and possess a large panel of oxidative/hydrolytic enzymes that could participate to pesticide degradation (Baldrian 2006; Artigas et al. 2012). Fungal species associated to submerged



**Table 3.** Species richness and Shannon-Weaver's diversity indices calculated on relative band intensities obtained from PCR-DGGE analysis of fungal and bacterial communities before (day 0) and after (day 40) single exposure to nicosulfuron and to the three-herbicide cocktail. Values are means ( $n = 3$ ) and standard errors (in parenthesis) of diversity indices for the upstream and downstream microbial communities

		Species richness		Shannon-Weaver	
		Fungi	Bacteria	Fungi	Bacteria
Day 0	Upstream	15 (1) <sup>b</sup>	35 (1) <sup>a</sup>	2.1 (0.0) <sup>b</sup>	3.2 (0.0) <sup>a</sup>
Day 40	Upstream single exposure	16 (4) <sup>b</sup>	25 (3) <sup>ab</sup>	2.3 (0.2) <sup>ab</sup>	2.9 (0.1) <sup>ab</sup>
Day 40	Upstream cocktail exposure	24 (4) <sup>ab</sup>	23 (1) <sup>b</sup>	2.7 (0.2) <sup>ab</sup>	2.8 (0.1) <sup>ab</sup>
Day 0	Downstream	13 (2) <sup>b</sup>	28 (1) <sup>ab</sup>	2.1 (0.2) <sup>b</sup>	3.0 (0.1) <sup>ab</sup>
Day 40	Downstream single exposure	38 (8) <sup>a</sup>	20 (1) <sup>b</sup>	3.2 (0.1) <sup>a</sup>	2.6 (0.1) <sup>a</sup>
Day 40	Downstream cocktail exposure	22 (5) <sup>ab</sup>	24 (3) <sup>b</sup>	2.7 (0.2) <sup>ab</sup>	2.8 (0.2) <sup>ab</sup>

Significant differences between conditions are indicated by letters in superscript (a > b, Tukey's test,  $P < 0.05$ )

leaves could therefore be mostly responsible for the transformation of nicosulfuron. However, the degradation potential of nicosulfuron by fungi described in our study is probably time- and site-specific. Therefore, integrating the influence of environmental conditions (including seasonality, leaf species, sites) will permit to assess the variability of nicosulfuron transformation potential by leaf-associated microbial communities in contaminated streams.

The exposure history of aquatic microbial communities to pesticides (e.g., diuron) and/or metals (e.g., zinc) has been observed to increase their tolerance (Pesce et al. 2010; Tlili et al. 2011). This acquisition of tolerance can be explained by (i) the physiological acclimation during the exposure to a toxicant and/or (ii) the genetic adaptation in populations exposed over generations to a toxic contaminant (Amiard-Triquet et al. 2011). Our study showed that downstream leaf-associated microbial communities (historically exposed to nicosulfuron) exhibit greater transformation potential of nicosulfuron than upstream communities (less polluted site) even if these microbial communities were not exposed to nicosulfuron during colonization experiments (Table 1). It could be also hypothesized that other pesticide molecules with similar degradation pathways (i.e., organophosphorus hydrolysis (Singh and Walker 2006)), and present in stream water during leaves colonization, would promote the enzymatic machinery necessary to transform nicosulfuron. However, this hypothesis remains to be tested.

Single and cocktail exposure of leaf-associated microbial communities to herbicides did not alter biotransformation potential of nicosulfuron although the concentration of the three herbicides in cocktail was high. However, a stronger impact of the cocktail was observed on the microbial community structure (especially fungi). From a functional point of view, similar nicosulfuron dissipation percentages were observed with different fungal community structures (i.e., single versus cocktail exposures in downstream communities), suggesting a certain functional redundancy (Allison and Martiny 2008; Dopheide et al. 2015). However, this functional redundancy

on nicosulfuron transformation was less evident in bacterial communities. Common pesticide degradation pathways have been described for different microbial strains (including fungi and bacteria) which also suggest functional redundancy for pesticide biotransformation (Lu et al. 2012; Song et al. 2013; Sviridov et al. 2015). Besides, the absence of cocktail effects on nicosulfuron transformation potential also reflects the ability of degrading communities to tolerate the other molecules of the cocktail, even at the high concentrations tested. It could also be assumed that S-metolachlor and mesotrione had no effect (inhibition or induction) on microbial capacities to degrade nicosulfuron.

## Conclusion

The present study showed the biotransformation potential of leaf-associated microbial communities for the herbicide nicosulfuron (alone or in cocktail with S-metolachlor and mesotrione). This transformation could be explained by the supply of organic carbon from leaf litter which supports the development of fungal and bacterial communities and prompts microbial co-metabolism of nicosulfuron. This ability was enhanced in the downstream sites, exhibiting greater exposure history to pesticides, and reflecting greater microbial acclimation. The shifts in fungal and bacterial community diversity in response to nicosulfuron exposure were different between single and cocktail exposure.

Nevertheless, leaves are ephemeral/transient substrates in river ecosystems (mostly associated to seasonality) suggesting a discontinuous contribution of these microbial communities able to transform nicosulfuron. Further studies are thus required to better assess the effect of herbicide concentration, environmental factors (i.e., temperature, nutrients, leaf species) and biological interactions (i.e., protozoans) in microbial potential to transform nicosulfuron. Understanding the ecology of leaf-associated microbial communities (i.e., the potential

use of herbicides as additional nutrient sources) can improve assessment of the fate of pesticides in contaminated streams.

**Acknowledgements** This project has been funded by the project EcoBioPest (Agence de l'Eau Loire-Bretagne), the Région Auvergne, the French Ministry for Higher Education and Research and the European Regional Development Fund.

#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- ADES (2016) Portail national d'accès aux données sur les eaux souterraines. <http://www.ades.eaufrance.fr/ExportData.aspx?paramFrance=ok>. Accessed 8 Feb 2016
- Agritox (2016) Base de données sur les substances actives phytopharmaceutiques. <http://www.agritox.anses.fr/php/sa.php?sa=1104>. Accessed 3 Feb 2016
- Alexander M (1994) Growth-linked biodegradation. In: Biodegradation and bioremediation. Academic Press, pp 8–15
- Aliferis KA, Materzok S, Pazirotou GN, Chrysai-Tokousbalides M (2009) *Lemna minor* L. as a model organism for ecotoxicological studies performing  $^1\text{H}$  NMR fingerprinting. *Chemosphere* 76:967–973
- Allison SD, Martiny JB (2008) Resistance, resilience, and redundancy in microbial communities. *Proc Natl Acad Sci* 105:11512–11519
- Amiard-Triquet C, Rainbow PS, Romeo M (2011) Tolerance to environmental contaminants. CRC Press
- Arthur EL, Anhalt JC, Anderson TA, Coats JR (1997) Enhanced degradation of deethylatrazine in an atrazine-history soil of Iowa 1. *J Environ Sci Health Part B* 32:599–620
- Artigas J, Romani AM, Sabater S (2008) Relating nutrient molar ratios of microbial attached communities to organic matter utilization in a forested stream. *Fundam Appl Limnol Für Hydrobiol* 173:255–264
- Artigas J, Gaudes A, Munoz I et al (2011) Fungal and bacterial colonization of submerged leaf litter in a Mediterranean stream. *Int Rev Hydrobiol* 96:221–234
- Artigas J, Majerhole J, Foulquier A et al (2012) Effects of the fungicide tebuconazole on microbial capacities for litter breakdown in streams. *Aquat Toxicol* 122:197–205
- Augustin T, Schlosser D, Baumbach R et al (2006) Biotransformation of 1-naphthol by a strictly aquatic fungus. *Curr Microbiol* 52:216–220
- Baldrian P (2006) Fungal laccases—occurrence and properties. *FEMS Microbiol Rev* 30:215–242
- Batissou I, Crouzet O, Besse-Hoggan P et al (2009) Isolation and characterization of mesotrine-degrading *Bacillus* sp. from soil. *Environ Pollut* 157:1195–1201
- Battaglin WA, Rice KC, Focazio MJ et al (2009) The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005–2006. *Environ Monit Assess* 155:281–307
- Berger BM, Wolfe NL (1996) Hydrolysis and biodegradation of sulfonyleurea herbicides in aqueous buffers and anaerobic water-sediment systems: assessing fate pathways using molecular descriptors. *Environ Toxicol Chem* 15:1500–1507
- Cessna AJ, Donald DB, Bailey J, Waiser M (2015) Persistence of the sulfonyleurea herbicides sulfosulfuron, rimsulfuron, and nicosulfuron in farm dugouts (ponds). *J Environ Qual* 44:1948–1955
- Chaabane H, Vulliet E, Calvayrac C et al (2008) Behaviour of sulcotriene and mesotrine in two soils. *Pest Manag Sci* 64:86–93
- Cook ME, Moore PA (2008) The effects of the herbicide metolachlor on agonistic behavior in the crayfish, *Orconectes rusticus*. *Arch Environ Contam Toxicol* 55:94–102
- Davila-Vazquez G, Tinoco R, Pickard MA, Vazquez-Duhalt R (2005) Transformation of halogenated pesticides by versatile peroxidase from *Bjerkandera adusta*. *Enzym Microb Technol* 36:223–231
- de Lafontaine Y, Beauvais C, Cessna AJ et al (2014) Sulfonyleurea herbicides in an agricultural catchment basin and its adjacent wetland in the St. Lawrence River basin. *Sci Total Environ* 479:1–10
- de Oliveira TA, dos Santos JB, Camelo GN et al (2009) Effect of sequential nicosulfuron and chlorpyrifos application on seed bank and soil microbial characteristics. *Rev Bras Ciênc Solo* 33:563–570
- Debenest T, Pinelli E, Coste M et al (2009) Sensitivity of freshwater periphytic diatoms to agricultural herbicides. *Aquat Toxicol* 93:11–17
- Dopheide A, Lear G, He Z et al (2015) Functional gene composition, diversity and redundancy in microbial stream biofilm communities. *PLoS One* 10:e0123179
- Durand S, Amato P, Sancelme M et al (2006) First isolation and characterization of a bacterial strain that biotransforms the herbicide mesotrine. *Lett Appl Microbiol* 43:222–228
- Dyson JS, Beulke S, Brown CD, Lane MCG (2002) Adsorption and degradation of the weak acid mesotrine in soil and environmental fate implications. *J Environ Qual* 31:613–618
- El-Dib MA, Abou-Waly HF (1998) Biodegradation of some triazines and phenylureas in surface waters. *Water Res* 32:1881–1887
- Elias D, Bernot MJ (2014) Effects of atrazine, metolachlor, carbaryl and chlorothalonil on benthic microbes and their nutrient dynamics. *PLoS One* 9:e109190
- Fort DJ, Rogers R, Copley H et al (1999) Effect of sulfometuron methyl and nicosulfuron on development and metamorphosis in *Xenopus laevis*: impact of purity. *Environ Toxicol Chem* 18:2934–2940
- Gessner MO, Gulis V, Kuehn KA et al (2007) Fungal decomposers of plant litter in aquatic ecosystems. *Environ Microb Relatsh* 4:301–324
- Gulis V, Suberkropp K (2003) Effect of inorganic nutrients on relative contributions of fungi and bacteria to carbon flow from submerged decomposing leaf litter. *Microb Ecol* 45:11–19
- Heuer H, Hartung K, Wieland G et al (1999) Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. *Appl Environ Microbiol* 65:1045–1049
- Hieber M, Gessner MO (2002) Contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates. *Ecology* 83:1026–1038
- Joly P, Besse-Hoggan P, Bonnemoy F et al (2012) Impact of maize formulated herbicides mesotrine and S-metolachlor, applied alone and in mixture, on soil microbial communities. *ISRN Ecol* 2012:1–9
- Joly P, Bonnemoy F, Charvy J-C et al (2013) Toxicity assessment of the maize herbicides S-metolachlor, benoxacor, mesotrine and nicosulfuron, and their corresponding commercial formulations, alone and in mixtures, using the Microtox® test. *Chemosphere* 93(10):2444–2450
- Joly P, Misson B, Perrière F et al (2014) Soil surface colonization by phototrophic indigenous organisms, in two contrasted soils treated by formulated maize herbicide mixtures. *Ecotoxicology* 23:1648–1658
- Joly P, Bonnemoy F, Besse-Hoggan P et al (2015) Responses of limagne “clay/organic matter-rich” soil microbial communities to realistic formulated herbicide mixtures, including S-metolachlor, mesotrine, and nicosulfuron. *Water Air Soil Pollut* 226:1–15

- Junghanns C, Moeder M, Krauss G et al (2005) Degradation of the xenoestrogen nonylphenol by aquatic fungi and their laccases. *Microbiologia* 151:45–57
- Karpouzias DG, Kandeler E, Bru D et al (2014a) A tiered assessment approach based on standardized methods to estimate the impact of nicosulfuron on the abundance and function of the soil microbial community. *Soil Biol Biochem* 75:282–291
- Karpouzias DG, Papadopoulou E, Ipsilantis I et al (2014b) Effects of nicosulfuron on the abundance and diversity of arbuscular mycorrhizal fungi used as indicators of pesticide soil microbial toxicity. *Ecol Indic* 39:44–53
- Kaushik NKK, Hynes HBN (1971) Fate of the dead leaves that fall into streams. *Arch Hydrobiol* 68:465–515
- Krauss G-J, Solé M, Krauss G et al (2011) Fungi in freshwaters: ecology, physiology and biochemical potential. *FEMS Microbiol Rev* 35: 620–651
- Kreutz LC, Barcellos LJG, Silva TO et al (2008) Acute toxicity test of agricultural pesticides on silver catfish (*Rhamdia quelen*) fingerlings. *Ciênc Rural* 38:1050–1055
- Liu D, Maguire RJ, Lau YL et al (2000) Factors affecting chemical biodegradation. *Environ Toxicol* 15:476–483
- Liu J, Chen S, Ding J et al (2015) Sugarcane bagasse as support for immobilization of *Bacillus pumilus* HZ-2 and its use in bioremediation of mesotriene-contaminated soils. *Appl Microbiol Biotechnol* 99:10839–10851
- Lu XH, Kang ZH, Tao B et al (2012) Degradation of nicosulfuron by *Bacillus subtilis* YB1 and *Aspergillus niger* YF1. *Appl Biochem Microbiol* 48:460–466
- Martin C, Corvini PF, Vinken R et al (2009) Quantification of the influence of extracellular laccase and intracellular reactions on the isomer-specific biotransformation of the xenoestrogen technical nonylphenol by the aquatic hyphomycete *Clavariopsis aquatica*. *Appl Environ Microbiol* 75:4398–4409
- Martins PF, Martinez CO, de Carvalho G et al (2007) Selection of microorganisms degrading S-metolachlor herbicide. *Braz Arch Biol Technol* 50:153–159
- Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. *Phytochemistry* 60:551–565
- Nélieu S, Perreau F, Guichon R et al (2005) Monitoring and effects of nicosulfuron in aquatic mesocosms: development of a simple analytical procedure and evidence for low toxicity to phytoplankton communities. *Anal Bioanal Chem* 382:108–114
- Neves M, Castro BB, Vidal T et al (2015) Biochemical and populational responses of an aquatic bioindicator species, *Daphnia longispina*, to a commercial formulation of a herbicide (Primextra® gold TZ) and its active ingredient (S-metolachlor). *Ecol Indic* 53:220–230
- Ni Y, Lai J, Wan J, Chen L (2014) Photosynthetic responses and accumulation of mesotriene in two freshwater algae. *Environ Sci Process Impacts* 16:2288–2294
- Oliveira RS, Koskinen WC, Ferreira FA (2001) Sorption and leaching potential of herbicides on Brazilian soils. *Weed Res* 41:97–110
- Pesce S, Margoum C, Montuelle B (2010) *In situ* relationships between spatio-temporal variations in diuron concentrations and phototrophic biofilm tolerance in a contaminated river. *Water Res* 44:1941–1949
- Petric I, Karpouzias DG, Bru D et al (2016) Nicosulfuron application in agricultural soils drives the selection towards NS-tolerant microorganisms harboring various levels of sensitivity to nicosulfuron. *Environ Sci Pollut Res* 23:4320–4333
- Phyt’eauvergne (2015) Groupe régional d’action contre les pollutions par les produits phytosanitaires. [http://www.fredon-auvergne.fr/phyteauevergne/carto\\_web/Fiche\\_ESU/Auzon\\_fiche.html](http://www.fredon-auvergne.fr/phyteauevergne/carto_web/Fiche_ESU/Auzon_fiche.html). Accessed 11 Dec 2015
- Pileggi M, Pileggi SAV, Olchanheski LR et al (2012) Isolation of mesotriene-degrading bacteria from aquatic environments in Brazil. *Chemosphere* 86:1127–1132
- Prenafeta-Boldu FX, Summerbell R, De Hoog GS (2006) Fungi growing on aromatic hydrocarbons: biotechnology’s unexpected encounter with biohazard? *FEMS Microbiol Rev* 30:109–130
- Providenti MA, Lee H, Trevors JT (1993) Selected factors limiting the microbial degradation of recalcitrant compounds. *J Ind Microbiol* 12:379–395
- Romdhane S, Devers-Lamrani M, Martin-Laurent F et al (2015) Isolation and characterization of *Bradyrhizobium* sp. SR1 degrading two  $\beta$ -triketone herbicides. *Environ Sci Pollut Res* 23:4138–4148
- Saglio P, Breteau S, Rivot E, Olsén KH (2003) Chemobehavioral changes induced by short-term exposures to prochloraz, nicosulfuron, and carbofuran in goldfish. *Arch Environ Contam Toxicol* 45:515–524
- Šantrić L, Radivojević L, Stanković-Kalezić R, Đorđević S (2004) Microbiological activity of soil treated with nicosulfuron. *Pestic Fitomedicina* 19:55–60
- Sarmah AK, Sabadie J (2002) Hydrolysis of sulfonylurea herbicides in soils and aqueous solutions: a review. *J Agric Food Chem* 50:6253–6265
- Seguin F, Lehoullanger C, Rimet F et al (2001) Effects of atrazine and nicosulfuron on phytoplankton in systems of increasing complexity. *Arch Environ Contam Toxicol* 40:198–208
- Singh BK, Walker A (2006) Microbial degradation of organophosphorus compounds. *FEMS Microbiol Rev* 30:428–471
- SMVVA (2014) Contrat territorial de la vallée de l’Auzon - Bilan milieux. 39–43
- SOeS (2013) Ministère de l’environnement, de l’énergie et de la mer. <http://www.statistiques.developpement-durable.gouv.fr/essentiel/ar/246/211/contamination-globale-cours-deau-pesticides.html>. Accessed 27 Mar 2016
- SOeS (2015) Liste des pesticides recherchés dans les cours d’eau de France métropolitaine en 2013. [www.statistiques.developpement-durable.gouv.fr/fileadmin/documents/Produits\\_editoriaux/L\\_essentiel\\_sur/Environnement/Eau/Les\\_pesticides\\_dans\\_les\\_eaux/2016/pesticides1c-2016-b.xls](http://www.statistiques.developpement-durable.gouv.fr/fileadmin/documents/Produits_editoriaux/L_essentiel_sur/Environnement/Eau/Les_pesticides_dans_les_eaux/2016/pesticides1c-2016-b.xls). Accessed 20 May 2016
- Song J, Gu J, Zhai Y et al (2013) Biodegradation of nicosulfuron by a *Talaromyces flavus* LZM1. *Bioresour Technol* 140:243–248
- Sviridov AV, Shushkova TV, Ermakova IT et al (2015) Microbial degradation of glyphosate herbicides (review). *Appl Biochem Microbiol* 51:188–195
- Tlili A, Corcoll N, Bonet B et al (2011) *In situ* spatio-temporal changes in pollution-induced community tolerance to zinc in autotrophic and heterotrophic biofilm communities. *Ecotoxicology* 20:1823–1839
- Topp E, Tessier L, Gregorich EG (1996) Dairy manure incorporation stimulates rapid atrazine mineralization in an agricultural soil. *Can J Soil Sci* 76:403–409
- Valiente Moro C, Bricheux G, Portelli C, Bohatier J (2012) Comparative effects of the herbicides chlortoluron and mesotriene on freshwater microalgae. *Environ Toxicol Chem* 31:778–786
- van der Meer JR (1994) Genetic adaptation of bacteria to chlorinated aromatic compounds. *FEMS Microbiol Rev* 15:239–249
- Whaley CM, Armel GR, Wilson HP, Hines TE (2006) Comparison of mesotriene combinations with standard weed control programs in corn. *Weed Technol* 20:605–611
- White TJ, Bruns T, Lee S et al (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protoc Guide Methods Appl* 18:315–322
- Youness M (2013) Impact de la formulation et du mélange de deux pesticides (mesotriene et tébuconazole) sur leur biodégradation et la croissance de microorganismes. Thesis, DU2378, University Blaise Pascal, Clermont-Ferrand, France
- Zemolin CR, Avila LA, Cassol GV et al (2014) Environmental fate of S-metolachlor: a review. *Planta Daninha* 32:655–664
- Zhang H, Mu W, Hou Z et al (2012) Biodegradation of nicosulfuron by the bacterium *Serratia marcescens* N80. *J Environ Sci Health Part B* 47:153–160
- Zhao W, Wang C, Xu L et al (2015) Biodegradation of nicosulfuron by a novel *Alcaligenes faecalis* strain ZWS11. *J Environ Sci* 35:151–162

