Université de Lorraine
Laboratoire des Interactions
Ecotoxicologie Biodiversité Ecosystèmes
LIEBE – CNRS UMR 7146

Ecole Doctorale Sciences et Ingénierie
Ressource Procédés Produits
Environnement
RP2E ED410

Thèse présentée par

Hugues CLIVOT

En vue de l'obtention du Doctorat de l'Université de Lorraine

Mention Ecotoxicité, Biodiversité, Ecosystèmes

Acidification et restauration d'écosystèmes forestiers : effets sur les communautés microbiennes et sur des processus fonctionnels associés

Soutenue publiquement le 15 novembre 2012 devant le jury composé de

Virginie BALDY	Maître de Conférences HDR, Université Aix-Marseille	Rapporteur
Robert DURAN	Professeur, Université de Pau et des Pays de l'Adour	Rapporteur
Isabelle BERTRAND	Chargée de Recherche, INRA Reims	Examinateur
Eric CHAUVET	Directeur de Recherche, CNRS Ecolab Toulouse	Examinateur
Etienne DAMBRINE	Professeur, Université de Savoie	Examinateur
François GUEROLD	Professeur, Université de Lorraine	Directeur de Thèse
Christophe PAGNOUT	Maître de Conférences, Université de Lorraine	Encadrant de Thèse

Remerciements

Je tiens à remercier en premier lieu mes encadrants François Guérold et Christophe Pagnout, qui m'ont donné l'opportunité de réaliser cette thèse. Merci pour les bonnes conditions de travail dans lesquelles se sont déroulées ces quelques années mais aussi pour les libertés que vous m'avez laissées prendre. Je vous suis profondément reconnaissant de m'avoir guidé et fait partager votre expérience ainsi que pour tous les moyens mis à ma disposition pour mener à bien cette thèse.

J'adresse mes plus sincères remerciements à Virginie Baldy et Robert Duran pour avoir accepté d'être rapporteurs de ce travail de thèse et je remercie également vivement Isabelle Bertrand, Eric Chauvet et Etienne Dambrine d'avoir accepté de faire partie du jury de cette thèse.

Merci à Pascal Poupin pour avoir encadré une partie de mes travaux ainsi que pour son aide et ses nombreux conseils.

Un grand merci à Michael Danger pour les très nombreuses discussions, aides et pour ses conseils. Ce fut un vrai plaisir de travailler avec toi.

Je souhaite faire part de ma profonde reconnaissance à Philippe Wagner, dont l'aide précieuse et l'expérience ont fait que les sorties sur le terrain se sont toujours déroulées dans les meilleures conditions possibles malgré la neige, le froid et les nombreuses péripéties qui ont agrémentées nos escapades vosgiennes… Merci pour tous ces bons moments passés dans les Vosges et à Metz.

Merci également à Philippe Rousselle qui m'a grandement aidé en réalisant de nombreuses analyses physico-chimiques.

Je remercie également les toulousains Eric Chauvet et Arnaud Elger pour cette belle collaboration ainsi que Julien Cornut, avec qui j'ai pu passer notamment d'innombrables heures au téléphone.

Je souhaite remercier les enseignant-chercheurs du LIEBE avec qui j'ai pu travailler : Pascale Bauda, Delphine Aran, Vincent Felten et Simon Devin.

Je remercie l'ensemble des personnes du LIEBE pour leur aide et pour leur soutien et plus particulièrement « les filles d'en bas » Marie-Andrée, Chantal et Rosy ainsi que Maryline pour sa précieuse aide administrative et Mireille pour son « assistance bibliographique » et bien entendu l'infodoc. Je remercie également Etienne et Nelly pour leur aide à la préparation des TP que j'ai pu encadrer. Merci à toutes les autres personnes (en particulier Sandrine, Florence, Françoise, Bénédicte et j'en oublie sûrement) pour tous les bons moments passés dans ce laboratoire et surtout pour avoir écouté (et même demandé !) toutes mes pires blagues.

Merci à Faustine pour le travail qu'elle a réalisé lors de son stage de Master que j'ai pu encadrer.

J'ai une pensée particulière pour Jean-Nicolas avec qui j'ai pu partager un mémorable Flying Trip to US, ainsi que pour Philippe UP avec qui nous avons passé une semaine ô combien dépaysante à Santa Fe. J'en profite au passage pour remercier encore François de m'avoir donné l'occasion de faire des congrès et des voyages enrichissants.

Je tiens à remercier les autres doctorants et stagiaires avec qui j'ai pu travailler ou ceux avec qui j'ai partagé mon quotidien : Apolline, Emilie, Julio, Karen, Anne-Sophie, Yann, Pierre, Fanny, Olivier, Hela, Céline, Mael, Renaud, Louis, Chris, Maxime.

Une mention spéciale à mes deux compères de Mic : Audrey, qui aura réussi à survivre à mes sournoiseries et Stéphane, qui est le seul à penser ne pas être un geek. Merci à tous les deux pour tous ces grands moments qui ont animé nos journées.

Merci à Eric pour son amitié et tous les bons moments partagés au labo et en dehors et pour m'avoir permis de squatter de nombreuses fois son canapé quand j'étais SDFM (sans domicile fixe messin).

Je ne remercierais jamais assez mes parents de m'avoir soutenu et permis de faire les études qui m'ont mené jusqu'ici. Je remercie également ma famille et mes proches pour tous leurs encouragements.

Enfin, je tenais à remercier tout particulièrement Aurélie d'être à mes côtés et pour tout son soutien, son aide et ses encouragements au quotidien.

Table des matières

Liste des figures et tableaux

Chapitre I : Contexte de l'étude

Figures

Chapitre II : Cas du massif vosgien et présentation des sites d'étude

Figures

Tableaux

Chapitre III : Effets de la restauration de bassins versants acidifiés sur les communautés microbiennes des sols

III.1. Effets de la restauration de bassins versants forestiers acidifiés sur les communautés bactériennes des sols

Figures

Tableaux

III.2. Diversité des communautés bactériennes oxalotrophes dans des sols forestiers acides

Figures

Tableaux

Chapitre IV : Effets de l'acidification anthropique des cours d'eau de tête de bassin sur la diversité et les activités microbiennes associées à la décomposition des litières

IV.1. Effets de l'acidification anthropique sur la décomposition de la litière et les communautés de décomposeurs associées dans les zones benthiques et hyporhéiques des cours d'eau de tête de bassin

IV.1.1. Effets de l'acidification sur la décomposition des litières dans les zones benthiques et hyporhéiques de cours d'eau forestiers

Figures

Tableaux

IV.1.2. Effets de l'acidification sur la diversité fongique associée aux litières en décomposition dans les zones benthiques et hyporhéiques de cours d'eau forestiers

Figures

Tableaux

IV.2. Effets de l'acidification des cours d'eau de tête de bassin sur les activités microbiennes associées à la litière en décomposition

Figures

Tableaux

IV.3. Approche en microcosmes pour l'étude des effets de l'Aluminium et de la limitation en Phosphore sur les activités fongiques associées à la décomposition des litières

Figures

Tableaux

Communication des travaux de recherche

Publications

Cornut J., **Clivot H.**, Chauvet E., Elger A., Pagnout C., Guérold F. 2012. Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams. Water Research, accepted 08/09/12.

Clivot H., Danger M., Pagnout C., Wagner P., Rousselle P., Poupin P., Guérold F. 2012. Impaired leaf litter processing in acidified streams: learning from microbial enzyme activities. Microbial Ecology, accepted 02/08/12.

Clivot H., Pagnout C., Aran D., Devin S., Bauda P., Poupin P., Guérold F. 2012. Changes in soil bacterial communities following liming of acidified forests. Applied Soil Ecology, 59: 116-123.

Communications orales

Jomini S., **Clivot H.**, Bauda P., Pagnout C., 2011. Effects of manufactured TiO2 nanoparticles on the planktonic bacterial communities from the Moselle river (France). HENVI2011. International Conference on Health and Environment. Frontiers in Environmental Health: Challenges to Water Safety. Luxembourg, 28/10/2011.

Clivot H., Cornut J., Gierlinski P., Danger M., Pagnout P., Poupin P., Elger A., Chauvet E., Guérold F., 2011. Acidification des cours d'eau de tête de bassin : Etude de la diversité fongique et des activités microbiennes associées à la litière végétale en décomposition. Colloque des Zones Ateliers. Rennes, 04-07/10/2011.

Clivot H., Cornut J., Gierlinski P., Danger M., Pagnout P., Poupin P., Elger A., Chauvet E., Guérold F., 2011. Diversity and activities of fungal communities associated with decaying leaves in acidified headwater streams. 12th Symposium on Aquatic Microbial Ecology. Rostock, Germany, 28/08-02/09/2011.

Clivot H., Pagnout C., Rousselle P., Wagner P., Danger M., Poupin P., Guérold F., 2010. Acidification of headwater streams: structural and functional assessment of microbial communities associated with decaying leaves. 2010 ASLO/NABS Meeting. Aquatic sciences: global changes from the center to the edge. Santa Fe, New Mexico, USA, 06-11/06/2010.

Clivot H., Pagnout C., Rousselle P., Wagner P., Poupin P., Guérold F., 2009. Etude des changements de diversité microbienne liés aux phénomènes d'acidification de bassins versants. ZAM. Séminaire annuel de la Zone Atelier Moselle. Champenoux, 29-30/09/2009.

Clivot H., Aran D., Rousselle P., Auclerc A., Devin S., Nahmani J., Wagner P., Bauda P., Poupin P., Pagnout C., Guérold F., 2009. Restauration de bassins versants acidifiés : effet sur la diversité des communautés bactériennes du sol. 10èmes Journées d'Etude des Sols. Strasbourg, 12-14/05/2009.

Communications affichées

Arce Funck J., **Clivot H.**, Felten V., Guérold F., Danger M., 2012. Does phosphorus limitation increase the toxic effect of silver on aquatic fungi and leaf litter decomposition? 6th SETAC World Congress. Berlin, Germany, 20-24/05/2012.

Jomini S., **Clivot H.**, Bauda P., Pagnout C., 2011. Effets des nanoparticules de dioxyde de titane sur les communautés bactériennes planctoniques de la Moselle. Colloque des Zones Ateliers. Rennes, 04-07/10/2011.

Charmasson F., **Clivot H.**, Felten V., Boudot J.P., Rousselle P., Guérold F., Danger M., 2011. Anthropogenic acidification of headwater streams: limiting factors for the decomposition of organic matter in the impacted streams. 12th Symposium on Aquatic Microbial Ecology. Rostock, Germany, 28/08-02/09/2011.

Auclerc A., Nahmani J., Aran D., Masfaraud J.F., Pagnout C., **Clivot H.**, Rousselle P., Wagner P., Guérold F., 2010. Impact d'un amendement calco-magnésien sur la macrofaune du sol dans les Vosges. Actes du Séminaire de l'Ecole doctorale RP2E. Nancy, 28/01/2010.

Clivot H., Aran D., Rousselle P., Auclerc A., Devin S., Nahmani J., Wagner P., Bauda P., Poupin P., Pagnout C., Guérold F., 2009. Impact d'un amendement calco-magnésien sur la diversité bactérienne de sols acidifiés. AFEM. 4ème Colloque d'Ecologie microbienne Lyon, 31/08-03/09/2009.

Introduction générale

Les activités anthropiques peuvent bouleverser profondément et durablement les écosystèmes, en fragmentant et dégradant les milieux naturels, en surexploitant les ressources naturelles, en modifiant le climat ou encore via l'introduction d'espèces exotiques. Près de la moitié des surfaces émergées de la planète a ainsi été transformée par les activités humaines, qui exploitent également plus de la moitié des ressources disponibles en eaux douces ce qui engendre aussi des modifications majeures de l'atmosphère et des cycles biogéochimiques (Vitousek et al., 1997).

La dégradation des milieux naturels, qu'elle soit physique ou chimique, se traduit par de profondes modifications de la composition et de la structure des communautés biologiques. L'actuelle érosion de la biodiversité, que l'on qualifie volontiers de 6^{ième} crise d'extinction des espèces, en est certainement la conséquence la plus manifeste (Pimm et al., 1995). Ces changements de biodiversité peuvent profondément modifier les processus fonctionnels qui participent au fonctionnement des écosystèmes et en retour altérer les services écologiques que les écosystèmes procurent à l'Homme (Hooper et al., 2005).

Parmi les principales atteintes environnementales, l'acidification anthropique des écosystèmes terrestres et aquatiques a depuis longtemps été reconnue comme étant un problème environnemental majeur dans de nombreuses régions de l'hémisphère nord, notamment en raison de ses effets délétères sur la biodiversité et le fonctionnement des écosystèmes terrestres et aquatiques (Gorham, 1998; Driscoll et al., 2001). Les révolutions industrielles des siècles derniers sont à l'origine des émissions polluantes et des dépôts atmosphériques qui ont entrainé l'acidification et l'altération de nombreux écosystèmes sensibles, principalement en Amérique du Nord et en Europe. La prise de conscience politique des conséquences écologiques engendrées par ce phénomène a conduit, depuis le début des années 1980, à une réduction d'une partie des polluants atmosphériques grâce à la mise en place de la convention sur la pollution atmosphérique transfrontalière à longue distance[1]. Cependant, bien que des signes de restauration spontanée aient pu être observés dans certaines régions (Likens et al., 1996; Stoddard et al., 1999; Skjelkvåle et al., 2001), de nombreux écosystèmes ne présentent aucun signe d'amélioration et subissent toujours les effets délétères engendrés par des décennies de dépôts atmosphériques acidifiants (Alewell et al., 2000; Driscoll et al., 2001). De plus, des phénomènes d'acidification commencent à apparaitre dans d'autres régions du monde et particulièrement en Asie (Larssen et al., 1999), en raison de

[1] Convention on Long-range Transboundary Air Pollution - 1979 -

l'augmentation des émissions polluantes dues à l'essor économique et démographique de pays en voie de développement (Streets et al., 2001).

Le massif des Vosges fait partie des régions françaises les plus sensibles à l'acidification (Party, 1999). Dans le massif vosgien, l'acidification prononcée des sols et des eaux de surface a ainsi entrainé d'importants dépérissements forestiers (Landmann & Bonneau, 1995), une dégradation marquée de la qualité des eaux de surface mais également une sévère érosion de la biodiversité et du fonctionnement des cours d'eau (Guérold et al., 2000; Dangles et al., 2004; Baudoin et al., 2008).

En attendant une restauration spontanée de ces écosystèmes et afin de pallier à leur dégradation et à celle des services associés (production de bois, production d'une ressource en eau de qualité), le recours à l'épandage d'amendements calco-magnésiens peut représenter une alternative permettant de reminéraliser les sols et les eaux de surface et ainsi restaurer ces écosystèmes acidifiés (Bonneau et al., 1992). Les objectifs de ces efforts de restauration forcée sont de rétablir la qualité physico-chimique des sols et des cours d'eau afin d'améliorer durablement l'état de santé des peuplements forestiers, mais également de restaurer la biodiversité et le fonctionnement des écosystèmes terrestres et aquatiques.

Les travaux de cette thèse s'inscrivent donc dans ce contexte et plus précisément dans le cadre d'un programme ANR Biodiversité (RECOVER) débuté en 2008, qui visait à évaluer les effets de l'acidification et d'une restauration par amendements calco-magnésiens sur la biodiversité et le fonctionnement des écosystèmes forestiers.

Cette étude s'appuie sur un précédent programme de recherche ayant mis en place des sites ateliers sur deux bassins versants amendés en 2003, l'un situé dans les Vosges gréseuses et l'autre dans les Vosges granitiques. Ces sites sont les premiers en France à avoir été amendés à l'échelle du bassin versant et présentent la particularité d'avoir chacun un bassin versant témoin contigu non amendé, permettant ainsi d'évaluer par comparaison les effets de l'amendement (Nys et al., 2004; Angéli, 2006).

Des études pré- et post-amendements ont montré des améliorations de la qualité physico-chimique des sols (Angéli, 2006) et de l'état sanitaire des arbres dans les bassins versants traités (Messant et al., 2008). Dans le cours d'eau drainant le bassin versant granitique, une amélioration de la qualité de l'eau a également été enregistrée et a permis d'observer une augmentation du processus de décomposition des litières de feuilles et de la diversité d'hyphomycètes aquatiques associée. En revanche, aucune amélioration de la qualité de l'eau,

de la décomposition des feuilles ou de la diversité fongique n'a été observée dans le cours d'eau drainant le bassin versant gréseux (Baudoin, 2007).

Ces résultats offrent ainsi deux opportunités de recherche intéressantes concernant les communautés microbiennes:

- l'étude des effets de l'amendement sur les caractéristiques édaphiques et les communautés des sols
- l'étude des relations entre les caractéristiques physico-chimiques des cours d'eau et la diversité et activités des communautés impliquées dans le processus de décomposition des litières

Les travaux présentés dans cette thèse s'intéresse à ces deux aspects et plus particulièrement aux communautés bactériennes et fongiques. En effet, ces micro-organismes sont impliqués dans de nombreux processus biogéochimiques et sont reconnues comme étant des indicateurs potentiels de la qualité des sols (Schloter et al., 2003). De plus, les micro-organismes hétérotrophes sont des acteurs majeurs de la dégradation des litières dans les cours d'eau et sont d'importants intermédiaires pour les transferts de nutriments et d'énergie au sein des réseaux trophiques (Bärlocher & Kendrick, 1976).

Dans ce contexte, le premier objectif de ces travaux de thèse sera d'évaluer si l'épandage d'amendements calco-magnésiens a induit des changements de diversité au sein des communautés microbiennes et, le cas échéant, si des indicateurs microbiens peuvent être associés à l'amélioration de la qualité des sols amendés.

Le deuxième objectif, quant à lui, sera d'améliorer la compréhension des facteurs responsables de l'altération de la décomposition des litières dans les cours d'eau acidifiés en étudiant la diversité et les activités microbiennes associées à ce processus.

Ce manuscrit de thèse est structuré en quatre chapitres :

Le premier chapitre replace cette étude dans son contexte. Le rôle des micro-organismes au sein des écosystèmes forestiers et les services rendus par ces derniers sont tout d'abord exposés. L'origine du processus d'acidification et ses effets sur les écosystèmes forestiers et les micro-organismes sont ensuite abordés avant de terminer cette partie sur les évolutions récentes des émissions atmosphériques et les tentatives de restauration des écosystèmes acidifiés.

Le deuxième chapitre expose les caractéristiques du massif vosgien et sa sensibilité à l'acidification, ainsi que les conséquences des dépôts atmosphériques acidifiants sur ce massif. Dans une deuxième partie seront présentés les sites expérimentaux spécifiques à notre étude et leur localisation sur les bassins versants amendés et témoins ainsi que sur les cours d'eau dans lesquels se sont déroulées les expérimentations sur la décomposition des litières.

Dans le troisième chapitre sont présentés les résultats de deux études sur les communautés bactériennes des sols des bassins versants amendés et témoins, la première étude se focalisant sur l'ensemble des groupes bactériens et la deuxième s'intéressant à la diversité des bactéries oxalotrophes.

Dans le quatrième chapitre sont exposés les résultats de trois expérimentations traitant des effets de l'acidification des cours d'eau sur la décomposition des litières. La première se propose d'étudier ce processus et la diversité des organismes associés dans les zones benthiques et hyporhéiques de cours d'eau le long d'un gradient d'acidification. La deuxième étude traite des effets de l'acidification sur les activités microbiennes associées à la litière en décomposition. Dans la troisième partie, des facteurs limitant potentiellement la décomposition des litières ont été isolés et leurs effets ont été évalués *in vitro* par une approche en microcosmes.

Enfin, une conclusion générale et des perspectives de recherche sont proposées dans la dernière partie du manuscrit.

Chapitre I :

Contexte de l'étude

I.1. Les micro-organismes au sein des écosystèmes forestiers

I.1.1. Services rendus par les écosystèmes forestiers

Les forêts couvrent plus de 30 % de la surface des terres émergées de notre planète, s'étendant ainsi sur près de 4 milliards d'hectares. En France, la surface qu'elles occupent s'accroit depuis plus d'un siècle et représente aujourd'hui plus d'un quart des terres de notre territoire correspondant à plus de 15 millions d'hectares de forêts.

Depuis toujours, l'homme est dépendant des services rendus par la nature et notamment des services environnementaux et sociaux rendus par les écosystèmes forestiers.

Ainsi, les bassins versants forestiers jouent un rôle particulièrement important dans la préservation des ressources hydrologiques aussi bien quantitativement, en régulant les débits des cours d'eau et en approvisionnant les nappes phréatiques, que qualitativement, dans leur fonction d'épuration de l'eau (Postel & Thompson, 2005). Cette eau fournie par les écosystèmes forestiers est importante pour l'agriculture, la production d'électricité, la consommation, les loisirs mais elle est aussi essentielle en tant qu'habitat pour la faune aquatique.

La végétation des forêts à une fonction importante dans la stabilisation des sols, en protégeant les sols en pente contre l'érosion et les glissements de terrain. Elle participe aussi au piégeage de poussières et de particules fines d'origine atmosphérique et améliore ainsi la qualité de l'air (Beckett et al., 1998). La végétation joue également un rôle primordial en séquestrant de grandes quantités de CO_2 par la photosynthèse (Goodale et al., 2002). Dans les écosystèmes forestiers, la quantité de CO_2 fixée étant supérieure à celle rejetée, ces derniers participent ainsi à la lutte contre le réchauffement climatique en piégeant une partie de ce gaz à effet de serre (Hansen et al., 1981).

Les forêts sont les écosystèmes terrestres biologiquement les plus diversifiés. La biodiversité assure le maintien et l'équilibre des populations animales et végétales et des chaines trophiques, les forêts étant aussi le lieu de reproduction de nombreuses espèces. De plus, les écosystèmes forestiers abritent un potentiel génétique qui peut être important pour certains services de production.

La forêt a également une valeur récréative par l'esthétique de ses paysages et est un lieu de détente pour l'homme. Ces activités peuvent avoir une valeur économique importante, à l'instar de la production de bois. De nombreux autres produits issus des écosystèmes

forestiers ont aussi une valeur marchande non négligeable, comme, par exemple, les produits de consommation d'origines animales et végétales ou encore les fleurs, les champignons ou les plantes médicinales.

Tous ces services rendus par la forêt participent ainsi au bien-être humain. Leurs définitions, classifications et valeurs (Costanza et al., 1997; Fisher et al., 2009) sont d'importants paramètres à évaluer pour les prises de décision concernant la protection ou la réhabilitation de ces écosystèmes et des services qu'ils rendent. Dans le contexte économique actuel et celui du changement climatique, le maintien de ces services s'oppose de plus en plus à la demande en énergie couplée à la productivité grandissante de notre société, posant ainsi un questionnement crucial quant au mode de gestion à adopter pour préserver l'intégrité des écosystèmes et de leurs services.

I.1.2. Rôles des micro-organismes dans le fonctionnement des écosystèmes forestiers et intérêt de leur étude

I.1.2.1. Les micro-organismes des sols

Dans les écosystèmes forestiers, la biomasse totale de micro-organismes dans les sols est relativement importante et représenterait environ 1% de la biomasse des plantes. De plus, elle excède largement celle de la faune du sol qui ne correspondrait en moyenne qu'à 2% de la biomasse microbienne (Fierer et al., 2009).

Au sein des sols, les micro-organismes, représentés essentiellement par les bactéries et les champignons, jouent un rôle fondamental dans la décomposition de la matière organique provenant majoritairement des résidus d'origines végétales (Swift et al., 1979; Hättenschwiler et al., 2005). Ils peuvent aussi intervenir dans la sécrétion (Landeweert et al., 2001) et la biodégradation d'acides organiques (Van Hees et al., 2002) et ainsi jouer un rôle dans le contrôle de l'altération des minéraux (Lundström & Öhman, 1990), de la mobilisation des nutriments (Jones, 1998) ou encore de certains processus pédogénétiques (Lundström et al., 2000). Ainsi, les micro-organismes participent au recyclage des éléments mais ils interviennent également dans la formation et la structuration des sols (Tisdall, 1994; Feeney et al., 2006; Rillig & Mummey, 2006).

Les bactéries et champignons sont des intermédiaires essentiels dans le flux des nutriments, particulièrement ceux de l'azote (Davidson et al., 1992; Hart et al., 1994) et du phosphore (Halder et al., 1990; Gyaneshwar et al., 2002), qui sont les deux principaux

facteurs limitant la croissance des plantes. Ainsi, les micro-organismes peuvent être des régulateurs de la productivité et de la diversité des plantes en tant que pourvoyeurs de nutriments mais aussi en tant que compétiteurs pour leur utilisation, leur transformation ou encore en tant qu'agents pathogènes (Van Der Heijden et al., 2008). Les invertébrés qui sont aussi des acteurs majeurs des processus dans les sols (Lavelle et al., 2006), peuvent affecter les processus réalisés par les micro-organismes. En effet, la microfaune, la mésofaune et la macrofaune peuvent induire des changements au sein des communautés bactériennes et fongiques et influencer leurs activités soit directement, en se nourrissant de micro-organismes, soit indirectement, en influençant leur environnement (Anderson, 1988; Wall & Moore, 1999).

Les micro-organismes sont donc à l'origine et au centre de nombreux processus dans les sols. Interagissant avec leur environnement et les autres acteurs impliqués dans ces processus, les communautés microbiennes peuvent être considérées comme le reflet des différentes composantes biotiques et abiotiques de l'écosystème et de son fonctionnement. De plus, les micro-organismes ont des capacités de réponse rapide aux changements environnementaux. Ainsi, certaines caractéristiques leur étant associées tels que leur biomasse, leurs activités et leur diversité sont considérées comme des indicateurs pouvant permettre d'évaluer la qualité des sols (Schloter et al., 2003) et le succès de restauration de sols dégradés (Harris, 2003).

I.1.2.2. Les micro-organismes dans les cours d'eau de tête de bassin

Figure 1. Accumulation de litière de feuilles dans un cours d'eau forestier de tête bassin (Photo P. Wagner)

Dans les cours d'eau de tête de bassin, les zones d'accumulation de matières organiques abritent une part importante de la biomasse microbienne (Findlay et al., 2002), les micro-organismes hétérotrophes y étant les intermédiaires de la majorité des flux de matière et d'énergie. En effet, en raison de nombreuses contraintes écologiques telles que la faible luminosité due à la densité du couvert forestier, la faible minéralisation ou encore la force du courant, la productivité primaire dans les cours d'eau de tête de bassin est relativement faible. Le fonctionnement de ces cours d'eau est ainsi basé sur l'hétérotrophie, leur principale source d'énergie provenant de l'apport de matière organique allochtone, essentiellement sous forme de feuilles mortes (Vannote et al., 1980; Cummins et al., 1989; Webster & Meyer, 1997) (Figure 1).

Dans ces écosystèmes aquatiques, la décomposition des litières d'origines végétales est donc un processus clé, dans lequel les micro-organismes, principalement les champignons et dans une moindre mesure les bactéries, jouent un rôle prépondérant. En effet, il a été démontré que les champignons dominent la biomasse et la productivité microbienne sur les feuilles en décomposition (Baldy & Gessner, 1997; Baldy et al., 2002; Hieber & Gessner, 2002), mais aussi qu'ils contribuent majoritairement aux activités enzymatiques impliquées dans ce processus, en dépit d'interactions complexes avec les bactéries (Romani et al., 2006). Les micro-organismes associés à la litière interviennent également au niveau du cycle des nutriments, en immobilisant de l'azote et du phosphore de la colonne d'eau (Gessner et al., 1998; Webster et al., 2009), les feuilles n'étant naturellement pas assez riches en ces éléments et ces derniers pouvant être des facteurs limitant le processus de décomposition (Grattan & Suberkropp, 2001).

Les hyphomycètes aquatiques, découverts par Ingold (1942), sont des formes à reproduction asexuée (anamorphes) de champignons Ascomycètes et Basidiomycètes (Bärlocher, 1992) et sont considérés comme étant les micro-organismes les plus importants dans le processus de décomposition des feuilles dans les cours d'eau (Gessner & Chauvet, 1994). De plus, ces champignons sont particulièrement adaptés à la colonisation des débris végétaux dans les écosystèmes aquatiques. En effet, ils se développent sous forme mycélienne, puis, par l'intermédiaire de conidiophores, les hyphomycètes aquatiques produisent des spores asexuées (conidies) de formes variées et spécifiques, qui sont ensuite disséminées dans le milieu, leur permettant de coloniser de nouveaux substrats.

Les hyphomycètes aquatiques jouent également un rôle essentiel au sein des réseaux trophiques en étant à la base du conditionnement de la matière foliaire pour les organismes

des niveaux supérieurs. Ainsi, des études ont montré que la colonisation de feuilles par ces champignons pouvait améliorer la qualité de la ressource pour les macro-invertébrés détritivores (Arsuffi & Suberkropp, 1988; Bärlocher, 1985; Graça, 2001), mais aussi que les activités enzymatiques fongiques associées à la décomposition pouvaient améliorer la digestibilité des feuilles pour les invertébrés (Bärlocher, 1982).

Il a été démontré que la décomposition des litières pouvait être un indicateur de l'intégrité fonctionnelle des cours d'eau (Gessner & Chauvet, 2002). En parallèle, il a été suggéré que les hyphomycètes aquatiques associés à cette litière pouvaient également être des bioindicateurs potentiels de perturbations d'origine anthropique (Solé et al., 2008), mettant ainsi en avant des outils particulièrement pertinents pour l'évaluation de la qualité des cours d'eau.

I.2. Acidification anthropique des écosystèmes forestiers

I.2.1. Origines des émissions polluantes atmosphériques et des dépôts acidifiants

Le terme de « pluies acides » a été mentionné pour la première fois par le français Ducros en 1845 dans un article intitulé « Observation d'une pluie acide » (Ducros, 1845). Il fut relayé par Smith, qui mena des études plus détaillées en Angleterre sur les pluies acides et leurs effets potentiels en 1872 (Smith, 1872). Mais ce n'est que bien plus tard qu'une prise de conscience plus générale des problèmes écologiques associés est apparue, alarmée notamment par les travaux d'Odén dans les années 1960 (Odén, 1968), qui établirent que le phénomène d'acidification à grande échelle des eaux de surface en Suède pouvait être attribué à des pollutions provenant du Royaume-Uni et de l'Europe central.

Depuis le 19ème siècle et les révolutions industrielles, l'émission croissante de polluants atmosphériques sont ainsi à l'origine de l'acidification des écosystèmes (Driscoll et al., 2001), les émissions d'origines naturelles (volcans et processus biogéochimiques) ne contribuant que minoritairement aux dépôts acidifiants. Les pollutions sont ainsi principalement générées par les émissions de dioxyde de soufre (SO_2) et d'oxydes d'azote (NO_X) provenant de la combustion des énergies fossiles et des transports, mais également dans une moindre mesure par les émissions d'ammoniaque (NH_3) résultant de l'intensification des activités agricoles. Dans l'atmosphère, ces polluants vont subir des réactions complexes d'hydratation et d'oxydation conduisant à la formation d'acides sulfurique (H_2SO_4) et nitrique (HNO_3). L'ammoniaque sera, quant à lui, transformé dans l'atmosphère en ammonium (NH_4^+), qui,

lors du processus de nitrification par les bactéries du sol, entrainera la libération de protons (H^+) et l'acidification des sols.

Les dépôts atmosphériques acidifiants peuvent être de trois types différents et se faire sous forme de pluies et de neiges (dépôts humides), de poussières, de gaz et d'aérosols (dépôts secs) ou encore sous forme de brouillards et de rosées (dépôts occultes) (Irwin, 1989).

I.2.2. Acidification des écosystèmes forestiers et facteurs aggravants

Les polluants atmosphériques peuvent être transportés sur de plus ou moins longues distances et ainsi, les dépôts acidifiants présentent la particularité de pouvoir affecter des écosystèmes comme les bassins versants forestiers, qui sont pourtant situés en amont de toute perturbation anthropique directe, à l'exception des activités forestières.

Les écosystèmes les plus sensibles à l'acidification sont les sols et les eaux présentant un faible pouvoir tampon et plus précisément une faible capacité à neutraliser les acides (ANC, Acid-Neutralizing Capacity). Dans les sols, les dépôts acides entrainent une augmentation des concentrations en protons, en anions mobiles d'acides forts, i.e. sulfates (SO_4^{2-}) et nitrates (NO_3^-), qui va conduire à son tour à un lessivage progressif des cations basiques (Ca, Mg, Na, K) (Gobran & Bosatta, 1988; Lawrence et al., 1999; Edwards et al., 2002; Fernandez et al., 2003) et à une mobilisation de métaux tels que l'Al par dissolution de la roche mère (Cronan & Schofield, 1979). Sous l'effet de cette déminéralisation, l'ANC des sols initialement pauvres en cations échangeables va ainsi diminuer, les sols vont alors perdre leur pouvoir tampon et voir leur pH diminuer. Les ions H^+, Al^{3+}, NO_3^- et SO_4^{2-}, en excès dans les sols acidifiés, vont ensuite être transférés vers les eaux de surface (Reuss et al., 1987; Boudot et al., 2000; Kawakami et al., 2001), l'acidification des sols d'un bassin versant engendrant celle des eaux courantes drainant celui-ci (Probst et al., 1999).

Les sols se développant sur des roches mères pauvres en minéraux altérables (gneiss, granites, grès) sont ainsi peu résistants à l'acidification. Cependant, ce phénomène peut être exacerbé par d'autres facteurs aggravants tels que le climat, l'altitude, la végétation ou les activités forestières. En effet, les régions en altitude sont souvent plus exposées aux précipitations, aux brouillards et aux vents et donc à l'érosion et aux dépôts atmosphériques, de fortes pluies pouvant notamment accentuer le drainage des cations basiques vers les eaux de surface. Les forêts de résineux sont aussi plus sensibles, leur feuillage persistant pouvant collecter plus de dépôts atmosphériques que celui des feuillus (Mayer, 1998) et la litière de certains résineux (e.g. Epicéa commun, Pin sylvestre) étant acidifiante. Une gestion intensive

peut aussi accentuer les effets de l'acidification à travers l'exploitation du bois des forêts, particulièrement de celles à croissance rapide, ou encore par le prélèvement des rémanents forestiers qui entrainent l'appauvrissement des sols par l'exportation des cations nutritifs (Olsson et al., 1993; Adams, 1999; Olsson, 1999; Adams et al., 2000).

I.2.3. Altération des écosystèmes forestiers et de la biodiversité

Le phénomène d'acidification a engendré des dépérissements forestiers dans de nombreuses régions de l'hémisphère nord, essentiellement en Amérique du Nord et en Europe (Johnson & Siccama, 1983; Johnson & Taylor, 1989; Schulze, 1989). Des symptômes de défoliation, de jaunissement des aiguilles et de décoloration des feuilles ont ainsi été observés, ces derniers pouvant aboutir à un affaiblissement des arbres et à leur détérioration. De plus, ces effets peuvent être amplifiés par la combinaison des stress acides, climatiques (e.g. sécheresses) et parasitaires.

Ces dépérissements forestiers sont principalement dus au lessivage des nutriments des sols vers les eaux de surface, altérant la nutrition des végétaux en induisant des carences en Ca et Mg (Landmann & Bonneau, 1995) exacerbées par la mise en solution de l'Al (Joslin & Wolfe, 1988; Kinraide, 1991; Boudot et al., 1994), qui est un métal particulièrement toxique pour les organismes vivants et qui peut interférer dans l'acquisition des nutriments par les plantes (Ericsson et al., 1995). Le phénomène d'acidification des sols couplé à l'importance des dépôts azotés favorise alors le développement des espèces acidophiles et nitrophiles au dépend des végétaux acido-sensibles, entrainant ainsi des changements au sein des communautés végétales (Falkengren-Grerup, 1989; Dupouey et al., 1999).

Des études ont montré que l'acidification des sols pouvait induire des changements au sein des groupes constituant la faune du sol dont les vers de terre, les nématodes, les acariens ou encore les collemboles (Bååth et al., 1980; Rusek & Marshall, 2000). Au niveau terrestre, des effets impactant la présence de certaines espèces d'oiseaux ont aussi été rapportés (Buckton et al., 1998; Hames et al., 2002) .

L'acidification des cours d'eau et l'augmentation en parallèle des concentrations en Al (Campbell et al., 1983) sont également à l'origine d'effets délétères sur la diversité de nombreux organismes aquatiques (Gorham, 1998) parmi lesquels les poissons (Driscoll et al., 1980; Heard et al., 1997), les mammifères (Mason & MacDonald, 1989), les amphibiens (Harte et al., 1994), les algues (Meegan & Perry, 1996), les bryophytes (Thiebaut et al., 1998),

les macrophytes (Farmer, 1990) ou encore les invertébrés benthiques (Rosemond et al., 1992; Dangles & Guérold, 2000; Guérold et al., 2000).

I.2.4. Effets de l'acidification sur les communautés microbiennes et les processus associés

A l'échelle microbienne, des études ont rapporté que l'acidification des sols pouvait entrainer une diminution de la biomasse fongique, du nombre de bactéries et de la taille de leurs cellules (Bååth et al., 1979, 1980). Des résultats ont également prouvé que les taux de croissance des bactéries pouvaient être réduits et que le principal changement au sein des communautés bactériennes était caractérisé par une augmentation de bactéries gram-positives (Pennanen et al., 1998). L'acidification peut aussi ralentir la décomposition de résidus végétaux tels que les aiguilles, les racines (Bååth et al., 1980) ou la litière de feuilles (Francis, 1982). Les travaux de Francis (Francis, 1982; Francis, 1986) ont également montré que les processus d'ammonification, de nitrification et de dénitrification pouvaient être significativement réduits dans les sols acidifiés, induisant un ralentissement du recyclage de l'azote. Il démontra également que l'abondance des bactéries fixant l'azote était plus faible dans les sols acidifiés.

Figure 2. Comparaison de l'aspect général de feuilles d'aulne ayant séjourné 83 jours dans un cours d'eau acide (à droite) et dans un cours d'eau témoin neutre (à gauche) (d'après Baudoin, 2007)

Dans les cours d'eau, de nombreuses études ont montré que la décomposition des litières (Figure 2) était aussi affectée par l'acidification (Mulholland et al., 1987; Chamier, 1987; Griffith et al., 1995; Meegan et al., 1996; Niyogi et al., 2001; Dangles et al., 2004; Baudoin, 2007), en agissant sur la diversité des macro-invertébrés impliqués dans ce processus (Dangles & Guérold, 1998; Dangles & Guérold, 2001) mais également sur les micro-organismes et leurs activités.

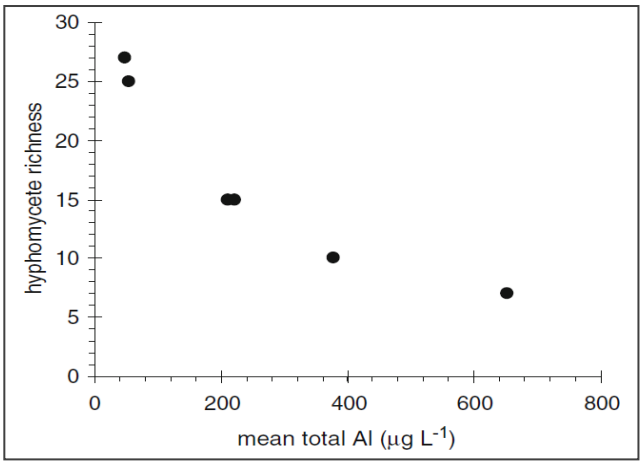

Figure 3. Relation entre la richesse totale d'espèces sporulantes d'hyphomycètes aquatiques et les concentrations en Al dans les cours d'eau (d'après Baudoin et al., 2008)

En effet, des travaux ont révélé que la respiration microbienne associée à la litière en décomposition était plus faible dans les cours d'eau acidifiés (Mulholland et al., 1987; Groom & Hildrew, 1989; Mulholland et al., 1992; Niyogi et al., 2001; Dangles et al., 2004). Certains auteurs ont démontré que l'acidification pouvait réduire la biomasse fongique associée à la litière en décomposition (Griffith & Perry, 1994), en revanche, ces résultats s'opposent à ceux qui n'ont observé aucun effet sur le développement mycélien (Dangles & Chauvet, 2003; Baudoin et al., 2008). D'un autre côté, des études ont montré que l'acidification et plus particulièrement les fortes concentrations en Al pouvaient altérer la croissance et la capacité à sporuler des hyphomycètes aquatiques (Chamier & Tipping, 1997), ainsi que la diversité d'espèces sporulantes associée aux litières en décomposition (Chamier, 1987; Baudoin et al., 2008) (Figure 3).

Enfin, la diminution de l'activité de décomposition des feuilles par les micro-organismes a principalement été attribuée à la diminution de l'activité de dégradation des pectines en présence de faibles concentrations en Ca et à pH acide (Chamier & Dixon, 1982; Jenkins & Suberkropp, 1995), en dépit du fait que la plupart des enzymes impliquées dans la décomposition des feuilles ont des optimums d'activité à pH acides (Sinsabaugh et al., 1981, 2002; Turner, 2010).

I.2.5. Evolution des émissions et des dépôts atmosphériques

Face aux désastres écologiques engendrés par l'acidification des écosystèmes, des mesures ont été prises afin de réduire les émissions atmosphériques polluantes. La conférence des Nations Unies sur l'Environnement à Stockholm en 1972 fut à l'origine de nombreuses lois pour la protection de l'environnement. Puis, la convention sur la pollution atmosphérique transfrontalière à longue distance (CLRTAP, Convention on Long-Range Transboundary Air

Pollution), signée à Genève en 1979, fut à la base de la réduction des émissions atmosphériques et d'autres protocoles visant à réduire plus précisément les émissions de soufre (Helsinki, 1985 ; Oslo, 1994) et d'oxydes d'azote (Sofia, 1988) ou plus généralement de l'ensemble des polluants acidifiants (Göteborg, 1999).

Depuis les années 80, l'application de mesures suivant ces protocoles a permis une réduction significative des émissions de dioxyde de soufre d'environ 70% en Europe et jusqu'à 50% en Amérique du Nord, alors que les émissions d'oxydes d'azote ont plus faiblement diminué pour atteindre une réduction moyenne d'environ 25% en Europe.

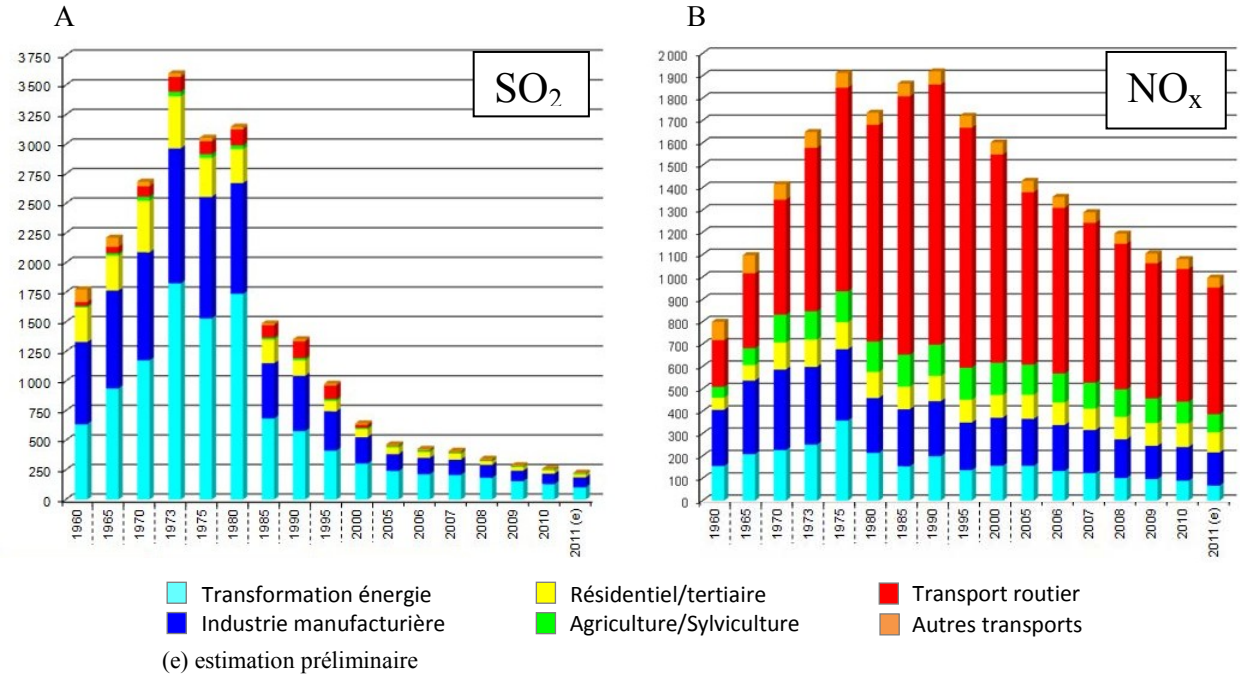

Figure 4. Emissions atmosphériques de SO_2 (A) et de NOx (B) entre 1960 et 2011 par secteur en France métropolitaine en kilotonne (Source : CITEPA).

En France, la réduction des émissions suit cette tendance et montre une plus forte diminution des émissions de dioxyde de soufre (Figure 4A) que d'oxydes d'azote (Figure 4B), ces dernières étant principalement la résultante des émissions issues des transports routiers. Des études ont permis de montrer que la baisse de ces émissions avait ainsi entrainé la diminution des dépôts acidifiants (Likens et al., 2001; Prechtel et al., 2001).

Cependant, la restauration spontanée de nombreux écosystèmes est très lente, voire inexistante (Likens et al., 1996; Alewell et al., 2000), des décennies de dépôts acidifiants ayant épuisé les réserves des sols les plus sensibles. Il convient de noter que la diminution des émissions polluantes a entrainé la réduction conjointe de l'émission de poussières alcalines,

qui constituaient une importante source de cations alcalins pour les écosystèmes à faibles intrants (Hedin et al., 1994). De plus, la diminution des émissions n'est pas un phénomène global. Une importante augmentation des émissions a ainsi été observée dans les pays en voie de développement et notamment en Asie (Streets et al., 2001), où des phénomènes d'acidification commencent à apparaitre (Larssen et al., 1999; Qin & Huang, 2001; Tang et al., 2001; Larssen et al., 2006).

I.2.6. Restauration d'écosystèmes acidifiés

Des études ont mis en évidence une restauration spontanée de certains écosystèmes acidifiés en Amérique du nord et en Europe (Stoddard et al., 1999; Skjelkvåle et al., 2001). Cependant, les effets délétères de l'acidification continuent d'altérer de nombreux écosystèmes où aucune amélioration significative n'a été observée (Driscoll et al., 2001; Wright et al., 2005). De nombreux travaux ont démontré l'intérêt des amendements, notamment sous forme d'apports calco-magnésiens, pour faire face aux effets persistants de l'acidification sur les écosystèmes terrestres (Huettl & Zoettl, 1993; Lundström et al., 2003; Löfgren et al., 2009) et aquatiques (Henrikson et al., 1995; Schindler, 1997; Hindar et al., 2003; Clair & Hindar, 2005). Généralement, ces apports engendrent une augmentation de l'ANC, des concentrations en cations et du pH des sols (Kreutzer, 1995; Ingerslev, 1997) et des cours d'eau ainsi qu'une diminution des concentrations en Al. Ces apports peuvent ainsi être un remède au dépérissement des forêts (Bonneau et al., 1992) et peuvent aussi permettre le retour d'espèces de poissons ou d'invertébrés acido-sensibles suite à l'amélioration de la qualité chimique des eaux (Fjellheim & Raddum, 1992; Hesthagen & Larsen, 2003).

Des études ont également montré que la qualité et la morphologie des humus des sols forestiers pouvaient être modifiées par des amendements, en agissant notamment sur les organismes impliqués dans leur formation/transformation tels que les vers de terre, les collemboles et les micro-organismes (Kreutzer, 1995). En particulier, des travaux sur ces derniers ont révélé que des sols traités présentaient des activités microbiennes plus importantes en comparaison avec des sols acidifiés non amendés. En effet, il a été rapporté que des amendements pouvaient augmenter la respiration et la biomasse microbienne des sols mais aussi le recyclage de l'azote (Shah et al., 1990; Illmer & Schinner, 1991; Badalucco et al., 1992; Smolander & Mälkönen, 1994). Des changements au sein des communautés bactériennes ont été observés et caractérisés par l'augmentation de bactéries acido-sensibles (Shah et al., 1990; Neale et al., 1997) et par une augmentation des bactéries gram-négatives

favorisées au dépend des gram-positives (Frostegård et al., 1993). Des travaux plus récents ont également révélé que les amendements calco-magnésiens pouvaient induire des changements durables au sein des communautés de champignons ectomycorhiziens, des espèces généralistes remplaçant les espèces acidophiles (Rineau & Garbaye, 2009; Rineau et al., 2010).

Chapitre II :

Cas du massif vosgien et présentation des sites d'étude

II.1. Cas du massif vosgien

II.1.1. Caractéristiques du massif vosgien et sensibilité à l'acidification

Le massif des Vosges est un massif de moyenne montagne présentant une altitude moyenne de 500 m (point culminant à 1400 m) et s'étendant au nord-est de la France sur près de 7000 km^2. Le massif vosgien connait un climat semi-continental de type montagnard-inférieur et son orientation Nord-Sud, arrêtant les nuages venant par l'ouest, explique les précipitations abondantes qui peuvent dépasser les 2000 mm/an dans les zones les plus exposées (e.g. les sommets du massif). La température moyenne annuelle dans le massif vosgien est proche de 8°C et on observe en moyenne 70 jours de neige par an à 700 m d'altitude et jusqu'à 140 jours de neige par an au niveau des crêtes.

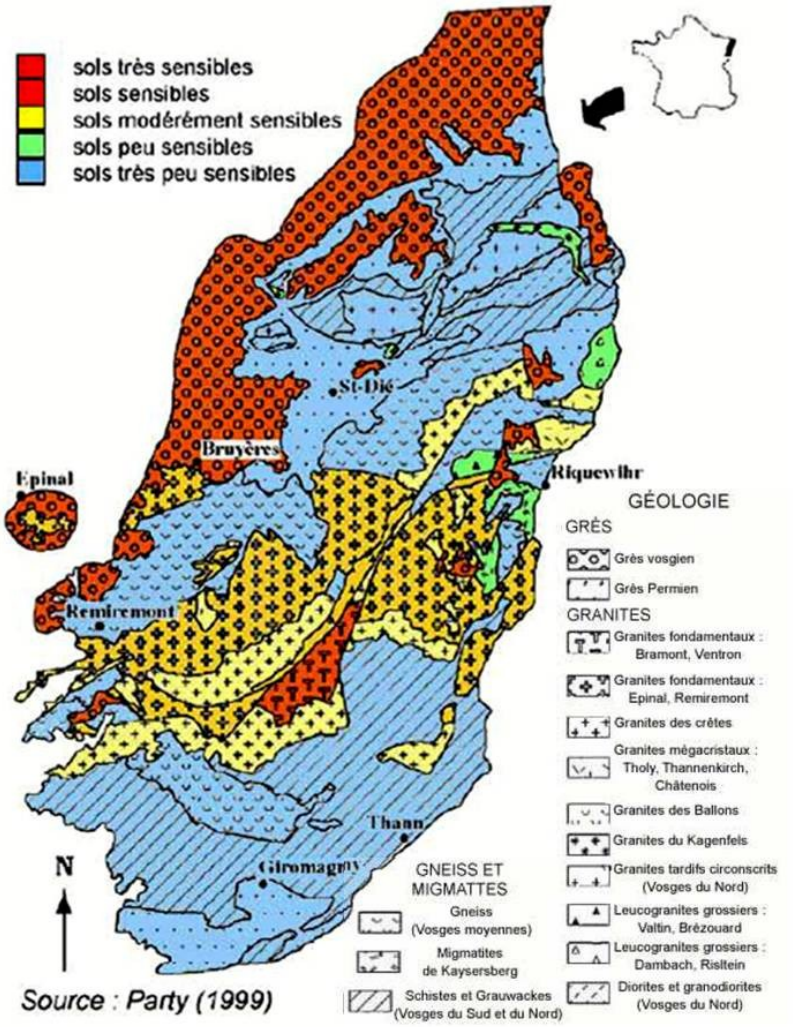

Figure 5. Carte géologique et sensibilité à l'acidification des sols du massif vosgien (d'après Party, 1999)

Le massif des Vosges est un massif ancien hercynien (plissement de l'ère primaire), rehaussé par l'orogénèse alpine et ayant subi de nombreux bouleversements géologiques responsables d'une géologie particulièrement complexe (Figure 5). Elle se caractérise par des roches mères gréseuses au nord et à l'ouest et des roches mères cristallines (gneiss, granite et schiste) plus au sud et à l'est.

Le massif vosgien présente une importante couverture forestière principalement dominée par la présence naturelle de sapins et de hêtres, et par des épicéas, dont la majorité a été introduite par des aménagements sylvicoles, les résineux dominant ainsi les forêts du massif.

Le réseau hydrographique du massif est dominé par des cours d'eau de premier ordre drainant de petits bassins versants forestiers, les plus grandes rivières (la Meurthe et la Moselle) se développant sur le versant ouest.

En France, les Vosges, les Ardennes, les Landes et l'est du massif central font partie des zones géologiques les plus sensibles à l'acidification (Party, 1999), les Vosges étant également une des régions les plus touchées par les dépôts atmosphériques acidifiants (Dambrine et al., 1995). Les sols du massif vosgien qui se développent sur des roches mères pauvres en cations basiques (grès et granite) sont ainsi particulièrement sensibles à l'acidification (Figure 5). Les fortes précipitations, la faible température, une couverture forestière dominée par les résineux et la sylviculture intensive sont autant de facteurs aggravant fortement les phénomènes d'acidification dans les Vosges.

Des décennies de pollutions atmosphériques acidifiantes associées à ces différents facteurs sont ainsi à l'origine de l'acidité prononcée des sols dans de nombreuses régions du massif (Figure 6), mais aussi de celle des cours d'eau drainant les bassins versants acidifiés (Probst et al., 1999). Une étude des caractéristiques physico-chimiques de près de 400 cours d'eau a été réalisée dans le département des Vosges à l'automne 1995 (Guérold et al., 1997). Les résultats ont montré que près de la moitié de ces cours d'eau présentait un pH inférieur à 5,5, et ce, même en période d'étiage, c'est-à-dire à la période pendant laquelle les cours d'eau sont les plus minéralisés en raison des faibles débits (Figure 7). Les concentrations en sulfates et en Al dans certains cours d'eau ont diminué ces dernières années, mais aucune évolution notable du pH n'a été rapportée (Angéli et al., 2009), les dépôts atmosphériques acidifiants du siècle dernier ayant entrainé une déminéralisation marquée des sols et des eaux courantes dans de nombreuses zones du massif.

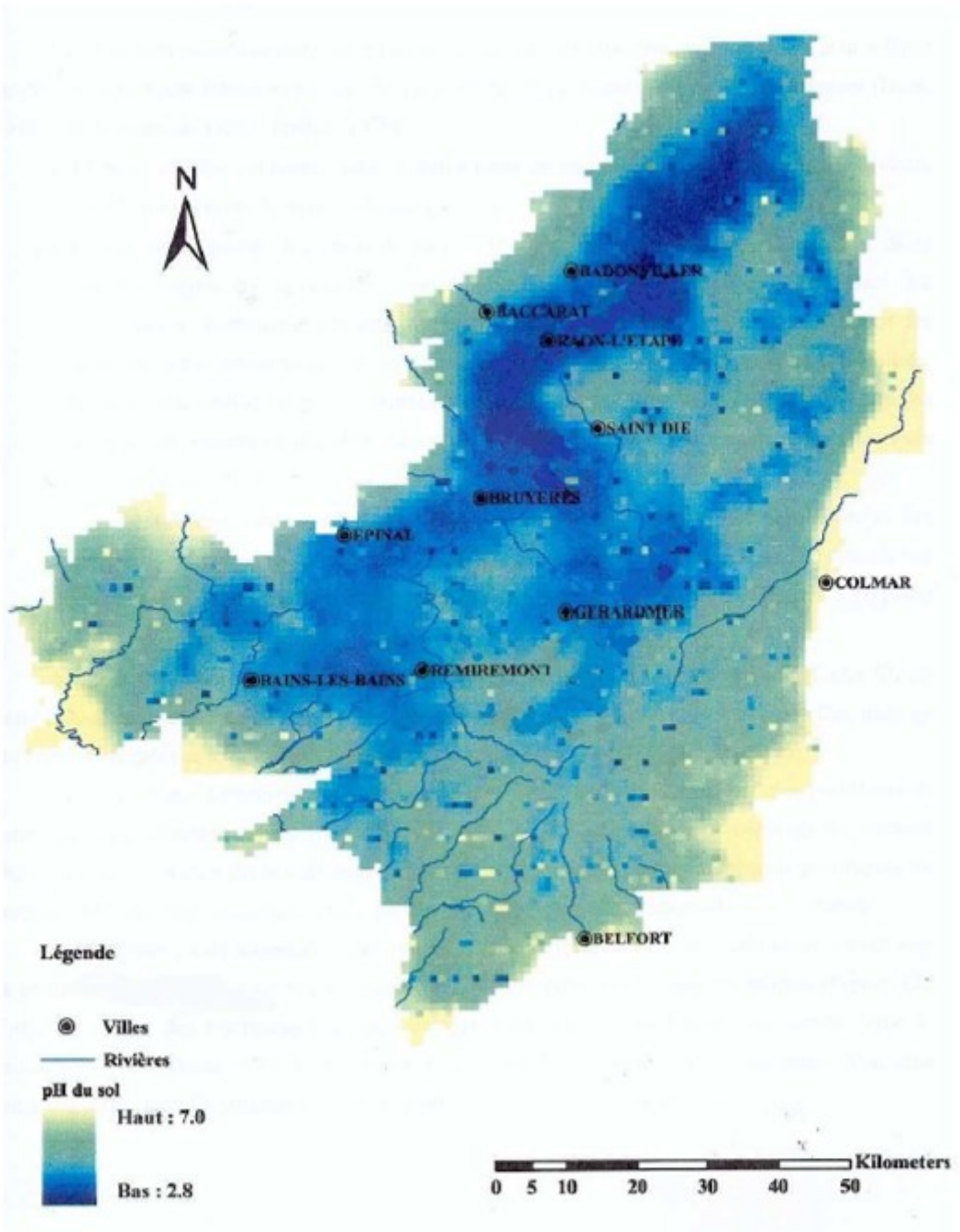

Figure 6. pH des sols du massif vosgien déduits des valeurs indicatrices des relevés floristiques par techniques du krigeage (d'après Gégout & Piedallu, 2002)

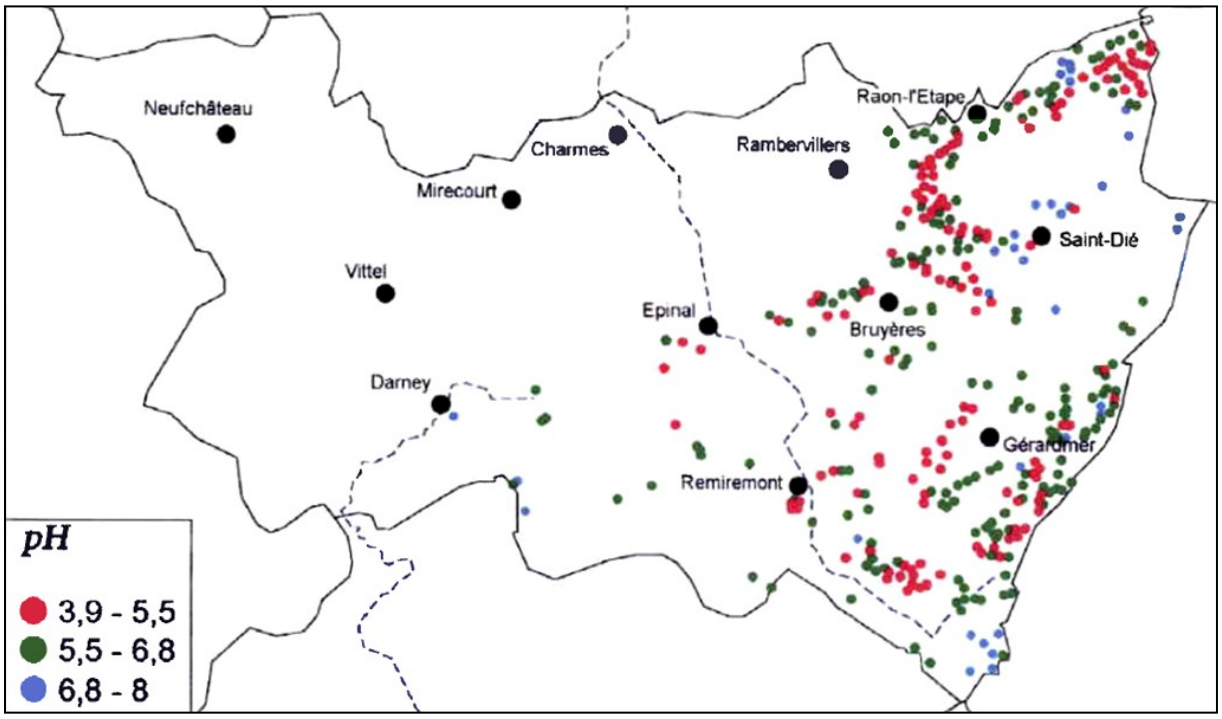

Figure 7. Gamme de pH de cours d'eau échantillonés en 1995 à l'étiage automnal dans le département des Vosges (d'après Guérold et al., 1997)

II.1.2. Résultantes de l'acidification des écosystèmes forestiers vosgiens

Nisbet en 1958, fut le premier à rapporter l'acidité d'un cours d'eau vosgien, qu'il attribua à la pauvreté de la roche mère gréseuse en Ca et à la présence de tourbières acides (Nisbet, 1958). Dans les années 1970, Bourrié étudia une cinquantaine de cours d'eau dans la partie granitique des Vosges (Bourrié, 1978). Il établit que la composition chimique des eaux était la résultante de nombreux facteurs écologiques tels que la nature de la roche, le type de sol ou encore l'acidité des dépôts atmosphériques.

Au début des années 1980, des dépérissements forestiers sont apparus dans le massif vosgien (Landmann & Bonneau, 1995). Des observations datant de 1983 et 1984 ont révélé des défoliations et un jaunissement des feuillages chez le sapin, l'épicéa, le hêtre et le pin sylvestre, ces symptômes pouvant s'accompagner d'une réduction de la croissance des arbres ainsi que d'une diminution et d'une altération de leur appareil racinaire (Bonneau & Fricker, 1985). Ces phénomènes ont ainsi été attribués aux dépôts atmosphériques acidifiants, mais il a été aussi suggéré que l'état de santé des arbres avait pu être fragilisé par des périodes de sécheresse et notamment celle de 1976, accélérant ainsi leur dépérissement.

En parallèle, d'autres études se sont intéressées à l'acidification des eaux de surface (Massabuau et al., 1987; Probst et al., 1987; Kreiser et al., 1995; Probst et al., 1995; Probst et al., 1999) et à ses effets sur les organismes aquatiques. Les travaux de Massabuau et al. (1987), suivis de ceux de Probst et al. (1990), ont notamment mis en évidence la disparition de truites dans des cours d'eau acidifiés. Des études ont également révélé une modification des communautés de macrophytes (Thiebaut et al., 1998) et des effets délétères sur la structure des communautés de macro-invertébrés benthiques (Dangles & Guérold, 2000; Guérold et al., 2000), sur la diversité des hyphomycètes aquatiques (Baudoin et al., 2008) ainsi que sur le processus de décomposition des litières dans lequel ces organismes sont impliqués (Dangles et al., 2004).

L'acidification des eaux de source dédiées à la consommation a aussi eu un impact sur la santé humaine, de nombreux cas de saturnisme ayant été rapportés dans les Vosges dans les années 1970 et 1980. En effet, il a été démontré que des teneurs en plomb élevées pouvaient être mobilisées dans les canalisations par des eaux faiblement minéralisées (Dambrine et al., 1999).

II.2. Sites d'étude de la restauration de bassins versants acidifiés

II.2.1. Description des sites et de l'opération d'amendement

Dans les Vosges, les effets marqués de l'acidification sur les écosystèmes forestiers et leur fonctionnement ont entrainé la mise en place d'un programme de recherche pluridisciplinaire impliquant des scientifiques (CNRS, INRA, Université de Metz), des financeurs et des gestionnaires (Conseil Général des Vosges, Agence de l'eau Rhin-Meuse, DGFAR/DSF, DRAF/DERFOB, DIREN, Région Lorraine, ONF). Ce programme a permis la réalisation, pour la première fois en France, d'amendements calco-magnésiens à l'échelle de bassins versants.

Deux sites ont été choisis pour recevoir les amendements, un site sur roche mère gréseuse (Massif du Donon, grès vosgien), caractéristique du nord du massif et un site sur roche mère granitique (Massif du Ventron, granite du Ventron type II), caractéristique du sud du massif, permettant ainsi d'évaluer les réponses à l'amendement de deux types de substratum géologique différents. Les bassins versants sélectionnés ont été particulièrement touchés par les dépôts atmosphériques acidifiants et se situent sur des roches mères pauvres en cations basiques. Les cours d'eau et les sols de ces bassins versants étaient particulièrement acides et

déminéralisés. De plus, d'importants taux de défoliation et des jaunissements avaient été observés sur les peuplements forestiers de ces bassins versants (Nys et al., 2004; Angéli, 2006).

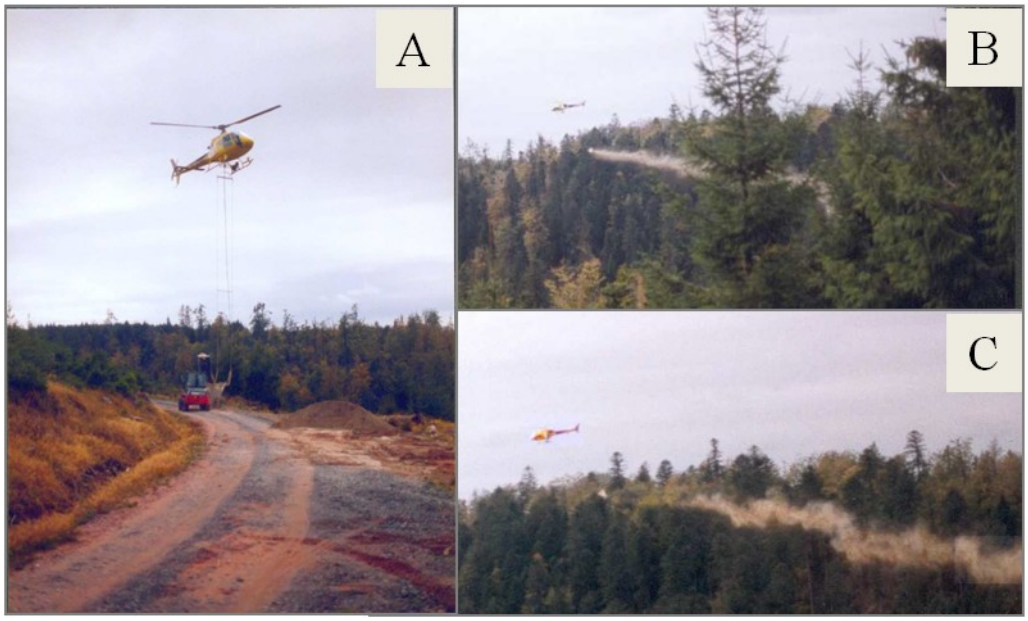

Figure 8. Opération d'amendement du bassin versant gréseux en octobre 2003. Chargement de l'amendement dans la nacelle d'épandage (A) et épandages par hélicoptère (B et C) (d'après Baudoin, 2007)

L'entreprise de restauration forcée de ces écosystèmes visait à rétablir la qualité physico-chimique (i.e. augmentation du pH, de l'ANC et des concentrations en cations basiques) des sols et des cours d'eau drainant les bassins versants traités, les objectifs étant d'améliorer l'état de santé des peuplements forestiers mais également de restaurer la biodiversité et le fonctionnement des cours d'eau. Les résultats d'études antérieures (Nys, 1981; Bonneau et al., 1992; Bonneau & Nys, 1997) et l'utilisation du logiciel REGESOL ont permis d'évaluer les besoins en nutriments des peuplements et de déterminer les doses et les types de produits à utiliser pour reminéraliser les sols et les cours d'eau et donc contrer les effets de l'acidification de ces écosystèmes. Les amendements se sont ainsi déroulés du 6 octobre au 21 novembre 2003 et environ 2,5 t/ha d'amendements calco-magnésiens ont été épandus par hélicoptère sur les bassins versants (figure 8). Les produits d'épandage étaient constitués d'environ 70% de $CaCO_3$, 17% de $MgCO_3$, 10% de $CaSO_4$ et de 3% de KCl, principalement sous forme d'un mélange de roches calcaires et de gypse broyés. La combinaison de ces deux types de roches permet d'apporter des éléments peu solubles comme le $CaCO_3$, qui permet de refertiliser durablement les horizons de surface, et des éléments plus solubles comme le

$CaSO_4$, qui s'infiltre plus rapidement dans les sols pour reminéraliser les eaux de surface. Enfin, le Cl sert de traceur pour suivre le cheminement des eaux au sein des bassins versants.

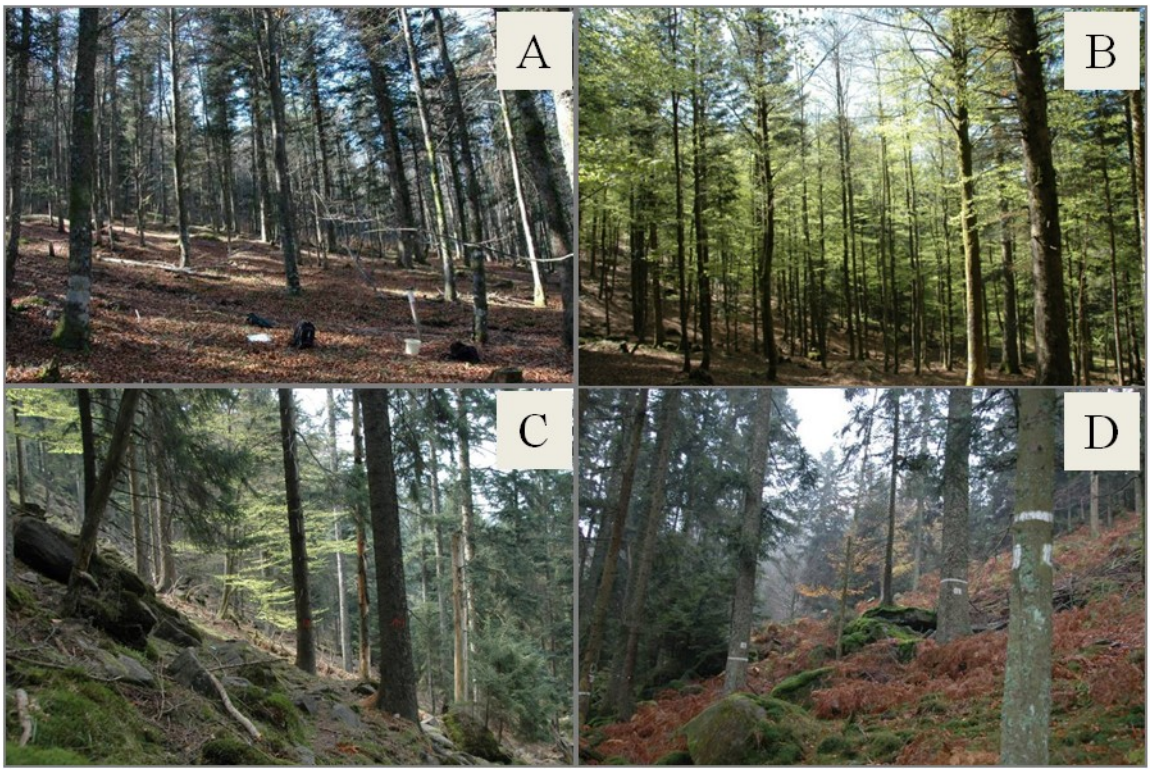

Figure 9. Photographies des 4 sites d'étude sur les bassins versants granitiques témoin (A) et amendé (B) et sur les bassins versants gréseux témoin (C) et amendé (D) (Photos P. Wagner)

Des sites ateliers ont été mis en place sur ces bassins versants amendés ainsi que sur des bassins versants témoins, situés à proximité et présentant des caractéristiques similaires en terme de nature de la roche mère, physico-chimie des sols, type de sol, couverture forestière, altitude et orientation (Nys et al., 2004; Angéli, 2006). L'analyse comparative des caractéristiques physico-chimiques et biologiques entre les bassins versants traités et témoins, doit ainsi permettre d'évaluer l'évolution des effets de l'amendement. Des photographies des sites de prélèvements des études du chapitre III sont présentées figure 9. La localisation des bassins versants est spécifiée dans la figure 10.

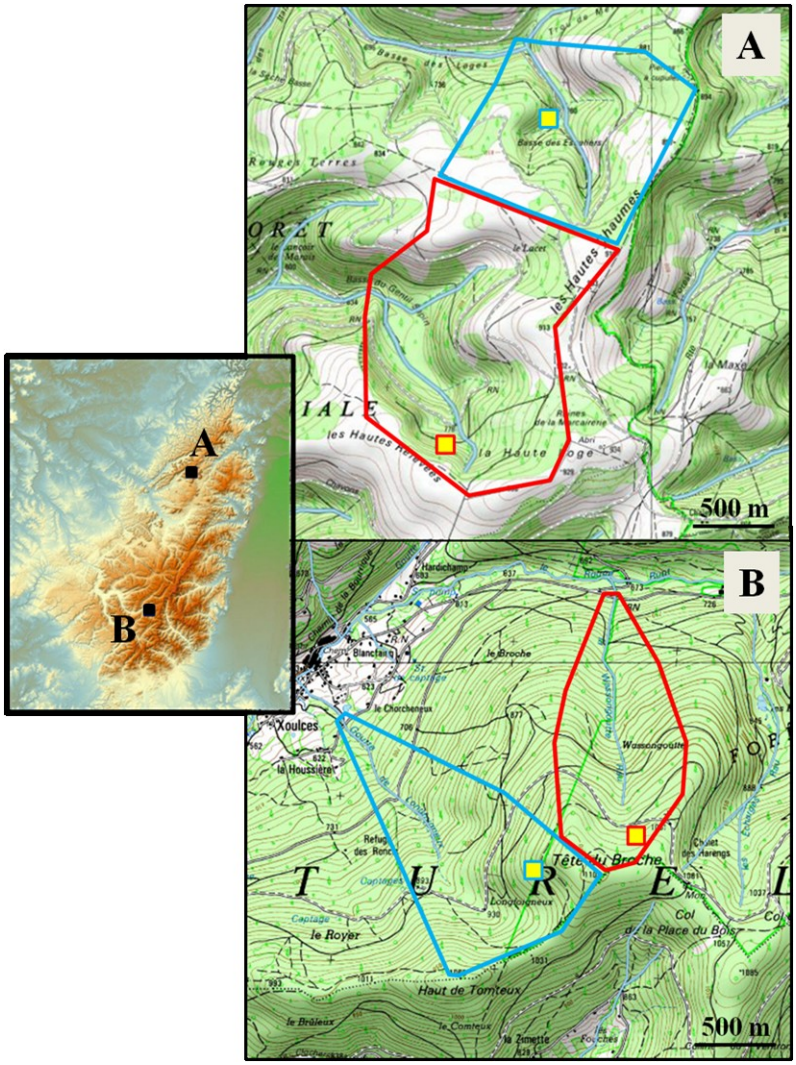

Figure 10. Situations géographiques au sein du massif vosgien des bassins versants amendés (en bleu) et témoins (en rouge) sur roches mères gréseuses (A) et granitiques (B) et emplacements (carrés jaunes) des centres des transects de prélèvements de cette étude.

Le bassin versant gréseux amendé couvre une surface de 123 ha avec une altitude moyenne de 829 m. Il est recouvert de 57% de chaumes et de 43% de forêts, dominées par des résineux (sapins et épicéas) et des hêtres. Une part de 30% du couvert forestier a subi des dégâts lors de la tempête de 1999. Ce bassin versant est drainé par le ruisseau de la Basse des Escaliers (BE).

Le bassin versant gréseux témoin couvre une surface de 222 ha avec une altitude moyenne de 786 m. Il est recouvert de 48% de chaumes et de 52% de forêts, dominées essentiellement par des résineux (sapins et épicéas). Une part de 16% du couvert forestier a subi des dégâts lors de la tempête de 1999. Ce bassin versant est drainé par le ruisseau du Gentil Sapin (GS).

Le bassin versant granitique amendé couvre une surface de 124 ha avec une altitude moyenne de 861 m. Il est recouvert à 95% de forêts, dominées par des résineux (sapins et épicéas) et des hêtres. Ce bassin versant est drainé par le ruisseau de Longfoigneux (LF).

Le bassin versant granitique témoin couvre une surface de 124 ha avec une altitude moyenne de 895 m. Il est recouvert à 96% de forêts, dominées par un mélange de résineux (sapins et épicéas) et de hêtres. Ce bassin versant est drainé par le ruisseau de Wassongoutte (WS).

Pour chaque bassin versant et son témoin, les prélèvements des études du chapitre III ont été réalisés à la même altitude le long de transects situés sur des zones présentant des caractéristiques similaires de pente et de végétation. Les sols sur les sites de prélèvements sont des podzosols ocriques. Les sites de prélèvements se situent à environ 810 m d'altitude sur les deux bassins versants gréseux, à 1050 m d'altitude sur le bassin versant granitique amendé et 1060 m d'altitude sur son bassin versant témoin. Les emplacements des centres des transects de prélèvements sont indiqués dans la figure 10 et leurs coordonnées sont 48°27'45''N, 7°05'50''E (grès amendé) ; 48°26'38''N, 7°05'25''E (grès témoin) ; 47°57'24''N, 6°52'55''E (granite amendé) et 47°57'40''N, 6°53'05''E (granite témoin).

II.2.2. Etudes post-amendement

Les travaux de thèse de Nicolas Angéli (2006) ont révélé qu'un an après les amendements, les concentrations en Ca et Mg ainsi que le pH avaient augmenté dans les sols des bassins versants amendés en comparaison aux sols des bassins versants témoins.

La majorité du traceur (Cl) a été évacué en 17 mois dans le cours d'eau LF, drainant le bassin versant granitique amendé. Une diminution des concentrations en Al, ainsi qu'une augmentation de la conductivité et des concentrations en Ca et Mg ont été observées dans ce ruisseau. Une stabilisation du pH a été observée par rapport au ruisseau WS, drainant le bassin versant témoin. Cette amélioration de la qualité de l'eau a permis d'observer une augmentation du processus de décomposition de litières de feuilles, de la diversité d'espèces sporulantes d'hyphomycètes aquatiques associée aux litières (Baudoin, 2007) ainsi que le retour de la truite (Angéli, 2006).

En revanche, aucune amélioration de la qualité de l'eau, de la décomposition des feuilles ou de la diversité fongique n'a été observée dans le cours d'eau BE, drainant le bassin versant gréseux amendé (Angéli, 2006; Baudoin, 2007). Le cheminement de l'eau dans ce sol est

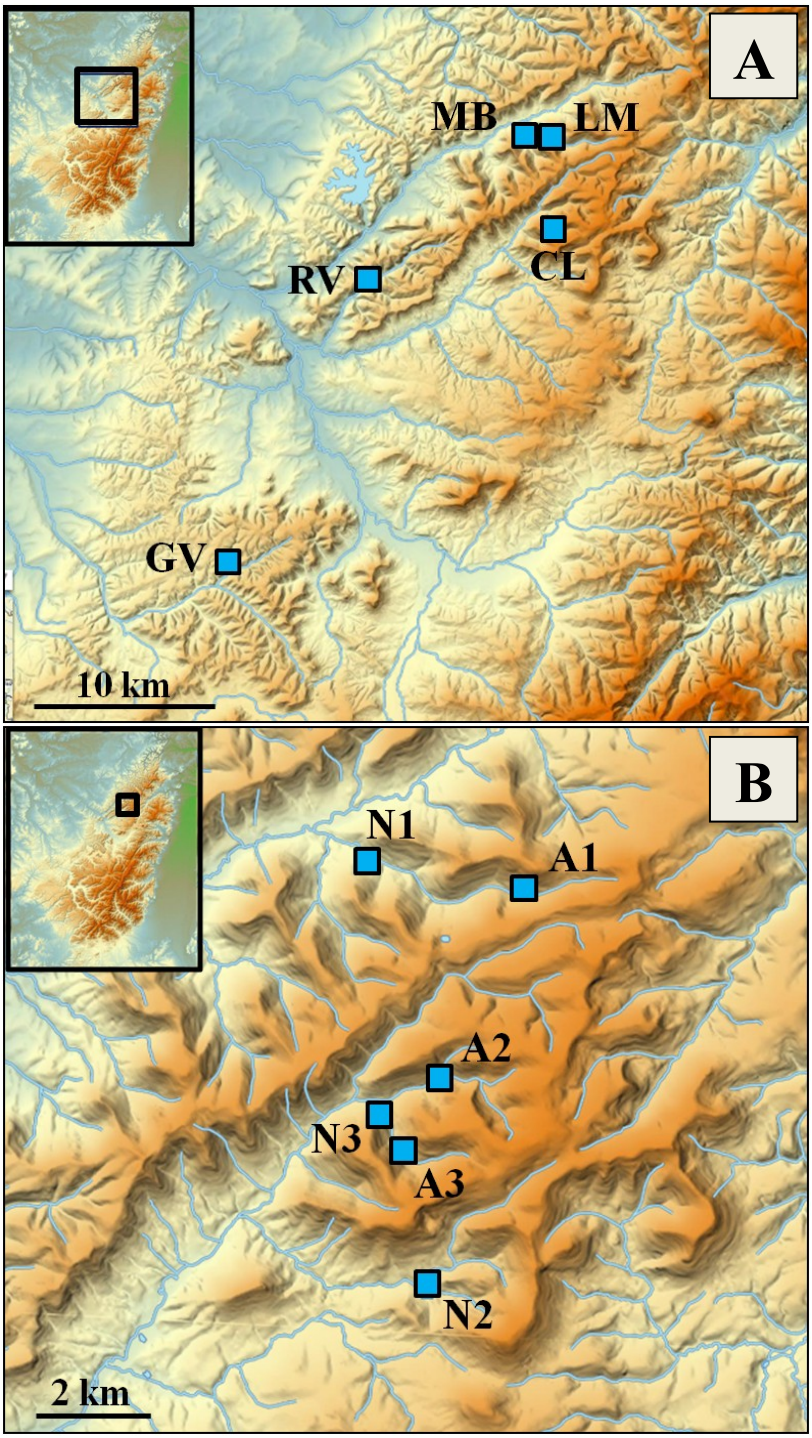

Figure 11. Situations géographiques au sein du massif vosgien des 5 sites retenus pour l'étude hivernale 2008-2009 (A : partie IV.1) et des 6 sites retenus pour l'étude hivernale 2009-2010 (B : partie IV.2)

probablement plus long que dans celui de la roche mère granitique, un suivi régulier ayant montré que le traceur n'était, à ce jour, toujours pas ressorti. L'importance du temps de résidence de l'eau dans ce bassin versant pose ainsi la question de l'intérêt du traitement des sols pour la restauration conjointe de la qualité du cours d'eau qui se fera peut-être à plus long terme. Enfin, les différences de réponses entre les bassins versants des deux types de roches mères démontrent aussi l'intérêt d'étudier les effets de l'amendement sur différentes géologies.

Deux ans après les amendements, une étude a également montré que l'état sanitaire des arbres s'était amélioré dans les bassins versants traités, se traduisant par une diminution sensible de la défoliation ainsi que du jaunissement des aiguilles (Messant et al., 2008).

II.3. Sites d'étude des effets de l'acidification sur la décomposition des litières de feuilles en cours d'eau

En raison d'une géologie relativement complexe, le massif vosgien présente la particularité d'avoir aussi bien des cours d'eau acidifiés que des cours d'eau neutres dans des zones géographiquement très proches. En effet, certains bassins versants se trouvent sur des roches mères plus riches en cations basiques et sont donc moins sensibles aux effets de l'acidification. Cette proximité de cours d'eau présentant différents degrés d'acidification, mais des conditions environnementales similaires, fait que le massif vosgien offre un terrain d'étude particulièrement adapté pour évaluer les effets de l'acidification sur la décomposition des litières et sur les organismes associés.

Les cours d'eau étudiés lors des deux campagnes hivernales 2008-2009 et 2009-2010 se situent dans la partie nord du massif vosgien sur roche mère gréseuse (figure 11). Ce sont des cours d'eau de premier ou de second ordre, présentant une largeur d'environ 2 à 3 m, à l'exception du cours d'eau de Ravines (RV), dont la largeur peut atteindre 5 m dans certaines zones. La hauteur d'eau est généralement comprise entre 40 et 60 cm et la pente moyenne de ces cours d'eau se situe entre 6 et 10%. La végétation rivulaire de ces ruisseaux est généralement composée de sapins, de hêtres, d'épicéas, d'aulnes glutineux, d'érables sycomores ou encore de pelouses ou prairies dans certaines zones. Ces cours d'eau se situent en amont de toute perturbation anthropique directe à l'exception des activités forestières, ces dernières étant toutefois limitées à proximité des zones d'étude.

Figure 12. Photographies des sites d'études des cours d'eau neutre de La Maix (A: sites LM et N1) et acide de Courbeligne (B: sites CL et A3) durant l'hiver 2009-2010 (Photos P. Wagner)

Des photographies des sites de référence en cours d'eau neutre (La Maix, LM et N1) et acidifié (Courbeligne, CL et A3) sont présentées figure 12. Les caractéristiques et la localisation des différents sites d'étude sont présentées dans le tableau 1.

Tableau 1. Caractéristiques, localisations, et variables physico-chimiques moyennes (n=7, périodes de 10 semaines) des sites retenus pour les études des effets de l'acidification des cours d'eau sur la décomposition de litières.

Cours d'eau	Code	Roche mère	Coordonnées des sites	Altitude (m)	Distance à la source (m)	pH	Cond ($\mu S.cm^{-1}$)	ANC ($\mu eq.L^{-1}$)	NO_3^- ($mg.L^{-1}$)	SO_4^{2-} ($mg.L^{-1}$)	Ca^{2+} ($mg.L^{-1}$)	Mg^{2+} ($mg.L^{-1}$)	Al_{total} ($\mu g.L^{-1}$)
Etude hiver 2008-2009 (partie IV.1)													
La Maix	LM	Grès permien	48°29'00"N 07°04'13"E	460	3700	7.4	71	448	3.2	4.6	6.7	3.2	26
Menombru	MB	Grès vosgien	48°29'05"N 07°02'48"E	400	3000	7.0	53	204	2.8	6.2	4.0	1.9	93
Gravelle	GV	Grès vosgien	48°16'22"N 06°49'17"E	440	2300	6.4	36	53	1.6	5.9	2.4	0.8	32
Ravines	RV	Grès vosgien	48°24'47"N 06°55'22"E	350	5200	6.1	37	25	2.6	7.0	2.2	1.0	105
Courbeligne	CL	Grès vosgien	48°26'24"N 07°03'54"E	590	1300	4.6	34	-27	4.4	4.6	1.2	0.4	697
Etude hiver 2009-2010 (partie IV.2)													
La Maix	N1	Grès permien	48°29'00"N 07°04'13"E	460	3700	7.4	70	440	3.1	4.3	6.1	3.1	60
Plaineux	N2	Grès permien	48°25'03"N 07°04'16"E	490	1200	7.6	95	674	2.9	4.8	8.5	4.8	99
Courbeligne	N3	Grès vosgien	48°26'56"N 07°03'33"E	500	2300	6.8	43	157	4.2	4.2	3.2	1.5	242
La Maix	A1	Grès vosgien	48°28'59"N 07°05'34"E	590	1800	4.7	32	-20	2.5	4.3	1.3	0.5	337
Gentil Sapin	A2	Grès vosgien	48°27'05"N 07°04'12"E	560	2500	4.6	30	-21	3.6	4.1	1.0	0.4	535
Courbeligne	A3	Grès vosgien	48°26'24"N 07°03'54"E	590	1300	4.5	35	-32	4.4	4.6	1.1	0.4	580

Cond : conductivité de l'eau

ANC : capacité à neutraliser les acides

La première étude (hiver 2008-2009, partie IV.1) portait sur les effets de l'acidification des cours d'eau sur la décomposition des litières et les communautés de décomposeurs associées dans les compartiments benthiques et hyporhéiques. Ainsi, cinq cours d'eau présentant des zones d'accumulation de sédiments et présentant différents degrés d'acidification ont été choisis. Les sites LM et MB sont situés dans des cours d'eau neutres et ont respectivement des pH de 7,4 et 7,0. GV et RV sont des sites légèrement plus acides avec respectivement des pH de 6,4 et 6,1, alors que CL est le site le plus acidifié avec un pH de 4,6. L'ANC et les concentrations en Ca et Mg diminuent le long de ce gradient d'acidification, alors qu'à l'inverse les concentrations en Al augmentent, passant de 26 $\mu g.L^{-1}$ dans LM à 697 $\mu g.L^{-1}$ dans CL. Notons une exception à ces tendances dans le site GV, qui présente une ANC et une minéralisation relativement faibles mais toutefois des concentrations faibles en Al (32 $\mu g.L^{-1}$).

Tableau 2. Distribution (%) en classes de tailles des sédiments des 5 sites de l'étude hivernale 2008-2009 présentée en partie IV.1

	Classes de sédiments (mm)							
Site	> 32	32-18	18-10	10-5	5-2	2-1	1-0.5	0.5-0.2
LM	4.2	8.1	11.3	18.1	13.4	8.1	21.3	15.5
MB	0.0	9.7	15.0	21.9	14.9	9.6	22.5	6.4
GV	4.3	21.7	23.0	15.8	8.7	4.7	18.8	3.0
RV	0.0	9.9	10.5	4.5	2.8	1.5	46.8	23.9
CL	2.9	15.4	11.2	7.1	4.8	5.2	30.2	23.1

Dans cette première étude, la décomposition des litières étant suivie dans les compartiments benthiques mais aussi hyporhéiques, une analyse de la granulométrie des sédiments des 5 sites a été réalisée, cette dernière étant présentée dans le tableau 2. Ces sédiments sont ainsi majoritairement composés de graviers et de sables fins et grossiers.

La deuxième étude (hiver 2009-2010, partie IV.2) portait sur les effets de l'acidification des cours d'eau sur les activités microbiennes associées à la décomposition de la litière. Contrairement à la première étude qui présentait les contraintes de trouver des sites avec un gradient d'acidification et des zones d'accumulation de sédiments, cette seconde étude, concernant exclusivement le compartiment benthique des cours d'eau, a pu se réaliser dans un périmètre plus restreint (Figure 11B). Les sites N1, N2 et N3 ont des pH neutres compris entre

7,4 et 6,8 et les sites A1, A2 et A3 ont des pH particulièrement acides étant inférieurs à 5. Ces sites sont classés le long d'un gradient d'Al, du site le moins impacté N1 (60 µg.L^{-1}) au site le plus impacté A3 (580 µg.L^{-1}). Notons que ces deux sites de références sont les mêmes que ceux utilisés dans la première étude.

Une originalité de cette étude réside dans le fait que certaines stations acides et neutres se trouvaient dans différentes zones d'un même cours d'eau. En effet, les stations N1 et A1 sont respectivement des sites neutre et acide du ruisseau de La Maix, et les stations N3 et A3 sont respectivement des sites neutre et acide du ruisseau de Courbeligne.

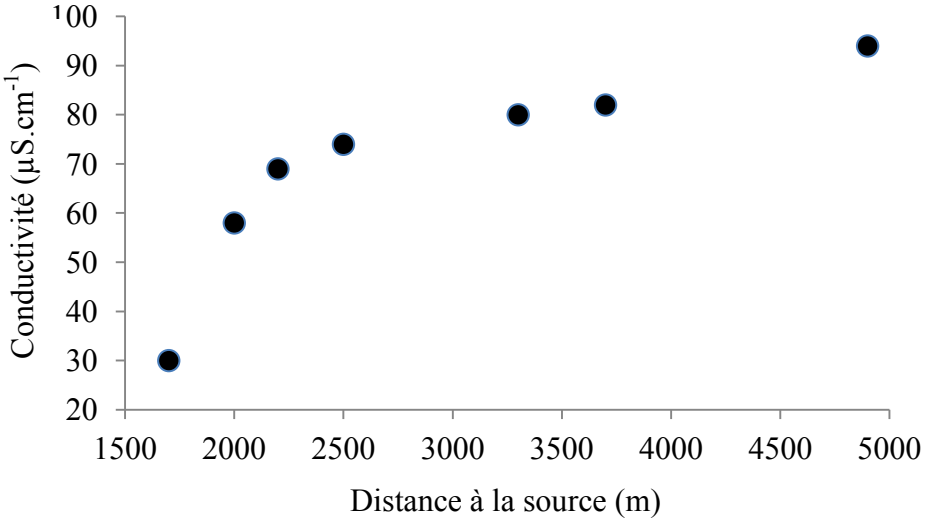

Figure 13. Evolution de la conductivité de l'eau le long du cours d'eau de La Maix le 16/09/2009

Cette particularité a pour origine des changements de substratum géologique et/ou des infiltrations et petits affluents alcalins, issus de roches beaucoup plus riches en cations basiques, qui vont alimenter certains ruisseaux le long de leur cours. Ces derniers passent ainsi d'une eau acide en amont à une eau à pH neutre et beaucoup plus minéralisée dans sa partie aval. Les analyses physico-chimiques d'un de ces petits affluents ont révélé un pH de 7,5, une ANC supérieure à 800 µeq.L^{-1} et des concentrations de 10 mg.L^{-1} de Ca et de 5 mg.L^{-1} de Mg. Des mesures de conductivité de l'eau, reflétant le degré de minéralisation, sont présentées figure 13 et illustrent bien cette reminéralisation du ruisseau de La Maix le long de son cours. L'étude de différentes stations d'un même cours d'eau permet ainsi de travailler sur des sites présentant des conditions environnementales pratiquement identiques mais aussi d'entrevoir les effets d'une « restauration naturelle » sur le processus de décomposition de litières et les communautés microbiennes associées.

Chapitre III :

Effets de la restauration de bassins versants acidifiés sur les communautés microbiennes des sols

III.1. Effets de la restauration de bassins versants forestiers acidifiés sur les communautés bactériennes des sols

Résumé

Peu d'études ont porté sur l'amélioration de la qualité des sols en milieu forestier par rapport au domaine agricole. Quelques travaux se sont cependant intéressés à l'impact d'amendements forestiers sur les communautés bactériennes du sol, mais aucune étude récente n'a caractérisé ces changements, notamment au niveau taxonomique, et dans des conditions réalistes à l'échelle du bassin versant. Les objectifs de ces travaux étaient ainsi d'évaluer si un amendement raisonné pouvait avoir un effet durable sur des sols forestiers et si des indicateurs microbiens pouvaient être associés à l'amélioration de la qualité de ces sols.

Quatre à cinq ans après une opération d'amendement (2,5 t/ha), des prélèvements de sols ont été réalisés à deux saisons (automne et printemps) dans des bassins versants traités et dans des bassins versants témoins sur deux types de roches mères différentes (gréseuse et granitique). Les résultats obtenus par PCR-DGGE et analyses Biolog ont permis de montrer que les communautés bactériennes des sols amendés étaient différentes de celles des sols témoins. De plus, en comparaison avec les analyses Biolog des sols témoins, les sols amendés présentaient des réponses significativement supérieures pour un plus grand nombre de substrats carbonés. La caractérisation au niveau taxonomique des différences observées a été réalisée par clonages et séquençages. Les résultats ont révélé que la diversité taxonomique était légèrement inférieure dans les sols amendés, la diversité d'*Acidobacteria*, d'*Actinobacteria* et de *Firmicutes* étant inférieure dans ces sols par rapport aux témoins. En revanche, la diversité en *Proteobacteria* était comparativement supérieure dans les sols traités. Le ratio entre les proportions relatives de *Proteobacteria* et d'*Acidobacteria* était également plus élevé dans les sols amendés et les proportions relatives d'*Actinobacteria* et de *Firmicutes* étaient comparativement plus élevées dans les sols témoins.

Ces résultats ont permis de montrer que quatre à cinq ans après un amendement raisonné, les communautés bactériennes de sols amendés étaient différentes de celles de sols témoins non traités. Les analyses Biolog ont ainsi montré que les sols amendés possédaient une diversité plus importante d'utilisation potentielle des substrats carbonés. La caractérisation des communautés bactériennes des sols témoins et amendés a également confirmé que le ratio entre *Proteobacteria* et *Acidobacteria* pouvait être un indicateur microbien de l'amélioration de la qualité des sols.

Applied Soil Ecology 59 (2012) 116–123

Contents lists available at SciVerse ScienceDirect

Applied Soil Ecology

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

Changes in soil bacterial communities following liming of acidified forests

Hugues Clivot, Christophe Pagnout*, Delphine Aran, Simon Devin, Pascale Bauda, Pascal Poupin, François Guérold

Laboratoire Interactions Ecotoxicologie, Biodiversité, Ecosystèmes, UMR CNRS 7146, Université Paul Verlaine-Metz, rue du Général Delestraint, 57070 Metz, France

ARTICLE INFO

Article history:
Received 22 June 2011
Received in revised form
23 September 2011
Accepted 24 September 2011

Keywords:
Soil bacterial communities
Liming
Forest management
DGGE
CLPP
16S rRNA genes

ABSTRACT

To counteract forest decline due to anthropogenic acidification, two catchments located on granite and on sandstone bedrock were limed (2.5 t/ha). The catchments are located in strongly affected areas in the Vosges Mountains of northeastern France. Four years after liming, structure of soil bacterial communities and community-level physiological profiling (CLPP) were investigated at two seasons. Bacterial communities were compared with those of two reference adjacent catchments to highlight changes induced by liming. Denaturing gradient gel electrophoresis (DGGE) and Biolog Ecoplate™ analysis revealed that liming modified the structure of bacterial communities and the CLPPs, suggesting that moderate large-scale liming has a sustainable impact on bacterial communities of forest soils. Moreover, significantly higher responses from limed soils were observed for a larger number of Biolog Ecoplate substrates compared to control soils. Analysis of 16S rRNA gene clone libraries showed that taxonomic diversity was slightly lower in limed soils, particularly within acidobacterial and Gram-positive groups, whereas proteobacterial diversity increased in limed soils. The relative abundance of dominant taxa showed that the ratio between *Proteobacteria* and *Acidobacteria* was higher in limed soils. This large-scale field study documents the effects of a four year old liming on bacterial communities of forest soils by providing a common indicator based on analysis of metabolic potential activities of heterotroph communities and by identifying major taxonomic changes in bacterial community structure. The results confirm previous observations showing that the ratio between *Proteobacteria* and *Acidobacteria* could be a microbial indicator of soil quality improvement.

1. Introduction

Decades of atmospheric acid depositions have severely acidified ecosystems in numerous sensitive regions throughout the Northern Hemisphere and, despite recent reductions in SO_2 emissions, acidification remains a widespread problem (Alewell et al., 2000). Acidification of ecosystems leads to severe impacts on the production of wood biomass (Schulze, 1989) as well as water quality (Probst et al., 1999), meaning that ecological services provided by forest ecosystems are seriously threatened by acidification. The long-term resilience of strongly affected ecosystems has led forest managers to use several palliative treatments while awaiting spontaneous recovery. Thus, for the past three decades, liming has been a widely used method to restore acidified ecosystems (Lundström et al., 2003). Limestone has been applied to forests and freshwaters in order to improve and sustain productivity as well as water quality. Liming raises the pH and the base cation (Ca and Mg) content of soils and concomitantly decreases heavy metal toxicity (Ingerslev, 1997; Kreutzer, 1995). Liming has been shown to modify soil organic matter composition by influencing dissolved organic carbon concentrations, C turnover and humus storage (Kreutzer, 1995) and such changes are probably linked to modifications of the root systems and of activities of microbial communities, which are inseparable from soil functions. Persistent effects of soil acidification and the associated metal toxicity can have repercussions on ecosystem functioning over long periods and also on microbial communities, which are involved in nutrient availability and in plant health. Indeed, it has been previously reported that acidification reduces bacterial number and cell size (Baath et al., 1980) and alters nutrient recycling in the forest ecosystem (Francis, 1982). Several studies have revealed that compared to limed soils, untreated soils have lower biological activities. Notably, it has been shown that liming increases respiration, microbial biomass and N turnover (Badalucco et al., 1992; Illmer and Schinner, 1991; Shah et al., 1990; Smolander and Mälkönen, 1994). Changes in bacterial communities have been characterized by the proliferation of acid-intolerant bacteria (Neale et al., 1997;

Abbreviations: ANOSIM, analysis of similarity; AUC, area under the absorbance *vs* time curves; AWCD, average well color development; CLPP, community-level physiological profiling; DGGE, denaturing gradient gel electrophoresis; NMDS, non-metric multidimensional scaling; OTU, operational taxonomic unit; PCA, principal components analysis.

* Corresponding author. Tel.: +33 387 378 513.
E-mail address: pagnout@univ-metz.fr (C. Pagnout).

0929-1393/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.apsoil.2011.09.010

Shah et al., 1990) and by a shift to more Gram-negative and less Gram-positive bacteria (Frostegård et al., 1993).

Convenient methods, developed over the past two decades, among which PCR-DGGE and CLPP, can provide useful information on structural and potential functional changes in microbial communities. Moreover, to our knowledge, no molecular taxonomic study has attempted to characterize the liming effects on soil bacterial communities of forested systems.

In the Vosges Mountains in northeastern France, anthropogenic acidification has been shown to represent a major threat to forests (Dambrine et al., 1995, 1998) and surface waters (Angéli et al., 2009; Guérold et al., 2000). In the present study, two catchments in the Vosges Mountains were limed in fall 2003, one located on sandstone and the other on granite. Four years later, treated soils exhibited improved chemical characteristics and microbial communities were thought to be adapted to the experimentally changed environmental conditions. In this context, the objectives of this study were, first to investigate if liming induced medium-term changes in the structure and metabolic potential of bacterial communities and second to highlight and characterize the possible impact of liming on the dominant taxa of soil bacterial populations. The results obtained could provide microbial indicators of soil quality and thus useful information for forest managers. To this end, we compared the medium-term community responses from limed and acidic control catchments in fall 2007 and in spring 2008. Changes in bacterial communities were assessed by using PCR-DGGE and analysis of 16S rRNA gene clone library sequencing and we investigated CLPP by using Biolog Ecoplate™.

2. Material and methods

2.1. Study site and sample collection

The study was conducted in the Vosges Mountains (N-Eastern France). In fall 2003, two watersheds were limed by helicopter with 2.5 t/ha of $CaMg(CO_3)_2$. One site was located on granite (124 ha, Longfoigneux, Ventron Massif) and the other on sandstone (123 ha, Basse des Escaliers, Donon Massif). For each type of rock, an adjacent acidified watershed was selected as a control on granite (124 ha, Wassongoutte, Ventron Massif) and on sandstone (222 ha, Gentil Sapin, Donon Massif). Limed and their control sites were similar in terms of forest cover and of geological and pedological characteristics according to a study of sites prior to the lime treatment (Nys et al., 2004). Moreover, the latter study showed that soil chemistry was not significantly different between limed and their control sites. The sites are mountain forests dominated by silver fir (*Abies alba*), Norway spruce (*Picea abies*) and common beech (*Fagus sylvatica*). Two years after liming, a significant decrease in needle loss and needle yellowing was observed in conifers and a decrease of beech defoliation was recorded (Messant et al., 2008).

Soils were sampled on two occasions, in fall (October) 2007 and spring (May) 2008 to overcome seasonal fluctuations. According to World Reference Base for Soil Resources (IUSS Working Group WRB, 2006), the soils belong to Entic Podzol. The location, the elevation and the soil chemistry of the sites are described in Table 1. At the same elevation, soil chemistry was performed on 10 soil samples (0–15 cm deep) distributed along a 200 m transect. Soils were air dried and sieved (2-mm mesh size) and pH was measured in water (AFNOR NF ISO 10390). Exchangeable cations were extracted by the cobaltihexamine saturation method (Orsini and Rémy, 1976) modified by AFNOR NF X 31-130. Total C and N were determined by the dry-combustion method (AFNOR NF ISO 10694).

At each site along the transect, 15 soil samples (0–15 cm deep) were collected for microbial analysis, each sample resulting from three pooled core samples to overcome potential spatial heterogeneity. All soils were stored on ice upon collection and transported to labs. All 120 soil samples were homogenized by sieving (2-mm mesh size), fresh subsamples were analyzed in the Biolog Ecoplate™ and the other parts were stored at −80 °C until later processing.

2.2. DNA extraction and PCR amplification

Soil DNA samples were extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Bacterial universal primers 341F-GC and 907R (Muyzer et al., 1993, 1998) were used for partial PCR amplification of 16S rRNA genes. PCR mixture (100 μl) contained 6 U of Taq DNA Polymerase (5 PRIME, Hamburg, Germany), 1× Taq buffer (5 PRIME) formulated to automatically adjust the Mg^{2+} concentration, 200 μM of each dNTP, 0.5 μM of each primer and 50 ng of extracted DNA as template. The PCR protocol consisted of 5 min at 95 °C, followed by 20 cycles of 30 s at 95 °C, 30 s at 65–55 °C (touchdown −0.5 °C per cycle), 35 s at 72 °C, and by 10 cycles of 30 s at 95 °C, 30 s at 55 °C and 35 s at 72 °C, followed by 7 min (for DGGE) or 10 min (for cloning) final extension at 72 °C.

2.3. DGGE analysis

The amplification products were subjected to quality control on 1% (w/v) agarose gel and separated with the DCODE mutation detection system (Bio-Rad, Hercules, CA). 15 μl per sample was loaded on 7% (w/v) polyacrylamide gels in 1× TAE buffer with a denaturing gradient from 40 to 60% (100% denaturant corresponds to 40% (v/v) formamide and 7 M urea). The gels were run in 1× TAE buffer at 65 V and 60 °C for 16 h and stained with ethidium bromide. The gels were imaged under UV light using a GelDoc EQ transilluminator (Bio-Rad) and Quantity One 1-D Analysis Software (Bio-Rad).

2.4. Community-level physiological profiling

Biolog Ecoplate™ (Biolog Inc., Hayward, CA) analyses were used to assess community-level physiological profiling. Briefly, 5 g of each fresh subsample were homogenized in 45 ml of sterile physiological water (0.85% saline solution) and shaken 30 min at 150 rpm. The suspensions were decanted during 2 h and 150 μl of supernatant was inoculated into plates. Plates were incubated at 25 °C in the dark and $OD_{600\,nm}$ were recorded every 12 h using an Expert Microplate Reader (ASYS, Eugendorf, Austria).

2.5. Cloning and sequencing of partial amplified 16S rRNA gene

The 15 soil DNA extracts from each transect were pooled into a composite DNA sample. PCR amplification was performed as described in Section 2.2. The corresponding PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Cergy Pontoise, France) and 144 individual clone colonies by sample were sequenced by GATC Biotech (Konstanz, Germany) using the T3 vector primer. The sequences were checked and only quality sequences were kept for phylogenetic analyses. These were deposited in Genbank under accession numbers HQ628650–HQ629549.

2.6. Data analysis

For soil chemical characteristics, significant differences between each treatment were determined by the Wilcoxon test.

GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was used to normalize and compare DGGE profiles. To this end, internal control samples were loaded on each gel. A tolerance in the band position of 1% was applied. Cluster analysis of DGGE pattern

Table 1
Location and main chemical characteristics of the four study sites.

Bedrock (Massif)	Location	Elevation (m)	Treatment (October 2003)	Sampling date	pH	Exchangeable cations (cmol/kg)					C/N
						Ca	Mg	K	Na	Al	
Granite (Ventron)	47°57′40″N; 6°53′05″E	1060	Control	October 2007	3.91	0.78	0.32	0.25	0.06	4.37	18.9
				May 2008	3.89	0.90	0.36	0.33	0.06	3.83	18.1
Granite (Ventron)	47°57′24″N; 6°52′55″E	1050	$CaMg(CO_3)_2$ (2.5 t/ha)	October 2007	4.18*	1.00	0.46*	0.22	0.06	6.37*	19.3
				May 2008	4.12*	1.10	0.51	0.22	0.06	4.96	17.1
Sandstone (Donon)	48°26′38″N; 7°05′25″E	810	Control	October 2007	3.86	0.30	0.11	0.10	0.03	1.93	18.6
				May 2008	3.96	0.28	0.12	0.10	0.02	1.32	15.9
Sandstone (Donon)	48°27′45″N; 7°05′50″E	810	$CaMg(CO_3)_2$ (2.5 t/ha)	October 2007	4.25**	0.73*	0.26*	0.10	0.03	1.56	19.6
				May 2008	4.03	0.70*	0.22*	0.12	0.02	0.80	15.3

Significant differences between each treated and their control samples were determined by the Wilcoxon test. Mean values, $n = 10$.
* Significant at $p < 0.05$.
** Significant at $p < 0.001$.

profiles was performed using the unweighted pair group method with arithmetic mean (UPGMA) based on the dice similarity coefficient (band based). A correlation matrix was generated for each comparison and the data were analyzed with non-metric multidimensional scaling (NMDS) using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL), since NMDS has been shown to be a robust and powerful ordination technique for the analysis of community ecology (Minchin, 1987). Analysis of similarity (ANOSIM) was performed using PAST software (Hammer et al., 2001) to characterize differences between samples. An *R*-value of 1 indicates completely dissimilar groups whereas an *R*-value of 0 indicates no difference. The *R*-value was statistically significant at a *p*-value lower than 0.05.

For community-level physiological profiling, each Ecoplate™ provided triplicates, which were used to calculate average values. Data were normalized by the average well color development (AWCD) following Weber et al. (2007). Kinetic of microbial activity was assessed through the area under the absorbance *vs* time curves (AUC) (Guckert et al., 1996). Those AUC were obtained from plate readings at 36 h of incubation and served for further analyses. Principal components analysis (PCA) is the most commonly used ordination method for the analysis of CLPP (Garland, 1997). PCA (R Development Core Team, 2008) was thus performed on sample responses for the 31 carbon sources and another comparative evaluation was conducted. Shannon index (H') and Simpson index ($1/D$) were calculated to estimate diversity of substrate utilization. The AUC values and the diversity indices of the 15 limed samples were compared with their 15 control soils. Significantly higher responses (at $p < 0.05$ and at $p < 0.001$) between treated and non-treated samples were determined by the Wilcoxon test. The substrates were clustered into chemical guilds according to Choi and Dobbs (1999).

For sequencing analysis, DNA sequences were compared with sequences deposited in the Ribosomal Database Project (Cole et al., 2009) and compared to the GenBank database using the Blast algorithm (Altschul et al., 1990) for identification. Multiple

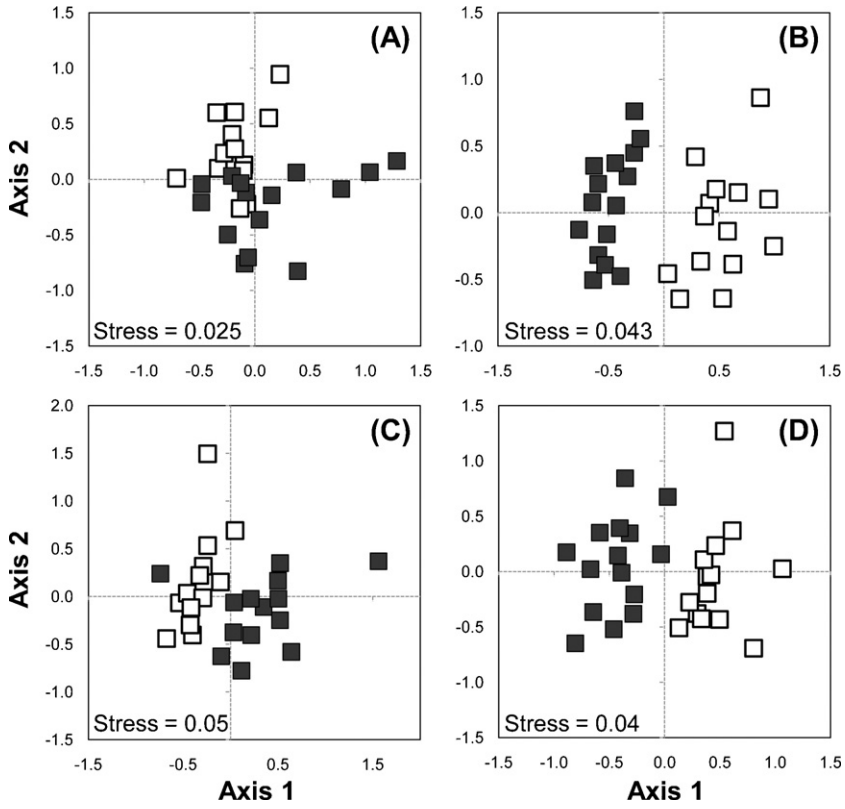

Fig. 1. NMDS plots of DGGE bacterial community profiles from limed soils (white square) and control soils (black square). (A) Granite bedrock in fall, (B) granite in spring, (C) sandstone in fall and (D) sandstone in spring.

sequence alignments were done using CLUSTALW (Larkin et al., 2007) at default settings. Distance matrices were constructed using DNAdist (default parameters) in the PHYLIP package (Felsenstein, 2005). Operational taxonomic units (OTUs) were defined at a 97% sequence similarity cut-off in the DOTUR software (Schloss and Handelsman, 2005). Shannon index ($H' = -\sum_{i=1}^{Sobs} p_i \times \log 2(p_i)$) and Simpson index ($1/D = 1/[\sum n_i \times (n_i - 1)/(N \times (N - 1))]$) were calculated to estimate diversity at 97% similarity cut-off and each OTU was affiliated to the phylum level. The relative abundances of bacterial phyla were defined by calculating the sequenced clone proportion of total sequences from each condition. Comparisons of 16S rRNA gene sequence libraries between limed and control soils were assessed using the LIBSHUFF program (Singleton et al., 2001). According to the authors, differences are statistically significant with a confidence of 95% at a *p*-value lower than or equal to 0.025.

3. Results

Four years after liming, the effect of the treatment ($CaMg(CO_3)_2$) is still noticeable on soil chemical characteristics. Although comparisons of soil parameters were all not significantly different, trends can be observed for pH and both Ca and Mg contents, which were overall higher in limed soils (Table 1). NMDS ordinations of PCR-DGGE fingerprint profiles illustrating bacterial community structure distances between samples showed differences among communities in limed sites and the adjacent control sites (Fig. 1). ANOSIM performed on the DGGE profiles confirmed that soil bacterial communities of limed soils were different from those of acidic controls on sandstone in both seasons (in fall: $R = 0.61$, $p < 0.0001$ and in spring: $R = 0.85$, $p < 0.0001$) and on granite in spring ($R = 0.97$, $p < 0.0001$) whereas the effects of liming were less pronounced on granite in fall ($R = 0.31$, $p = 0.0001$).

Community-level physiological profiles were compared using Biolog Ecoplates. For the 31 substrate utilization, the first two principal components of the PCA explain 39% (Fig. 2A: granite, fall), 43% (Fig. 2B: granite, spring), 34% (Fig. 2C: sandstone, fall) and 42% (Fig. 2D: sandstone, spring) of the variance. Responses to Biolog substrates from the 15 samples of limed soils were clearly separated from the 15 control samples for soils located on granite in fall and spring and for soils located on sandstone for both sampling periods.

Table 2 shows that the responses were also significantly higher for a larger number of substrates in limed soils compared to the control soils on granite (11 *vs* 6 substrates in fall and 13 *vs* 9 substrates in spring, respectively) and sandstone (10 *vs* 6 substrates in fall and 10 *vs* 6 substrates in spring, respectively). Diversity of substrate utilization calculated by Shannon (H') and Simpson (1/D) indices was significantly higher for limed soil compared to non-treated ones on granite (H' was 4.61 *vs* 4.51 in fall and 4.63 *vs* 4.41 in spring; $1/D$ was 21.35 *vs* 19.54 in fall and 22.12 *vs* 18.42 in spring). No significant differences were found on sandstone in fall. However, diversity of substrate utilization was significantly higher for treated samples compared to control ones on sandstone in spring (H' was 4.69 *vs* 4.59; $1/D$ was 23.35 *vs* 20.85) (Table 2).

For 16S rRNA gene sequence library analysis, richness and diversity indices (determined after defining the OTU phylogenetic resolution at a 97% sequence similarity cut-off) (Table 3) showed that the total number of OTUs was higher in control sites compared to their limed counterparts in granite soils and in sandstone soils. The diversity indices that were influenced either by rare taxa such as the Shannon index H' or by the most abundant taxa such as the Simpson index ($1/D$) confirmed a moderate reduction of diversity in limed soils. Comparisons between limed and control sites revealed a lower diversity in *Acidobacteria*, *Firmicutes* and *Actinobacteria* phyla and a higher diversity in *Proteobacteria* in limed soils (Table 3). Finally, OTU richness in *Bacteroidetes* was higher in granite and in sandstone limed soils in fall.

Table 2
Comparative analysis of soil sample responses to Biolog Ecoplate substrates and diversity indices of substrate utilization after 36 h of incubation.

	Granite		Sandstone	
	Fall	Spring	Fall	Spring
Amines				
Phenylethylamine	++	++	++	+
Putrescin	−−	−	+	ns
Amino acids				
Glycyl-L-glutamic Acid	ns	+	ns	ns
L-Arginine	++	ns	ns	++
L-Asparagine	ns	+	ns	+
L-Phenylalanine	ns	ns	−	−
L-Serine	++	++	ns	+
L-Threonine	ns	ns	ns	−−
Carbohydrates				
α-D-Lactose	ns	ns	−−	ns
β-Methyl-D-glucoside	ns	−	ns	+
D-Cellobiose	++	−	−	ns
D-Galactonic acid γ-Lactone	ns	−	ns	ns
D,L-α-Glycerol phosphate	−	ns	ns	ns
D-Mannitol	ns	−	ns	ns
D-Xylose	+	−−	−−	ns
Glucose-1-phosphate	−	−−	−	−−
i-Erythritol	+	+	++	+
N-Acetyl-D-glucosamine	ns	−−	ns	ns
Carboxylic acids				
α-Ketobutyric Acid	ns	+	++	−
D-Galacturonic Acid	+	−	−−	ns
D-Glucosaminic Acid	++	+	ns	−−
D-Malic Acid	++	++	ns	++
γ-Hydroxybutyric acid	ns	ns	+	ns
Itaconic acid	+	++	ns	ns
Pyruvic acid methyl ester	ns	ns	ns	−−
Phenolic compounds				
2-Hydroxy benzoic acid	ns	ns	++	ns
4-Hydroxy benzoic acid	++	++	ns	++
Polymers				
α-Cyclodextrin	−	+	+	++
Glycogen	−	ns	++	++
Tween 40	ns	++	+	ns
Tween 80	−	++	+	ns
H'	++	++	ns	++
$1/D$	++	++	ns	++

AUC values and diversity indices of limed samples were compared to their controls ($n = 15$). Significant differences were determined by the Wilcoxon test (ns: non-significant; (+) limed higher than control at $p < 0.05$; (++) limed higher than control at $p < 0.001$; (−) limed lower than control at $p < 0.05$; (−−) limed lower than control at $p < 0.001$).

LIBSHUFF comparison of 16S rRNA gene sequence libraries from limed and control soils revealed no significant differences on granite in fall ($p = 0.057$) but composition of communities were significantly different on granite in spring ($p = 0.010$) and on sandstone ($p < 0.001$ and $p = 0.003$ in fall and in spring, respectively).

Overall, 16S rRNA gene sequencing revealed that *Acidobacteria* and *Proteobacteria* largely dominate the bacterial communities, jointly representing 85% of total sequences, followed to a lesser extent by *Firmicutes* (6%), *Actinobacteria* (5%) and *Bacteroidetes* (2%) (Table 3). Discrete taxonomic groups, such as *Gemmatimonadetes*, *Verrucomicrobia*, *Chloroflexi*, *Cyanobacteria* or *Planctomycetes* were also found in the soils. In parallel to the changes in diversity induced by the treatment, we observed a higher proportion of proteobacterial sequences concomitant to a lower abundance of Gram-positive groups (*Actinobacteria* and *Firmicutes*) in each limed site compared to its control (Table 3).

Table 3
Intra-phylum diversity, OTU richness and diversity indices (at 97% sequence similarity) and relative abundances of bacterial phyla based on 16S rRNA gene clone libraries of control (C) and limed (L) soils.

	Intra-phylum diversity (no. of OTUs)								Relative abundance (% of sequenced clones)							
	Granite				Sandstone				Granite				Sandstone			
	Fall		Spring		Fall		Spring		Fall		Spring		Fall		Spring	
	C	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L
Acidobacteria	44	30	30	27	37	32	42	34	50.8	35.0	36.9	39.0	51.0	45.4	41.9	43.6
Proteobacteria	34	43	31	35	25	26	28	36	33.9	52.0	50.5	55.2	29.8	37.1	35.9	46.2
Firmicutes	6	5	6	4	7	4	6	3	5.6	4.0	5.8	3.8	8.7	4.6	8.5	3.4
Actinobacteria	8	4	3	1	5	4	8	1	8.1	3.3	3.9	1.0	5.8	4.6	8.5	0.9
Bacteroidetes	1	5	1	1		3	3	3	0.8	4.9	1.0	1.0		2.8	2.5	2.5
Gemmatimonadetes					1	1							1.0	0.9		
Verrucomicrobia			1			1					1.0			0.9		
Chloroflexi							1									2.5
Cyanobacteria							1								0.9	
Planctomycetes							1								0.9	
Unclassified	1	1	1		2	4	1	1	0.8	0.8	0.9		3.7	3.7	0.9	0.9
Total no. of OTUs	94	88	73	68	77	75	90	79								
H'	6.40	6.11	5.98	5.89	6.13	6.08	6.31	6.11								
$1/D$	179	73	103	101	153	134	151	117								

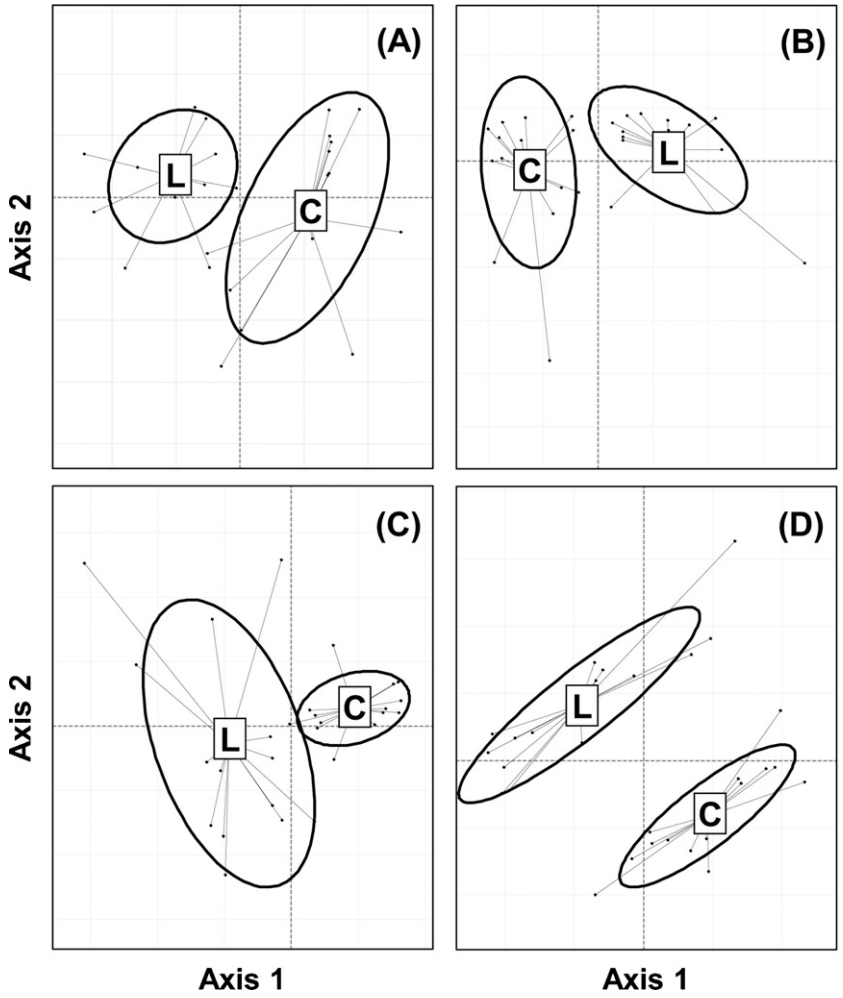

Fig. 2. Results of the principal component analyses (PCAs) performed on the Biolog Ecoplate profiles (30 samples × 31 substrata) from limed [L] and control [C] soils after 36 h of incubation. Ordination of samples (black dots) on the first factorial plane of each PCA: (A) granite bedrock in fall (axes 1 and 2 explain, respectively, 22% and 17% of the variance), (B) granite in spring (axes 1 and 2 explain, respectively, 29% and 14% of the variance), (C) sandstone in fall (axes 1 and 2 explain, respectively, 18% and 16% of the variance) and (D) sandstone in spring (axes 1 and 2 explain, respectively, 24% and 18% of the variance). Each black dot represents the mean position of the three Biolog replicates constituting a given sample. Lines link dots to the weighted average of their corresponding group (i.e. [L] *vs* (C]). Inertia ellipses summarizing each group dispersion and were drawn to contain 67% of this group according to a Gaussian distribution.

4. Discussion

Four years after liming, soil parameters showed that treated soils tended to have a higher pH and higher base cation contents than soils from control watersheds. Despite the small increase in soil pH induced by liming, differences were significant and non-negligible for acidified soils. Indeed, since pH is a logarithmic scale, an increasing of 0.3 pH units corresponds to a decrease by half of the proton concentration in the soils. Concurrently, the DGGE analyses showed that fingerprinting patterns were markedly different between limed and control sites, revealing differences in the bacterial community structures except for those sampled in fall on granite soils, where differences were less pronounced. This latter observation has been confirmed by the LIBSHUFF comparison of 16S rRNA gene sequence libraries. Biolog Ecoplate™ analyses revealed that community-level physiological profiles from limed and control samples are separated in both seasons and in both types of soil, confirming that liming induced changes in soil microbial communities. These results confirm the discriminatory capacity of the Ecoplate™ for environmental sample investigations (Insam, 1997). While limed and control soils had lower pH compared to the media of Biolog plates, Yao et al. (2000) showed that for soils along a pH gradient and particularly for a forest soil, AWCD were generally higher in modified plates buffered to neutral pH than for acid pH ones. However, the higher responses observed for a larger number of substrates in limed soils could be due to bacteria more adapted to the media pH of Ecoplates. Thus, the higher diversity of substrate utilization cannot be related to a higher functional diversity in such analyses. Furthermore, the shifts observed by analysis of the 16S rRNA gene sequences and community-level physiological profiles are both assumed to result from the lime treatment. It would be then tempting to link both responses described separately, but only a minor part of communities, especially some culturable fast-growing bacteria, are able to use sole-carbon-sources. However, both methods revealed complementary responses by providing informations on major changes in community structure and on metabolic potential of a heterotrophic part of the bacterial community. The global function of the communities could not be characterized by a Biolog analysis. Nevertheless, such analyses reveal bacterial responses suitable for a comparative study dependent on the representativeness of the substrates used and we observed a common response of all limed treatments characterized by significantly higher responses for a larger number of substrates compared to their control conditions.

Thus, our results show that a four-year-old moderate treatment (2.5 t/ha) has a sustainable impact on chemical properties of soils. In turn, these effects induce changes in bacterial populations inhabiting soils underlain by granite or sandstone, which are the major geologic substrata in the Vosges Mountains. This tends to corroborate the findings of Fierer and Jackson (2006) who observed that soil pH was a good predictor of bacterial diversity and community composition. However, other biotic and abiotic characteristics not investigated in the present study, such as the vegetation, fungal activities, nutrient availability, or even the past land use of soils, could affect the bacterial communities and could provide interesting data in order to predict their ecology.

Bacterial diversity, investigated by OTU richness and diversity indices, was slightly higher in control acidified soils compared with limed ones; findings that contrasted with previous studies that showed a positive relationship between pH and bacterial diversity (Fierer and Jackson, 2006; Hartman et al., 2008). By contrast, our results were consistent with those obtained by Kennedy et al. (2004) after a lime treatment of acidic grassland soils in a microcosm-based study. The latter authors hypothesized that liming could act as a selective factor for neutrophilic species. The observed decrease of bacterial diversity in our study can be explained by a lower diversity in both *Acidobacteria* and Gram-positive taxa (*Firmicutes* associated with *Actinobacteria*), whereas we showed higher proteobacterial diversity in limed soils compared to their control ones.

In our study, the *Acidobacteria* and *Proteobacteria* phyla represent an average of 44% (ranging from 35 to 52%) and 41% (ranging from 27 to 54%), respectively, of all sequences and dominate the soil bacterial communities. Both phyla are among the most diversified groups within the bacterial domain. Encompassing numerous culturable and well-described species, the *Proteobacteria* are characterized by a high metabolic diversity and are known to be involved in biogeochemical cycles in soils. By contrast, because of their recalcitrant culturability, the functions of the *Acidobacteria* in the ecosystems are not yet completely understood. Nevertheless, recent studies (Lee et al., 2008; Ward et al., 2009) revealed that the *Acidobacteria* are metabolically active in soils and suggested that members of this phylum might contribute greatly to biogeochemical processes.

In most soil 16S rRNA gene libraries, *Proteobacteria* are commonly found to dominate bacterial communities (Janssen, 2006), but the soil acidity in the present study (pH ranging from 3.86 to 4.25) has probably led to an important abundance of *Acidobacteria* as was previously observed in other acid soils (Jones et al., 2009). Since the pH and the *Proteobacteria* abundance were higher in limed soils compared to their acidified control ones, the changes observed here support the finding that the relative proportion of *Proteobacteria* seems to be positively related to pH (Rousk et al., 2010). Combined with the lower relative proportion of *Firmicutes* and *Actinobacteria* in limed soils, such modifications could be involved in the shift previously observed toward more Gram-negative and fewer Gram-positive bacteria following soil liming (Frostegård et al., 1993).

We noticed that the ratio between the relative abundance of *Proteobacteria* and *Acidobacteria* was higher in limed soils compared to the adjacent untreated soils (1.49 *vs* 0.67 in fall and 1.42 *vs* 1.37 in spring on granite; 0.82 *vs* 0.58 in fall and 1.06 *vs* 0.86 in spring on sandstone). Following Smit et al. (2001), who have suggested that this increasing ratio could be indicative of the nutrient status of soils from oligotrophic to high-nutrient ecosystems, we could then hypothesize that plants interacting with microorganisms in limed soils were probably under less limiting nutrient conditions than forests developing on strongly acidified soils. Other studies on bacterial communities have reported changes of this ratio in root-associated soils. Recently, Thomson et al. (2009) found an increased ratio in vegetated acid grassland soils compared to bare ones. Moreover, a meta-analysis of published clone libraries (Fierer et al., 2007) revealed that *Proteobacteria* are more abundant and Gram-positive bacteria less abundant in rhizosphere soils compared to bulk ones. The changes in dominant phyla observed in our study could then indicate that bacterial communities from limed soils seem to have been modified toward communities more characteristic of revegetated soils or more active rhizospheric soils. Further investigations are needed to specify if composition of bacterial communities resulted from changes in root-associated activities and/or from a direct impact of liming on soil chemical properties.

To counteract the depletion of base cations in soils due to the anthropogenic acidification of forested ecosystems, liming represents an interesting management strategy that has been already applied over large-scale areas in Europe. In France, only small surfaces have been limed in the Vosges Mountains, but over the past several years, signs of forest decline are increasing and large scale liming operations are now planned to sustain forest productivity. Moreover, in France as in many other countries, growing concerns about climate change combined with the need to reduce fossil fuel consumption are encouraging the use of alternative and renewable sources of energy, such as wood biomass. Increasing the harvest

of wood biomass from forests located in acid sensitive areas will unfortunately increase the deficiency of base cations in soils. To face this threat, it seems obvious that liming represents a major alternative for forest managers. In this context and considering the fact that microbial communities represent a key component in the functioning of forested ecosystems, it appears important to better assess the effects of liming on microbial diversity. It has been previously suggested that the success of restoration could be estimated by measures of soil microbial communities (Harris, 2003) and that major bacterial taxa in soils, especially ratio between *Proteobacteria* and *Acidobacteria*, could be promising indicators of restoration (Hartman et al., 2008). Thus, this study contributes to confirm the usefulness of microbial indicators by providing a common indicator of liming effects based on analysis of CLPPs and by identifying the major taxonomic changes of bacterial communities, showing notably an increasing ratio between *Proteobacteria* and *Acidobacteria* in limed soils.

Acknowledgements

The present study was financed by the French ANR program (ANR Biodiversité, RECOVER-Project). We thank the Conseil Général des Vosges, the Office National des Forêts and the Zone Atelier Moselle for their support. We also thank two anonymous reviewers for comments on the manuscript.

References

AFNOR NF ISO 10390, 1999. Détermination du pH. NF ISO 10390 Novembre 1994. Qualité des sols, vol. 1. Association Française de Normalisation, Paris, France, pp. 339–347.

AFNOR NF ISO 10694, 1999. Détermination du carbone organique et du carbone total après combustion sèche (analyse élémentaire). NF ISO 10694 Juin 1995. Qualité des sols, vol. 1. Association Française de Normalisation, Paris, France, pp. 361–371.

AFNOR NF X 31-130, 1999. Détermination de la capacité d'échange cationique (CEC) et des cations extractibles. NF X 31-130 Mai 1993. Qualité des sols, vol. 1. Association Française de Normalisation, Paris, France, pp. 265–278.

Alewell, C., Manderscheid, B., Meesenburg, H., Bittersohl, J., 2000. Environmental chemistry: is acidification still an ecological threat? Nature 407, 856–857.

Altschul, S., Gish, W., Miller, W., Myers, E., Lipman, D., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Angéli, N., Dambrine, E., Boudot, J.P., Nedeltcheva, T., Guérold, F., Tixier, G., Probst, A., Party, J.P., Pollier, B., Bourrié, G., 2009. Evaluation of streamwater composition changes in the Vosges Mountains (NE France): 1955–2005. Sci. Total Environ. 407, 4378–4386.

Baath, E., Berg, B., Lohm, U., Lundgren, B., Lundvist, H., Rosswall, T., Oederstroem, B., Wiren, A., 1980. Effects of experimental acidification and liming on soil organisms and decomposition in a Scots pine forest. Pedobiologia 20 (2), 85–100.

Badalucco, L., Grego, S., Dell'Orco, S., Nannipieri, P., 1992. Effect of liming on some chemical, biochemical, and microbiological properties of acid soils under spruce (*Picea abies* L.). Biol. Fertil. Soils 14, 76–83.

Choi, K.H., Dobbs, F.C., 1999. Comparison of two kinds of Biolog microplates (GN and ECO) in their ability to distinguish among aquatic microbial communities. J. Microbiol. Methods 36, 203–213.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37, 141–145.

Dambrine, E., Pollier, B., Poszwa, A., Ranger, J., Probst, A., Viville, D., Biron, P., Granier, A., 1998. Evidence of current soil acidification in spruce stands in the Vosges Mountains, north-eastern France. Water Air Soil Pollut. 105, 43–52.

Dambrine, E., Ulrich, E., Cénac, N., Durand, P., Gauquelin, T., Mirabel, P., Nys, C., Probst, A., Ranger, J., Zéphoris, M., 1995. Atmospheric deposition in France and possible relation with forest decline. In: Landmann, G., Bonneau, M. (Eds.), Forest Decline and Atmospheric Deposition Effects in the French Mountains. Springer, Berlin, Heildeberg, New York, pp. 177–199.

Felsenstein, J., 2005. PHYLIP (Phylogeny Inference Package) Version 3.6. Distributed by the Author. Department of Genome Sciences, University of Washington, Seattle.

Fierer, N., Bradford, M.A., Jackson, R.B., 2007. Toward an ecological classification of soil bacteria. Ecology 88, 1354–1364.

Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. U.S.A. 103, 626–631.

Francis, A., 1982. Effects of acidic precipitation and acidity on soil microbial processes. Water Air Soil Pollut. 18, 375–394.

Frostegård, Å., Bååth, E., Tunlid, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol. Biochem. 25, 723–730.

Garland, J.L., 1997. Analysis and interpretation of community level physiological profiles in microbial ecology. FEMS Microbiol. Ecol. 24, 289–300.

Guckert, J.B., Carr, G.J., Johnson, T.D., Hamm, B.G., Davidson, D.H., Kumagai, Y., 1996. Community analysis by Biolog: curve integration for statistical analysis of activated sludge microbial habitats. J. Microbiol. Methods 27, 183–197.

Guérold, F., Boudot, J.P., Jacquemin, G., Vein, D., Merlet, D., Rouiller, J., 2000. Macroinvertebrate community loss as a result of headwater stream acidification in the Vosges Mountains (N-E France). Biodivers. Conserv. 9, 767–783.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4 (1), 9.

Harris, J.A., 2003. Measurements of the soil microbial community for estimating the success of restoration. Eur. J. Soil Sci. 54, 801–808.

Hartman, W.H., Richardson, C.J., Vilgalys, R., Bruland, G.L., 2008. Environmental and anthropogenic controls over bacterial communities in wetland soils. Proc. Natl. Acad. Sci. U.S.A. 105, 17842–17847.

Illmer, P., Schinner, F., 1991. Effects of lime and nutrient salts on the microbiological activities of forest soils. Biol. Fert. Soils 11, 261–266.

Ingerslev, M., 1997. Effects of liming and fertilization on growth, soil chemistry and soil water chemistry in a Norway spruce plantation on a nutrient-poor soil in Denmark. For. Ecol. Manage. 92, 55–66.

Insam, H., 1997. A new set of substrates proposed for community characterization in environmental samples. In: Insam, H., Rangger, A. (Eds.), Microbial Communities. Springer-Verlag, Heidelberg, pp. 259–260.

IUSS Working Group WRB, 2006. World reference Base for Soil Resources 2006, 2nd edition. World Soil Resources Reports No. 103.

Janssen, P.H., 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. 72, 1719–1728.

Jones, R.T., Robeson, M.S., Lauber, C.L., Hamady, M., Knight, R., Fierer, N., 2009. A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. ISME J. 3, 442–453.

Kennedy, N., Brodie, E., Connolly, J., Clipson, N., 2004. Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms. Environ. Microbiol. 6, 1070–1080.

Kreutzer, K., 1995. Effects of forest liming on soil processes. Plant Soil 168–169, 447–470.

Larkin, M., Blackshields, G., Brown, N., Chenna, R., McGettigan, P., McWilliam, H., Valentin, F., Wallace, I., Wilm, A., Lopez, R., Thompson, J., Gibson, T., Higgins, D., 2007. Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) 23, 2947–2948.

Lee, S.H., Ka, J.O., Cho, J.C., 2008. Members of the phylum Acidobacteria are dominant and metabolically active in rhizosphere soil. FEMS Microbiol. Lett. 285, 263–269.

Lundström, U.S., Bain, D.C., Taylor, A.F.S., van Hees, P.A.W., 2003. Effects of acidification and its mitigation with lime and wood ash on forest soil processes: a review. Water Air Soil Poll.: Focus 3, 5–28.

Messant, D., Baudoin, J.M., Bock, J., Dambrine, E., Guérold, F., Nys, C., 2008. Restauration de deux bassins versants acidifiés des Vosges par amendement calco-magnésien. Rendez-vous Tech. 22, 3–13.

Minchin, P.R., 1987. An evaluation of the relative robustness of techniques for ecological ordination. Vegetation 69, 89–107.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H., Wawer, C., 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Akkermans, A.D.L., van Elsas, J.D., de Bruijn, F.J. (Eds.), Molecular Microbial Ecology Manual. , 3rd ed. Kluwer Academic Publishers, Dordrecht, The Netherlands, 3.4.4, pp. 1–27.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Neale, S.P., Shah, Z., Adams, W.A., 1997. Changes in microbial biomass and nitrogen turnover in acidic organic soils following liming. Soil Biol. Biochem. 29, 1463–1474.

Nys, C., Picart, J.F., Dambrine, E., 2004. Restauration de la qualité des sols et des eaux de deux bassins versants situés dans les Vosges gréseuses et les Vosges cristallines. Caractérisation initiale des sites ateliers d'amendement calco magnésien. Bassins versants de Cornimont et Senones. INRA Nancy ONF, Nancy.

Orsini, L., Rémy, J.C., 1976. Utilisation du chlorure de cobaltihexamine pour la détermination simultanée de la capacité d'échange et des bases échangeables des sols. Science du sol 4, 269–275.

Probst, A., Party, J.P., Fevrier, C., Dambrine, E., Thomas, A.L., Stussi, J.M., 1999. Evidence of springwater acidification in the Vosges Mountains (North-East of France): influence of bedrock buffering capacity. Water Air Soil Pollut. 114, 395–411.

R Development Core Team, 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, URL http://www.R-project.org.

Rousk, J., Baath, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., Fierer, N., 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J. 4, 1340–1351.

Schloss, P.D., Handelsman, J., 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. 71, 1501–1506.

Schulze, E.D., 1989. Air pollution and forest decline in a spruce (*Picea abies*) forest. Science 244, 776–783.
Shah, Z., Adamst, W.A., Haven, C.D.V., 1990. Composition and activity of the microbial population in an acidic upland soil and effects of liming. Soil Biol. Biochem. 22, 257–263.
Singleton, D.R., Furlong, M.A., Rathbun, S.L., Whitman, W.B., 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol. 67, 4374–4376.
Smit, E., Leeflang, P., Gommans, S., van den Broek, J., van Mil, S., Wernars, K., 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. 67, 2284–2291.
Smolander, A., Mälkönen, E., 1994. Microbial biomass C and N in limed soil of norway spruce stands. Soil Biol. Biochem. 26, 503–509.
Thomson, B.C., Ostle, N., McNamara, N., Bailey, M.J., Whiteley, A.S., Griffiths, R.I., 2009. Vegetation affects the relative abundances of dominant soil bacterial taxa and soil respiration rates in an upland grassland soil. Microb. Ecol. 59, 335–343.
Ward, N.L., Challacombe, J.F., Janssen, P.H., Henrissat, B., Coutinho, P.M., Wu, M., Xie, G., Haft, D.H., Sait, M., Badger, J., Barabote, R.D., Bradley, B., Brettin, T.S., Brinkac, L.M., Bruce, D., Creasy, T., Daugherty, S.C., Davidsen, T.M., DeBoy, R.T., Detter, J.C., Dodson, R.J., Durkin, A.S., Ganapathy, A., Gwinn-Giglio, M., Han, C.S., Khouri, H., Kiss, H., Kothari, S.P., Madupu, R., Nelson, K.E., Nelson, W.C., Paulsen, I., Penn, K., Ren, Q., Rosovitz, M.J., Selengut, J.D., Shrivastava, S., Sullivan, S.A., Tapia, R., Thompson, L.S., Watkins, K.L., Yang, Q., Yu, C., Zafar, N., Zhou, L., Kuske, C.R., 2009. Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils. Appl. Environ. Microbiol. 75, 2046–2056.
Weber, K.P., Grove, J.A., Gehder, M., Anderson, W.A., Legge, R.L., 2007. Data transformations in the analysis of community-level substrate utilization data from microplates. J. Microbiol. Methods 69, 461–469.
Yao, H., He, Z., Wilson, M., Campbell, C., 2000. Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. Microb. Ecol. 40, 223–237.

III.2. Diversité des communautés bactériennes oxalotrophes dans des sols forestiers acides

Résumé

Les acides organiques de faible poids moléculaire, comme l'acide oxalique, sont supposés jouer un rôle important dans les sols pauvres et acidifiés (Jones 1998), dans lesquels les faibles concentrations en cations basiques et en P et les concentrations élevées en Al sont les principaux facteurs limitant la croissance des plantes. En effet, les champignons ectomycorhiziens et les plantes sont reconnus pour excréter ces acides afin de mobiliser les nutriments présents en faibles quantités dans ces sols. De plus, il a été démontré qu'un amendement pouvait induire des changements des capacités de biodégradation de l'acide oxalique par les micro-organismes du sol. Ainsi, les sols forestiers acides pourraient potentiellement renfermer une importante diversité de bactéries oxalotrophes, cette diversité pouvant elle-même être modifiée par l'apport d'amendements calco-magnésiens au travers de ses effets sur la qualité physico-chimique des sols.

A notre connaissance, un nombre limité de bactéries oxalotrophes a été identifié à ce jour. Un marqueur moléculaire de la fonction oxalotrophe a cependant été récemment développé afin d'étudier la diversité des communautés bactériennes impliquées dans le catabolisme de l'acide oxalique dans les sols (Khammar et al., 2009). L'amplification et le clonage-séquençage d'un gène fonctionnel *frc* codant pour la formyl-CoA-transferase (enzyme impliquée dans la voie de dégradation bactérienne de l'acide oxalique) ont été réalisés sur des échantillons provenant des sols granitiques et gréseux amendés et des sols témoins non amendés, afin d'étudier la diversité des communautés bactériennes oxalotrophes dans ces sols.

Les résultats ont montré que les librairies de clones étaient significativement différentes entre les quatre sols analysés. Le profil des courbes de raréfaction ainsi que l'important nombre de séquences uniques et de séquences non-communes aux quatre librairies de clones ont révélé que la diversité de bactéries oxalotrophes était relativement importante dans ces sols, dépassant largement celle estimée dans une étude antérieure par PCR-DGGE (Khammar et al., 2009), cette diversité étant également légèrement inférieure dans les sols amendés par rapport aux sols témoins. Une partie des séquences obtenues étaient proches de celles d'Alphaproteobacteria, de Betaproteobacteria et d'Actinobacteria, mais la majorité des séquences de clones (59%) étaient phylogénétiquement distantes de gènes d'espèces connues, indiquant ainsi la présence dans ces sols de nombreuses bactéries oxalotrophes encore non identifiées.

En parallèle, dix souches bactériennes ont été isolées après enrichissement des sols en acide oxalique et ont été identifiées comme étant des espèces de genres *Burkholderia* et *Collimonas*. Les séquences de leurs gènes *frc* étaient proches de celles de souches de Burkholderiales déjà connues, mais différentes de celles de la majorité des séquences des librairies de clones issues de nos sols.

Cette étude présentait ainsi l'originalité d'être la première s'intéressant à la diversité moléculaire des communautés bactériennes oxalotrophes par analyses de librairies de clones. Les résultats obtenus ont montré que leur diversité dans les sols était encore loin d'être exhaustivement décrite. L'isolement de nouvelles souches oxalotrophes et davantage d'études moléculaires sont ainsi nécessaires pour améliorer nos connaissances sur ces communautés qui peuvent jouer un rôle important en étant les médiateurs d'une partie des flux de carbone et de nutriments dans les sols.

Diversity of oxalate-oxidizing bacterial communities in acid forest soils.

Hugues Clivot[1,2], Christophe Pagnout[1,2], François Guérold[1,2] and Pascal Poupin[1,2].

[1]Université de Lorraine; Laboratoire des interactions Ecotoxicologie Biodiversité Ecosystèmes (LIEBE); UMR 7146; Campus Bridoux rue du Général Delestraint, 57070 Metz, France

[2]CNRS; LIEBE; UMR 7146; 57070 Metz, France

Abstract

Oxalate fate in forest soils has been a subject of interest due to its important role in rhizosphere and pedogenic processes. However, far less is known on the diversity of microorganisms able to metabolize oxalate, particularly in acid forest soils, where oxalate can be produced by fungi and plant roots to alleviate scarcity of nutrients and Al toxicity. By using functional gene (formyl-CoA-transferase, *frc*) clone libraries, the diversity of oxalotrophic bacteria was investigated in acid forest soils lying on granite and sandstone bedrocks and on similar soils treated with lime. Libshuff comparisons showed that the clone libraries from the four acid soils differed significantly (p-values < 0.001). The patterns of rarefaction curves and the high number of unique and unshared sequences found in these soils revealed a high diversity of *frc* gene and then a high diversity of oxalotrophs, exceeding that estimated in previous studies. A part of clone sequences obtained were close to *frc* gene from Alpha- and Betaproteobacteria and Actinobacteria. However, the majority (59%) of them was phylogenetically distant from *frc* gene of any known species, indicating the presence of numerous unknown oxalate oxidizers in soils. Ten bacterial strains were isolated from soil enrichment cultures with oxalate and were identified as *Burkholderia* and *Collimonas* species. Their *frc* gene sequences were close to those of already known members of Burkholderiales but were distant from most of clone sequences. This study is the first report on molecular diversity of oxalotroph in acid forest soils. Our data highlights that for now, our knowledge are probably limited by cultivation-dependent approaches and that the diversity of oxalatrophic bacteria in soils is far from being exhaustively described.

Keywords : *oxalate, bacterial communities, acid soils, diversity, clone libraries.*

Introduction

Oxalate is a low molecular weight organic acid commonly found in forest soils (Stevenson, 1967; Van Hees et al., 2000; Westergaard & Hansen, 1998; Comerford & Fox, 1990). In particular, oxalate is supposed to play an important role in acid soils (Jones, 1998), where toxic levels of Al and suboptimal concentrations of Ca, Mg and P are the main limiting factors for plant growth (Godbold et al., 1988; Van Praag et al., 1997; Schaberg et al., 2000). Indeed, owing to their high chelating and weathering properties, organic acids such as oxalate are produced by fungi and plant roots and are known to influence metal and nutrient availability in soils (Bolan et al., 1994; Gadd, 1999; Landeweert et al., 2001; Ström et al., 2002; Jones, 1998; Ahonen-Jonnarth et al., 2000). Therefore, oxalotrophic bacteria, which can use and degrade oxalate, may be considered as key mediators of C and nutrient cycling in soils (Morris & Allen, 1994; Daniel et al., 2007).

A limited number of oxalotrophic bacteria has been previously described, some of them being notably isolated from aquatic and terrestrial ecosystems (Sahin, 2003). But to date, most of studies were focused on bacteria involved in human oxalate homeostasis, due to pathologies resulting from oxalate accumulation (Abratt & Reid, 2010; Williams & Wandzilak, 1989). However, a molecular marker was recently developed to study bacterial communities involved in oxalate catabolism in soils (Khammar et al., 2009), thus providing a useful tool to assess the genetic diversity structure of oxalotrophic communities. Despite the role that they could play, we are not aware of any molecular study that attempted to assess the diversity of oxalotrophic communities in acid soils.

In addition, it has been shown that liming, a widely used method to restore acidifed soils (Lundström et al., 2003), can induce changes in bacterial communities (Neale et al., 1997; Shah et al., 1990; Frostegård et al., 1993; Clivot et al., 2012) and soil chemical characteristics (Kreutzer, 1995). Moreover, Van Hees et al. (2003) reported that liming can also affect biodegradation rates of oxalate in forest soils. These results suggest that liming, through its impact on bacterial communities and/or on soil chemical properties, could induce in turn changes in oxalotrophic communities.

In this context, the aim of this work was to assess oxalotrophic bacterial diversity in acid soils by using the *frc* gene proposed by Khammar et al. (2009) as a molecular marker. To this end, total DNA were extracted from anthropogenically acidified soils of the Vosges Mountains (North-eastern France). By using clone libraries of amplified *frc* genes, we compared the diversity and the structure of oxalate-oxidizing communities between soils

coming from catchments lying on sandstone and granite bedrocks. Limed soils were sampled on adjacent catchments and their oxalotrophic communities were concurrently assessed. Enrichment cultures from these soils were additionally performed to isolate culturable oxalotrophic bacteria.

Methods

Study site, sample collection and soil chemistry

The study was conducted in the Vosges Mountains (N-Eastern France), where decades of atmospheric acid depositions have led to forest decline and to soil and freshwater acidification (Landmann & Bonneau, 1995; Dambrine et al., 1998, 1995). The sites are mountain forests dominated by silver fir (*Abies alba*), Norway spruce (*Picea abies*) and common beech (*Fagus sylvatica*). One site was located on a granite watershed (124 ha, Wassongoutte, Ventron Massif) and the other on a sandstone watershed (222 ha, Gentil Sapin, Donon Massif). For each type of rock, limed sites were selected on adjacent watersheds on granite (124 ha, Longfoigneux, Ventron Massif) and on sandstone (123 ha, Basse des Escaliers, Donon Massif). In fall 2003, these two watersheds were limed by helicopter with 2.5 t/ha of $CaMg(CO_3)_2$. Limed and non-treated sites were similar in terms of forest cover and of geological and pedological characteristics according to a study of sites prior to the lime treatment (Nys et al., 2004).

Soils were sampled in May 2008 and belong to Entic Podzol, according to World Reference Base for Soil Resources (IUSS Working Group WRB, 2006). At each site, 5 soil samples distributed along a 50 m transect (0–15 cm deep) were collected at the same elevation.

For soil chemistry, soil subsamples were air dried and sieved (2-mm mesh size) and pH was measured in water (AFNOR NF ISO 10390). Exchangeable cations were extracted by the cobaltihexamine saturation method (Orsini & Rémy, 1976) modified by AFNOR NF X 31-130). Organic C and N were determined by the dry-combustion method (AFNOR NF ISO 10694). To determined oxalate content in soils, oxalate was extracted following the method described in Certini et al. (2000) and oxalate concentrations were assessed using an enzymatic kit (Libios, Bully, France). Main chemical characteristics of soils are described in Table 1. The four soils have acidic pH at around pH 4.0, but limed soils exhibited higher Ca and Mg contents and lower Al saturation compared to their non-treated counterparts. Similar oxalate

concentrations were found in the four soils when oxalate content was expressed as a function of organic matter (OM) mass instead of soil dry mass.

Table 1. Location and main chemical characteristics (mean values, n=5) of the four study sites.

	Granite		Sandstone	
	Control	Limed	Control	Limed
Location	47°57'40"N 6°53'05"E	47°57'24"N 6°52'55"E	48°26'38"N 7°05'25"E	48°27'45"N 7°05'50"E
pH	3.82	4.16	4.03	4.04
Ca (cmol/kg)	0.64	1.16	0.22	0.58
Mg (cmol/kg)	0.28	0.54	0.10	0.20
K (cmol/kg)	0.26	0.25	0.09	0.11
Na (cmol/kg)	0.05	0.06	0.02	0.02
Al (cmol/kg)	3.18	4.21	0.75	0.66
Al saturation (%)	72.5	67.9	49.8	42.0
C/N	18.3	17.9	13.9	13.9
OM (%)	19.4	20.1	6.3	5.9
Oxalate (mmoles/kg of soil)	1.32	1.21	0.25	0.34
Oxalate (mmoles/kg of OM)	6.9	6.4	5.4	5.3

For oxalotrophic bacterial community analysis, 5 soil subsamples (0–15 cm deep) were stored on ice upon collection and transported to labs, where they were homogenized by sieving (2-mm mesh size). An amount of 1 g of sieved soil was used for enrichment cultures and the other part was stored at -80°C for further molecular analysis.

Enrichment cultures

Liquid enrichment cultures were performed using mineral salt medium (MMK) supplemented with 250 mg/L of oxalate as a sole carbon source (MMK-oxalate). The composition of MMK medium was as follows: KH_2PO_4 1 g/L, K_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L and $FeCl_3 \cdot 6H_2O$ 0.0004 g/L. A soil amount of 1 g was added to 10 ml of MMK-oxalate medium and incubated on a rotary shaker for 10 days at 20°C. Enrichment cultures were then plated on MMK-oxalate Agar solid media for isolation of oxalotrophic bacteria.

DNA extraction and PCR amplification

Soil DNA samples were extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) and DNA from 10 bacteria isolated from enrichment cultures

were extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The formyl-CoA-transferase (*frc*) gene was used as a molecular marker of the oxalotrophic function (Khammar et al., 2009). Primers *frc*171-F and *frc*627-R were used for amplification of *frc* genes from soil and bacterial DNA (Khammar et al., 2009). Primers W01 and W02 were used for partial amplification of 16S rRNA gene from bacterial DNA (Stahl & Amann, 1991). PCR mixtures (100 µl) contained 6 U of Taq DNA Polymerase (5 PRIME, Hamburg, Germany), 1x Taq buffer (5 PRIME) formulated to automatically adjust the Mg^{2+} concentration, 200 µM of each dNTP, 0.5 µM of each primer and 50 ng of extracted DNA as template. The PCR protocol for *frc* gene amplification consisted of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, and 30 s at 74°C, followed by 10 min final extension at 74°C. The PCR protocol for partial 16S rRNA gene amplification consisted of 5 min at 95°C, followed by 35 cycles of 40 s at 95°C, 30 s at 52°C, and 45 s at 68°C, followed by 10 min final extension at 68°C.

Cloning and sequencing

PCR products of the 5 *frc* gene amplifications from each site were equally pooled into a composite sample. PCR products from soil composite sample or from isolated bacteria were cloned using the Clone JET PCR Cloning Kit (Fermentas, Villebon sur Yvette, France). A total of 5 individual clone colonies by bacteria and 96 by soil composite sample were sequenced by GATC Biotech (Konstanz, Germany) using T3 primer. Sequences were checked and only quality sequences encoding formyl-CoA-transferase were kept for phylogenetic analyses.

To identify isolated oxalotrophic bacteria, PCR products of 16S rRNA gene sequences were also sequenced by GATC Biotech (Konstanz, Germany) using W01 primer.

Data analysis

For phylogenetic analysis, multiple sequence alignments were performed using CLUSTALW (Larkin et al., 2007) at default settings. Distance matrices were constructed using DNAdist (default parameters) in the PHYLIP package (Felsenstein, 2005). Number of OTUs, Shannon (H') and Simpson (1/D) indices as well as rarefaction curves were calculated and generated by the MOTHUR software (Schloss et al., 2009) to estimate diversity at 100, 90 and 75% sequence similarity cut-offs. Comparisons between *frc* gene clone libraries were performed using the Libshuff program (Singleton et al., 2001).

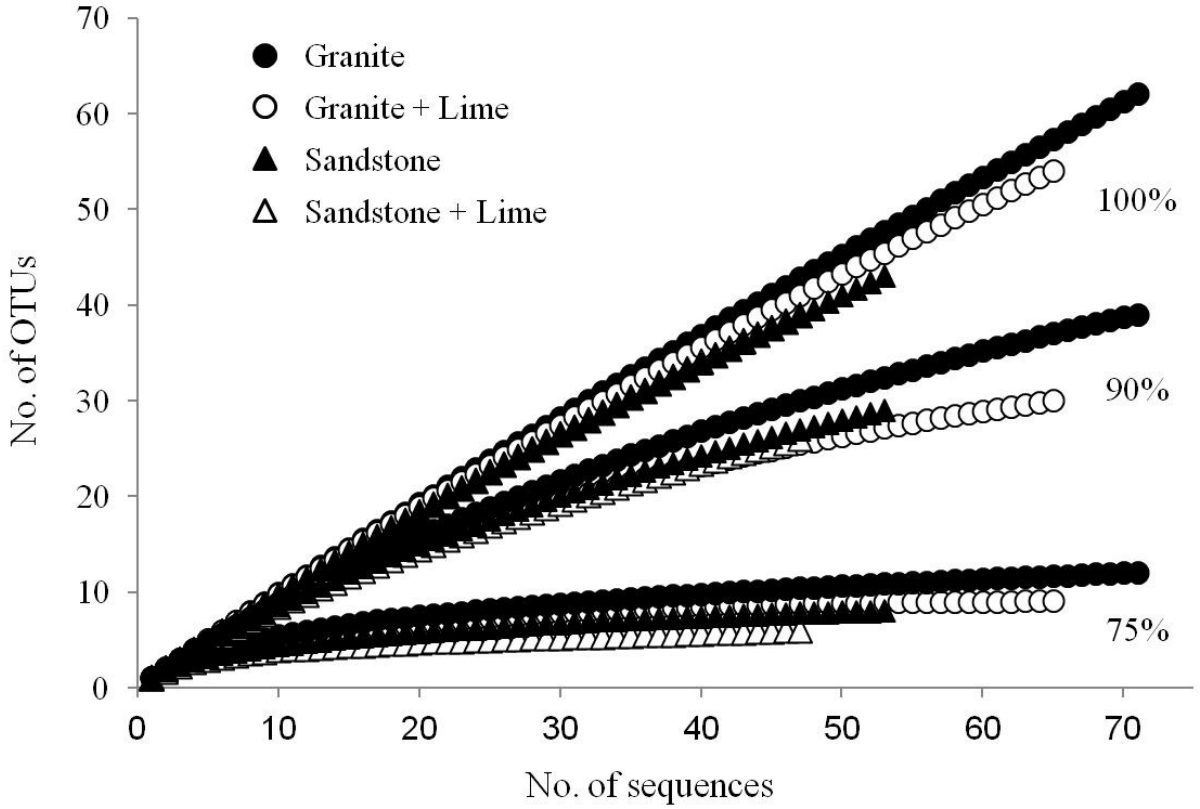

Figure 1. Rarefaction analysis of bacterial *frc* gene libraries from the four acid soils at three different sequence similarity cut-offs.

Table 2. OTU richness and diversity indices at three different sequence similarity cut-offs based on bacterial *frc* gene libraries from the four acid soils.

		Granite		Sandstone	
	Cut-off	Control	Limed	Control	Limed
No. of OTUs	100%	62 (58)	54 (49)	43 (39)	39 (36)
(unshared OTUs)	90%	39 (19)	30 (12)	29 (19)	26 (17)
	75%	12 (3)	9 (1)	8 (3)	6 (1)
H'	100%	4.07	3.92	3.67	3.58
	90%	3.43	3.18	3.13	3.01
	75%	1.97	1.73	1.59	1.37
1/D	100%	208.3	149.3	98.0	98.0
	90%	34.5	27.0	26.5	23.5
	75%	5.8	4.3	4.2	3.4
No. of sequences		71	65	53	47

Amplified *frc* genes and 16S rRNA genes were compared with sequences deposited in the Genbank database using the Blast algorithm (Altschul et al., 1990). Sequences close to *frc* genes obtained from both soil samples and isolated bacteria were collected. The MEGA software (Tamura et al., 2007) was used to align all sequences and to generate a phylogenetic tree with a neighbor-joining algorithm (default parameters).

Results

Libshuff comparisons revealed that bacterial *frc* gene libraries from granite soils differed significantly from those of sandstone and that those from limed soils also differed significantly from those of non-treated ones (all p-values < 0.001).

Diversity of the four *frc* gene clone libraries was assessed at three genetic distances, which were used to define OTUs at 100, 90 and 75% sequence similarity cut-offs.

At the highest level of resolution (100% sequence similarity cut-off), rarefaction curves showed that additional clone sequencing would significantly lead to increased number of unique sequences in the four soils (Figure 1). At intermediary 90% sequence similarity cut-off, rarefaction curves had not yet reached a plateau phase, whereas a low increase of diversity would be expected from additional sequencing at 75% sequence similarity cut-off. At each level of resolution, rarefaction curves of the four gene libraries predicted a similar level of diversity, while that of non-treated granite soil seemed to be slightly higher at the three sequence similarity cut-offs.

A total of 236 sequences were analyzed, including 189 unique sequences among which 182 that were not shared between the four soils (Table 2). At 90% sequence similarity cut-off, 90 different OTUs were found including 67 unshared OTUs. At 75% sequence similarity cut-off, a total of 17 different OTUs were found including 8 unshared OTUs and 4 OTUs common to the four soils, the four latter ones encompassing 80% of all sequences. At the three sequence similarity cut-offs, OTU richness and diversity indices showed that the diversity was slightly higher in the granite non-treated soil than in the granite limed one, whereas less pronounced differences were found between diversity of sandstone soils.

Phylogenetic analysis showed that *frc* genes obtained in this study fell into three main clusters (Figure 2, I-III). The majority of our clone sequences (85%) was affiliated to the cluster I, which notably contained *frc* gene sequences from Alpha- and Betaproteobacteria. In this cluster, 23% of clone sequences were affiliated to a subcluster close to *frc* genes of

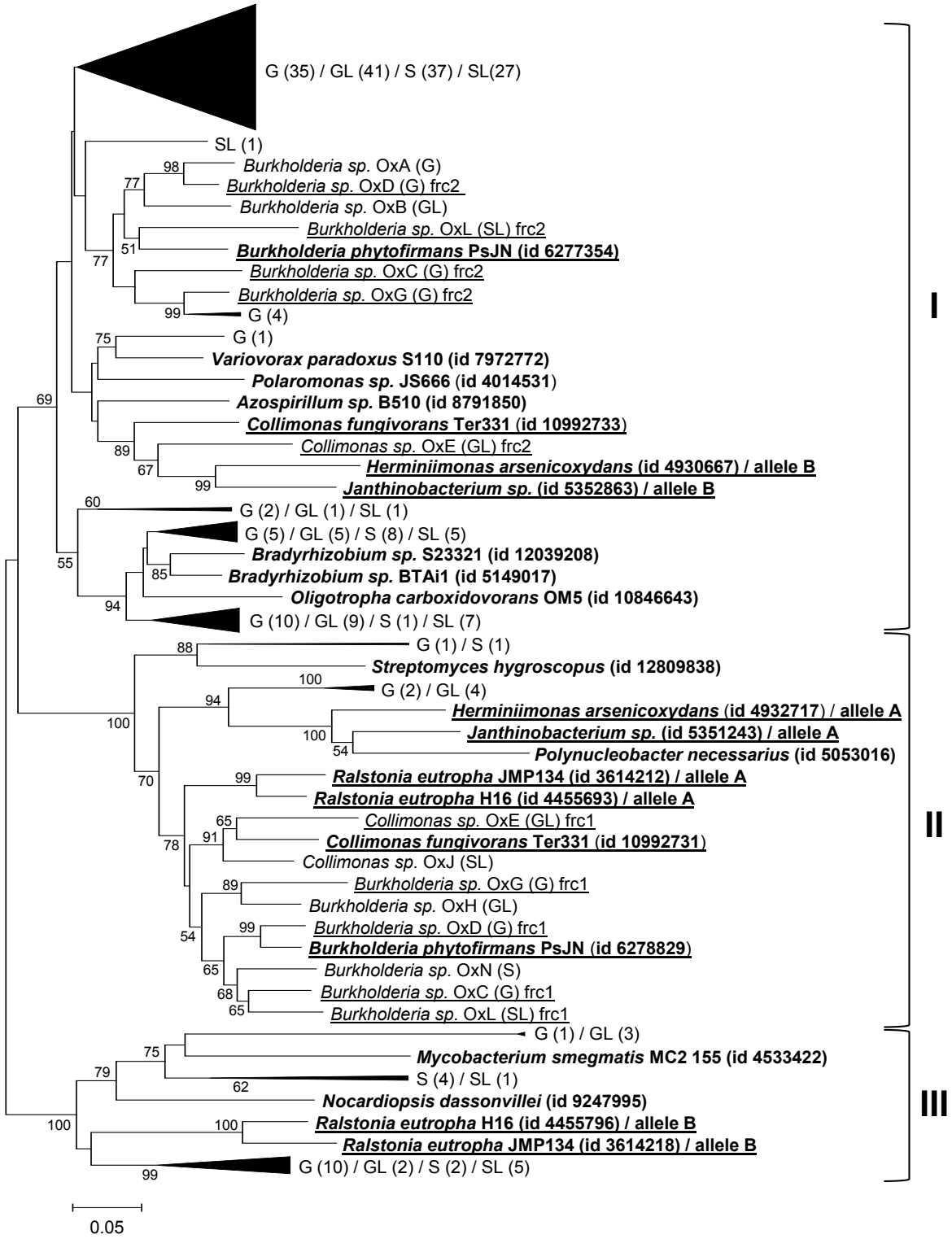

Figure 2. Neighbor-joining phylogenetic tree of *frc* gene sequences from the four clone libraries (site codes are G=Granite, GL=Granite+Lime, S=Sandstone and SL=Sandstone+Lime, number of sequences in brackets), from the ten isolated strains (site code) and from sequences retrieved from NCBI database (indicated in bold, accession numbers in brackets). Bacteria with two *frc* gene copies are underlined. Bootstrap values (1000 replicates) greater than 50% are indicated. The three main clusters are indicated in roman numbers.

Bradyrhizobiales (Alphaproteobacteria), whereas the greatest part of our clone sequences (59%) was not closely related to *frc* genes from any known species. Only 3% of clone sequences were affiliated to cluster II, which contained sequences from Actinobacteria and Betaproteobacteria. Cluster III contained a low proportion (12%) of our clone sequences and *frc* genes from some Actinobacteria and Betaproteobacteria.

Some Betaproteobacteria strains are known to have two different *frc* gene copies. The *frc* gene allele A has been defined as the gene in juxtaposition with the *oxc* gene (gene encoding the oxalyl coenzyme A decarboxylase), allele B being more distant in the genome. In the phylogenetic analysis, all *frc* gene allele A grouped together in the cluster II, whereas allele B sequences were found in cluster I and III.

The ten isolated oxalotrophic strains were identified as Burkholderiales (Betaproteobacteria), eight of them being identified at the genus level as *Burkholderia* and two of them as *Collimonas*. Five strains were found to have only one *frc* gene, while the five others (4 *Burkholderia* and 1 *Collimonas*) were found to have two different copies of this gene. Their amplified *frc* genes were affiliated to cluster I or II and were closely related to *frc* genes of *Burkholderia phytofirmans* and *Collimonas fungivorans*. For the strains with two *frc* gene copies, sequences named “frc1” were close to allele A and affiliated to cluster II and those named “frc2” were close to allele B and affiliated to cluster I.

Discussion

The fate of oxalate in soils and its biodegradation by microorganisms has been a subject of interest due its role in plant nutrition and in pedogenesis (Van Hees et al., 2003; Van Hees et al., 2005; Morris & Allen, 1994), but far less is known on the diversity of microorganisms able to mediate this process. The present study is the first report that attempted to assess the molecular diversity of oxalotrophic bacterial communities in soils by using functional gene clone libraries. Results obtained suggest that a high *frc* gene diversity could occur in acid forest soils, as evidenced by the rarefaction curves and the number of both unshared and unique sequences found in these soils. To date, a similar level of *frc* gene diversity has never been recorded and exceeds that from previous soil DGGE analyses performed with the same PCR primers, which was comprised between 17 and 31 DGGE bands (Khammar et al., 2009). Because some strains may have two different copies of *frc* gene, the diversity of oxalotrophic bacteria should be probably lower than that evaluated by *frc* gene. Nevertheless, our results showed that 97% of clone sequences were affiliated with allele B in cluster I and III, whereas

only 3% of clone sequences were close to allele A and affiliated to cluster II. This suggests that a low proportion of bacteria from the four studied soils would have two different copies of *frc* gene and that these soils potentially harboured a large variety of bacteria able to degrade oxalate.

Most of described oxalotrophic bacteria are members of Proteobacteria, Actinobacteria and Firmicutes phyla (Sahin, 2003). We showed that clone sequences of our study were grouped with *frc* gene of Alpha- and Betaproteobacteria and Actinomycetes. However, as taxonomically distant bacteria may have *frc* genes that are clustered together, it cannot be excluded that other bacterial groups could be involved in oxalate catabolism in acid soils. The *frc* gene sequences from isolated strains were close to those of known species, whereas most of environmental clone sequences were phylogenetically more distant, indicating the presence of numerous not easily culturable oxalatrophs in soils. Current knowledge on oxalotrophs is thus probably limited by our inability to isolate and cultivate the majority of microorganisms (Ward et al., 1990; Dunbar et al., 1999). Indeed, enrichment cultures with oxalate performed in our study or in that of Khammar et al. (2009) have led, respectively, to a low phylogenetic diversity of bacteria and to less complex DGGE profiles than those from initial soils. Interestingly, we noticed that sequences from DGGE bands obtained from their soil enrichment cultures showed high identity with *frc* gene sequences from Burkholderiales and that our isolated strains were also identified as Burkholderiales. Members of this bacterial order have been previously reported to be particularly active in taking up root-derived carbon (Vandenkoornhuyse et al., 2007; Lu et al., 2006). Moreover, Weisskopf et al. (2011) showed that *Burkholderia* species are the dominant bacterial group of White Lupin cluster roots, whose development represents an efficient strategy to access nutrients. In addition, the latter authors reported that *Burkholderia* isolates from these cluster roots were more tolerant to acidity (growth at pH 4) than non-*Burkholderia* strains and that almost only *Burkholderia* isolates were able to grow on oxalate. These results showed that some members of Burkholderiales can play an essential role in soils particularly under acidic conditions and that they have a high ability to metabolize oxalate. This could partly explain why strains of this bacterial order have been isolated from the four acid soils of this study.

Libshuff analysis and the high proportion of unshared sequences between the four sites revealed that different oxalotroph assemblages can be found in these soils. Even if a lower number of sequences was obtained from limed soils and was used for phylogenetic analyses, slightly lower *frc* gene diversity has been observed in the limed soils compared to their non-

treated counterparts. In a previous study (Clivot et al., 2012), we reported that liming could decrease the diversity of bacterial communities and these effects could have a direct repercussion on oxalotrophic communities. In addition, Van Hees et al. (2003) showed that liming can reduce the biodegradation rates of oxalate in limed forest soils. The latter authors hypothesize that oxalate was probably unavailable for microorganisms due to adsorption and precipitation of Ca-oxalate. All these results suggest that liming, through its effects on microbial communities and oxalate availability, could induce changes in oxalotroph assemblages. However, soils are complex and heterogeneous systems and thus further investigations are needed to ascertain these observations.

Our data highlights that oxalotroph diversity in soils is far from being exhaustively described. Therefore, further molecular studies and isolation of new oxalotrophic strains are required to better understand the role of oxalotrophic bacteria in soils and to predict their ecology.

Acknowledgements

The present study was financed by the French ANR program (ANR Biodiversité, RECOVER-Project). We thank the Conseil Général des Vosges, the Office National des Forêts and the Zone Atelier Moselle for their support.

References

Abratt, V.R., Reid, S.J., 2010. Chapter 3 - Oxalate-Degrading Bacteria of the Human Gut as Probiotics in the Management of Kidney Stone Disease. In: Advances in Applied Microbiology, Vol. Volume 72, Academic Press, p. 63-87.

AFNOR NF ISO 10390, Détermination du pH. NF ISO 10390 Novembre 1994. Qualité des sols, Volume 1. Association Française de Normalisation, Paris, France, 1999 339-347.

AFNOR NF ISO 10694, Détermination du carbone organique et du carbone total après combustion sèche (analyse élémentaire). NF ISO 10694 Juin 1995. Qualité des sols, Volume 1. Association Française de Normalisation, Paris, France, 1999 361-371.

AFNOR NF X 31-130, Détermination de la capacité d'échange cationique (CEC) et des cations extractibles. NF X 31-130 Mai 1993. Qualité des sols, Volume 1. Association Française de Normalisation, Paris, France, 1999 265-278.

Ahonen-Jonnarth, U., Hees, Van Hees, P.A.W., Lundström, U.S., Finlay, R.D., 2000. Organic acids produced by mycorrhizal Pinus sylvestris exposed to elevated aluminium and heavy metal concentrations. New Phytologist 146, 557–567.

Altschul, S., Gish, W., Miller, W., Myers, E., Lipman, D., 1990. Basic local alignment search tool. Journal of molecular biology 215, 403-410.

Bolan, N.S., Naidu, R., Mahimairaja, S., Baskaran, S., 1994. Influence of low-molecular-weight organic acids on the solubilization of phosphates. Biology and Fertility of Soils 18, 311-319.

Certini, G., Corti, G., Ugolini, F.C., 2000. Vertical trends of oxalate concentration in two soils under Abies alba from Tuscany (Italy). Journal of Plant Nutrition and Soil Science 163, 173–177.

Clivot, H., Pagnout, C., Aran, D., Devin, S., Bauda, P., Poupin, P., Guérold, F., 2012. Changes in soil bacterial communities following liming of acidified forests. Applied Soil Ecology 59, 116-123.

Comerford, N.B., Fox, T.R., 1990. Low-Molecular-Weight Organic Acids in Selected Forest Soils of the Southeastern USA. Soil Science Society of America Journal 54, 1139-1144.

Dambrine, E., Pollier, B., Poszwa, A., Ranger, J., Probst, A., Viville, D., Biron, P., Granier, A., 1998. Evidence of current soil acidification in spruce stands in the Vosges Mountains, north-eastern France. Water, Air, & Soil Pollution 105, 43-52.

Dambrine, E., Ulrich, E., Cénac, N., Durand, P., Gauquelin, T., Mirabel, P., Nys, C., Probst, A., Ranger, J., Zéphoris, M., 1995. Atmospheric deposition in France and possible relation with forest decline. In: Forest decline and atmospheric deposition effects in the French mountains, (eds G. Landmann, M. Bonneau), Springer, Berlin, Heildeberg, New York. 177-199.

Daniel, S.L., Pilsl, C., Drake, H.L., 2007. Anaerobic oxalate consumption by microorganisms in forest soils. Research in Microbiology 158, 303-309.

Dunbar, J., Takala, S., Barns, S.M., Davis, J.A., Kuske, C.R., 1999. Levels of Bacterial Community Diversity in Four Arid Soils Compared by Cultivation and 16S rRNA Gene Cloning. Applied and Environmental Microbiology 65, 1662-1669.

Felsenstein, J., 2005. PHYLIP (Phylogeny Inference Package) version 3.6.Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Frostegård, Å., Bååth, E., Tunlid, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology and Biochemistry 25, 723-730.

Gadd, G.M., 1999. Fungal Production of Citric and Oxalic Acid: Importance in Metal Speciation, Physiology and Biogeochemical Processes. In:Advances in Microbial Physiology, Vol. Volume 41, Academic Press, p. 47-92.

Godbold, D. L., Fritz, E., Hüttermann, A., 1988. Aluminum toxicity and forest decline. PNAS 85, 3888-3892.
IUSS Working Group WRB., 2006. World reference base for soil resources 2006. 2nd edition. World Soil Resources Reports No. 103.
Jones, D.L., 1998. Organic acids in the rhizosphere – a critical review. Plant and Soil 205, 25-44.
Khammar, N., Martin, G., Ferro, K., Job, D., Aragno, M., Verrecchia, E., 2009. Use of the frc gene as a molecular marker to characterize oxalate-oxidizing bacterial abundance and diversity structure in soil. Journal of Microbiological Methods 76, 120-127.
Kreutzer, K., 1995. Effects of forest liming on soil processes. Plant and Soil 168-169, 447-470.
Landeweert, R., Hoffland, E., Finlay, R.D., Kuyper, T.W., Van Breemen, N., 2001. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. Trends in Ecology & Evolution 16, 248-254.
Landmann, G., Bonneau, M., 1995. Forest decline and atmospheric deposition effects in the French mountains. Springer-Verlag, Berlin, 461 pp.
Larkin, M., Blackshields, G., Brown, N., Chenna, R., McGettigan, P., McWilliam, H., Valentin, F., Wallace, I., Wilm, A., Lopez, R., Thompson, J., Gibson, T., Higgins, D., 2007. Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) 23, 2948, 2947.
Lu, Y., Rosencrantz, D., Liesack, W., Conrad, R., 2006. Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. Environmental Microbiology 8, 1351–1360.
Lundström, U.S., Bain, D.C., Taylor, A.F.S., Van Hees, P.A.W., 2003. Effects of Acidification and its Mitigation with Lime and Wood Ash on Forest Soil Processes: A Review. Water, Air, & Soil Pollution: Focus 3, 5-28.
Morris, S.J., Allen, M.F., 1994. Oxalate-metabolizing microorganisms in sagebrush steppe soil. Biology and Fertility of Soils 18, 255-259.
Neale, S.P., Shah, Z., Adams, W.A., 1997. Changes in microbial biomass and nitrogen turnover in acidic organic soils following liming. Soil Biology and Biochemistry 29, 1463-1474.
Nys, C., Picart, J.F., Dambrine, E., 2004. Restauration de la qualité des sols et des eaux de deux bassins versants situés dans les Vosges gréseuses et les Vosges cristallines. Caractérisation initiale des sites ateliers d'amendement calco magnésien. Bassins versants de Cornimont et Senones. INRA Nancy ONF, Nancy.
Orsini, L., Rémy, J.C., 1976. Utilisation du chlorure de cobaltihexamine pour la détermination simultanée de la capacité d'échange et des bases échangeables des sols. Science du Sol 4, 269-275.
Sahin, N., 2003. Oxalotrophic bacteria. Research in Microbiology 154, 399-407.
Schaberg, P.G., DeHayes, D.H., Hawley, G.J., Strimbeck, G.R., Cumming, J.R., Murakami, P.F., Borer, C.H., 2000. Acid mist and soil Ca and Al alter the mineral nutrition and physiology of red spruce. Tree Physiology 20, 73-85.
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Horn, D.J.V., Weber, C.F., 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Applied and Environmental Microbiology 75, 7537-7541.
Shah, Z., Adamst, W.A., Haven, C.D.V., 1990. Composition and activity of the microbial population in an acidic upland soil and effects of liming. Soil Biology and Biochemistry 22, 257-263.

Singleton, D.R., Furlong, M.A., Rathbun, S.L., Whitman, W.B., 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Applied and Environmental Microbiology 67, 4374-4376.

Stahl, A.D., Amann, R., 1991. Development and application of nucleic acid probes. In:Nucleic acid techniques in bacterial systematics (eds E. Stackebrandt, M. Goodfellow), John Wiley and Sons, New York. p. 205-248.

Stevenson, FJ., 1967. Organic acids in soil. In:Soil Biochemistry, Vol. 1, (eds A.D. McLaren, G.H. Peterson), New York, p. 119-149.

Ström, L., Owen, A.G., Godbold, D.L., Jones, D.L., 2002. Organic acid mediated P mobilization in the rhizosphere and uptake by maize roots. Soil Biology and Biochemistry 34, 703-710.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24, 1596-1599.

Van Hees, P.A.W, Lundström, U.S., Giesler, R., 2000. Low molecular weight organic acids and their Al-complexes in soil solution—composition, distribution and seasonal variation in three podzolized soils. Geoderma 94, 173-200.

Van Hees, P.A.W., Jones, D.L., Godbold, D.L., 2003. Biodegradation of Low Molecular Weight Organic Acids in a Limed Forest Soil. Water, Air, & Soil Pollution: Focus 3, 121-144.

Van Hees, P.A.W., Jones, D.L., Nyberg, L., Holmström, S.J.M., Godbold, D.L., Lundström, U.S., 2005. Modelling low molecular weight organic acid dynamics in forest soils. Soil Biology and Biochemistry 37, 517-531.

Van Praag, H.J., Weissen, F., Dreze, P., Cogneau, M., 1997. Effects of aluminium on calcium and magnesium uptake and translocation by root segments of whole seedlings of Norway spruce (Picea abies Karst.). Plant and Soil 189, 267-273.

Vandenkoornhuyse, P., Mahé, S., Ineson, P., Staddon, P., Ostle, N., Cliquet, J.B., Francez, A.J., Fitter, A.H., Young, J.P.W., 2007. Active root-inhabiting microbes identified by rapid incorporation of plant-derived carbon into RNA. PNAS 104, 16970-16975.

Ward, D.M., Weller, R., Bateson, M.M., 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345, 63-65.

Weisskopf, L., Heller, S., Eberl, L., 2011. Burkholderia Species Are Major Inhabitants of White Lupin Cluster Roots. Applied and Environmental Microbiology 77, 7715-7720.

Westergaard, B., Hansen, H.C.B., 1998. Determination of anions in soil solutions by capillary zone electrophoresis. The Analyst 123, 721-724.

Williams, H.E., Wandzilak, T.R., 1989. Oxalate synthesis, transport and the hyperoxaluric syndromes. Journal of Urology 141, 742-749.

Chapitre IV :

Effets de l'acidification anthropique des cours d'eau de tête de bassin sur la diversité et les activités microbiennes associées à la décomposition des litières

IV.1. Effets de l'acidification anthropique sur la décomposition de la litière et les communautés de décomposeurs associées dans les zones benthiques et hyporhéiques des cours d'eau de tête de bassin

IV.1.1. Effets de l'acidification sur la décomposition des litières dans les zones benthiques et hyporhéiques de cours d'eau forestiers.

Résumé

L'acidification d'origine anthropique est notamment reconnue pour avoir des effets délétères sur la structure et le fonctionnement des cours d'eau de tête de bassin, et particulièrement sur la décomposition des litières de feuilles et les organismes associés à ce processus.

Des études ont mis en évidence le rôle important joué par la zone hyporhéique dans le fonctionnement des cours d'eau forestiers, de grandes quantités de matière organique pouvant être stockées dans ce compartiment qui est en interaction avec le compartiment benthique en surface, par de nombreux échanges d'eau, de matière organique et de nutriments. Ainsi, l'analyse des deux compartiments permettrait d'évaluer, de manière plus intégrée, les effets d'une perturbation sur le fonctionnement des cours d'eau de tête de bassin.

Cette étude visait donc à évaluer les effets de l'acidification sur la décomposition des litières et sur les assemblages associés d'hyphomycètes aquatiques et de macro-invertébrés déchiqueteurs dans les compartiments benthiques et hyporhéiques des cours d'eau.

Durant 70 jours, des sachets de litières ont été disposés dans les deux compartiments de cinq cours d'eau le long d'un gradient d'acidification compris entre un cours d'eau de référence neutre (pH 7,4) et un cours d'eau très acide (pH 4,7). Globalement, les effets de l'acidification suivaient les mêmes tendances dans les deux compartiments, bien que les effets soient moins prononcés dans les zones hyporhéiques. Les taux de décomposition des feuilles étaient plus importants dans les zones benthiques et hyporhéiques du cours d'eau neutre (k = 0,0534 et 0,0068 j^{-1}, respectivement) que dans celles du cours d'eau le plus impacté (k = 0,0055 et 0,0016 j^{-1}, respectivement). La décomposition des litières était ainsi comparativement moins affectée par le gradient d'acidification dans la zone hyporhéique, probablement en raison d'effets plus marqués sur les populations de macro-invertébrés plus abondantes en surface. La biomasse fongique ne semblait pas affectée par l'acidification

contrairement à la diversité d'espèces sporulantes d'hyphomycètes aquatiques, qui était significativement réduite dans les deux compartiments du cours d'eau le plus impacté.

Ces résultats ont montré que les hyphomycètes aquatiques étaient les principaux décomposeurs des feuilles enfouies dans les zones hyporhéiques des cours d'eau, et qu'en dépit des effets délétères de l'acidification sur le processus de décomposition des litières et les communautés de décomposeurs, la subsistance des hyphomycètes aquatiques permettait le maintien des flux de carbone et de nutriments dans les écosystèmes impactés, leur assurant ainsi un potentiel de résilience.

Erratum :

La figure 5 de l'article est erronée, il convient de la remplacer par celle présentée ci-dessous.

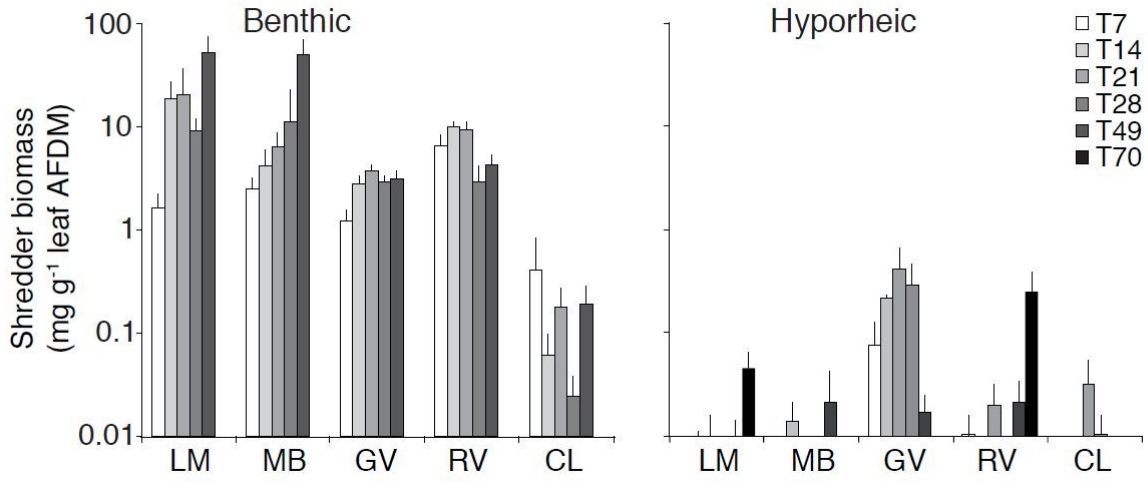

Fig. 5 – Total shredder biomass associated with alder leaves in the two compartments of five streams along an acidification gradient at six incubation dates (mean + SE, n = 4). Note the logarithmic scale. LM: La Maix, MB: Ménombru, GV: Gravelle, RV: Ravines, CL: Courbeligne.

WATER RESEARCH XXX (2012) 1–15

Available online at www.sciencedirect.com

SciVerse ScienceDirect

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

Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams

Julien Cornut [a,b,*], *Hugues Clivot* [c], *Eric Chauvet* [a,b], *Arnaud Elger* [a,b], *Christophe Pagnout* [c], *François Guérold* [c]

[a] *Université de Toulouse, INP, UPS, Laboratoire Ecologie Fonctionnelle et Environnement, UMR CNRS 5245, 118 Route de Narbonne, 31062 Toulouse cedex 9, France*

[b] *CNRS, EcoLab, 31062 Toulouse, France*

[c] *Université de Lorraine, Laboratoire des Interactions Ecotoxicologie, Biodiversité, Ecosystèmes, UMR CNRS 7146, Campus Bridoux, rue du Général Delestraint, F-57070 Metz, France*

ARTICLE INFO

Article history:
Received 13 April 2012
Received in revised form
3 September 2012
Accepted 8 September 2012
Available online xxx

Keywords:
River sediment
Aquatic fungi
Shredders
pH
Organic matter breakdown
Ecosystem functioning

ABSTRACT

Anthropogenic acidification has deleterious effects on both structure and functioning of surface water ecosystems. This study examined how it may affect the leaf decomposition rate and the community structure and activity of decomposers in both benthic and hyporheic zones of five headwater streams along an acidification gradient from highly acidic (pH 4.6) to circumneutral (pH 7.4). Overall, responses to acidification in hyporheic zones were less pronounced, but followed the same pattern as in their benthic counterparts. Leaf decomposition was much faster in the circumneutral stream, both in the hyporheic and benthic zones (k = 0.0068 and 0.0534 d^{-1}, respectively), than in the most acidic one (k = 0.0016 and 0.0055 d^{-1}, respectively), and correlated well with the acidic gradient in both compartments. Interestingly, leaf litter decomposition was less affected by acidification in hyporheic compared to benthic compartments, likely due to the relatively low sensitivity of fungi, which were the main decomposers of buried coarse particulate organic matter. These results argue in favour of conserving hyporheic habitats in acidified streams as they can maintain matter and species fluxes that are essential to the ecosystem.

1. Introduction

Over the last two decades there has been an increased recognition of the role of the hyporheic compartment – *i.e.*, the permeable sediments through which there is significant exchange of water with surface zones – in the ecological functioning of stream systems (Brunke and Gonser, 1997; Boulton et al., 1998; Hancock et al., 2005; Boulton et al., 2010). It is now clearly demonstrated that as water flows through the hyporheic zone, the physical and chemical characteristics prevailing in this latter can affect a wide range of ecological processes, including nutrient (Duff and Triska, 1990; Triska et al., 1993; McKnight et al., 2004) and carbon cycling (Metzler and Smock, 1990; Naegeli et al., 1995; Jones et al.,

* *Corresponding author*. Present address: Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Department Limnology of Stratified Lakes, Alte Fischerhütte 2, 16775 Stechlin-Neuglobsow, Germany. Tel.: +49 (0) 33082 699 69; fax: +49 (0) 33082 699 17.

E-mail addresses: cornut@igb-berlin.de (J. Cornut), hugues.clivot@umail.univ-metz.fr (H. Clivot), chauvet@univ-tlse3.fr (E. Chauvet), arnaud.elger@univ-tlse3.fr (A. Elger), pagnout@univ-metz.fr (C. Pagnout), guerold@univ-metz.fr (F. Guérold).

0043-1354/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.watres.2012.09.023

1995; Cornut et al., 2010), and also influence the behaviour of solutes and organisms (Boulton et al., 1998) such as microbes (Hendricks, 1993; Pusch et al., 1998; Storey et al., 1999; Fischer and Pusch, 2001; Findlay et al., 2003; Cornut et al., 2010) and invertebrates (Godbout and Hynes, 1982; Strommer and Smock, 1989; Palmer et al., 2000; Storey and Williams, 2004).

Acidification of ecosystems resulting from atmospheric pollution is one of the most demonstrative illustrations that human activities can strongly impair terrestrial and aquatic ecosystems even in remote areas. During the three last decades, international efforts aiming at reducing atmospheric emissions have led to a marked decreased of sulphate deposition, both in North America and Europe (EMEP, 1999; Evans et al., 2001; Likens et al., 2001), while nitrate and ammonia remained stable (Cooper, 2005) or decreased (Barrett et al., 2000; Vuorenmaa, 2004) depending on the regions. Despite these abatements, acidification remains a major threat in numerous areas, notably to headwater streams (Alewell et al., 2001; Evans et al., 2001; Baudoin et al., 2008). In addition, acid rain is now being reported from Asia where economies and populations are expanding (Tang et al., 2001; Larssen et al., 2006) leading to an acidification of freshwater ecosystems in the near future.

Acidification has been shown to induce severe deleterious effects on both structure and functioning of surface water ecosystems (Mulholland et al., 1992; Dangles and Guerold, 2001; Dangles et al., 2004; Baudoin et al., 2008; Simon et al., 2009), with low-order streams being particularly impacted. Leaf litter decomposition is a key process driving the flows of energy and nutrients in woodland stream ecosystems (Cummins et al., 1989). Several studies have shown the extent to which this ecological process is depressed by atmospheric acidification (Burton et al., 1985; Chamier, 1987; Groom and Hildrew, 1989; Mulholland et al., 1992; Griffith and Perry, 1993; Dangles and Guerold, 2001; Dangles et al., 2004; Baudoin et al., 2008). However, a substantial portion of leaf litter entering running waters is subject to burial into the streambed as a consequence of flood events (Herbst, 1980) and sediment movement (Metzler and Smock, 1990; Naegeli et al., 1995). For the streams in which both benthic and hyporheic coarse particulate organic matter (CPOM) amounts have been measured, the latter accounts for 25–90% of total stored organic matter (Cummins et al., 1983; Metzler and Smock, 1990). In the same line, a recent study from Cornut et al. (2012) reported that the total CPOM content in the interstitial zone of headwater streams can be up to one order of magnitude higher than that stored at the sediment surface. Under the control of abiotic processes and decomposers' activity, the hyporheic zone may thus act as a sink or a source of detrital organic matter to surface waters. We have recently shown that, compared to the benthic habitat, leaf litter decomposition in the hyporheic zone tends to be slower with the decomposer community being dominated by aquatic fungi (Cornut et al., 2010). The extent to which this major process and the associated biota in the hyporheic zone are affected by an anthropogenic stressor like acidification is however unknown, even though this is essential to encompass both benthic and hyporheic compartments when assessing stream ecosystem impairment and elaborating appropriate management measures in accordance with the Water Framework Directive (Smith, 2005).

The aim of this study was to assess the effects of acidification on the leaf-associated decomposer communities and on leaf litter decomposition according to the location of leaf litter within the streambed (*i.e.*, on the surface or buried). On one hand, burial within the substratum generally reduces the access of decomposers to leaves with the small interstices of the gravelly sediment acting as a physical constraint, especially for the largest shredders. On the other hand, numerous studies have emphasized the deleterious effects of freshwater acidification on macroinvertebrate communities. In particular the high sensitivity of numerous macroinvertebrate species including efficient shredders such as gammaridae, has been repeatedly demonstrated in North America and Europe, while Trichoptera showed a more varied response to acidification (Guérold et al., 2000). In contrast, based on numerous studies that have investigated fungal growth, richness and activity of aquatic hyphomycete species within a relatively broad pH region, Krauss et al. (2011) in their review concluded that low pH values *per se* do not appear to be harmful to aquatic hyphomycetes. Therefore, if shredders are more sensitive to acidity compared to aquatic fungi, the impact of acidification will be more pronounced in the benthic than in the hyporheic zone. As a consequence, we expected the discrepancy in decomposition rates between the two stream compartments to be lowest at the lower end and highest at the higher end of the pH range. We tested these hypotheses by conducting an experimental study where leaf-associated microbial and macroinvertebrate communities, as well as leaf decomposition rates and the activity of microbial decomposers, were compared in the benthic and hyporheic zones of five low-order streams along a pronounced acidification gradient.

2. Materials and methods

2.1. Study sites

The experiment was conducted in the Vosges Mountains (North-eastern France) where anthropogenic deposition of acidifying substances has adversely affected surface waters during the last decades (Dambrine et al., 1998; Probst et al., 1999). Such impacts of acidification have been observed in many areas of the world since the end of the 19th century, due to long-range atmospheric transport and subsequent deposition of acidifying sulphur and nitrogen compounds (Schindler, 1988; Driscoll et al., 2001). Forestry was the only other potential anthropogenic disturbance, although limited within the study area. Five first and second-order streams were selected along an acidification gradient. They differed in pH and total Al concentration, but exhibited similar hydrological and morphological characteristics. Courbeligne (CL, 48°26′24″N; 7°03′54″E) was the most acidified stream. Ravines (RV, 48°24′47″N; 6°55′22″E) and Gravelle (GV, 48°16′22″N; 6°49′17″E) were two moderately acidic streams, differing by their concentrations in total Al and NO_3^-, and acid neutralising capacities (ANC). Two non-acidified streams, Ménombru (MB, 48°29′05″N; 7°02′48″E) and La Maix (LM, 48°29′00″N; 7°04′13″E), showed a circumneutral pH. These two streams exhibited rather similar physical and chemical characteristics, but strongly differed regarding their ANC and total Al

Please cite this article in press as: Cornut, J., et al., Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams, Water Research (2012), http://dx.doi.org/10.1016/j.watres.2012.09.023

concentrations (448 *vs.* 204 μEq/L and 26 *vs.* 93 μg/L, respectively; Table 1). Vegetation in the selected catchments was dominated by silver fir *Abies alba* Mill., Norway spruce *Picea abies* L. and beech *Fagus sylvatica* L. Beech and alder (*Alnus glutinosa* (L.) Gaertn.) were by far the most common deciduous tree species adjacent to streams. The five streams had similar substratum, which was unconsolidated and mostly made of gravel and coarse/fine sand sediments.

2.2. Stream physico-chemistry

Water chemistry was determined on the seven dates when leaf bags were introduced into or retrieved from the streams. Samples of surface and interstitial water were collected from each stream, stored in pre-rinsed polyethylene bottles, and placed in an icebox until they were returned to the laboratory to be analysed within 48 h. Interstitial water was pumped from plexiglas minipiezometers using a hand-held vacuum pump. Stream pH was measured in the laboratory using a microprocessor pH meter (pH 3000, WTW), and acid-neutralizing capacity (ANC) was determined by Gran's titration. Conductivity was measured with a Metrohm Herisau Conductometer E518 (Herisau, Switzerland) at 25 °C. Concentrations of Ca^{2+} and total Al (after acidification with HNO_3) were determined by atomic absorption spectrophotometry (Aanalyst 100; Perkin Elmer and Varian SpectrAA-300) and concentrations of NO_3^- by ion chromatography (Dionex 1500i with a AS 4 A SC column; Sunnyvale, USA). During the leaf litter decomposition study, the temperature of surface and interstitial waters was recorded every 4 h (SmartButton, ACR System Inc., BC, Canada).

2.3. Leaf decomposition

We selected alder as one of the commonest riparian tree species across stream sites. In October 2008, leaves were collected from trees at abscission and air-dried for 15 days at 20–22 °C. Leaf bags consisted of 3.00 g (mean air-dry mass ± 0.03 g) of leaves enclosed in plastic net bags (15 × 10 cm, 5 mm mesh) to simulate natural accumulations of leaf detritus in the stream. Before being enclosed into bags, the leaves were moistened with distilled water from a vaporizer to prevent breakage during handling and transport. A total of 48 leaf bags per stream were introduced at the head of riffles (downwelling zones) on 18th November 2008. The upstream end of riffles was chosen i) to circumvent the local spatial variability in surface and subsurface hydrology within riffle scale affecting leaf litter breakdown rates and ii) because it represents a downwelling area (infiltration of surface water into the hyporheic zone, Hendricks and White, 1991) fed with oxygen and nutrients, resulting in a higher breakdown rate (Tillman et al., 2003). Leaf bags were subject to two treatments: benthic and hyporheic (*i.e.*, buried in the sediment). Benthic leaf bags were placed on the sediment surface and secured to the bank with plastic-coated wire, which was anchored to the stream bottom with large boulders. Hyporheic leaf bags were embedded approximately 15–20 cm below the sediment surface using a small shovel, with a coloured plastic wire attached to facilitate localization and retrieval (Cornut et al., 2010). Four replicate bags were randomly retrieved from each zone of the five streams after 7, 14, 21, 28, 49, and 70 days of exposure, immediately placed and stored individually in plastic zip-lock bags with stream water and transported to the laboratory in a cool box. During leaf bag sampling, a Surber net (500 μm mesh size) was used to minimize invertebrate loss due to passive or active drift. In the laboratory, leaves were washed individually with water from the respective stream to remove sediments, exogenous organic matter and macroinvertebrates, which were collected in a 500 μm screen sieve and then preserved in 70% ethanol until processing. One set of five 12 mm diameter discs and another of ten were cut from leaves of each bag retrieved, avoiding the central vein. The set of five leaf discs was promptly frozen at −20 °C until processing for ergosterol extraction, and the set of ten was immediately used for incubation in aerated microcosms (see below). The remaining leaf litter was dried at 105 °C to

Table 1 – Mean values of the physico-chemical parameters of water of the five streams from November 2008 to January 2009 (n = 7). Minimum and maximum values are given in brackets. DO = dissolved oxygen; ANC = acid neutralizing capacity.

	Streams	LM	MB	GV	RV	CL
pH	Benthic	7.4 (7.3/7.4)	7.0 (6.8/7.2)	6.4 (6.2/6.5)	6.1 (5.9/6.3)	4.6 (4.5/4.6)
	Hyporheic	7.4 (7.3/7.6)	6.9 (6.6/7.1)	6.4 (6.3/6.5)	6.3 (6.2/6.7)	4.7 (4.5/5.1)
Temperature (°C)	Benthic	6.0 (4.7/7.0)	5.8 (4.3/7.3)	6.6 (5.3/7.6)	5.6 (3.8/6.8)	4.2 (2.0/5.7)
	Hyporheic	5.9 (4.7/6.9)	5.8 (4.5/7.1)	6.6 (5.3/7.8)	5.5 (3.8/6.7)	4.2 (2.4/5.9)
[DO] (mg/L)	Benthic	11.8 (11.3/12.4)	11.8 (10.1/12.5)	11.3 (10.8/11.8)	12.1 (11.7/12.8)	12.0 (10.7/12.9)
	Hyporheic	10.3 (9.4/10.9)	10.1 (9.2/11.1)	9.9 (9.4/10.4)	10.2 (9.9/10.6)	11.0 (10.1/12.4)
Conductivity (μS/cm)	Benthic	71.3 (60.0/76.0)	52.7 (41.5/60.5)	35.5 (34.9/36.0)	37.0 (36.5/37.8)	33.8 (30.9/35.5)
	Hyporheic	75.9 (62.5/83.0)	61.7 (48.5/75.5)	36.2 (35.0/37.0)	39.0 (37.5/40.0)	33.4 (30.0/35.5)
ANC (μEq/L)	Benthic	448 (348/512)	204 (113/286)	53 (44/63)	25 (15/33)	−27 (−32/−13)
	Hyporheic	486 (372/548)	302 (198/509)	60 (48/72)	43 (31/76)	−19 (−33/−3)
[Total Al] (μg/L)	Benthic	26 (11/67)	93 (72/110)	32 (23/53)	105 (44/310)	697 (630/844)
	Hyporheic	140 (29/237)	249 (145/427)	67 (32/119)	360 (70/868)	682 (144/1962)
[Ca^{2+}] (mg/L)	Benthic	6.7 (5.8/8.4)	4.0 (3.0/4.7)	2.4 (2.3/2.5)	2.2 (2.2/2.3)	1.2 (1.0/1.5)
	Hyporheic	7.4 (5.8/8.4)	5.4 (4.0/7.4)	2.4 (2.3/2.6)	2.4 (2.2/2.6)	1.4 (1.2/1.6)
[NO_3^-](mg/L)	Benthic	3.2 (3.0/3.4)	2.8 (2.5/2.9)	1.6 (1.6/1.7)	2.6 (2.5/2.8)	4.4 (4.1/4.7)
	Hyporheic	3.2 (3.0/3.4)	2.3 (1.0/2.8)	1.6 (1.5/1.6)	2.6 (2.5/2.7)	4.4 (4.2/4.7)

constant mass and weighed to the nearest 0.01 g. The leaf material was ground using a micro hammer mill (Culatti, Zurich, Switzerland) with a 0.5 mm mesh. Portions of leaf material of about 500 mg were ashed at 550 °C for 4 h in a muffle furnace and weighed to determine the organic matter content. The leaf mass remaining in each bag was determined by adding the mass of oven-dried litter to those of leaf discs (see below). The leaf mass remaining was expressed as the ratio of the ash-free dry mass (AFDM) between the final and initial leaf litter. Four unexposed batches of leaf litter were used to determine the initial AFDM and oven-dried mass to air-dried mass ratio of leaves according to the procedures above.

2.4. Fungal diversity

Once cut, the batches of ten fresh leaf discs were quickly placed in aerated microcosms (Suberkropp, 1991) filled with 40 mL filtered (Glass fibre GF/F, Whatman) water from the respective stream and incubated at 10 °C. After 48 h, a 20 mL aliquot of spore suspension from each microcosm was transferred into a 30 mL glass tube and preserved with 1.5 mL of 37% formalin. The leaf discs were then lyophilized and weighed to the nearest 0.1 mg. 0.5 mL of Triton X-100 (0.5% solution) was added to each spore suspension, which was stirred gently to ensure uniform distribution of spores. A 5 mL aliquot was then filtered through a membrane filter (5 μm pore size, diameter 25 mm; Millipore Corporation, Bedford, MA, USA), and the spores on the filter were stained with 0.1% Trypan blue in 60% lactic acid (Iqbal and Webster, 1973). Spores were counted and identified under the microscope (×200).

2.5. Fungal biomass

Mycelial biomass in leaves was assessed through the content in ergosterol (Gessner and Chauvet, 1993). Leaf material was lyophilised and weighed to the nearest 0.1 mg, and then lipids were extracted with alkaline methanol heated at 80 °C for 30 min. Extracts were purified using solid-phase extraction cartridges (Oasis HLB, 60 mg, 3 cc, Waters, Milford, MA, USA) and ergosterol was quantified by high-performance liquid chromatography (procedure slightly modified from Gessner (2005)). The ergosterol amount was corrected for the extraction efficiency (87–100%), which was measured for each sample series on controls to which known amounts of ergosterol were added. Ergosterol was converted into fungal biomass using a conversion factor of 5.5 mg ergosterol g^{-1} fungal dry mass (Gessner and Chauvet, 1993).

2.6. Macroinvertebrates

Macroinvertebrates retained over a 500-μm mesh sieve were counted and identified to the lowest practicable level. Identification was to the genus/species level whenever possible, except for Oligochaeta and Diptera (family and sub-family or tribe, respectively), and individuals were assigned to shredders, grazers or others (Tachet et al., 2000). For each leaf sample, the biomass of macroinvertebrates assigned to the shredder group was determined to the nearest 0.01 mg after drying to constant mass at 60 °C.

2.7. Fine particulate organic matter production

After removal of the 20 mL aliquot for spore production and fungal diversity determinations (see above), the contents of the microcosms were filtered through a 1-mm mesh screen to retain coarse particles. The 20 mL remaining were then filtered through a membrane filter (0.45 μm pore size, diameter 25 mm; Millipore Corporation), washed three times beforehand with 10 mL of pure water, dried at 80 °C and preweighed to the nearest μg. The fine particulate organic matter (FPOM) release due to microbial activity was determined to the nearest μg after drying to constant mass at 80 °C.

2.8. Statistical analysis

Principal components analysis (PCA) was carried out to ordinate streams with respect to physical and chemical variables. PCA was performed using the following variables: pH, ANC, conductivity, total Al, DO, Ca^{2+} and NO_3^-. Leaf decomposition rates (k) for each treatment were estimated by using the exponential decay model, $M_t = M_o \cdot e^{-kt}$, where M_o is the initial AFDM, M_t is the AFDM remaining at time t, and t is the time in days (Petersen and Cummins, 1974). The k values were determined from linear regression of the log-transformed relationship. An analysis of covariance (ANCOVA) was used to compare k values among the five streams and the two exposure treatments, followed by a multiple-comparison (Tukey HSD test). A three-way factorial ANOVA was used to assess differences in microbial and macroinvertebrate parameters with stream, treatment (*i.e.*, benthic or hyporheic) and exposure time as the main effects. Physicochemical differences among streams and between the hyporheic and benthic zone of streams, and the potential interactions between these factors were examined using a two-way factorial ANOVA, with stream and treatment as categorical predictors. Data were log transformed to improve homoscedasticity when necessary. When significant differences were detected between treatments, Tukey HSD tests were carried out for post hoc pairwise comparisons.

The Simpson's dominance index and the Simpson's diversity index were computed for fungal communities associated with decomposing leaves from each compartment of the five streams. Non-metric multidimensional scaling (NMDS) analyses of sporulation data and shredder abundances were used to assess differences in species composition among sites. This ordination method is a robust procedure for analysing ecological data (Minchin, 1987). The Bray–Curtis coefficient was used to quantify the dissimilarity among sites based on joint occurrence and abundance of taxa. NMDS attempts to maximise the fit between measured dissimilarities and distance between resulting data points within a predefined number of spatial dimensions (Legendre and Legendre, 1998; Legendre and Marti, 1999). Stress function for each NMDS plot indicates the fitness of representation of differences among sites. Stress values range from 0 to 1, with values close to zero indicating a good fit. Axes from the NMDS analysis were correlated (Spearman rank correlation) with

Please cite this article in press as: Cornut, J., et al., Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams, Water Research (2012), http://dx.doi.org/10.1016/j.watres.2012.09.023

physical and chemical data to identify variables most strongly corresponding to among-site differences in aquatic hyphomycete and shredder assemblages (Hawkins et al., 1997; Baudoin et al., 2008). PCA was performed using R software 2.6.0 (R development Core Team, 2007). NMDS analysis was performed with PRIMER 6 (Clarke and Gorley, 2001). STATISTICA 6.0 (StatSoft Inc., 2001) was used for all other statistical analyses. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Physico-chemical conditions in benthic and hyporheic zones

The five headwater streams ranged from circumneutral (mean pH = 7.4, in both benthic and hyporheic zones of LM) to strongly acidified (mean pH = 4.6 and 4.7 in CL benthic and hyporheic zones, respectively). The factorial plane defined by axes 1 and 3 of the PCA explained 72.2% of the total variance (Fig. 1). The first axis of the PCA was primarily defined by the ANC, Ca^{2+}, pH and conductivity (Fig. 1a). It explained 57.5% of the total variance and strongly separated the five streams along the acidification gradient (Fig. 1b). An additional 14.7% of the total variance was explained by the third axis, which discriminated among benthic and hyporheic zones of streams. Hyporheic zones of the five streams were mainly characterized by lower dissolved oxygen concentrations than their benthic counterparts. Although, the second axis (mainly defined by NO_3^-) explained 21.6% of the total variance in the data set, the first and third axes together discriminated much better the variation among the streams and between benthic vs. hyporheic zones. The second axis was actually mostly influenced by CL, the stream showing the highest NO_3^- concentration. All five streams significantly varied in acidity ($F_{4,60} = 756.8$, $P < 10^{-3}$ and HSD test) and each stream exhibited quite similar values in benthic and hyporheic zones (mean pH range = 4.6–7.4 and 4.7–7.4, respectively), except RV whose pH was slightly higher in hyporheic than in benthic zone ($F_{4,60} = 2.6$, $P = 0.04$ and HSD test; Table 1). Not surprisingly, the most acidified stream (CL) and the two moderately acidic streams (RV and GV) presented typical chemical patterns of acid-stressed headwater streams, *i.e.*, low conductivity, negative or low positive ANC and low Ca^{2+} concentration ($F_{4,60} = 229.30$, $P < 10^{-3}$; $F_{4,60} = 287.68$, $P < 10^{-3}$; $F_{4,60} = 3011.11$, $P < 10^{-3}$ and HSD test, respectively). As a result of high acid deposition in the region during the last decades, total Al concentration strongly varied between streams ($F_{4,60} = 19.70$, $P < 10^{-3}$) and showed a trend, although non-significant, of being higher in the hyporheic zone than in the benthic one ($F_{4,60} = 0.82$, $P = 0.52$; Table 1). The concentration in atmospheric-derived nitrates differed greatly among streams ($F_{4,60} = 259.88$, $P < 10^{-3}$ and HSD test) with the most acidic stream exhibiting higher concentrations (Table 1), but was quite similar between the benthic and hyporheic zones ($F_{4,60} = 2.40$, $P = 0.06$). Water temperature was not different between the two stream compartments ($F_{4,60} = 0.024$, $P = 0.99$), but significantly varied among streams mainly due to the lower temperature of the most acidic stream (CL; $F_{4,60} = 14.19$, $P < 10^{-3}$ and HSD test; Table 1). The five streams were always well oxygenated throughout the experiment and presented similar values of dissolved oxygen ($F_{4,60} = 1.66$, $P = 0.17$) while these latter were significantly lower in the hyporheic than in the benthic zones, showing a reduction by ca. 12% ($F_{1,60} = 132.28$, $P < 10^{-3}$; Table 1).

3.2. Litter decomposition

In both benthic and hyporheic zones, a significant decrease in leaf decomposition rates was observed under acidic conditions. After 10 weeks, the percentage of remaining AFDM in the benthic zone was less than 2% in the circumneutral LM and MB, while it was more than 30% in the GV and RV and 60%

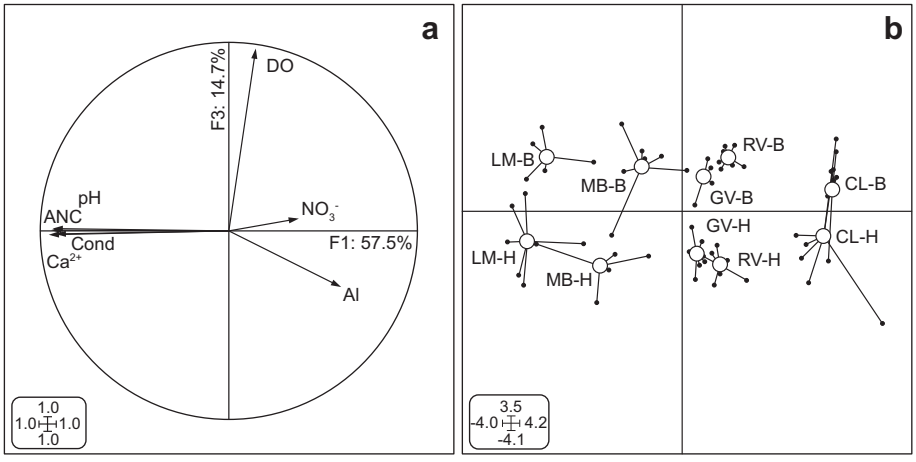

Fig. 1 – PCA of the physico-chemical variables in the benthic and hyporheic zones of five streams along an acidification gradient. LM: La Maix, MB: Ménombru, GV: Gravelle, RV: Ravines, CL: Courbeline. a Correlation circle on the F1 × F3 factorial plane. b Ordination of benthic (B) and hyporheic (H) zones of five streams on the F1 × F3 factorial plane. Small filled circles represent the sample position at each sampling date. Open circles denote the weighted average of all samples taken from a given zone in a given stream. Lines link samples to their weighted average.

in the most acidic stream CL. As a result, leaf decomposition rates at the sediment surface in LM and MB were significantly higher than in the other streams ($F_{9,229} = 74.08$, $P < 10^{-3}$ and HSD test, all comparisons, $P < 10^{-3}$).

The differences among streams in the hyporheic zone were less pronounced (Fig. 2), with percentages of remaining AFDM varying between 55 and 79% for the circumneutral LM and the most acidic CL streams, respectively. As a consequence, the ratio of circumneutral-to-acidic decomposition rates (LM:CL) for the hyporheic zone was 4.34 while it was 9.76 in the benthic counterpart.

Since water temperature of the most acidic stream was significantly lower than the four other ones, leaf litter decomposition rates were normalized for temperature by referring to degree-days (sum of daily mean water temperatures over the decomposition time) instead of days. Leaf litter decomposition rate patterns among streams and compartments however remained unchanged ($F_{9,229} = 75.89$, $P < 10^{-3}$ and HSD test, all comparisons, $P < 10^{-3}$) demonstrating the pre-eminence of the acidic stress over temperature effect.

3.3. *Fungal diversity and biomass*

A total of 34 sporulating species of aquatic hyphomycetes was detected during leaf decomposition (Table 2). The highest fungal diversity (cumulated number of species from benthic and hyporheic zones) was found in the circumneutral stream LM (24 species) and the lowest (15 species) in the two most acidic ones, RV and CL. Whatever the stream, the fungal species richness associated with decomposing leaf-litter was always lower in the hyporheic than in the benthic zone (Table 2). This difference appeared relatively small for the circumneutral stream (*i.e.*, 18 and 17 species for the benthic and hyporheic zones, respectively). In contrast, the leaf-associated species richness from the hyporheic zone of the most acidic stream was less than half of that from the benthic zone (6 *vs.* 13 species). Throughout the experiment, the most striking difference between aquatic hyphomycete assemblages from decomposing leaves was the dominance of *Flagellospora curvula* in the most acidic stream CL, both in the benthic and hyporheic zones (accounting for 96% and 81% of the pool of conidia produced, respectively). In contrast, four to six species (*i.e.*, *F. curvula*, *Heliscus lugdunensis*, *Tetrachaetum elegans*, *Lemonniera cornuta*, *Alatospora pulchella*, *Alatospora acuminata*) were codominant on leaves from the circumneutral stream LM in both the hyporheic and benthic zones. The NMDS ordination highlighted the overall differences in aquatic hyphomycete assemblages between the five streams and the two compartments (Fig. 3). It revealed marked differences in species assemblages along the acidification gradient: Axis 2 strongly correlated with pH ($r = 0.83$, $P = 0.001$). The position along this axis reflected the acidification status of the streams and distinctly separated the acidic streams with lower fungal community richness and evenness from circumneutral streams (Table 2). The NMDS ordination also separated well aquatic hyphomycete assemblages from the benthic and hyporheic zones, with Axis 1 correlating with the concentration in dissolved oxygen ($r = 0.80$, $P = 0.003$).

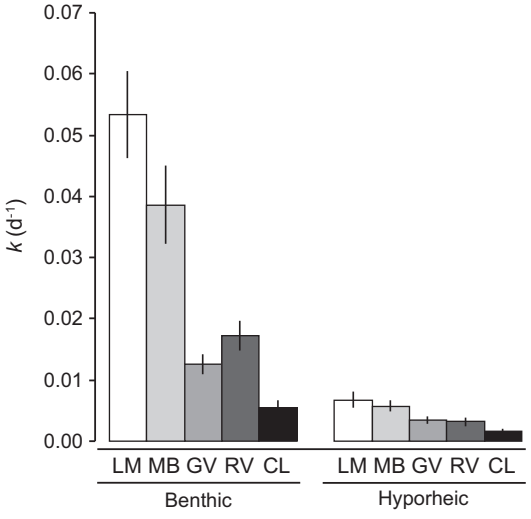

Fig. 2 – Leaf decomposition rate in the benthic and hyporheic zones of five streams along an acidification gradient (mean ± SE, $n = 24$). LM: La Maix, MB: Ménombru, GV: Gravelle, RV: Ravines, CL: Courbeline.

Freshly collected leaves contained minute amounts of ergosterol, indicating that fungal colonization was negligible at the beginning of the experiment. Fig. 4 shows that fungal biomass associated with decomposing leaves at the sediment surface increased rapidly within the first 7 weeks, except for the circumneutral stream LM, which reached a maximum of 28 mg g^{-1} AFDM after 3 weeks, then rapidly declined. Leaves exposed at the sediment surface in the most acidic stream CL differed from this general pattern in that mycelial biomass continuously increased to reach a maximum of 91 mg g^{-1} AFDM after 10 weeks, whereas leaves in the four other streams were decomposed almost entirely before the last sampling date. Consequently, biomass associated with decomposing leaves in these streams was determined from the first five sampling dates for MB, GV and RV and from the first four sampling dates only for LM (Fig. 4).

The mycelial biomass showed no particular trend regarding the acidification gradient, except that maxima were higher in the most acidic stream, both in benthic and hyporheic zones. The maximum reached during the course of the experiment differed significantly between streams in the benthic ($F_{4,12} = 5.38$, $P < 10^{-3}$) and hyporheic zones ($F_{4,15} = 7.99$, $P < 10^{-3}$). The mycelial biomass from the first four sampling dates differed significantly between compartments ($F_{1,149} = 25.12$, $P < 10^{-3}$) and among streams ($F_{4,149} = 2.60$, $P < 10^{-3}$). The maximum biomass on leaves exposed in the hyporheic zone, except for the most acidic stream CL, was delayed by 3–7 weeks in comparison with the benthic zone. Surprisingly, the most acidic stream CL, exhibiting also the highest total Al concentrations both in benthic and hyporheic zones (means of 697 and 682 μg/L, respectively), showed similar or higher fungal biomass compared to the other streams.

3.4. *Shredders*

The shredder biomass differed significantly among streams ($F_{4,210} = 5.58$, $P < 10^{-3}$) and between location within the

Table 2 – Relative abundance (%) of the leaf-associated aquatic hyphomycete species for the two compartments of the five streams, determined from the cumulative conidial production over the six sampling dates.

Species	Benthic					Hyporheic				
	LM	MB	GV	RV	CL	LM	MB	GV	RV	CL
Alatospora acuminata Ingold	1.66	0.05	–	–	0.29	2.59	–	–	0.02	–
Alatospora flagellata (Gönczöl) Marvanová	–	–	–	–	0.10	0.13	–	–	–	–
Alatospora pulchella Marvanová	2.48	0.03	–	–	–	0.67	–	–	–	–
Anguillospora crassa Ingold	–	–	0.06	–	–	–	–	–	–	–
Anguillospora filiformis Greathead	–	<0.01	0.31	0.37	0.71	0.20	–	6.18	0.07	2.06
Anguillospora furtiva Descals & Marvanová	–	–	0.06	–	–	–	–	–	–	0.02
Anguillospora longissima (Sacc. & Syd.) Ingold	0.30	–	–	–	0.06	–	–	–	0.02	0.45
Anguillospora rosea Descals & Marvanová	0.03	0.07	0.75	0.02	0.09	–	0.01	–	–	–
Articulospora tetracladia Ingold	0.87	0.12	1.71	0.15	0.07	0.10	10.34	0.55	1.58	16.34
Clavariopsis aquatica De Wild.	0.90	0.23	0.10	–	–	–	–	–	–	–
Clavatospora longibrachiata (Ingold) Marvanová & Nilsson	0.12	9.41	2.02	0.28	0.90	0.38	2.44	0.58	0.05	–
Culicidospora aquatica Petersen	0.33	–	0.06	–	–	–	–	0.17	–	–
Flagellospora curvula Ingold	38.08	59.68	28.20	95.74	96.24	66.30	77.17	50.45	76.79	81.12
Fontanospora eccentrica (Petersen) Dyko	0.22	<0.01	0.03	–	1.12	–	–	–	–	–
Fusarium like	0.02	–	–	0.13	–	3.13	–	–	7.29	–
Heliscella stellata (Ingold & Cox) Marvanová & Nilsson	–	0.11	–	–	–	–	0.03	–	–	–
Heliscus lugdunensis Sacc. & Thérry	7.98	1.14	0.20	0.22	0.02	21.30	6.03	35.61	10.81	–
Lemonniera aquatica De Wild.	0.02	0.80	25.76	1.24	0.33	–	0.24	0.54	0.69	–
Lemonniera centrosphaera Marvanová	–	0.03	0.23	–	–	–	–	–	–	–
Lemonniera cornuta Ranzoni	2.44	0.75	1.39	–	–	0.39	0.23	–	–	–
Lemonniera terrestris Tubaki	0.41	0.09	3.33	0.43	–	–	–	–	–	–
Mycocentrospora sp. (cf. *angulata* (Petersen) Iqbal)	–	–	–	–	–	–	0.10	–	–	–
Stenocladiella neglecta Marv. & Descals	0.06	–	0.08	–	–	0.44	–	–	–	–
Tetrachaetum elegans Ingold	43.93	27.38	35.23	1.32	–	3.26	3.00	5.15	2.65	<0.01
Tetracladium marchalianum De Wild.	–	–	–	–	–	0.26	–	–	–	–
Tetracladium furcatum Descals	–	–	–	–	0.03	–	–	0.39	–	–
Tricellula aquatica Webster	–	–	–	0.01	0.03	–	–	–	–	–
Tricladium chaetocladium Ingold	0.15	0.10	0.49	0.09	–	0.09	0.06	–	–	–
Tripospermum camelopardus Ingold, Dann & McDougall	–	–	–	–	–	–	0.16	–	0.03	–
Tripospermum myrti (Lind.) Hughes	–	–	–	–	–	–	–	0.39	–	–
Tumularia aquatica (Ingold) Marvanová & Descals	–	–	–	–	–	0.04	–	–	–	–
Tumularia tuberculata (Gönczöl) Descals & Marvanová	–	0.01	–	–	–	–	0.20	–	–	–
Sigmoid (<60 μm)	–	–	–	–	–	0.62	–	–	–	–
Sigmoid (60–120 μm)	–	–	–	–	–	0.08	–	–	–	–
Total number of species	18	18	18	12	13	17	13	10	11	6
Simpson's diversity index	0.70	0.57	0.69	0.03	0.00	0.49	0.26	0.51	0.56	0.01
Simpson's dominance index	0.30	0.43	0.31	0.97	1.00	0.51	0.74	0.49	0.44	0.99

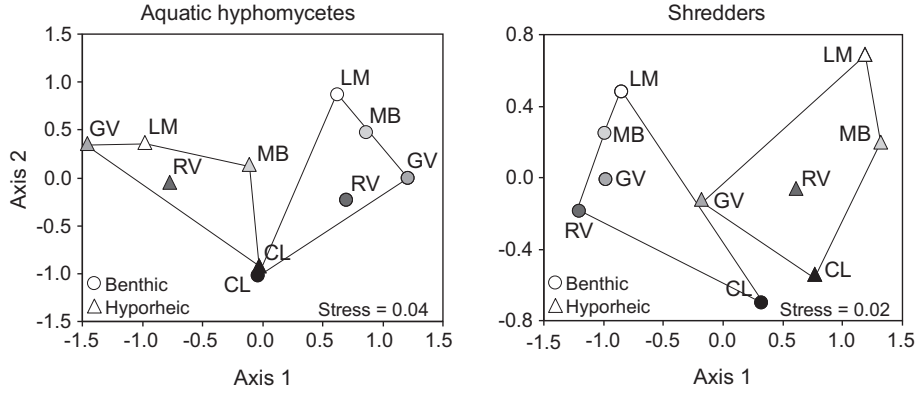

Fig. 3 – NMDS plot of sites based on (a) aquatic hyphomycete and (b) shredder species associated with leaves decomposing in the benthic and hyporheic zones of five streams along an acidification gradient. LM: La Maix, MB: Ménombru, GV: Gravelle, RV: Ravines, CL: Courbeline.

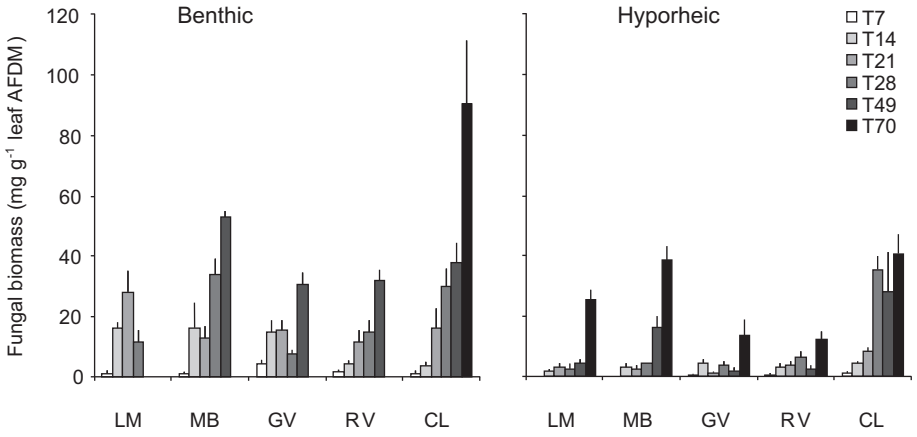

Fig. 4 – Fungal biomass associated with leaves decomposing in the benthic and hyporheic zones of five streams along an acidification gradient at six incubation dates (mean + SE, $n = 4$). LM: La Maix, MB: Ménombru, GV: Gravelle, RV: Ravines, CL: Courbeline.

stream ($F_{1,210} = 34.74$, $P < 10^{-3}$). The highest biomasses on benthic leaves were found in the two circumneutral streams LM (mean of 20.2 mg g^{-1} AFDM) and MB (15.0 mg g^{-1} AFDM), where they differed significantly from their hyporheic counterpart (0.004 and 0.007 mg g^{-1}, respectively, Fig. 5). A considerable reduction in shredder biomass on benthic leaves was observed along the acidification gradient, with values from the two circumneutral streams LM and MB being significantly higher than in the three acidic streams (CL: 0.145, RV: 7.060 and GV: 2.297 mg g^{-1} AFDM, Fig. 5). In the hyporheic zone, shredder biomass was similar among streams and mostly dominated by *Leuctra* spp. and *Amphinemura sulcicollis* (Fig. 5 and Table 3). The peaks in shredder biomass observed on benthic leaves at T49 only occurred in the two circumneutral streams LM and MB and were mostly attributable to individuals of *Gammarus fossarum* and Trichoptera (*Sericostoma personatum*, *Potamophylax* sp. and unidentified Limnephilinae), *i.e.*, the largest dominant shredder taxa in the stream (Fig. 5 and Table 3). However, these taxa were generally found at low densities. In acidic streams GV and RV, shredders were represented by stoneflies, mainly *Leuctra* spp., *A. sulcicollis*, *Protonemura* sp. and *Nemoura* sp., often in abundance (Table 3). In the most acidic stream CL, shredder assemblages in both benthic and hyporheic zones were dominated, in biomass and numbers, by *Leuctra* spp (Table 3). Shredder assemblages among the five streams were well discriminated on Axis 2 of the NMDS analysis (Fig. 3), which was correlated with pH ($r = 0.75$, $P = 0.007$). Similarly, shredder assemblages associated with leaves from the benthic and hyporheic zones were distinguished along Axis 1, which was well correlated with the concentration in dissolved oxygen ($r = -0.79$, $P = 0.003$). The discrimination between macroinvertebrate communities along the acidification gradient held for both benthic and hyporheic zones of the streams. In particular, communities from benthic and hyporheic zones were found to converge in the most acidic stream whereas they diverged the most in the two circumneutral streams. This overall pattern was remarkably similar to that of fungal assemblages (Fig. 3).

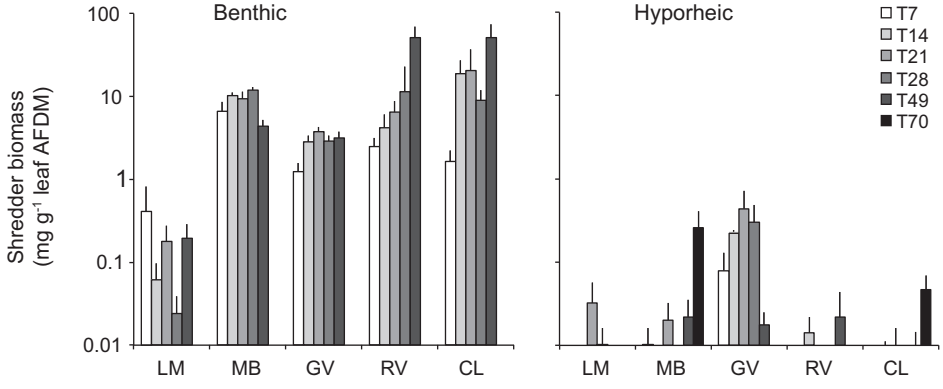

Fig. 5 – Total shredder biomass associated with alder leaves in the two compartments of five streams along an acidification gradient at six incubation dates (mean + SE, $n = 4$). Note the logarithmic scale. LM: La Maix, MB: Ménombru, GV: Gravelle, RV: Ravines, CL: Courbeline.

Table 3 – Relative occurrence (%) of shredder genera associated with alder leaves in the two compartments of the five streams (all sampling dates and replicates combined).

	Benthic					Hyporheic				
	LM	MB	GV	RV	CL	LM	MB	GV	RV	CL
Average per leaf bag per date	13.3	28.0	51.4	315.1	1.8	0.4	0.3	7.2	1.5	0.7
Plecoptera										
Amphinemura	0.9	3.4	53.0	44.1	2.3	11.1	37.5	39.0	65.7	–
Capnia	–	–	–	–	4.5	–	–	–	–	–
Leuctra	19.7	23.4	22.5	39.3	77.3	22.2	25.0	54.1	28.6	94.1
Nemoura	0.3	0.6	1.5	0.7	4.5	–	–	–	–	–
Protonemura	39.7	36.8	14.9	15.1	9.1	–	12.5	1.2	2.9	5.9
Trichoptera										
Adicella	–	–	0.1	–	–	–	–	–	–	–
Crunoecia	0.3	0.4	–	–	–	–	–	–	–	–
Limnephilinae	2.8	2.2	6.1	0.7	–	–	–	–	–	–
Potamophylax	1.6	1.8	0.2	0.1	2.3	–	–	–	–	–
Sericostoma	1.3	1.5	0.1	–	–	44.4	25.0	2.9	2.9	–
Indetermined	–	–	–	–	–	–	–	0.6	–	–
Diptera										
Tipula	0.3	–	–	–	–	–	–	–	–	–
Amphipoda										
Gammarus	33.1	29.8	1.6	–	–	22.2	–	1.7	–	–

3.5. *Microbial FPOM*

The temporal pattern of microbial FPOM production from decomposing leaves was comparable across streams and treatments, however with contrasted dynamics (Fig. 6). Overall, the release of FPOM by microbial activity increased significantly with time ($F_{4,150} = 315.42$, $P < 10^{-3}$) and decreased in a consistent trend along the acidification gradient ($F_{4,150} = 116.65$, $P < 10^{-3}$) in both compartments ($F_{4,150} = 4.13$, $P < 10^{-3}$). At any date and for any stream, the FPOM release in the benthic zone exceeded that from the hyporheic zone. The mean FPOM production in the benthic zone varied from 11.0 to 50.2 mg g^{-1} litter d^{-1} in the most acidic stream CL and the circumneutral one LM, respectively, and from 8.5 to 42.3 mg g^{-1} litter d^{-1} for the hyporheic zone of the same streams.

4. Discussion

4.1. *Acidic stress and ecosystem function in benthic and hyporheic zones*

To our knowledge, the present study provides the first comparative data set on benthic and hyporheic leaf-decomposer communities and litter decomposition in streams affected by anthropogenic acidification. This study presents evidence that the anthropogenic acidification reduced decomposition of leaves both in benthic and hyporheic zones of headwater streams. However, the overall response to acidification in the hyporheic zones was less pronounced than in their benthic counterparts, which fully supports our initial hypothesis. Although a substantial decrease in decomposition rates of

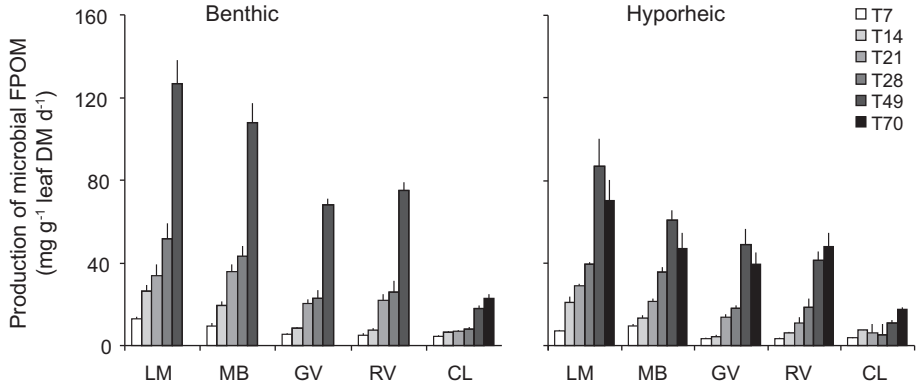

Fig. 6 – Rate of microbial production of fine particulate organic matter from leaves decomposing in the benthic and hyporheic zones of five streams along an acidification gradient at six incubation dates (mean + SE, $n = 4$). LM: La Maix, MB: Ménombru, GV: Gravelle, RV: Ravines, CL: Courbeline.

leaves was observed under acidic conditions, this effect was not primarily due to changes in fungal biomass (Fig. 4). Fungal biomass has generally been shown to increase to a maximum and then decline during leaf decomposition (Gessner and Chauvet, 1994). In contrast, fungal biomass associated with leaves decomposing in both benthic and hyporheic zones generally increased over the whole experiment for the five streams whatever the level of constraint generated by acidic deposition. As already reported by Dangles and Chauvet (2003), a significant decline following a peak only occurred in the benthic zone of the circumneutral stream (Fig. 4), which was the least affected by acidic deposition and exhibited the highest decomposition rates both in benthic and hyporheic zones. Our findings thus corroborated the results from Dangles and Chauvet (2003), Baudoin et al. (2008) and Simon et al. (2009) showing that fungal biomass was unresponsive to pH for streams of the Vosges mountains in France and of the Western Virginia in USA, but contrasted markedly with the substantial reduction in fungal biomass on oak and maple leaves in an acidified West Virginia stream, while the extent of acidic stress was quite similar (Griffith and Perry, 1994).

Moreover, studies that have attempted to link aquatic hyphomycete richness and activity and pH have reported ambiguous or contradictory patterns, while Krauss et al. (2011) suggested that aquatic hyphomycetes do not appear to be sensitive to low pH values *per se*. Bärlocher (1987), based on an investigation in ten streams of Nova Scotia and New Brunswick combined with an earlier study, revealed a significant negative correlation between aquatic hyphomycete species richness and stream water pH, and even concluded that "their tolerance of low pH values makes them one of the rare groups of stream organisms that may actually benefit from the effects of acid rain". In the same line, Wood-Eggenschwiler and Bärlocher (1983) found a clear decline in aquatic hyphomycete species richness with increasing pH in various European streams.

The studies of Iqbal and Webster (1977) and Shearer and Webster (1985) contrasted with these conclusions, as these studies reported impoverished fungal diversity in upland acidic water compared to lowland circumneutral sites. However, this may be due to a confounding effect of altitude, as Chamier (1987) found more species of aquatic hyphomycetes at lowland than at upland sites, regardless of pH (4.9–6.8) in seven streams of the English Lake District. Through a series of experiment on the physiological requirements of aquatic hyphomycetes, Rosset and Bärlocher (1985) reported that 10 species grew the best on solid media at pH values between 4 and 5, compared to pH around 7. In addition, some species even ceased to grow completely at pH $>$ 7. However, aquatic hyphomycete growth rates were always higher when grown on solid media amended with Ca^{2+}. In a laboratory stream experiment, Thompson and Bärlocher (1989) demonstrated that weight loss of maple leaves due to microbial activity peaked at a pH between 5.5 and 6.0. However, although these different examples indicate that lowered pH values are not necessarily detrimental to aquatic hyphomycetes, anthropogenic acidification can also raise Al concentration in stream water, which may severely depress fungal richness and activity (Krauss et al., 2011).

Surprisingly, the leaves exposed in the stream with the lowest pH in the present study (CL: 4.6 and 4.7 in benthic and hyporheic zones, respectively) and highest total Al concentrations (697 and 682 μg/L, respectively) showed the highest fungal biomass, both in benthic and hyporheic zones. As suggested by Dangles and Chauvet (2003), this pattern could be partly explained by the relatively high concentrations of atmospheric-derived nitrates in this stream (4.4 mg/L in the two zones), contrasting with the particularly low values reported by Griffith and Perry (1994), providing inorganic N to fungal production (Suberkropp, 1998) and thus compensating for the unfavourable acidic conditions. This difference might also result from discrepancies in substrate recalcitrance, which in the case of oak and maple (Griffith and Perry, 1994), led to a limited extent of development of fungal communities and therefore a substantial reduction in fungal biomass compared to alder (Danger et al., 2012).

Our results show that the peak of mycelial biomass in the hyporheic zone of the five streams was delayed by 7 weeks in comparison with the benthic zone, even though the latter also showed a strong increase at the final sampling date. Moreover, the amount of leaf-associated mycelial biomass was depressed in burial conditions, as reported from alder leaves in an analogous stream (Cornut et al., 2010) or from woody debris in a headwater mountain stream (Crenshaw et al., 2002). Surprisingly, in the present study, differences in dissolved oxygen between hyporheic and benthic zones remained low (from 1.0 to 1.9 mg/L) and the reduced fungal biomass in the hyporheic zone could only be partly explained by oxygen limitation, as supported by experimental data from hypoxic environments (Medeiros et al., 2009).

Also, we expect the number of conidia in water to drop from the surface to the deepest sediment layers. Even though fungal spores may disperse and allow small, short-lived colonies to develop on the substrates they encounter in the hyporheic zone (Bärlocher et al., 2006), the lower amount of conidia circulating in the sediment may have also contributed to the observed delay to reach a substantial accrual of mycelial biomass.

4.2. *Evidence for microbiological processes*

The contribution of shredders to leaf decomposition in the hyporheic zone of the present streams was rather low as reported in a recent study (Cornut et al., 2010). Shredders were rare on buried leaves whatever the level of acidification, although they were relatively abundant on leaf detritus at the surface of sediments. In addition, the only taxa with the appropriate morphology to penetrate the substratum in this study, *i.e., Leuctra spp.* and *A. sulcicollis*, were also the less efficient detritivores in leaf decomposition. The burial of leaves in the sandy substratum thus reduced the access of invertebrate decomposers to leaves due to the small interstices of the sediment and the subsequent bottom instability, particularly constraining the largest shredders. As a consequence, the contributions of fungi *vs.* shredders to leaf decomposition, as well as the level of their trophic interactions (Cornut et al., 2010), were contrasted in the two habitat types of the present acidified streams (Fig. 7). The absence of shredding invertebrates in the most acidic stream (CL) virtually suppressed the competition with fungi, and allowed the latter to grow to a higher extent than in the circumneutral stream (LM). This increased importance of

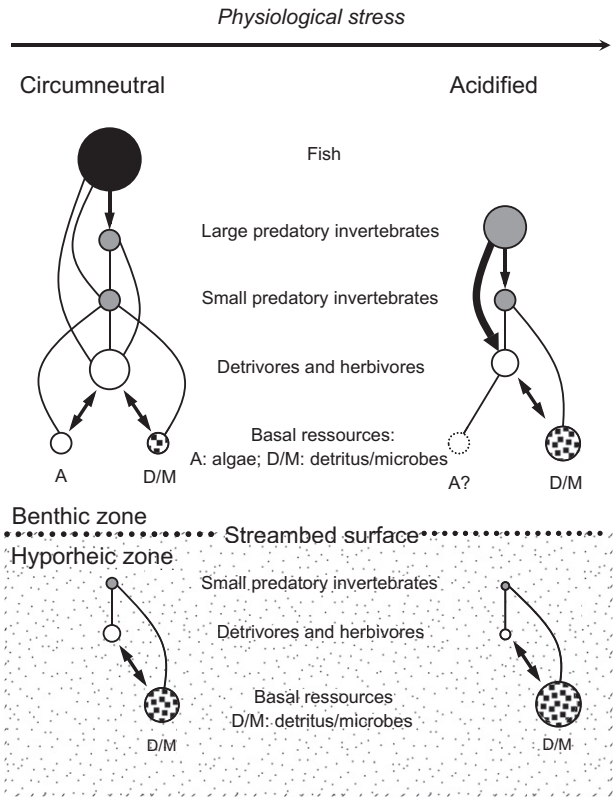

Fig. 7 – Conceptual scheme of how anthropogenic acidification affects trophic and structural relationships in stream food web both in the benthic and hyporheic zones. The figure displays a simplified food web for the benthos and hyporheos, consisting of five elements (symbolised by circles): fish, large and small predatory invertebrates, herbivorous/detritivorous invertebrates and basal resources (algae, organic matter, fungi and bacteria). Trophic linkages within the web are indicated by lines and arrows between circles. The circle diameter is proportional to the abundance of each group and the thickness of lines and arrows to the intensity of interactions (adapted from Hildrew, 1996).

aquatic fungi in litter decomposition in the hyporheic environment is in accordance with recent findings (Cornut et al., 2010). While the extent of fungal development was possibly related to differential abundance of shredding invertebrates, trophic interactions in both compartments were also modified (Fig. 7). Overall, this is discernible when extrapolating data beyond the final sampling date in the hyporheic zone that leaf litter remaining in this habitat (*i.e.*, more than 50% of initial mass in LM, the stream exhibiting the highest decomposition rates) can sustain further fungal growth in contrast to the benthic zone where the decomposition process is nearly completed after 70 days. Also, as suggested by Cornut et al. (2010), it is expected that fungal decomposers in the hyporheic zone compensate, if not fully, at least partly for the action of invertebrate detritivores in the benthic zone.

The acidification of freshwater ecosystems resulting from atmospheric pollution is often accompanied by increased concentrations of aqueous Al, which is considered a major environmental issue due to its high toxicity to aquatic organisms. Indeed, aqueous Al has been recognized as a main toxicant for aquatic animals (Gensemer and Playle, 1999), and several studies have also suggested a direct effect on microbial metabolism (Myrold and Nason, 1992; Pina and Cervantes, 1996; Chamier and Tipping, 1997) and diversity (Baudoin et al., 2008). The presence of aquatic hyphomycetes in our streams strongly contaminated by aqueous Al confirms that at least some species are resistant to this constraint, as already shown for other metals (Krauss et al., 2011).

Overall, our findings showed that the diversity of aquatic hyphomycetes on leaves in hyporheic zone, assessed through the release of conidia, was substantially lower than on the streambed. It is however important to note that these results were only based on conidia released during leaf decomposition. Thereby, non-sporulating fungal species may have not been detected. Molecular techniques might be fruitfully applied to fungal communities on plant detritus in streams to circumvent such obstacles, although still subject to uncertainties and limitations (Bärlocher, 2010).

Both aquatic hyphomycete and macroinvertebrate communities were strongly modified under acidic conditions, and they responded similarly to the acidification gradient across the benthic and hyporheic zones. However communities differed much more between the two compartments of circumneutral streams than those of the most acidic one (Fig. 3a and b). For invertebrates, this pattern is more likely due to the converging effects of physical (hyporheic) and chemical (acidic) constraints, which led to drastic reduction in their diversity and density (Fig. 7). The same pattern observed in fungal assemblages means that fungi responded in a similar way to both physical and chemical barriers, even though individual responses, perhaps exacerbated by interspecific interactions, differed among species (cf. Table 2). Environmental filtering of shredder and aquatic hyphomycete species assemblages through acidity and habitat conditions may therefore act synergistically, leading to lower leaf litter decomposition. The observed reduction in leaf litter decomposition rate along the acidic gradient for both benthic and hyporheic zones supports the contention that stream acidification has a profound impact on leaf litter decomposition, as previously reported (Chamier, 1987; Mulholland et al., 1992; Griffith et al., 1995; Dangles and Guerold, 2001; Dangles et al., 2004; Baudoin et al., 2008). However, as stressed by Dangles et al. (2004), it is not well defined whether this effect is mainly due to changes in community structure and taxonomic diversity of aquatic hyphomycetes and shredders and/or to a lower microbial activity. Dangles et al. (2004) reported that the abundance and biomass of a single acid-sensitive macroinvertebrate species, *G. fossarum*, in the same or similar streams were good predictors of leaf litter decomposition, explaining 80% and 73%, respectively, of variations in litter decomposition rate among streams. There are no counterpart studies for aquatic hyphomycete taxa to our knowledge. Experimental findings by Dang et al. (2005) and Duarte et al. (2006) however suggest that specific traits of certain species may have a greater influence on leaf decomposition rate than species richness of fungal assemblages.

Despite the lower fungal diversity in acidic hyporheic environments, leaf decomposition was relatively less affected by acidification in this environment compared with the benthic one, which was supported by the maintenance of a substantial biomass (Fig. 4) and activity (Fig. 6) of fungal decomposers. This argues for a lower sensitivity of aquatic fungi to the present physical and chemical stressors, occurring at the functional but not structural level. A distinction must however be made between the metabolic activity oriented towards the maintenance of mycelial biomass, the reproductive activity (*i.e.*, production of conidia) and the enzymatic activities leading to the production of FPOM and leaf decomposition (Suberkropp and Klug, 1980). All activities do not respond equally to different levels of stressors, with the maintenance of fungal biomass or reproductive activity being sometimes reported as insensitive to or even stimulated by certain stressor levels (*e.g.*, acidification, Baudoin et al., 2008; copper contamination, Roussel et al., 2008). The less severe effects of moderate pollution on fungal biomass and degradational activity than on fungal diversity have been reported in a review (Krauss et al., 2011), suggesting the occurrence of some compensation by resistant strains or species. The subsistence of active fungal decomposers in environments unoccupied by invertebrate detritivores represents insurance for impaired stream ecosystems to maintain carbon fluxes, with the hyporheic compartment acting as an important source of organic matter and propagules to downstream. More importantly, this biodiversity potentially confers resilience of the stream ecosystem functions to natural and anthropogenic disturbances. Nonetheless, the biodiversity and ecosystem services from the hyporheic zone are generally underestimated, jeopardising their effective protection and wise management (Boulton et al., 2010).

5. Conclusion

In both benthic and hyporheic zones, a significant decrease in leaf decomposition rates was observed under acidic conditions. Our study provides evidence that this reduced decomposition of leaves in acidified streams was not primarily due to changes in fungal biomass. Indeed, fungal biomass showed no particular trend regarding the acidification gradient, except that maxima were higher in the most acidic stream, likely resulting from the depressed competition and predation by shredders. In parallel, the structure of aquatic hyphomycete and leaf shredding macroinvertebrate communities was strongly modified in acidic conditions. Interestingly, leaf litter decomposition was less affected by acidification in the hyporheic zone compared with the benthic one, probably due to the lower sensitivity of at least some fungal decomposer taxa. The subsistence of active fungal decomposers in environments unoccupied by invertebrate detritivores is a strong insurance for impaired stream ecosystems to maintain carbon fluxes. Despite widespread interest in the concept of stream health (Meyer, 1997), the notion of hyporheic health has received little attention (Boulton et al., 2008). If an intact hyporheic zone may underpin stream health in some streams, assessment of ecological health of hyporheic zone of reaches where the streams are impacted by human activities is a logical direction for management applications (Boulton et al., 2010). Typically, river restoration focused on surface systems and their longitudinal and lateral connections, whereas the vertical dimension was largely ignored (Ward et al., 2001; Boulton, 2007). Within the Water Framework Directive, subsurface water is identified as a resource that requires specific management attention. In the light of our findings and in line with a new direction emerging since the last decade, we think that assessing the ecological integrity of streams by considering the health and good functioning of the hyporheic zone, in a manner equivalent to that already practiced or proposed for surface waters, is needed in a whole-ecosystem management perspective.

Statement of authorship

J.C., F.G., E.C., A.E. and C.P. designed the experiments, J.C. and H.C. carried out the experiments, and J.C., A.E., E.C. and F.G. wrote the manuscript.

Acknowledgements

We greatly appreciate the assistance of Pierre Gierlinski, Sylvain Lamothe, Philippe Wagner, Didier Lambrigot and Philippe Rouselle in the field and laboratory. Comments provided by 3 anonymous referees on an early version of the manuscript were most appreciated. This study was funded through the InBioProcess (ANR-06-BDIV-007) and Recover (ANR-07 BDIV-007-01) projects of the ANR Biodiversity programme, and the Zone Atelier Moselle.

REFERENCES

Alewell, C., Prechtel, A., Bittersohl, J., Moritz, K., Meesenburg, H., Armbruster, M., 2001. Are there signs of acidification reversal in freshwaters of the low mountain ranges of Germany? Hydrology and Earth System Sciences 5 (3), 367–378.

Bärlocher, F., 1987. Aquatic hyphomycete spora in 10 streams in New Brunswick and Nova Scotia. Canadian Journal of Botany 65 (1), 76–79.

Bärlocher, F., 2010. Molecular approaches promise a deeper and broader understanding of the evolutionary ecology of aquatic hyphomycetes. Journal of the North American Benthological Society 29 (3), 1027–1041.

Bärlocher, F., Nikolcheva, L.G., Wilson, K.P., Williams, D.D., 2006. Fungi in the hyporheic zone of a springbrook. Microbial Ecology 52 (4), 708–715.

Barrett, K., Aas, W., Hjellbrekke, A.G., Tarrason, L., Schaug, J., 2000. An evaluation of trends for concentrations in air and precipitation. In: Transboundary Acidification and Eutrophication in Europe, EMEP Summary Report 2000. Tarrason, L., Schaug, J. (Eds.), Norwegian Meteorological Institute, Oslo, Norway, pp. 41–48.

Baudoin, J.M., Guérold, F., Felten, V., Chauvet, E., Wagner, P., Rousselle, P., 2008. Elevated aluminium concentration in acidified headwater streams lowers aquatic hyphomycete diversity and impairs leaf-litter breakdown. Microbial Ecology 56 (2), 260–269.

Boulton, A.J., 2007. Hyporheic rehabilitation in rivers: restoring vertical connectivity. Freshwater Biology 52 (4), 632–650.
Boulton, A.J., Datry, T., Kasahara, T., Mutz, M., Stanford, J.A., 2010. Ecology and management of the hyporheic zone: stream-groundwater interactions of running waters and their floodplains. Journal of the North American Benthological Society 29 (1), 26–40.
Boulton, A.J., Fenwick, G., Hancock, P.J., Harvey, M.S., 2008. Biodiversity, functional roles and ecosystem services of groundwater invertebrates. Invertebrate Systematics 22 (2), 103–116.
Boulton, A.J., Findlay, S., Marmonier, P., Stanley, E.H., Valett, H.M., 1998. The functional significance of the hyporheic zone in streams and rivers. Annual Review of Ecology and Systematics 29, 59–81.
Brunke, M., Gonser, T., 1997. The ecological significance of exchange processes between rivers and groundwater. Freshwater Biology 37 (1), 1–33.
Burton, T.M., Stanford, R.M., Allan, J.W., 1985. Acidification effects on stream biota and organic matter processing. Canadian Journal of Fisheries and Aquatic Sciences 42 (4), 669–675.
Chamier, A.-C., 1987. Effect of pH on microbial degradation of leaf litter in seven streams of the English Lake District. Oecologia 71 (4), 491–500.
Chamier, A.-C., Tipping, E., 1997. Effects of aluminium in acid streams on growth and sporulation of aquatic hyphomycetes. Environmental Pollution 96 (3), 289–298.
Clarke, K.R., Gorley, R.N., 2001. Primer v5: User Manual/Tutorial. Primer-E Ltd, Plymouth, UK.
Cooper, D.M., 2005. Evidence of sulphur and nitrogen deposition signals at the United Kingdom acid waters monitoring network sites. Environmental Pollution 137 (1), 41–54.
Cornut, J., Elger, A., Greugny, A., Bonnet, M., Chauvet, E., 2012. Coarse particulate organic matter in the interstitial zone of three French headwater streams. Annales de Limnologie – International Journal of Limnology 48 (3), 303–313.
Cornut, J., Elger, A., Lambrigot, D., Marmonier, P., Chauvet, E., 2010. Early stages of leaf decomposition are mediated by aquatic fungi in the hyporheic zone of woodland streams. Freshwater Biology 55 (12), 2541–2556.
Crenshaw, C.L., Valett, H.M., Tank, J.L., 2002. Effects of coarse particulate organic matter on fungal biomass and invertebrate density in the subsurface of a headwater stream. Journal of the North American Benthological Society 21 (1), 28–42.
Cummins, K.W., Wilzbach, M.A., Gates, D.M., Perry, J.B., Taliaferro, W.B., 1989. Shredders and riparian vegetation. Bioscience 39 (1), 24–30.
Cummins, K.W., Sedell, J.R., Swanson, F.J., Minshall, G.W., Fisher, S.G., Cushing, C.E., Petersen, R.C., Vannote, R.L., 1983. Organic matter budgets for stream ecosystems: problems in their evaluation. In: Barnes, J.R., Minshall, G.W. (Eds.), Stream Ecology. Application and Testing of General Ecological Theory. Plenum Press, New York, pp. 299–353.
Dambrine, E., Pollier, B., Poszwa, A., Ranger, J., Probst, A., Viville, D., Biron, P., Granier, A., 1998. Evidence of current soil acidification in spruce stands in the Vosges Mountains, north-eastern France. Water, Air & Soil Pollution 104 (1–2), 43–52.
Dang, C.K., Chauvet, E., Gessner, M., 2005. Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. Ecology Letters 8 (11), 1129–1137.
Danger, M., Cornut, J., Elger, A., Chauvet, E., 2012. Effects of burial on leaf litter quality, microbial conditioning and palatability to three shredder taxa. Freshwater Biology 57 (5), 1017–1030.
Dangles, O., Chauvet, E., 2003. Effects of stream acidification on fungal biomass in decaying beech leaves and leaf palatability. Water Research 37 (3), 533–538.
Dangles, O., Gessner, M.O., Guérold, F., Chauvet, E., 2004. Impact of stream acidification on litter breakdown: implication for assessing ecosystem functioning. Journal of Applied Ecology 41 (2), 365–378.
Dangles, O., Guerold, F., 2001. Linking shredders and leaf litter processing: Insights from an acidic stream study. International Review of Hydrobiology 86 (4–5), 395–406.
Driscoll, C.T., Lawrence, G.B., Bulger, A.J., Butler, T.J., Cronan, C.S., Eagar, C., Lambert, K.F., Likens, G.E., Stoddard, J.L., Weathers, K.C., 2001. Acidic deposition in the northeastern United States: sources and inputs, ecosystem effects, and management strategies. BioScience 51 (3), 180–198.
Duarte, S., Pascoal, C., Cássio, F., Bärlocher, F., 2006. Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. Oecologia 147 (4), 658–666.
Duff, J.H., Triska, F.J., 1990. Denitrification in sediments from the hyporheic zone adjacent to a small forested stream. Canadian Journal of Fisheries and Aquatic Sciences 47 (6), 1140–1147.
EMEP, 1999. Transboundary Acid Deposition in Europe. EMEP Emission Data. Status Report 1999 of the European Monitoring and Evaluation Programme. EMEP/MSC-W Report 1/1999. http://www.emep.int/ladm.html.
Evans, C.D., Cullen, J.M., Alewell, C., Kopácek, J., Marchetto, A., Moldan, F., Prechtel, A., Rogora, M., Veselý, J., Wright, R., 2001. Recovery from acidification in European surface waters. Hydrology and Earth System Sciences 5 (3), 283–298.
Findlay, S.E.G., Sinsabaugh, R.L., Sobczak, W.V., Hoostal, M., 2003. Metabolic and structural response of hyporheic microbial communities to variations in supply of dissolved organic matter. Limnology and Oceanography 48 (4), 1608–1617.
Fischer, H., Pusch, M., 2001. Comparison of bacterial production in sediments, epiphyton and the pelagic zone of a lowland river. Freshwater Biology 46 (10), 1335–1348.
Gensemer, R.W., Playle, R.C., 1999. The bioavailability and toxicity of aluminum in aquatic environments. Critical Reviews in Environmental Science and Technology 29 (4), 315–450.
Gessner, M.O., 2005. Ergosterol as a measure of fungal biomass. In: Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.), Methods to Study Litter Decomposition: a Practical Guide. Springer, Dordrecht, The Netherlands, pp. 189–195.
Gessner, M.O., Chauvet, E., 1993. Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Applied and Environmental Microbiology 59 (2), 502–507.
Gessner, M.O., Chauvet, E., 1994. Importance of stream microfungi in controlling breakdown rates of leaf litter. Ecology 75 (6), 1807–1817.
Godbout, L., Hynes, H.B.N., 1982. The three dimensional distribution of the fauna in a single riffle in a stream in Ontario. Hydrobiologia 97 (1), 87–96.
Griffith, M.B., Perry, S.A., 1993. Colonization and processing of leaf-litter by macroinvertebrate shredders in streams of contrasting pH. Freshwater Biology 30 (1), 93–103.
Griffith, M.B., Perry, S.A., 1994. Fungal biomass and leaf-litter processing in streams of different water chemistry. Hydrobiologia 294 (1), 51–61.
Griffith, M.B., Perry, S.A., Perry, W.B., 1995. Leaf litter processing and exoenzyme production on leaves in streams of different pH. Oecologia 102 (4), 460–466.
Groom, A.P., Hildrew, A.G., 1989. Food quality for detritivores in streams of contrasting pH. Journal of Animal Ecology 58 (3), 863–881.
Guérold, F., Boudot, J.-P., Jacquemin, G., Vein, D., Merlet, D., Rouillet, J., 2000. Macroinvertebrate community loss as a result of headwater stream acidification in the Vosges Mountains (N-E France). Biodiversity and Conservation 9 (6), 767–783.
Hancock, P.J., Boulton, A.J., Humphreys, W.F., 2005. Aquifers and hyporheic zones: towards an ecological understanding of groundwater. Journal of Hydrogeology 13 (1), 98–111.

Please cite this article in press as: Cornut, J., et al., Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams, Water Research (2012), http://dx.doi.org/10.1016/j.watres.2012.09.023

Hawkins, C.P., Hogue, J.N., Decker, L.M., Feminella, J.W., 1997. Channel morphology, water temperature, and assemblage structure of stream insects. Journal of North American Benthological Society 16 (4), 728–749.
Hendricks, S.P., 1993. Microbial ecology of the hyporheic zone: a perspective integrating hydrology and biology. Journal of the North American Benthological Society 12 (1), 70–78.
Hendricks, S.P., White, D.S., 1991. Physicochemical patterns within a hyporheic zone of a Northen Michigan River, with comments on surface water patterns. Canadian Journal of Fisheries and Aquatic Sciences 48 (9), 1645–1654.
Herbst, G.N., 1980. Effects of burial on food value and consumption of leaf detritus by aquatic invertebrates in a lowland forest stream. Oikos 35 (3), 411–424.
Hildrew, A.G., 1996. Food webs and species interaction. In: Petts, G., Calow, P. (Eds.), River Biota: Diversity and Dynamics. Blackwell Science, London, UK.
Iqbal, S.H., Webster, J., 1973. The trapping of aquatic hyphomycete spores by air bubbles. Transactions of the British Mycological Society 60 (1), 37–48.
Iqbal, S.H., Webster, J., 1977. Aquatic hyphomycetes spora of some Dartmoor streams. Transactions of the British Mycological Society 65 (2), 233–241.
Jones, J.B., Fisher, S.G., Grimm, N.B., 1995. Vertical hydrologic exchange and ecosystem metabolism in a Sonoran Desert stream. Ecology 76 (3), 942–952.
Krauss, G.-J., Solé, M., Krauss, G., Schlosser, D., Wesenberg, D., Bärlocher, F., 2011. Fungi in freshwaters: ecology, physiology and biochemical potential. FEMS Microbiology Reviews 35 (4), 620–651.
Larssen, T., Lydersen, E., Tang, D.G., He, Y., Gao, J.X., Liu, H.Y., Duan, L., Seip, H.M., Vogt, R.D., Mulder, J., Shao, M., Wang, Y., Shang, H., Zhang, X., Solberg, S., Aas, W., Okland, T., Eilertsen, O., Angell, V., Li, Q., Zhao, D., Xiang, R., Xiao, J., Luo, J., 2006. Acid rain in China. Environmental Science & Technology 40 (2), 418–425.
Legendre, P., Legendre, L., 1998. Numerical Ecology. Elsevier, Amsterdam.
Legendre, P., Marti, J.A., 1999. Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. Ecological Monographs 69 (1), 1–24.
Likens, G.E., Buttler, T.J., Buso, D.C., 2001. Long- and short-term changes in sulfate deposition: effects of the 1990 clean air act amendments. Biogeochemistry 52 (1), 1–11.
McKnight, D.M., Runkel, R.L., Tate, C.M., Duff, J.H., Moorhead, D.L., 2004. Inorganic N and P dynamics of Antarctic glacial meltwater streams as controlled by hyporheic exchange and benthic autotrophic communities. Journal of the North American Benthological Society 23 (2), 171–188.
Medeiros, A.O., Pascoal, C., Graça, M.A.S., 2009. Diversity and activity of aquatic fungi under low oxygen conditions. Freshwater Biology 54 (1), 142–149.
Metzler, G.M., Smock, L.A., 1990. Storage and dynamics of subsurface detritus in a sandbottomed stream. Canadian Journal of Fisheries and Aquatic Sciences 47 (3), 588–594.
Meyer, J.L., 1997. Stream health: incorporating the human dimension to advance stream ecology. Journal of the North American Benthological Society 16 (2), 439–447.
Minchin, P.R., 1987. An evaluation of the relative robustness of techniques for ecological ordination. Plant Ecology 69 (1–3), 89–107.
Mulholland, P.J., Driscoll, C.T., Elwood, J.W., Osgood, M.P., Palumbo, A.V., Rosemond, A.D., Smith, M.E., Schofield, C., 1992. Relationship between stream acidity and bacteria, macroinvertebrates and fish: a comparison of north temperate and south temperate mountain streams, USA. Hydrobiologia 239 (1), 7–24.
Myrold, D.D., Nason, G.E., 1992. Effect of acid rain on soil microbial processes. In: Mitchell, R. (Ed.), Environmental Microbiology. Wiley–Liss, Inc, New York, pp. 59–81.
Naegeli, M.W., Hartmann, U., Meyer, E.I., Uehlinger, U., 1995. POM-dynamics and community respiration in the sediments of a floodprone prealpine river (Necker, Switzerland). Archiv für Hydrobiologie 133 (3), 339–347.
Palmer, M.A., Covich, A.P., Lake, S., et al., 2000. Linkages between aquatic sediment biota and life above sediments as potential drivers of biodiversity and ecological processes. Bioscience 50 (12), 1062–1075.
Petersen, R.C., Cummins, K.W., 1974. Leaf processing in a woodland stream. Freshwater Biology 4 (4), 343–368.
Pina, R.G., Cervantes, C., 1996. Microbial interactions with aluminium. BioMetals 9 (3), 311–316.
Probst, A., Party, J.P., Fevrier, C., Dambrine, E., Thomas, A.L., Stussi, J.M., 1999. Evidence of springwater acidification in the Vosges Mountains (North-East of France): Influence of bedrock buffering capacity. Water, Air & Soil Pollution 114 (3–4), 395–411.
Pusch, M., Fiebig, D., Brettar, I., Eisenmann, H., Ellis, B.K., Kaplan, L.A., Lock, M.A., Naegeli, M.W., Traunspurger, W., 1998. The role of micro-organisms in the ecological connectivity of running waters. Freshwater Biology 40 (3), 453–495.
R Development Core Team, 2007. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. Available at: http://www.R-project.org.
Roussel, H., Chauvet, E., Bonzom, J., 2008. Alteration of leaf decomposition in copper contaminated freshwater mesocosms. Environmental Toxicology and Chemistry 27 (3), 637–644.
Rosset, J., Bärlocher, F., 1985. Aquatic hyphomycetes: influence of pH, Ca^{2+} and HCO_3^- on growth in vitro. Transactions of the British Mycological Society 84 (1), 137–145.
Schindler, D.W., 1988. Effects of acid rain on freshwater ecosystems. Science 239 (4836), 149–157.
Shearer, C.A., Webster, J., 1985. Aquatic hyphomycete communities in the River Teign. I. Longitudinal distribution patterns. Transactions of the British Mycological Society 84 (3), 509–518.
Simon, K.S., Simon, M.A., Benfield, E.F., 2009. Variation in ecosystem function in Appalachian streams along an acidity gradient. Ecological Applications 19 (5), 1147–1160.
Smith, J.W.N., 2005. Groundwater–surface Water Interactions in the Hyporheic Zone. Agency Science Report SC030155/SR1. Environment Agency, Bristol, UK, 70 pp.
StatSoft Inc, 2001. Statistica 6.0: Electronic Statistics Textbook. Available at: http://www.statsoft.com/textbook/stathome.html.
Storey, R.G., Fulthorpe, R.R., Williams, D.D., 1999. Perspectives and predictions on the microbial ecology of the hyporheic zone. Freshwater Biology 41 (1), 119–130.
Storey, R.G., Williams, D.D., 2004. Spatial responses of hyporheic meiofauna to seasonal changes in environmental parameters. Freshwater Biology 49 (11), 1468–1486.
Strommer, J.L., Smock, L.A., 1989. Vertical distribution and abundance of riverine invertebrates within the sandy substrate of a low-gradient headwater stream. Freshwater Biology 22 (2), 262–274.
Suberkropp, K., 1991. Relationships between growth and sporulation of aquatic hyphomycetes on decomposing litter. Mycology Research 95 (7), 843–850.
Suberkropp, K., 1998. Effect of dissolved nutrients on two aquatic hyphomycetes growing on leaf litter. Mycological Research 102 (8), 998–1002.
Suberkropp, K., Klug, M.J., 1980. The maceration of deciduous leaf litter by aquatic hyphomycetes. Canadian Journal of Botany 58 (9), 1025–1031.
Tachet, H., Richoux, P., Bournaud, M., Usseglio-Polatera, P., 2000. Invertébrés d'eau douce. Systématique, biologie, écologie. CNRS Editions, Paris.

Please cite this article in press as: Cornut, J., et al., Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams, Water Research (2012), http://dx.doi.org/10.1016/j.watres.2012.09.023

Tang, D., Lydersen, E., Seip, H.M., Angell, V., Eilertsen, O., Larssen, T., Liu, G., Kong, G., Mulder, J., Semb, A., Solberg, S., Torseth, K., Vogt, R.D., Xiao, J., Zhao, D., 2001. Integrated monitoring program on acidification of Chinese terrestrial systems (impacts)—a Chinese–Norwegian collaboration project. Water, Air & Soil Pollution 130 (1–4), 1073–1078.

Thompson, P.L., Bärlocher, F., 1989. Effect of pH on leaf breakdown in stream and in the laboratory. Journal of the North American Benthological Society 8 (3), 203–210.

Tillman, D.C., Moerke, A.H., Ziehl, C.L., Lamberti, G.A., 2003. Subsurface hydrology and degree of burial affect mass loss and invertebrate colonisation of leaves in a woodland stream. Freshwater Biology 48 (1), 98–107.

Triska, F.J., Duff, J.H., Avanzino, R.J., 1993. The role of water exchange between a stream channel and its aquatic interface. Hydrobiologia 251 (1–3), 167–184.

Vuorenmaa, J., 2004. Long-term changes of acidifying deposition in Finland (1973–2000). Environmental Pollution 128 (3), 351–362.

Ward, J.V., Tockner, K., Uehlinger, U., Malard, F., 2001. Understanding natural patterns and processes in river corridors as the basis for effective river restoration. Regulated Rivers: Research and Management 17 (4–5), 311–323.

Wood-Eggenschwiler, S., Bärlocher, F., 1983. Aquatic hyphomycetes in sixteen streams in France, Germany and Switzerland. Transactions of the British Mycological Society 81 (2), 371–379.

Please cite this article in press as: Cornut, J., et al., Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams, Water Research (2012), http://dx.doi.org/10.1016/j.watres.2012.09.023

IV.1.2. Effets de l'acidification sur la diversité fongique associée aux litières en décomposition dans les zones benthiques et hyporhéiques de cours d'eau forestiers.

Résumé

Les résultats de l'étude présentée dans la partie précédente ont notamment montré que l'acidification pouvait entrainer une diminution conjointe de la décomposition des litières et de la diversité d'espèces sporulantes d'hyphomycètes aquatiques dans les compartiments benthiques et hyporhéiques des cours d'eau. Cette étude de la diversité fongique a été réalisée par méthode microscopique après sporulation. Cependant, l'utilisation de techniques moléculaires a révélé que cette méthode traditionnelle sous-estimerait la diversité des champignons associés à la décomposition des litières. De plus, à notre connaissance, aucune étude moléculaire n'a tenté d'identifier les changements taxonomiques induits par les effets d'une perturbation. Le but de ces travaux était donc d'évaluer de manière plus exhaustive les effets de l'acidification des cours d'eau sur les assemblages fongiques associés aux litières, en étudiant plus en détail les résultats obtenus par analyses microscopiques dans les deux compartiments des cours d'eau (*i.e.* benthique et hyporhéique), et en les combinant à ceux obtenus par des analyses moléculaires. Les ADN des communautés microbiennes associées aux litières introduites dans les compartiments benthiques et hyporhéiques des cinq cours d'eau le long du gradient d'acidification ont été extraits. Les ARNr 18S fongiques ont ensuite été amplifiés par PCR et analysés par DGGE. Des clonages-séquençage de ces gènes ont spécifiquement été réalisés pour les communautés microbiennes associées aux litières exposées dans les compartiments benthiques et hyporhéiques du cours d'eau de référence neutre (LM) et du cours d'eau le plus impacté (CL).

Contrairement à ce qui a pu être observé grâce aux analyses microscopiques, les résultats des études moléculaires n'ont pas révélé d'effets marqués du gradient d'acidification sur la diversité fongique. Les résultats des analyses microscopiques ont montré que *Flagellospora curvula* (FLCU) était l'espèce dominant la production de conidies (plus de 95%) dans les deux compartiments des deux cours d'eau les plus impactés. *Tetrachaetum elegans* (THEL) et *Lemonniera aquatica* (LEAQ), dans le compartiment benthique, et *Heliscus lugdunensis* (HULU), dans le compartiment hyporhéique, co-dominaient les assemblages avec FLCU dans les cours d'eau les moins touchés par l'acidification. La production conidienne était significativement réduite dans les zones hyporhéiques par rapport aux benthiques, ainsi que dans la zone benthique du cours d'eau présentant les plus fortes concentrations en Al (CL).

De plus, la production conidienne de THEL était significativement réduite par les concentrations croissantes en Al dans la zone benthique des cours d'eau. Ces résultats montrent que les espèces produisant des conidies de grandes tailles (*i.e.* THEL et LEAQ) seraient défavorisées aux dépends de celles produisant des conidies plus petites (*i.e.* THEL et LEAQ) dans les zones hyporhéiques et dans les cours d'eau impactés. Ces observations suggèrent que les espèces produisant de petites spores pourraient se disperser plus facilement dans le sédiment et que certaines contraintes prévalant dans la zone hyporhéique (*e.g.* faible oxygénation) et dans les cours d'eau impactés (*e.g.* fortes concentrations en Al) pourraient affecter davantage les espèces devant fournir un effort métabolique conséquent pour la production de conidies de grandes tailles.

Les séquençages, quant à eux, ont révélé que les communautés fongiques sur les feuilles étaient essentiellement dominées par les Ascomycètes (70 à 79% des séquences) et dans de moindres proportions par les Basidiomycètes (9 à 25%) et les Chytridiomycètes (5 à 11%). Les séquences proches de celles d'hyphomycètes aquatiques correspondaient à respectivement 39% et 8% du total des séquences provenant des feuilles exposées dans les zones benthiques et hyporhéiques de LM, et à respectivement 6% et 16% de celles des zones benthiques et hyporhéiques de CL. Une importante contribution de champignons d'origine terrestre a été observée sur les feuilles, et plus particulièrement celle d'espèces appartenant aux genres *Phoma*, *Cladosporium*, *Itersonilia*, et *Taphrina*. Cependant, ces espèces sont supposées être moins impliquées que les hyphomycètes aquatiques dans le processus de décomposition des litières. Il est justement intéressant de noter que les plus importants taux de décomposition ont été observés dans la zone benthique de LM et en présence de la plus grande diversité en hyphomycètes aquatiques, que ce soit par méthode microscopique ou moléculaire. De plus, en dépit du fait que la méthode microscopique sous-estime la diversité fongique, les analyses des assemblages déterminés par cette méthode montrent une meilleure discrimination des effets de l'acidification que les analyses faites par PCR-DGGE. Ces résultats confirment ainsi le rôle majeur joué par les hyphomycètes aquatiques dans la décomposition des litières mais également que l'analyse microscopique de leur diversité fournit un indicateur particulièrement pertinent pour l'évaluation d'une perturbation.

A l'heure actuelle, peu d'études moléculaires ont tenté de caractériser les communautés fongiques associées aux litières. Ainsi, d'autres études doivent être envisagées afin de mieux comprendre le rôle des différents champignons se succédant lors du processus de décomposition des litières. Une attention particulière devra notamment être apportée à la

compréhension de la persistance des espèces d'origine terrestre dans les cours d'eau impactés et dans les zones hyporhéiques mais également quelles interactions ces espèces peuvent avoir avec les hyphomycètes aquatiques.

Effects of acidification on leaf-associated fungi in benthic and hyporheic zones of streams: combining traditional and molecular approaches.

Hugues Clivot[1,2], Julien Cornut[3,4], Arnaud Elger[3,4], Pascal Poupin[1,2], François Guérold[1,2], Eric Chauvet[3,4] and Christophe Pagnout[1,2]

[1]Université de Lorraine; Laboratoire des interactions Ecotoxicologie Biodiversité Ecosystèmes (LIEBE); UMR 7146; Campus Bridoux rue du Général Delestraint, 57070 Metz, France

[2]CNRS; LIEBE; UMR 7146; 57070 Metz, France

[3]Université de Toulouse; INP, UPS; Laboratoire d'Ecologie Fonctionnelle et Environnement; 118 Route de Narbonne, 31062 Toulouse, France

[4]CNRS; EcoLab; 31062 Toulouse, France

Abstract

By combining microscopic and molecular methods, we studied leaf associated fungal assemblages exposed during four weeks in the benthic and hyporheic zones of five streams along an acidification gradient. We found that acidification and elevated Al concentrations strongly depressed sporulating aquatic hyphomycetes diversity in both zones of streams, while fungal diversity assessed by PCR-DGGE appeared unaffected. Clone library analyses revealed that leaf associated fungal communities were mainly dominated by taxa belonging to Ascomycetes and to a lesser extent to Basidiomycetes and Chytritiomycetes. An important contribution of terrestrial fungi (genera such as *Phoma*, *Cladosporium*, *Itersonilia*, and *Taphrina*) was observed in both zones of the most acidified stream and in the hyporheic zone of the reference circumneutral stream. Interestingly, the highest leaf breakdown rate was observed in the benthic zone of the circumneutral stream where occurred both the highest diversity of sporulating aquatic hyphomycetes and the highest contribution to clone libraries of sequences affiliated to aquatic hyphomycetes. Our findings underline the major role played by aquatic hyphomycetes in leaf decomposition process and demonstrated that molecular approaches, could not only bring out new highlights on the identity of leaf associated fungal communities but were also complementary with results obtained from traditional approach.

Keywords: *acidification, aquatic hyphomycetes, hyporheic zone, clone libraries, leaf litter*

Introduction

Fungi play a crucial role in detritus-based lotic ecosystems as main microbial mediators of allochthonous leaf litter decomposition (Findlay & Arsuffi, 1989; Baldy et al., 1995; Gulis & Suberkropp, 2003). Through their leaf-degrading enzyme production, aquatic fungi contribute to microbial leaf processing and to leaf conditioning for consumption by detritivores (Bärlocher & Kendrick, 1981; Arsuffi & Suberkropp, 1988). In particular, aquatic hyphomycetes, a polyphyletic group of fungi, are thought to dominate microbial communities growing on decaying leaves (Bärlocher, 1992; Suberkropp, 1992; Gessner et al., 2007).

Leaf litter breakdown can be a useful indicator to assess functional integrity of streams (Gessner & Chauvet, 2002) and leaf-associated aquatic hyphomycetes have been also considered as potential bioindicators of anthropogenic stress (Solé et al., 2008). Reduced leaf litter breakdown and a loss of aquatic hyphomycete species richness as a result of ecosystem alteration have been concurrently reported in numerous studies, notably under high concentrations of metals such as Al in acidified streams (Baudoin et al., 2008), Cd (Moreirinha et al., 2011), Ag (Pradhan et al., 2011), Zn (Niyogi et al., 2009; Fernandes et al., 2009) or even in context of multiple contaminations (Duarte et al., 2008). These observed decreases in species richness under anthropogenic stress have been traditionally assessed using microscopic method based on the morphological identification of conidia (asexual spores), which vary between species. Molecular methods, developed more recently, allow the study of fungal communities regardless of their reproductive form, including the mycelia, which constitutes the metabolically active part of fungal biomass on leaves (Bärlocher, 2007, 2010; Krauss et al., 2011). Molecular fingerprinting of microbial communities, namely terminal restriction fragment length polymorphism (T-RFLP) analysis and denaturing gradient gel electrophoresis (DGGE), first showed that traditional method (conidial identification) underestimates fungal diversity on leaves (Nikolcheva et al., 2003). Molecular approaches using taxon-specific fungal primers (Nikolcheva & Bärlocher, 2004) and sequencing (Seena et al., 2008) or comparison of fungal clone libraries (Harrop et al., 2009) revealed an important relative contribution of non-aquatic hyphomycete species to leaf-associated microbial assemblages. Some studies concurrently used both conidial identification and DGGE to estimate fungal richness on leaves under anthropogenic disturbance and revealed contrasted results on phylotype richness depending on situations, *i.e.* negative impact with exposure to Cd (Moreirinha et al., 2011) and to Cu and Ag (Pradhan et al., 2011), but no effects under Cu and/or Zn stress (Duarte et al., 2008; Fernandes et al., 2009). On the other

hand, phylotype richness always exceeded that of sporulating aquatic hyphomycete in these studies, confirming the underestimation of fungal diversity when only assessed by traditional method. Despite the differences observed, no study has attempted to investigate through molecular techniques the taxonomic changes occurring in altered sites subject to a loss of diversity of sporulating leaf-associated aquatic hyphomycetes.

Recent studies have reported the occurrence of aquatic hyphomycetes (Bärlocher et al., 2006) and their significant involvement in leaf decomposition in the hyporheic zone of streams (Cornut et al., 2010). Bärlocher et al. (2006) hypothesized that this stream compartment could be a long-term reservoir for dispersal of aquatic hyphomycetes. Large amounts of organic matter may be stored in the streambed (Jones, 1997; Cornut et al., 2012) and benthic and hyporheic zones are intimately connected through exchanges of water, organic matter and nutrients (Boulton et al., 1998). Therefore, investigating leaf colonization in the hyporheic and benthic zones may provide a more global view of fungal decomposer diversity involved in stream ecosystem functioning. For now, the most accomplished studies (see above) on the effects of an anthropogenic stress on leaf-associated fungi have been restricted to the benthic compartment of streams and to concurrent analyses of sporulating species and molecular fingerprinting without identifying species dominating fungal assemblages by molecular methods.

Recently, we reported the alteration of leaf litter decomposition in the benthic and hyporheic zones of streams subjected to anthropogenic acidification (Cornut et al., in press), and particularly, its negative impact on macroinvertebrate shredders and sporulating aquatic hyphomycete communities. We observed notably that the diversity of the latter was significantly reduced in both zones along the gradient of acidification, providing us interesting data that could be confronted to molecular analyses of leaf-associated fungal assemblages.

The aim of this study was thus to identify, by combining molecular analyses and results obtained by traditional method, the effects of an anthropogenic stress on fungal diversity and assemblages associated with decaying leaves in the benthic and hyporheic zones of streams. During initial stages of leaf decomposition, we followed leaf colonization in both zones of five low-order streams subjected to different levels of Al mobilized by anthropogenic acidification. The structure and diversity of fungal communities on leaves assessed by DGGE were compared to those previously evaluated by conidial identification (Cornut et al., in press). To identify differences in community composition between the reference non-

impacted stream and the most impacted one, fungal communities from both zones were then compared using analysis of 18S rRNA gene clone libraries.

Materials and methods

Site description

This study focused on the four first weeks of the litter bag experiment presented in Cornut et al. (in press), which was conducted in the Vosges Mountains (North-eastern France) from mid-November 2008 to January 2009 in a forested area sensitive to acidification. Five 1st and 2nd-order streams subjected to different levels of acidification were selected and ranged across an Al gradient. Site characteristics are described in Cornut et al. (in press) and main physicochemical parameters during the four first weeks are detailed in Table 1. Briefly, La Maix (LM) and Gravelle (GV) are, respectively, a circumneutral (mean pH of 7.3) and a moderately acidic (mean pH of 6.4) stream, exhibiting low total Al concentrations in the water column (means of 31 and 35 µg L^{-1} over the experimental period). Menombru (MB) and Ravines (RV) are, respectively, a circumneutral (mean pH of 7.0) and a moderately acidic (mean pH of 6.2) stream with intermediate Al concentrations (means of 100 and 113 µg L^{-1}). Courbeline (CL,) is the most impacted stream, exhibiting low pH (mean of 4.6) and high Al concentration (mean of 658 µg L^{-1}).

Field experiment and leaf litter processing

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

Physical and chemical analyses

Dissolved oxygen concentrations in surface and interstitial waters were measured in each stream (Multi 350i, WTW). Surface and interstitial waters were collected from each stream at each of the four sampling dates and when leaf bags were initially introduced into the streams. Detailed sampling protocol is described in Cornut et al. (in press). Stream pH was determined in the laboratory with a microprocessor pH meter (pH 3000, WTW). Acid-neutralizing capacity (ANC) was measured by Gran's titration. Total Al concentrations (after acidification with HNO_3) were determined by atomic absorption spectrophotometry (Aanalyst 100; Perkin Elmer and Varian SpectrAA-300).

Sporulation and conidial identification

Protocol for sporulation experiments is detailed in Cornut et al. (in press). Briefly, ten leaf discs were incubated at 10°C during 48 h in aerated microcosms (Suberkropp, 1991) in 40 mL of filtered water from their respective stream. Conidial suspension was filtered (5 µm pore size filter), after being preserved with formalin. Conidia on the filter were stained with Trypan blue (Iqbal & Webster, 1973). Conidia were identified and counted under the microscope (× 200). Conidial production was expressed as number of conidia released per milligram leaf AFDM per day or as total conidial biomass released per mg leaf AFDM per day. Conidial biomass released was calculated using individual conidial mass for the species mentioned in Chauvet and Suberkropp (1998) and those listed in (Bärlocher & Schweizer, 1983) with a conversion factor of 500 fg per μm^3 (Gessner & Chauvet, 1994). The average value of 200 pg per conidium was applied to the other species (Gessner, 1997).

DNA extraction and amplification

For molecular analysis, three leaf discs from each of the four replicates were pooled together for each date. The 12 leaf discs were finely ground with a micro-pestle and total DNA from each composite sample was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Fungal partial 18S rRNA gene fragments were amplified using a two-step PCR protocol (Oros-Sichler et al., 2006). The first amplification used primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and EF3 (5'-TCCTCTAAATGACCAAGTTTG-3'). Second amplification was performed using primers NS1 and FR1-GC (5'- CCCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGG CCGAICCATTCAATCGGTAIT -3'). PCR mixtures (100 µL) contained 6 U of Taq DNA Polymerase (5 PRIME, Hamburg, Germany), 1x Taq buffer (5 PRIME), 200 µM of each

dNTP, 0.5 µM of each primer and 50 ng of extracted DNA as template for the first PCR and a dilution of this first PCR amplicons (1:500) was used as template for the second amplification. The first PCR amplification protocol consisted of 5 min at 94°C, 25 cycles of 30 s at 94°C, 45 s at 47°C, and 3 min at 72°C, followed by a final extension of 10 min at 72°C. The second step was identical except that the number of cycles was lowered to 20 and the annealing temperature was 48°C.

Denaturing gradient gel electrophoresis

The amplification products were controlled on 1% (w/v) agarose gel and separated with the DCODE Mutation Detection System (Bio-Rad, Hercules, CA). A volume of 15 µL per sample was loaded on 6% (w/v) polyacrylamide gels in 1× TAE buffer with a denaturing gradient from 25 to 40% (100% denaturant corresponds to 40% (v/v) formamide and 7 M urea). The gels were run in 1× TAE buffer at 180 V and 58°C for 16 h and stained with SYBR Green I. The gels were imaged with a STARION FLA-9000 scanner (Fujifilm Life Sciences FSVT, Courbevoie, France).

Cloning and sequencing of amplified 18S rRNA gene

PCR products at the 4 dates were equally pooled into a composite sample for each stream and for each zone. PCR samples were then cloned using the Clone JET PCR Cloning Kit (Fermentas, Villebon sur Yvette, France) and 72 individual clone colonies by sample were sequenced by GATC Biotech (Konstanz, Germany) using NS1 primer. Sequences were checked and only quality sequences of approximately 530 bp were kept for phylogenetic analyses.

Flagellospora curvula 18S rRNA gene sequence

Flagellospora curvula (FLCU) dominated conidial production (number of conidia) in benthic and hyporheic zones of CL and LM. However, by contrast to other species that were frequently observed in these streams, 18S rRNA gene of FLCU was not sequenced yet. In order to identify sequences of our clone library belonging to this species, 18S rRNA genes of two FLCU strains (FLCU 130-1655 and FLCU 180-1662) were sequenced. The single conidial isolates of these two FLCU strains were obtained from foam samples originating from two streams of southwestern France, namely Le Lampy (43°25’07’’N; 2°11’15’’E) and Les Montauds (43°29’14’’N; 2°15’43’’E), respectively. Cultures were grown and maintained on 2% malt-agar plates. Both strains were then cultivated in 2% malt liquid medium. DNA extraction from mycelia and amplification of partial 18S rRNA gene were performed as

described above for leaf samples. PCR products were sequenced by GATC Biotech (Konstanz, Germany) using NS1 primer.

Phylogenetic analyses

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

Data analysis

Differences in physico-chemical variables, percentage of remaining leaf AFDM and rate of conidial production (logarithmically transformed data) were tested using a two-way analysis of variance (ANOVA) with stream and zone as factors and followed by post hoc multiple-comparisons (Tukey's HSD test).

GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was used to normalize and align DGGE profiles. To this end, internal control samples were loaded on each gel. For each stream and compartment, means of the four sampling dates were calculated using band relative intensity for DGGE and using relative contribution of each aquatic hyphomycete species to total conidial production to compare fungal assemblages on leaves. Bray-Curtis distance matrices were generated (Bray & Curtis, 1957). Cluster analysis of DGGE mean profiles and aquatic hyphomycete assemblages were performed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) and illustrated with dendrograms (Legendre & Legendre, 1998). For each condition, the phylotype richness was calculated as the total number of bands over the 4-week period. The R Software was used for all statistical analyses (R Development Core Team, 2008). For this study, the level of significance was set at $P = 0.05$.

Table 1. Main physico-chemical parameters over the 4-week study period, leaf mass loss after 4 weeks and cumulated richness of both sporulating aquatic hyphomycetesspecies and DGGE phylotypes associated with decaying alder leaves exposed in the benthic and hyporheic zones of the five streams. Values are means of physico-chemical variables (n=5) and leaf mass loss (n=4).

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

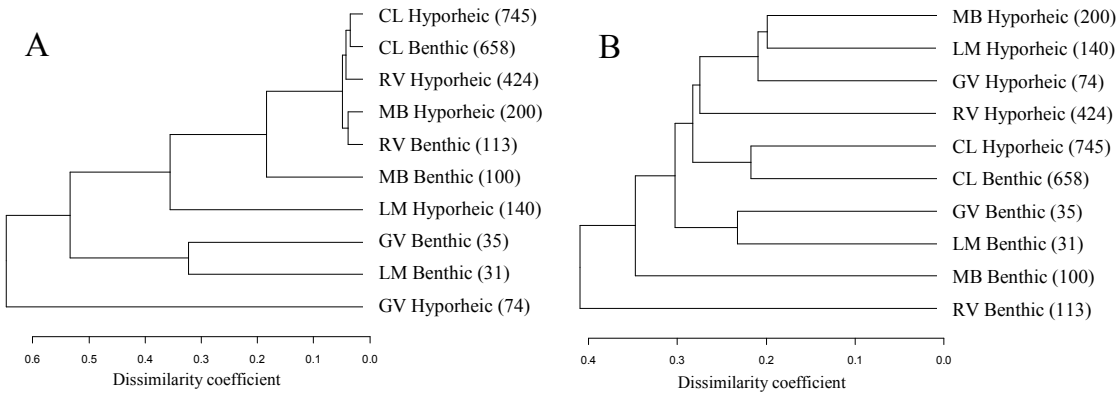

Figure 1. Cluster dendrograms of sporulating aquatic hyphomycete assemblages (A) and fungal communities investigated by DGGE (B) over the four sampling dates on leaves incubated in the benthic and hyporheic zones of the five streams. Values in brackets are mean total Al concentrations ($\mu g\ l^{-1}$)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

Results

Surface and interstitial water characteristics and leaf decomposition

Streams ranged across a gradient of increasing Al concentrations, from LM, *i.e.* the reference circumneutral stream, to CL, *i.e.* the most impacted one. Most of physico-chemical variables were quite similar between the benthic and hyporheic zones within each stream (Table 1). Although the streams were well oxygenated, two-way ANOVA (stream × zone) revealed that oxygen saturation was significantly lower in the hyporheic compared to the benthic zones of streams ($F_{1,40}$ = 97.3, P < 0.001). After four weeks, leaf mass loss differed significantly among streams (two-way ANOVA, $F_{4,30}$= 92.8, P < 0.001) and was the highest in LM (67.9% and 36.7% of leaf mass loss in the benthic and hyporheic zones, respectively) and the lowest in the most impacted stream CL (23.1% and 15.5% in the benthic and hyporheic zones, respectively) (Table 1). Leaf mass loss also differed among compartments of streams, being significantly lower in the hyporheic zones (two-way ANOVA, $F_{1,30}$ = 267.8, P < 0.001). However, the difference between zones was reduced for the most impacted streams (significant interaction zone × stream, $F_{4,30}$ = 13.3, P < 0.001).

Fungal assemblages

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

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

From conidial identification, FLCU was the dominant species in both zones of the most impacted streams CL and RV, contributing to more than 95% of conidial production in these streams (Figure 2). Together with FLCU, *Tetrachaetum elegans* (THEL) was the codominant species in the benthic zone of LM and GV, its contribution decreasing across the Al gradient

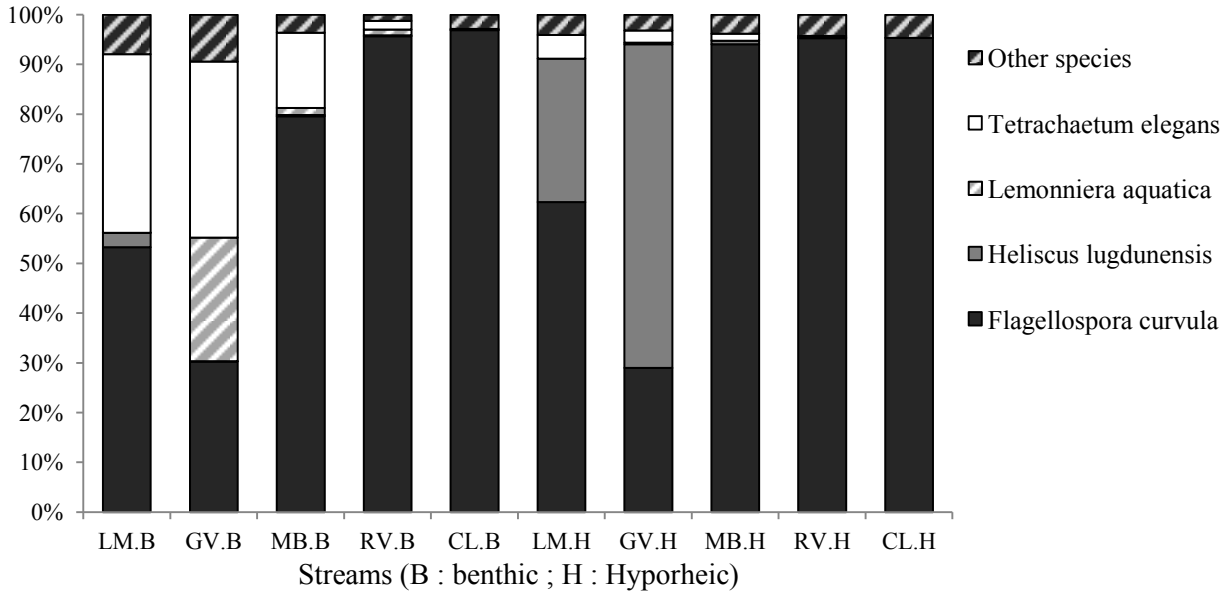

Figure 2. Contribution of the leaf-associated aquatic hyphomycete species to conidial production (%) for leaves exposed in the benthic and hyporheic zones of the five streams across the Al gradient. Species contributing to less than 5% of total conidial production were grouped together.

in both zones. However, THEL contribution was lower in the hyporheic zone of LM and GV, where *Heliscus lugdunensis* (HULU) was the codominant species with FLCU.

Conidial biomass production differed significantly between stream zones, being lower in the hyporheic one, except for CL (Figure 3A and Table 2). The differences observed were primarily due to a significant decrease of conidial production in the hyporheic zone of streams (Figure 3B and Table 2) and to a higher production of FLCU conidia in the benthic zone of RV compared to the hyporheic one (Figure 3C). In the benthic zones, a significant decrease of THEL conidial production was also observed across the Al gradient (Figure 3D).

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

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

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

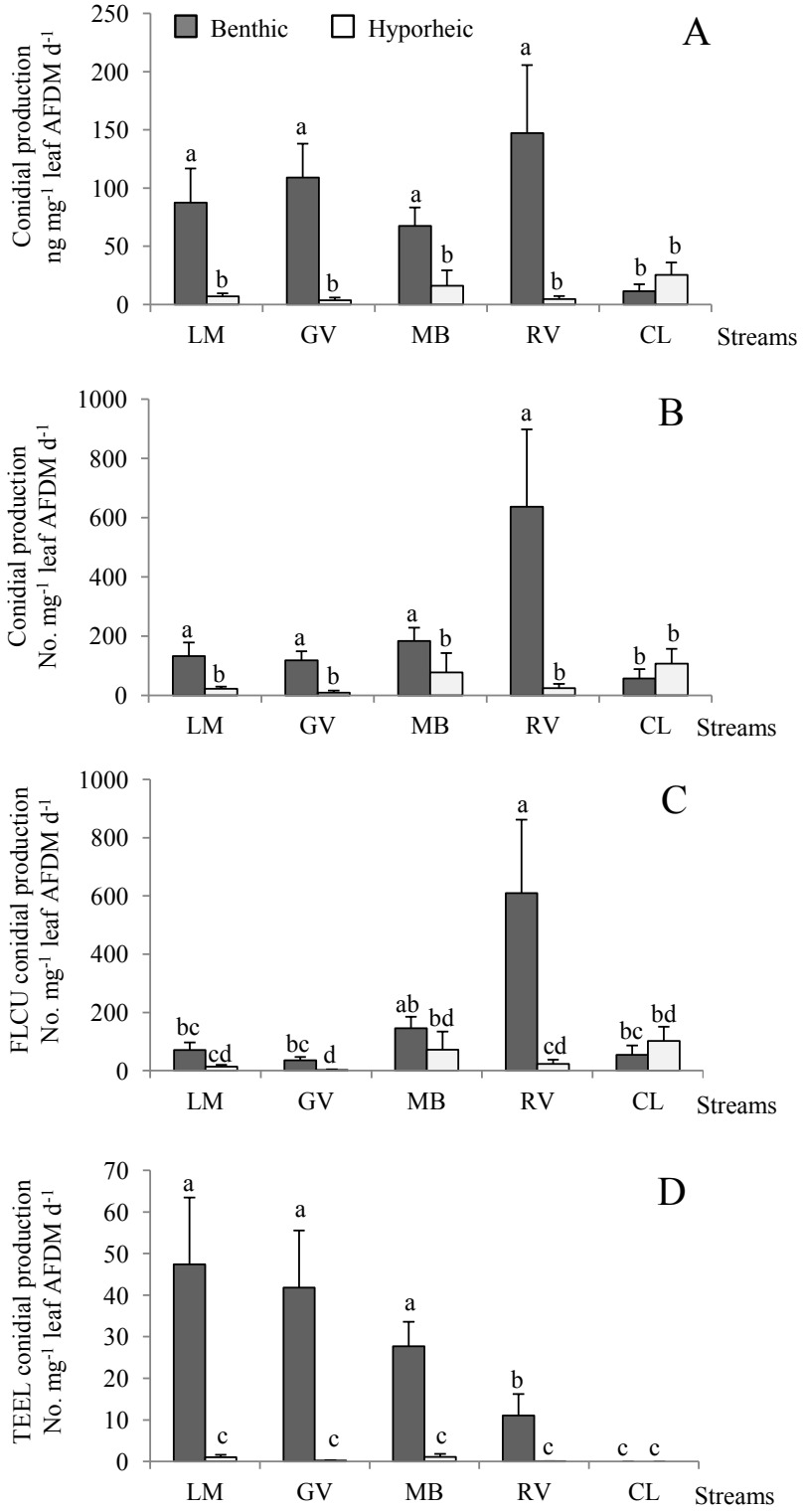

Figure 3. Conidial biomass (A) and total conidial (B) productions, and FLCU (C) and THEL (D) specific conidial productions over the four sampling dates for leaves exposed in the benthic and hyporheic zones of the five streams across the Al gradient. Values are means ± 1 SE (n=16). Different letters are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

Fungal diversity assessed through clone libraries

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

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

Clone sequences related to the Ascomycetes (mainly orders such as Pleosporales, Helotiales, Capnodiales and Taphrinales) were distributed in 24 OTUs (OTU1-24) and contribute to between 70% and 79% of the total sequences of the four clone libraries (Figure 4). In the benthic zone of LM, 39% of clone sequences were identified as belonging to the group of aquatic hyphomycetes, whereas lower proportions were found in the hyporheic zone of LM (8%) and in CL (6% and 16% in the benthic and hyporheic zones, respectively) (Figure 4).

Results showed that sequences close to FLCU and belonging to OTU9 (Table 3) contribute to 3% and 0% of total sequences in the benthic and hyporheic zones of LM, respectively, and to 4% and 9% in the benthic and hyporheic zones of CL, respectively. In OTU13 (Table 3), sequences that were close to THEL, the species co-dominating conidial production in the benthic zone of LM, contribute to 17% and 3% of total sequences in the benthic and hyporheic zones of LM, respectively, and to 0% and 2% in the benthic and hyporheic zones of CL, respectively. Sequences that had nearest blast hits to *Lemonniera terrestris* (LETE) and *Lemonniera aquatica* (LEAQ) were also found in this OTU and contribute to 3% of clone sequences from the benthic zone of LM.

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

A total of 6 OTUs (OTU25-30) had nearest Blast to Basidiomycetes and contribute to 9% (LM, hyporheic) to 25% (CL, benthic) of total clone sequences (Figure 4), most of them being

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

	LM		CL		Fungal order	Nearest BLAST hit (Accession No.)	% Similarity
	Benthic	Hyporheic	Benthic	Hyporheic			
Ascomycetes							
OTU1	2	6	3	3	*Taphrinales*	*Taphrina tosquinetii* (AJ496252.1)	100
OTU2	0	0	0	2	*Taphrinales*	*Taphrina wiesneri voucher* OSC:100047 (NG_013152.1)	99
OTU3	6	18	13	18	*Pleosporales*	*Phoma* sp. Zzz202 (HQ696111.1)	100
OTU4	2	2	0	1	*Pleosporales*	*Phaeosphaeria caricicola* strain CBS 603.86 (GQ387529.1)	100
OTU5	4	3	1	0	*Pleosporales*	*Alternaria alternata* strain HDJZ-zwm-34 (JN673371.1)	100
OTU6	1	0	1	0	*Pleosporales*	*Pleosporales* sp. PG170709B1 isolate PG170709B3 (JN397390.1)	99
OTU7	4	0	0	0	*Helotiales*	*Goniopila monticola* strain FNP 1 (AY357277.1)	100
OTU8	1	0	0	0	*Dothideales*	*Aureobasidium* sp. B23 (HQ696089.1)	99
OTU9	2	0	2	5	Not assigned	*Flagellospora curvula* 130-1655 and 180-1662	100
OTU10	4	0	0	1	*Helotiales*	*Alatospora acuminata* strain 102-280 (AY357261.1)	100
OTU11	1	0	3	2	*Helotiales*	*Botryotinia fuckeliana* strain DAOM 189076 (JN939020.1)	100
OTU12	0	0	1	0	*Helotiales*	*Satchmopsis brasiliensis* strain CPC 11017 (DQ195809.1)	99
OTU13*	13	2	0	1	Not assigned	*Tetrachaetum elegans* strain 105-326 (AY357281.1)	100
OTU14	0	0	1	0	Not assigned	*Phialea strobilina* strain CBS 643.85 (EF596820.1)	99
OTU15	0	0	1	1	*Helotiales*	*Tricladium patulum* strain 139-1063 (AY357285.1)	99
OTU16	0	0	0	1	*Pleosporales*	*Anguillospora filiformis* strain CCM F-20687 (AY178825.1)	99
OTU17	0	0	2	0	*Diaporthales*	*Sirococcus conigenus* strain CBS 113.75 (EU754115.1)	99
OTU18	0	0	0	1	*Diaporthales*	*Sirococcus conigenus* strain CBS 113.75 (EU754115.1)	99
OTU19	2	1	0	0	*Hypocreales*	*Fusarium* sp. Zzz612 (HQ696108.1)	100
OTU20	0	1	0	0	*Hypocreales*	*Nectria lugdunensis* strain CS-950 (AY357278.1)	99
OTU21	0	1	0	0	*Hypocreales*	*Fusarium* sp. Zzz612 (HQ696108.1)	99
OTU22	8	16	7	4	*Capnodiales*	*Cladosporium* sp. B01 (HQ696097.1)	100
OTU23	0	0	1	0	*Capnodiales*	*Cladosporium* sp. YK16 (JN546120.1)	99
OTU24	0	0	1	0	*Capnodiales*	*Cladosporium* sp. YK16 (JN546120.1)	99
Basidiomycetes							
OTU25	4	3	10	7	*Cystofilobasidiales*	*Itersonilia perplexans* (AB072228.1)	100
OTU26	0	2	1	1	*Tremellales*	*Cryptococcus skinneri* (AB032646.1)	99
OTU27	0	1	1	1	*Tremellales*	*Cryptococcus heveanensis* (AB032635.1)	99
OTU28	2	0	0	0	*Tremellales*	*Cryptococcus* sp. FYB-2007a strain AS 2.2653 (EF363152.1)	99
OTU29	0	0	1	0	*Filobasidiales*	*Bullera taiwanensis* (AB072234.1)	100
OTU30	0	0	0	1	*Tremellales*	*Cryptococcus terricola* strain RUB001 (JN938686.1)	99
Chytridiomycetes							
OTU31	0	0	2	3	Not assigned	Uncultured Chytridiomycota clone T3P1AeH07 (GQ995262.1)	98
OTU32	0	0	1	0	Not assigned	Uncultured Chytridiomycota clone T3P1AeH07 (GQ995262.1)	99
OTU33	0	3	0	0	*Chytridiales*	*Chytridiales* sp. JEL187 (AY635825.1)	100
OTU34	7	1	0	0	*Rhizophydiales*	*Rhizophydium* sp. JEL138 (AF164266.1)	96
Zygomycetes							
OTU35	0	0	0	1	*Mortierellales*	*Mortierella angusta* strain CBS 293.61 (HQ667443.1)	99
Other Eukaryotes					Taxonomic group		
OTU36	0	4	0	2	*Rhizaria*	Cercozoa sp. WA28p82t6LS (EU709189.1)	100
Total no. of clones	63	64	53	56			
Total no. of OTUs	16	15	19	19			

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

closely related to *Itersonilia perplexans* (OTU25, order Cystofilobasidiales) (Table 3). Clone sequences were also identified as belonging to the Chytridiomycete group (OTU31-34) and contribute to 5% to 11% of total sequences (Figure 4). Few other clone sequences related to Zygomycetes (OTU35) and to other eukaryotes (OTU36) were also identified in our clone libraries.

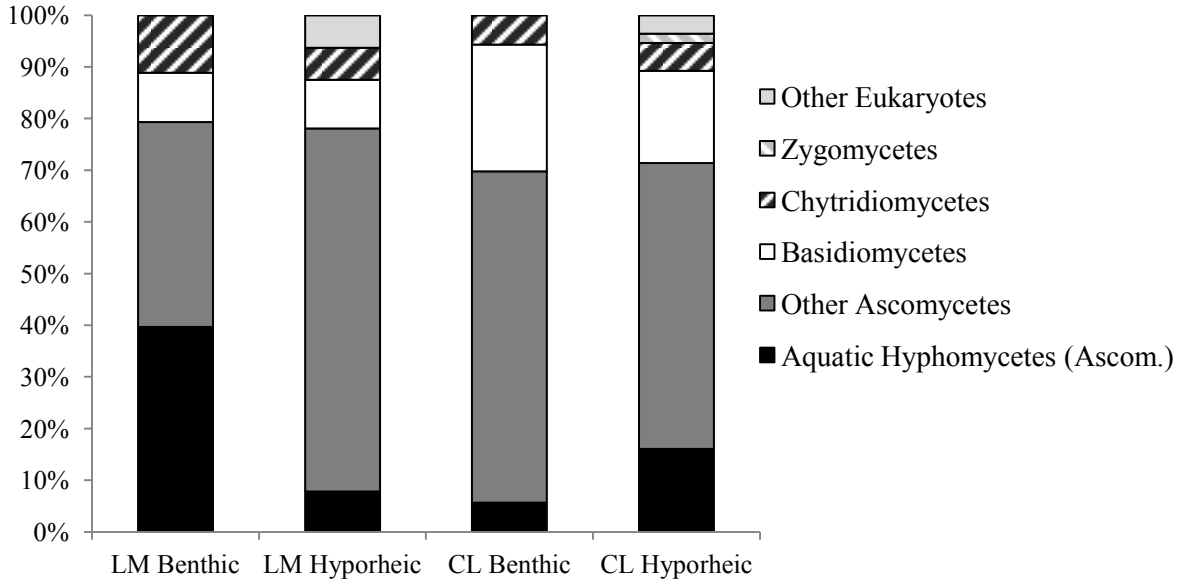

Figure 4. Taxonomic distribution of the nearest BLAST hits from 18S rRNA gene clone libraries of leaves exposed in the benthic and hyporheic zones of the non-impacted stream LM and the most impacted stream CL.

Discussion

Traditional versus molecular analyses

For the first time, this work combined traditional and molecular approaches to provide new insights on leaf-associated fungal communities in the benthic and hyporheic zones of streams subject to a gradient of anthropogenic disturbance.

Molecular analyses using DGGE and sequencing of clone libraries showed that the microscopic method underestimates the fungal diversity on leaves in both zones, particularly in impacted sites. However, comparison of fungal assemblages by conidial identification and DGGE revealed that the traditional method gave a better discrimination between impacted and non impacted sites. We noticed that analyses of clone libraries revealed the presence of sequences close to aquatic hyphomycetes (*e.g. Goniopila monticola*) not detected by conidial

identification. These results could be due to the fact that some species are growing on leaves without producing conidia or, on the other hand, to the poor representation of aquatic fungi in sequences databases. Indeed, many clone sequences have not revealed a perfect identity with database sequences, which precluded an unequivocal identification, as results previously obtained for OTU9 before FLCU sequencing. We observed that this species dominating (up to 96%) conidial production has never contribute to more than 9% of total sequences from clone libraries, revealing marked differences between sporulation and molecular analyses for the characterization of fungal communities. By contrast to sporulation analyses, results also showed that fungal richness assessed by molecular analyses was not depressed across the Al gradient, whatever the compartment of the stream. Phylotype richness, evaluated by DGGE, was slightly higher than OTU richness assessed through clone library analyses, which was supported by some sequences grouping into the same OTU being differentiated in DGGE. For example, sequences of both THEL and LETE were grouped into the same OTU at 99% similarity cut-off but gave distinct phylotypes on DGGE profiles when assessed with the specific DGGE method used in this study (data not shown). This could notably be due to divergences of sequences within an OTU by up to 1%, *i.e.* 5 nucleotides since sequence size used for our phylogenetic analyses was slightly higher than 500 bp.

Acidification effects on benthic and hyporheic sporulating aquatic hyphomycete assemblages

Sporulating aquatic hyphomycete richness was severely reduced in the hyporheic zone of streams and particularly in the most impacted one falling from 12 to only 4 species observed in CL over the 4-week period of the experiment. Various abiotic and biotic factors could affect the diversity of aquatic hyphomycetes and their ability to sporulate in the hyporheic zone of streams. While differences in dissolved oxygen concentrations never exceeded 15% between both zones, lower oxygen concentrations in the hyporheic zone could however have a direct effect on the sporulation of aquatic hyphomycetes as shown by Medeiros et al. (Medeiros et al., 2009), and/or exacerbate the previously observed effects of Al on the metabolism of aquatic hyphomycetes. In a previous study, Chergui & Pattee (1988) also demonstrated that sporulating aquatic hyphomycete richness and leaf breakdown were reduced due to lower dissolved oxygen concentrations and to lower water velocity in a part of a river network. Leaf decomposition being slower in the hyporheic zone, fungal succession could then be slowed down, but this could also result from the inability of conidia to easily access leaf litter. Lower turbulence, which may notably affect sporulation (Webster &

Towfik, 1972; Sanders & Webster, 1980), and sediment porosity could slow down conidia dispersal and thus delay fungal colonization in the hyporheic zone of streams.

Concerning sporulating aquatic hyphomycete assemblages, the important dominance of FLCU in impacted streams did not result from increasing FLCU conidial production but rather from decreasing conidial production of other species. Particularly, THEL conidial production was negatively affected across the Al gradient. Moreover, it is interesting to note that HULU and FLCU were the co-dominant species in the hyporheic zone of less-impacted streams LM and GV, whereas THEL and/or LEAQ were co-dominating together with FLCU in their benthic counterpart. In another area (Montagne Noire, SW France), Cornut et al. (2010) reported similar trends for the contribution of these species to conidial production in both zones. According to the specific morphology and size of their conidia, we could therefore hypothesize that species producing smaller conidia (*i.e.* FLCU and HULU) could probably disperse more readily within the sediment. Metabolic constraints due to elevated Al concentrations in impacted streams and lower oxygen concentrations in the hyporheic zone could also affect aquatic hyphomycete ability to produce large tetraradiate conidia (*e.g.* THEL and LEAQ), as supported by the lower conidial biomass produced.

New insights from molecular evidences

As previously observed in other molecular studies (Nikolcheva & Bärlocher, 2004; Seena et al., 2008), sequencing of clone libraries showed that Basidiomycetes and Chytridiomycetes may contribute significantly to leaf-associated fungal assemblages, which were largely dominated by Ascomycetes. Fungi on leaves affiliated to the latter phylum were mainly terrestrial fungi, which belong to Pleopsorales, Capnodiales or Taphrinales orders and aquatic hyphomycetes, which belong notably to Pleosporales, Helotiales or undefined orders. Despite the use of a two-step PCR protocol, sequences close to other eukaryotes were found in our clone libraries as reported by Seena *et al*. (2008), which also targeted small subunit rDNA gene for their fungal community analysis. However, in our study, sequences that resulted from this lack of primer specificity were in the minority and revealed the presence of eukaryotes close to *Cercozoa* species in the hyporheic zone of LM and CL. Heterotrophic protozoa have been already identified in the interstices of stream sediments, but their grazing activity has not been considered as a major link in the carbon flow within the hyporheic zone (Gücker & Fischer, 2003; Königs & Cleven, 2007). On the other hand, aquatic hyphomycetes are known to greatly contribute to organic matter cycle in streams as main microbial decomposers of leaf litter (Gessner & Chauvet, 1994). Clone library sequencing showed that

their relative contribution to fungal communities on leaves is particularly higher in the benthic zone of LM compared to its hyporheic counterpart and in both zones of CL. Surprisingly, it was also much higher than in previous studies (Seena et al., 2008; Harrop et al., 2009), in which numerous identification attempts have led to poor similarity with sequences of well identified fungi. Nevertheless, continuing advances in the characterization of microbial strains from environmental samples could now provide us more exhaustive database that potentially allow more precise identifications.

An important contribution of terrestrial fungi (genera such as *Phoma*, *Cladosporium*, *Itersonilia*, and *Taphrina*) was observed in both zones of CL and in the hyporheic zone of LM, indicating that these phyllosphere colonizers could persist longer on leaves as well in the hyporheic zone of streams as in impacted streams. These fungi may constitute a great part of fungal community on leaves but they are considered as less efficient leaf decomposers than aquatic hyphomycetes in freshwaters (Bärlocher & Kendrick, 1974). However, additionally to the effects of potential reduction of both sporulation and conidia dispersal in hyporheic zones and impacted streams, these fungi could outcompete aquatic hyphomycetes and thereby delay their colonization and growth on leaves.

Leaf ergosterol content is frequently assessed to estimate fungal living biomass associated with decaying leaves. In Cornut et al. (in press), we confirmed previous results, which revealed no effects of acidification on ergosterol leaf content (Dangles & Chauvet, 2003; Baudoin et al., 2008; Simon et al., 2009; Clivot et al., in press). In each case, this observation has led to the assumption that fungal biomass on leaves was probably not depressed by acidification. A mean ergosterol content of 5.1 mg per gram of fungal biomass was found for numerous and diverse species (Djajakirana et al., 1996) and was close to this of 5.5 found by Gessner and Chauvet (1993) for aquatic hyphomycetes. However, acidification, through its effects on leaf colonizer assemblages, could have an impact on other fungal species, which may contain variable amounts of ergosterol. Notably, we observed that the contribution to clone libraries of Chytridiomycetes, which do not contain ergosterol (Gessner et al., 1991; Gessner & Newell, 2002) is slightly higher on leaves in the benthic zone of LM than in CL. Moreover, higher contribution of Basidiomycetes has been observed in both zones of CL compared to those of LM and Pasanen et al. (1999) reported high amount of ergosterol (37-42 mg per gram of fungal biomass) in some species affiliated to this phylum. Therefore, it cannot be excluded that fungal biomass on leaves could have been underestimated in non-impacted streams and, in the same time, overestimated in impacted streams, potentially minimizing the

observed effects of acidification on mycelial growth. Further molecular analyses and isolation of other fungal strains to assess their specific ergosterol contents are needed to ascertain this hypothesis.

Significance of aquatic hyphomycete contribution to leaf processing

While leaf processing determined in our study did not only result from microbial contribution, it is interesting to note that the highest leaf breakdown rate occurred in presence of both the highest diversity of sporulating aquatic hyphomycetes and the highest contribution of aquatic hyphomycetes to clone libraries in the benthic zone of LM. Aquatic hyphomycete diversity and identity are known to affect leaf litter decomposition (Duarte et al., 2006) and their diversity may also increase functional stability of this ecosystem process under metal-induced stress (Pascoal et al., 2010; Krauss et al., 2011). In addition, their identity can influence leaf processing due to specific preferences of leaf-shredding invertebrates (Bärlocher & Kendrick, 1973; Arsuffi & Suberkropp, 1989). These results underline the major role of aquatic hyphomycetes in leaf decomposition, and show that constraints prevailing in the hyporheic zone and/or linked to anthropogenic disturbances could alter their efficiency to colonize and process plant matter. But, despite the alteration of leaf processing in the hyporheic and benthic zones of impaired streams, Cornut et al. (in press) suggested that aquatic hyphomycete subsistence insures a maintaining of organic matter recycling and a resilience potential of stream ecosystem functioning.

Conclusion

For now, few molecular studies have attempted to characterize leaf-associated fungi. Therefore, further investigations are needed to understand more precisely the contribution of each fungal taxa and what factors drive microbial assemblages and activities on decaying leaves. More attention on terrestrial fungi would be required owing to their significant subsistence on leaves in impacted streams as well as in the hyporheic zones. We showed that molecular responses were not necessarily contradictory but complementary with results obtained from traditional approach. Indeed, aquatic hyphomycete diversity and contribution appeared to be major factors associated with leaf processing. Even if sporulation analysis underestimates leaf-associated fungal diversity, we confirmed that this method may provide a valuable bioindicator of the effects of an anthropogenic disturbance. The combination of both methods and its extent to the study of hyporheic zone of streams is thus promising for deepen

our knowledge on interactions between microorganisms and their role in the functioning of ecosystems, particularly when subject to human-induced alterations.

Acknowledgements

We are grateful to Pierre Gierlinski, Didier Lambrigot, Philippe Rousselle and Philippe Wagner for field and laboratory assistance. The present study was financed by French ANR programs (ANR-06-BDIV-007 InBioProcess and ANR-07-BDIV-007-01 Recover projects). We thank the Conseil Général des Vosges, the Office National des Forêts and the Zone Atelier Moselle for their support.

References

Altschul, S., Gish, W., Miller, W., Myers, E., Lipman, D., 1990. Basic local alignment search tool. Journal of molecular biology 215, 403-410.

Arsuffi, T. L., Suberkropp, K., 1988. Effects of Fungal Mycelia and Enzymatically Degraded Leaves on Feeding and Performance of Caddisfly (Trichoptera) Larvae. Journal of the North American Benthological Society 7, 205-211.

Arsuffi, T. L., Suberkropp, K., 1989. Selective feeding by shredders on leaf-colonizing stream fungi: comparison of macroinvertebrate taxa. Oecologia 79, 30-37.

Baldy., Gessner, M. O., Chauvet, E., 1995. Bacteria, fungi and the breakdown of leaf litter in a large river. Oikos 74, 93-102.

Bärlocher, F., Nikolcheva, L. G., Wilson, K.P., Williams, D.D., 2006. Fungi in the Hyporheic Zone of a Springbrook. Microbial Ecology 52, 708-715.

Bärlocher, F., Schweizer, M., 1983. Effects of Leaf Size and Decay-Rate on Colonization by Aquatic Hyphomycetes. Oikos 41, 205-210.

Bärlocher, F., 2007. Molecular approaches applied to aquatic hyphomycetes. Fungal Biology Reviews 21, 19-24.

Bärlocher, F., 2010. Molecular approaches promise a deeper and broader understanding of the evolutionary ecology of aquatic hyphomycetes. Journal of the North American Benthological Society 29, 1027-1041.

Bärlocher, F., 1992. The Ecology of aquatic hyphomycetes. Springer-Verlag, Berlin.

Bärlocher, F., Kendrick, B., 1981. The role of aquatic hyphomycetes in the trophic structure of streams. In:The fungal community (eds D.T. Wicklow , G.C. Carroll), Marcel Dekker, New York, p. 743-760.

Bärlocher, F., Kendrick, B., 1973. Fungi and Food Preferences of Gammarus-Pseudolimnaeus. Archiv für Hydrobiologie 72, 501-516.

Bärlocher, F., Kendrick, B., 1974. Dynamics of the Fungal Population on Leaves in a Stream. Journal of Ecology 62, 761-791.

Baudoin, J., Guérold, F., Felten, V., Chauvet, E., Wagner, P., Rousselle, P., 2008. Elevated Aluminium Concentration in Acidified Headwater Streams Lowers Aquatic Hyphomycete Diversity and Impairs Leaf-Litter Breakdown. Microbial Ecology 56, 260-269.

Boulton, A.J., Findlay, S., Marmonier, P., Stanley, E.H., Valett, H.M., 1998. The Functional Significance of the Hyporheic Zone in Streams and Rivers. Annual Review of Ecology and Systematics 29, 59-81.

Bray, J.R., Curtis, J.T., 1957. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecological Monographs 27, 325-349.

Chauvet, E., Suberkropp, K., 1998. Temperature and Sporulation of Aquatic Hyphomycetes. Appl. Environmental Microbiology 64, 1522-1525.

Chergui, H., Pattee, E., 1988. The dynamics of Hyphomycetes on decaving leaves in the network of the River Rhone (France). Archiv für Hydrobiologie 114, 3-20.

Clivot, H., Danger, M., Pagnout, C., Wagner, P., Rousselle, P., Poupin, P., Guérold, F., 2012. Impaired Leaf Litter Processing in Acidified Streams. Microbial Ecology.

Cornut, J., Clivot, H., Chauvet, E., Elger, A., Pagnout, C., Guérold, F., 2012. Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams. Water Research.

Cornut, J., Elger, A., Greugny, A., Bonnet, M., Chauvet, E., 2012. Coarse particulate organic matter in the interstitial zone of three French headwater streams. Annales de Limnologie - International Journal of Limnology 48, 303-313.

Cornut, J., Elger, A., Lambrigot, D., Marmonier, P., Chauvet, E., 2010. Early stages of leaf decomposition are mediated by aquatic fungi in the hyporheic zone of woodland streams. Freshwater Biology 55, 2541-2556.
Dangles, O., Chauvet, E., 2003. Effects of stream acidification on fungal biomass in decaying beech leaves and leaf palatability. Water Research 37, 533-538.
Djajakirana, G., Joergensen, R.G., Meyer, B., 1996. Ergosterol and microbial biomass relationship in soil. Biology and Fertility of Soils 22, 299-304.
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., Bärlocher, F., 2006. Aquatic hyphomycete diversity and identity affect leaf litter decomposition in microcosms. Oecologia 147, 658-666.
Felsenstein, J., 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
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.
Findlay, S.E.G., Arsuffi, T.L., 1989. Microbial growth and detritus transformations during decomposition of leaf litter in a stream. Freshwater Biology 21, 261-269.
Gessner, M.O., Bauchrowitz, M., Escautier, M., 1991. Extraction and quantification of ergosterol as a measure of fungal biomass in leaf litter. Microbial Ecology 22, 285-291.
Gessner, M.O., 1997. Fungal biomass, production and sporulation associated with particulate organic matter in streams. Limnetica 33-44.
Gessner, M.O., Newell, S.Y., 2002. Biomass, growth rate, and production of filamentous fungi in plant litter. In: Manual of environmental microbiology (eds C.J. Hurst, R.L. Crawford, G. Knudsen, M. Mclnerney, L.D. Stetzenbach) ASM Press, Washington D.C., p. 390-408.
Gessner, M.O., Gulis, V., Kuehn, K.A., Chauvet, E., Suberkropp, K., 2007. Fungal decomposers of plant litter in aquatic ecosystems. In: The Mycota: a Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research, Vol. IV: Environmental and Microbial Relationships. 2nd edition (eds C.P. Kubicek and I.S. Druzhinia), Springer, Berlin, p. 301-324.
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., 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.
Gücker, B., Fischer, H., 2003. Flagellate and ciliate distribution in sediments of a lowland river: relationships with environmental gradients and bacteria. Aquatic Microbial Ecology 31, 67-76.
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.
Harrop, B.L., Marks, J.C., Watwood, M.E., 2009. Early bacterial and fungal colonization of leaf litter in Fossil Creek, Arizona. Journal of the North American Benthological Society 28, 383-396.
Iqbal, S.H., Webster, J., 1973. The trapping of aquatic hyphomycete spores by air bubbles. Transactions of the British Mycological Society 60, 37-48.

Jones, J.B., 1997. Benthic Organic Matter Storage in Streams: Influence of Detrital Import and Export, Retention Mechanisms, and Climate. Journal of the North American Benthological Society 16, 109-119.

Königs, S., Cleven, E.J., 2007. The bacterivory of interstitial ciliates in association with bacterial biomass and production in the hyporheic zone of a lowland stream. FEMS Microbiology Ecology 61, 54–64.

Krauss, G.J., Solé, M., Krauss, G., Schlosser, D., Wesenberg, D., Bärlocher, F., 2011. Fungi in freshwaters: ecology, physiology and biochemical potential. FEMS Microbiology Reviews 35, 620-651.

Larkin, M., Blackshields, G., Brown, N., Chenna, R., McGettigan, P., McWilliam, H., Valentin, F., Wallace, I., Wilm, A., Lopez, R., Thompson, J., Gibson, T., Higgins, D., 2007. Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) 23, 2948, 2947.

Legendre, P., Legendre, L., 1998. Numerical Ecology. Elsevier.

Medeiros, A.O., Pascoal, C., Graça, M.A.S., 2009. Diversity and activity of aquatic fungi under low oxygen conditions. Freshwater Biology 54, 142-149.

Moreirinha, C., Duarte, S., Pascoal, C., Cássio, F., 2011. Effects of Cadmium and Phenanthrene Mixtures on Aquatic Fungi and Microbially Mediated Leaf Litter Decomposition. Archives of Environmental Contamination and Toxicology 61, 211-219.

Nikolcheva, L., Bärlocher, F., 2004. Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. Mycological Progress 3, 41-49.

Nikolcheva, L.G., Cockshutt, A.M., Bärlocher, F., 2003. Determining Diversity of Freshwater Fungi on Decaying Leaves: Comparison of Traditional and Molecular Approaches. Applied and Environmental Microbiology 69, 2548-2554.

Niyogi, D.K., Cheatham, C.A., Thomson, W.H., Christiansen, J.M., 2009. Litter breakdown and fungal diversity in a stream affected by mine drainage. Fundamental and Applied Limnology / Archiv für Hydrobiologie 175, 39-48.

Oros-Sichler, M., Gomes, N.C.M., Neuber, G., Smalla, K., 2006. A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. Journal of Microbiological Methods 65, 63-75.

Pasanen, A.L., Yli-Pietila, K., Pasanen, P., Kalliokoski, P., Tarhanen, J., 1999. Ergosterol Content in Various Fungal Species and Biocontaminated Building Materials. Applied and Environmental Microbiology 65, 138-142.

Pascoal, C., Cássio, F., Nikolcheva, L., Bärlocher, F., 2010. Realized Fungal Diversity Increases Functional Stability of Leaf Litter Decomposition Under Zinc Stress. Microbial Ecology 59, 84-93.

Pradhan, A., Seena, S., Pascoal, C., Cássio, F., 2011. Can Metal Nanoparticles Be a Threat to Microbial Decomposers of Plant Litter in Streams? Microbial Ecology 62, 58-68.

R Development Core Team., 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Sanders, P.F., Webster, J., 1980. Sporulation responses of some 'aquatic hyphomycetes' in flowing water. Transactions of the British Mycological Society 74, 601-605.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Horn, D.J.V., Weber, C.F., 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Applied and Environmental Microbiology 75, 7537-7541.

Seena, S., Wynberg, N., Bärlocher, F., 2008. Fungal diversity during leaf decomposition in a stream assessed through clone libraries. Fungal Diversity 30, 1-14.

Simon, K.S., Simon, M.A., Benfield, E.F., 2009. Variation in ecosystem function in Appalachian streams along an acidity gradient. Ecological Applications 19, 1147–1160.
Solé, M., Fetzer, I., Wennrich, R., Sridhar, K.R., Harms, H., Krauss, G., 2008. Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. Science of The Total Environment 389, 557-565.
Suberkropp, K., 1992. Aquatic Hyphomycete communities. In:The Fungal Community: Its Organization and Role in the Ecosystem, 2nd edition, Marcel Dekker Inc., New-York, p. 729-747.
Suberkropp, K., 1991. Relationships between growth and sporulation of aquatic hyphomycetes on decomposing leaf litter. Mycological Research 95, 843-850.
Webster, J., Towfik, F.H., 1972. Sporulation of aquatic hyphomycetes in relation to aeration. Transactions of the British Mycological Society 59, 353-364.

IV.2. Effets de l'acidification des cours d'eau de tête de bassin sur les activités microbiennes associées à la litière en décomposition

Résumé

Les travaux précédents ont notamment révélé que la décomposition des litières et les communautés microbiennes impliquées dans celle-ci pouvaient être affectées par l'acidification des cours d'eau. Cependant, peu de choses sont connues sur ses effets potentiels sur les activités enzymatiques des micro-organismes décomposeurs. L'étude qui suit à pour objectif la compréhension des relations existantes entre les activités enzymatiques microbiennes et la diminution de la décomposition de la litière dans les cours d'eau impactés.

Durant 70 jours, des sachets de litières ont été disposés dans six sites le long d'un gradient d'acidification. Les résultats ont révélé que la décomposition des feuilles par les micro-organismes était significativement et négativement corrélée aux concentrations en Al (r= -0.99, p<0.001) et positivement corrélée aux concentrations en Ca (r=0.94, p=0.005) et au pH (r=0.93, p=0.008). Les analyses DGGE ont montré que les assemblages microbiens étaient différents entre les sites impactés et non impactés, alors que la biomasse fongique n'était pas affectée. Le contenu en nutriments des feuilles et les activités enzymatiques d'acquisition de C, N et P ont révélé que l'acquisition de N n'était pas altérée. En revanche, l'acquisition de P était significativement réduite le long du gradient d'acidification, le contenu en P des feuilles étant négativement corrélé aux concentrations en Al (r= -0.94, p<0.01) et positivement corrélée aux taux de décomposition (r= 0.95, p<0.01). Cette limitation potentielle en P des micro-organismes décomposeurs dans les sites impactés a été confirmée par de très importantes activités phosphatases et des ratios déséquilibrés entre les activités enzymatiques d'acquisition de C et de P.

Les formes toxiques de l'Al ont des effets reconnus sur les communautés aquatiques mais cette étude montre aussi que l'Al peut potentiellement affecter le cycle du P et les communautés microbiennes impliquées dans la décomposition de la litière. Ces effets peuvent avoir à leur tour un impact sur toute la chaine trophique et donc sur tout l'écosystème.

Microb Ecol
DOI 10.1007/s00248-012-0107-x

MICROBIOLOGY OF AQUATIC SYSTEMS

Impaired Leaf Litter Processing in Acidified Streams

Learning from Microbial Enzyme Activities

Hugues Clivot · Michael Danger · Christophe Pagnout · Philippe Wagner · Philippe Rousselle · Pascal Poupin · François Guérold

Received: 27 March 2012 / Accepted: 2 August 2012

Abstract Anthropogenic acidification in headwater streams is known to affect microbial assemblages involved in leaf litter breakdown. Far less is known about its potential effects on microbial enzyme activities. To assess the effects of acidification on microbial activities associated with decaying leaves, a 70-day litter bag experiment was conducted in headwater streams at six sites across an acidification gradient. The results revealed that microbial leaf decomposition was strongly and negatively correlated with total Al concentrations (r=−0.99, p<0.001) and positively correlated with Ca^{2+} concentrations (r=0.94, p=0.005) and pH (r=0.93, p=0.008). Denaturing gradient gel electrophoresis analyses showed that microbial assemblages differed between non-impacted and impacted sites, whereas fungal biomass associated with decaying leaves was unaffected. The nutrient content of leaf detritus and ecoenzymatic activities of carbon (C), nitrogen (N) and phosphorus (P) acquisition revealed that N acquisition was unaltered, while P acquisition was significantly reduced across the acidification gradient. The P content of leaf litter was negatively correlated with total Al concentrations (r=−0.94, p<0.01) and positively correlated with decomposition rates (r=0.95, p<0.01). This potential P limitation of microbial decomposers in impacted sites was confirmed by the particularly high turnover activity for phosphatase and imbalanced ratios between the ecoenzymatic activities of C and P acquisition. The toxic form of Al has well-known direct effects on aquatic biota under acidic conditions, but in this study, Al was found to also potentially affect microbially mediated leaf processing by interfering with the P cycle. These effects may in turn have repercussions on higher trophic levels and whole ecosystem functioning.

H. Clivot · M. Danger · C. Pagnout · P. Wagner · P. Rousselle · P. Poupin · F. Guérold (✉)
Université de Lorraine, Laboratoire des Interactions Ecotoxicologie Biodiversité Ecosystèmes (LIEBE), UMR 7146, Metz 57070, France
e-mail: guerold@univ-metz.fr

H. Clivot · M. Danger · C. Pagnout · P. Wagner · P. Rousselle · P. Poupin · F. Guérold
CNRS, Laboratoire des Interactions Ecotoxicologie Biodiversité Ecosystèmes (LIEBE), UMR 7146,
Metz 57070, France

Introduction

Many freshwater ecosystems are affected by anthropogenic acidification, which leads to severe damage to aquatic biota through reduced pH, elevated Al concentrations and base cation deficiencies. A dramatic decline in biodiversity is often observed in headwater streams draining forested watersheds [25, 28, 49]. The results of various studies have shown that the key process of leaf litter breakdown in these detritus-based streams is also severely reduced by acidification [7, 12, 35–37]. In particular, Dangles et al. [12] reported breakdown rates of beech (*Fagus sylvatica*) leaves that were in average ten times lower in acidified streams (pH <5.0) than in circumneutral streams. Leaves in streams are primarily conditioned by microbial decomposers, then processed by invertebrate shredders. Aquatic hyphomycetes are commonly considered to be the main microbial decomposers of leaf litter [21], and are essential to increasing leaf litter consumption by detritivores [1, 3]. Therefore, slower leaf litter breakdown in acidified streams could be explained by both changes in microbial and detritivore communities. It is now well established that leaf decomposition is related to the abundance of some acid-sensitive key shredders [11, 13, 48] and that aquatic hyphomycete diversity on leaves can be reduced in acidified streams and under elevated Al concentrations [4, 7]. However, mechanisms underlying alteration

Published online: 19 August 2012

of leaf litter breakdown at the microbial level are not yet fully understood.

Chamier [7] found that microbial decomposition of alder and oak leaves was reduced in streams exhibiting low pH (pH <5.5), and Mulholland et al. [37] suggested that low rates of decomposition were due to reduced activities of microorganisms. During leaf decomposition, microorganisms produce leaf-degrading extracellular enzymes that play an essential role in carbon and nutrient cycles. Through their enzymatic activity, they also release dissolved organic matter (DOM) and fine particulate organic matter (FPOM) within the streams. Decreasing pH can reduce the efficiency of pH-sensitive enzymes involved in pectin degradation of yellow poplar leaves [31]. Conversely, low pH does not appear to be limiting for microbial processing of maple leaves that peaked at pH values between 5.5 and 6.0 [55], and high activities of cellulolytic enzymes can be found on leaves in acidified streams [24, 48]. Chamier [7] suggested that other elements in low pH streams such as increasing monomeric Al could inhibit microbial metabolism. Chamier and Tipping [9] demonstrated that Al could alter the growth and capacity of sporulation of aquatic hyphomycetes, revealing negative effects on the metabolism of these fungi. More recently, Dangles et al. [12] showed that increasing concentrations of Al can decrease microbial respiration associated with decaying leaves. In this context, it seems obvious that Al mobilized by the acidification process can have a strong effect on microbial metabolism, which is tied to factors such as nutrient availability and enzymatic production.

According to Sinsabaugh et al. [52], nutrient acquisition by heterotrophic microbial communities generally shows balanced ecoenzymatic ratios as evidenced by relationships between activities of some extracellular enzymes (ecoenzymes), which release assimilable products from C, N and P organic sources. Alteration of the functional stoichiometry of microbial communities could be used to reveal some constraints of nutrient acquisition, and potentially nutrient limitations of microbial leaf litter processing in acidified streams.

By focusing on microbial decomposers, the aim of this study was to highlight changes in microbial activities that could explain reduced leaf litter breakdown in acidified streams. To this end, a 70-day litter bag experiment was conducted in the Vosges Mountains (Northeastern France) at six sites across an acidification gradient. We assessed the potential activities of extracellular enzymes related to leaf litter decomposition and N and P acquisition. Because microbial activities might be related to microbial community structure, the bacterial and fungal community structures were also investigated.

Materials and Methods

Site Description and Biological Material

The study was conducted in first-order streams in the Vosges Mountains (Northeastern France). The study area is underlain by sandstone bedrock and the streams are surrounded by mixed deciduous–coniferous forests. The six selected sites showed different levels of acidification (see Table 1).

Sites N1, N2 and N3 showed circumneutral pH (from 6.80 to 7.58), but N3 exhibited a higher mean total Al concentration (242 μg l^{-1} for N3 vs. 60 – 99 μg l^{-1} for N1 and N2, respectively). Ac1, Ac2 and Ac3 were strongly acidified (mean pH<4.7) and characterized by high mean Al concentrations (337, 535 and 580 μg l^{-1}, respectively).

Senescent leaves of Norway maple (*Acer platanoides*) were collected from a single tree just before abscission in the fall of 2009. Maple is a common tree species of riparian vegetation at the study sites and maple leaves were selected due to their relatively fast decomposition rate. Disks (18-mm diameter) were cut from the leaves while taking the vein in the middle of each disk. Leaf disks were air dried at room temperature (20–22 °C) for 3 weeks and then packed in

Table 1 Physicochemical variables and leaf litter decomposition rates ($-k$) for the six sites over the 70-day study period

Site	Location	pH	Conductivity (μS cm^{-1})	ANC (μeq l^{-1})	NO_3^- (mg l^{-1})	SO_4^{2} (mg l^{-1})	Ca^{2+} (mg l^{-1})	Mg^{2+} (mg l^{-1})	Al_{total} (μg l^{-1})	$-k$ (day^{-1})
N1	48°29′00″N; 07°04′13″E	7.43^{a}	70^{b}	440^{b}	3.1^{cd}	4.3^{ab}	6.1^{b}	3.1^{b}	60^{d}	0.0219^{a}
N2	48°25′03″N; 07°04′16″E	7.58^{a}	95^{a}	674^{a}	2.9^{cd}	4.8^{a}	8.5^{a}	4.8^{a}	99^{cd}	0.0175^{ab}
N3	48°26′56″N; 07°03′33″E	6.80^{b}	43^{cd}	157^{c}	4.2^{ab}	4.2^{b}	3.2^{c}	1.5^{c}	242^{bc}	0.0110^{bc}
Ac1	48°28′59″N; 07°05′34″E	4.66^{c}	32^{c}	-20^{d}	2.5^{d}	4.3^{ab}	1.3^{d}	0.5^{d}	337^{b}	0.0067^{c}
Ac2	48°27′05″N; 07°04′12″E	4.62^{c}	30^{c}	-21^{d}	3.6^{bc}	4.1^{b}	1.0^{d}	0.4^{d}	535^{a}	0.0046^{c}
Ac3	48°26′24″N; 07°03′54″E	4.48^{c}	35^{cd}	-32^{d}	4.4^{a}	4.6^{ab}	1.1^{d}	0.4^{d}	580^{a}	0.0048^{c}

Values are the mean of the physicochemical variables (n=7). Different letters represent statistical differences (ANOVA, p<0.05 followed by Tukey's tests for physicochemical variables and ANCOVA, p<0.001 for daily breakdown rates)

ANC acid-neutralizing capacity

nylon fine-mesh bags (0.4 mm mesh). The litter bags were submerged at each site in mid November 2009 and four litter bags were retrieved from each site after 7, 13, 21, 28, 49 and 70 days. At the beginning of the experiment, four replicate bags were returned to the laboratory to assess the initial conditions.

Water Chemistry

Water samples were collected at the beginning of the experiment and at each sampling date. The samples were kept cold until laboratory analysis within 48 h. Conductivity and pH were measured with a Metrohm Herisau Conductometer E518 (Herisau, Switzerland) at 25 °C and a microprocessor pH meter (pH 3000, WTW), respectively. Acid-neutralizing capacity (ANC) was measured by Gran's titration. The Ca^{2+}, Mg^{2+} and total Al (after acidification with HNO_3) concentrations were determined by atomic absorption spectrophotometry (Aanalyst 100; Perkin Elmer and Varian SpectrAA-300). The SO_4^{2-} and NO^{3-} concentrations were determined by ion chromatography (Dionex 1500i with an AS 4 A SC column; Dionex, Sunnyvale, CA).

Litter Bag Processing and Leaf Litter Decomposition

At each sampling date, four litter bag replicates were randomly retrieved from each site and placed in individual plastic bags with stream water. Ten leaf disks from each litter bag were immediately transferred into cryogenic tubes and stored in liquid nitrogen for enzyme activity analysis. Plastic bags were kept cold and transported to the laboratory, where the remaining leaf disks were stored at −80 °C until processing to determine leaf mass loss, to quantify nutrient and ergosterol content and to extract microbial DNA to further molecular analysis.

Twenty-four disks from each sample were used to assess the remaining mass of leaf litter. Leaf disks were oven-dried at 105 °C for 24 h to constant mass and then weighed (dry mass, DM) before being ignited in a muffle furnace at 550 °C for 4 h to determine the ash-free dry mass (AFDM).

Microbial Community Structure

For polymerase chain reaction (PCR)-DGGE analyses, three leaf disks from each of the four replicates were pooled for each condition. Leaf disks were finely ground and total DNA from each composite sample was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Fungal 18S rRNA gene fragments were amplified using a two-step PCR protocol [42]. Briefly, the first amplification was performed using NS1 (5′-GTAGTCA TATGCTTGTCTC-3′) and EF3 (5′-TCCTCTAAATGACC AAGTTTG-3′) primers. Diluted amplicons (1:500) obtained were used as the template for the second amplification, which was conducted with primers NS1 and FR1-GC (5′-CCCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA-CGGGCCG AICCATTCAATCGGTAIT-3′). Bacterial partial 16S rRNA gene fragments were amplified using the universal primers 341 F-GC (5′-CGCCCGCCGCGCG C G G C G G G C G G G G C G G G G G C A C G G G G G G GCCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTC AATTCMTTTGAGTTT-3′) [38, 39]. PCR mixtures (100 μl) contained 6 U of *Taq* DNA Polymerase (5 PRIME, Hamburg, Germany), 1× *Taq* buffer (5 PRIME) formulated to automatically adjust the Mg^{2+} concentration, 200 μM of each dNTP, 0.5 μM of each primer and 50 ng of extracted DNA as template. For the fungal communities, the first PCR amplification protocol consisted of 5 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 45 s at 47 °C, 3 min at 72 °C, and a final extension of 10 min at 72 °C. The second step was identical except that the number of cycles was reduced to 20 and the annealing temperature was 48 °C [42]. For bacterial communities, the PCR protocol consisted of 5 min at 95 °C, followed by 20 cycles of 30 s at 95 °C, 30 s at 65–55 °C (touchdown −0.5 °C per cycle), 35 s at 72 °C, and 10 cycles of 30 s at 95 °C, 30 s at 55 °C and 35 s at 72 °C, followed by 7 min of final extension at 72 °C (adapted from Muyzer et al. [38, 39]). The amplification products were subjected to quality control on 1 % (w/v) agarose gel and then separated with the DCODE Mutation Detection System (Bio-Rad, Hercules, CA). A total of 10 μl per sample were loaded onto 6 % (fungal PCR products) or 7 % (bacterial PCR products) (w/v) polyacrylamide gels in 1× Tris–acetate–EDTA (TAE) buffer with denaturing gradients ranging from 25 % to 40 % for the fungal PCR products and from 40 % to 60 % for the bacterial PCR products (100 % denaturant corresponds to 40 % (v/v) formamide and 7 M urea). The gels were run in 1× TAE buffer at 180 V and 58 °C for 16 h for fungal PCR products and at 100 V and 60 °C for 16 h for bacterial PCR products, after which they were stained with SYBR Green I and imaged with a STARION FLA-9000 scanner (Fujifilm Life Sciences FSVT, Courbevoie, France).

Fungal Biomass and Leaf Litter Nutrient Content

Five leaf disks were used to determine leaf-associated fungal biomass in each sample. The ergosterol concentration of the leaf litter was assessed using solid-phase extraction and reversed phase high performance liquid chromatography [22]. A conversion factor of 5.5 mg ergosterol g^{-1} of mycelial dry mass was used to determine the fungal biomass [20].

The nutrient contents of leaves were assessed using five leaf disks of each sample. Disks were oven-dried at 105 °C for 24 h and then finely ground. The N content was determined using a CHN elemental analyzer and the P content was determined using a colorimetric method after oxidation

by sodium persulfate [41]. The nutrient contents were expressed as percentages of DM.

Enzyme Activities

Ten leaf disks of each sample were ground in a mortar in 10 ml of cold 50 mM sodium acetate buffer (pH 5.0). The homogenate was then centrifuged for 20 min at 10,000×*g* and 4 °C, after which the supernatant was used as the enzyme extract.

The potential activities of phenoloxidase (EC 1.10.3.2), β-glucosidase (EC 3.2.1.21), β-*N*-acetylglucosaminidase (EC 3.2.1.52) and phosphatase (EC 3.1.3.2) were assessed using 30 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) [32], 5 mM pNP-β-D-glucopyranoside, 2 mM pNP-*N*-acetyl-β-D-glucosaminide and 5 mM pNP-phosphate, respectively [51] (all products were purchased from Sigma-Aldrich, St. Quentin Fallavier, France). For the phenoloxidase potential activity, 100 μl of enzyme extract, 100 μl of ABTS and 800 μl of 50 mM sodium acetate buffer (pH 5.0) were homogenized and the oxidation rate of substrate was measured at 420 nm for 5 min (ξ^{M}=36,000 M^{-1} cm^{-1}). The results were expressed as μmol of product formed per gram of dry mass (DM) and per minute. For other kinetic analyses, 150 μl of enzyme extract and 150 μl of substrate were homogenized and incubated at 22 °C as follows to determine the activities: 4 h for β-glucosidase, 5 h for β-*N*-acetylglucosaminidase and 1 h for phosphatase. The three controls consisted of 150 μl of enzyme extract with 150 μl of sodium acetate buffer, 150 μl of substrate with 150 μl of sodium acetate buffer and 300 μl of sodium acetate buffer as a blank. Reactions were stopped by adding 500 μl of 0.1 M NaOH, at which point the absorbance at 405 nm was read. The results were expressed as μmol of *p*-nitrophenol formed per gram of DM per hour.

Data Analysis

Differences in physicochemical variables between sites were tested using one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons (Tukey's) test. Leaf disk breakdown rates ($-k$) were determined by regression of the ln-transformed negative exponential model [44]. Decomposition rates were compared among sites using analysis of covariance (ANCOVA) followed by Tukey's post hoc multiple-comparisons test [2].

Two-way ANOVAs were carried out to test for the effects of site and time on logarithmically transformed data from fungal biomass, nutrient content of leaves and enzyme activities. Differences between sites were tested using Tukey's post-hoc test [56].

Pearson correlations were performed to investigate potential relationships between breakdown rates, means across all dates of physicochemical variables and means of extracellular enzyme activities, fungal biomass, N and P content of leaves over the study period.

For enzyme turnover activities, cumulative enzyme activities were first calculated by integrating potential activities over time. Linear regressions of leaf remaining mass as a function of cumulative enzyme activities were performed. The turnover activity (T_a), expressed in millimoles per gram, is the inverse of the slope (k_a) of each relationship. All regressions were statistically significant ($p<0.05$, R^2=0.71–0.98). The turnover activity represents the amount of enzyme activity required to completely process leaf litter [51].

In addition, the ecoenzymatic ratios of the β-glucosidase and β-*N*-acetylglucosaminidase activities and β-glucosidase and phosphatase activities were determined [52, 53]. Differences in ecoenzymatic ratios were assessed by one-way ANOVAs followed by post hoc multiple-comparisons (Tukey's) test.

GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was used to normalize DGGE profiles. To accomplish this, internal control samples were loaded onto each gel and the means of the relative band intensity for each site on the six sampling dates were calculated to compare assemblages on leaves. Bray–Curtis distance matrices were generated for bacterial and fungal assemblages [6]. Cluster analysis of DGGE mean profiles was performed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) and illustrated with dendrograms [14, 34]. The phylotype richness was calculated as the total number of distinct bands, by cumulating richness for the six dates over the 70-day period. R Software was used for all statistical analyses [46].

Results

Leaf Decomposition

Decomposition rates of maple leaf disks by microorganisms differed significantly among sites (ANCOVA; F=16.2, $p<0.001$) (Table 1). Rates of leaf litter decomposition ranged from 0.0046 in the most impacted site (Ac3) to 0.0219 day^{-1} in the circumneutral site with the lowest mean Al concentrations (N1). Leaf litter decomposition rate was strongly negatively correlated with total Al concentrations (log_{10} transformed; r=−0.99, $p<0.001$) (Table 2). Additionally, strong significant correlations were observed with the mean Ca^{2+} concentration (log_{10} transformed; r=0.94, p=0.005) and pH (r=0.93, p=0.008).

Microbial Assemblages

Cluster analyses of DGGE fingerprints showed that bacterial and fungal community structure on leaves differed between acidic and circumneutral sites (Fig. 1a and b, respectively).

Table 2 Pearson correlations between leaf litter decomposition rates (−*k*), total Al, Ca^{2+} concentrations, pH, fungal biomass, leaf nutrient contents and enzyme activities

	−*k*	Al_{total} [a]	Ca^{2+} [a]	pH	Fungal biomass	N	P	PO	BG	NAG	PH[a]
−*k*		**−0.99*****	**0.94****	**0.93****	0.63	0.68	**0.95****	−0.69	**−0.95****	**−0.91***	**−0.95****
Al_{total} [a]	–		**−0.94****	**−0.92***	−0.58	−0.66	**−0.94****	0.63	**0.95****	**0.88***	**0.95****
Ca^{2+} [a]	–	–		**0.98*****	0.40	0.40	**0.82***	−0.69	**−0.95****	**−0.96****	**−0.97****
pH	–	–	–		0.37	0.39	0.79	−0.65	**−0.97****	**−0.99*****	**−0.99*****
Fungal biomass	–	–	–	–		**0.91***	**0.82***	−0.74	−0.37	−0.45	−0.40
N	–	–	–	–	–		**0.83***	−0.47	−0.48	−0.42	−0.46
P	–	–	–	–	–	–		−0.76	−0.81	−0.80	**−0.82***
PO	–	–	–	–	–	–	–		0.51	0.72	0.60
BG	–	–	–	–	–	–	–	–		**0.94****	**0.99*****
NAG	–	–	–	–	–	–	–	–	–		**0.97****
PH[a]	–	–	–	–	–	–	–	–	–	–	

[a] $\log_{10}$ transformed data

Significant correlations are indicated in bold (levels of significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$)

PO phenoloxidase, *BG* β-glucosidase, *NAG* β-*N*-acetylglucosaminidase, *PH* phosphatase

The phylotype richness (*S*) of fungal communities was slightly lower in the three acidic sites than in the three circumneutral ones (14 in Ac1 and Ac3, 16 in Ac2 vs. 19 in N1 and 20 in N2 and N3). In contrast, bacterial phylotype richness was higher in the highly impacted sites Ac2 and Ac3 (28 and 26, respectively) than in the circumneutral sites, N1 and N2 (17 and 15, respectively). In sites N3 and Ac1, intermediate responses were observed for bacterial phylotype richness (*S*=22).

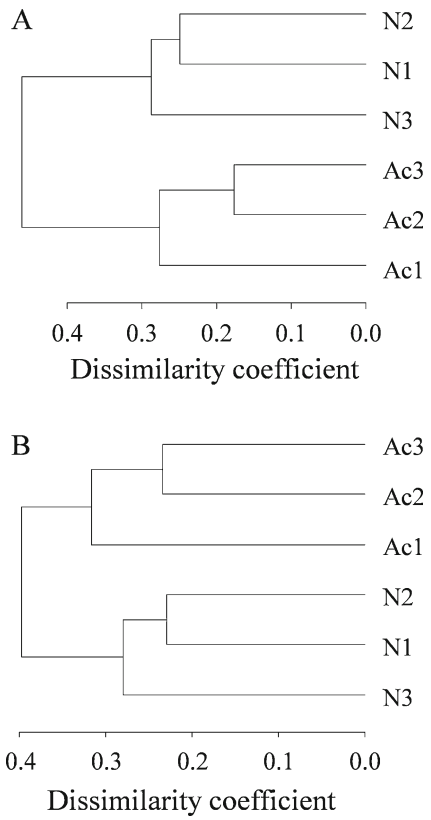

Figure 1 Cluster dendrograms of bacterial (**a**) and fungal (**b**) communities on leaves among the six sites over the 70-day study period

Fungal Biomass and Leaf Litter Nutrient Content

Fungal biomass differed significantly among sites, over time and for the interaction between both factors (two-way ANOVA, Table 3), but did not differ between the two most impacted sites (Ac2 and Ac3) and those showing the lowest Al concentrations (N1 and N2). However, we noticed that the biomass increased faster in the non impacted site N1 during the first weeks of the study (Fig. 2a). No significant correlations were observed between fungal biomass and decomposition rates or physicochemical variables (Table 2).

The N and P contents of leaves differed significantly among sites, over time and for the interaction between both factors (two-way ANOVAs, Table 3). No differences were observed in the leaf N content (Fig. 2b) of the three acidified sites and the circumneutral sites N2 and N3, but it was significantly higher in N1. No significant correlations were observed between N content and decomposition rates (Table 2). In contrast, the P content (Fig. 2c) differed significantly between impacted sites and the two sites with the lowest Al concentrations (Table 3). A strong but negative relationship was observed between P content in leaves and Al concentrations in water. Finally, we also observed a strong relationship between leaf P content and decomposition rates (Table 2).

Table 3 Results of two-way ANOVA analyses for fungal biomass, leaf litter nutrient content and enzyme activities

	F values — two-way ANOVAs[a]			Tukey tests[b]					
	Site (*df*=5)	Time (*df*=5)	Site×Time (*df*=25)	N1	N2	N3	Ac1	Ac2	Ac3
Fungal biomass	5.3	23.9	2.0	a	b	b	b	ab	ab
N	16.7	60.7	7.4	a	b	b	b	b	b
P	99.0	30.4	6.4	a	b	c	c	c	c
PO	16.1	242.3	6.6	bc	c	ab	a	b	b
BG	96.3	54.7	23.8	c	b	b	a	a	a
NAG	23.9	18.3	5.0	b	b	b	a	a	a
PH	307.0	10.1	28.5	d	c	b	a	a	a

PO phenoloxidase, *BG* β-glucosidase, *NAG* β-*N*-acetylglucosaminidase, *PH* phosphatase

[a] All tested effects were significant at $p<0.001$, except for the interaction between site and time for fungal biomass and BG where $p<0.01$

[b] Different letters represent statistical differences between sites ($p<0.05$)

Microbial Extracellular Enzyme Activities

For all enzyme activities studied, the potential extracellular enzyme activities differed significantly among sites, over time and for the interaction between both factors (two-way ANOVAs; Table 3). Specifically, potential phenoloxidase activities were higher during the initial portions of the experiment and decreased with time in all streams (Fig. 3a). No significant differences were observed between severely impacted sites and the non-impacted one N1 (Table 3). Conversely, β-glucosidase and β-*N*-acetylglucosaminidase activities, which exhibited similar patterns with time (Fig. 3b and c), differed significantly between circumneutral and acidic sites (Table 3). Potential activities decreased through time in circumneutral sites, while they remained at high levels in acidic sites.

Phosphatase activities showed a marked opposed pattern in acidified and circumneutral streams. Specifically, they increased with time at acidic sites, whereas they showed a constant decrease at circumneutral sites (Fig. 3d). In addition, the potential phosphatase activities measured for acidic sites reached values 4–5 times higher than those measured for the peak activity of the non-impacted sites. The potential activities of alkaline phosphatase were tested and were very low, even for circumneutral sites (data not shown).

Except for phenoloxidase activity, the potential extracellular enzyme activities over the study period were significantly and negatively correlated with decomposition rates, Ca^{2+} concentrations and pH, but positively correlated with total Al concentrations (Table 2).

For acquisition activities of C and N, the ecoenzymatic ratios ranged from 3.3 to 4.6 and did not differ significantly among sites (ANOVA, $F=1.7$, $p=0.14$) (Fig. 4a). However, the ratios between β-glucosidase and phosphatase activities were imbalanced and differed significantly among sites (ANOVA, $F=30.8$, $p<0.001$), being up to four times higher in the non-impacted sites (Fig. 4b).

For all enzymes studied, the non-impacted site N1 had the lowest turnover activities, whereas the highest turnover activities were observed in the most impacted sites, Ac2 and Ac3 (Table 4). Leaf litter decomposition required up to 6.2-fold more phenoloxidase, 15.2-fold more β-glucosidase and 9.3-fold more β-*N*-acetylglucosaminidase activities in impacted sites than in N1. Nevertheless, the most striking difference was observed for phosphatase turnover activity, which was 88.8-fold higher in Ac3 than in N1.

Discussion

Leaf litter processing has been successfully used to assess the integrity of stream functioning in numerous studies documenting the effects of anthropogenic stresses, notably in acidification and/or metal contamination contexts [12, 15, 19, 40, 48]. However, mechanisms underlying alteration of this functional process in acidified streams are not fully understood. In this study, total Al, Ca^{2+} concentrations, and pH were found to be the environmental parameters most closely correlated with the microbial decomposition rates of maple leaves. Similarly, Dangles et al. [12] reported that microbial respiration associated with decaying beech leaves was notably related to total Al, and that this microbial variable was positively related to leaf breakdown. Here, we showed that leaf litter decomposition by microorganisms decreased by about 5-fold between circumneutral streams and the most heavily impacted ones, confirming the effects of acidification on microbial activities involved in leaf decomposition.

Leaf litter decomposition is driven by the production of extracellular enzymes, which are involved in organic carbon

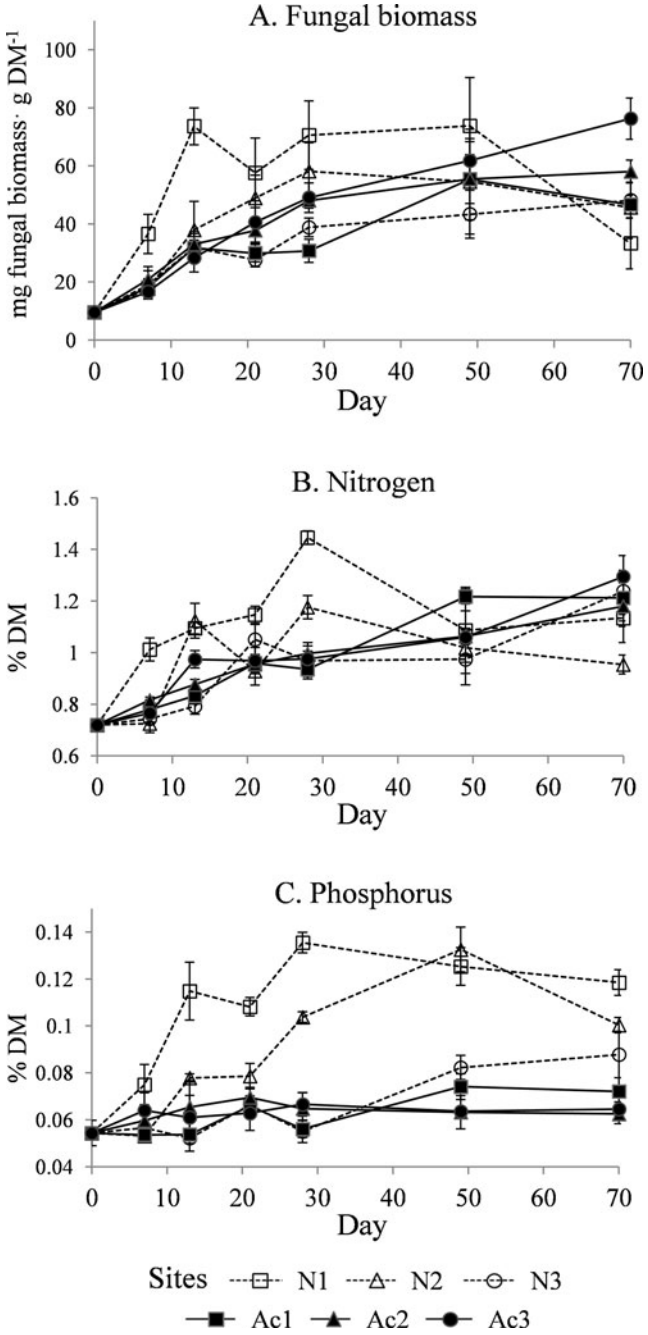

Figure 2 Fungal biomass and nutrient contents of decaying leaves on the six sites over the 70-day study period. Values are the mean of fungal biomass or nutrient content ± SE (n=4)

assimilation from plant polymers as well as in N and P acquisition from the surrounding environment. We did not observe significant effects of acidification on phenoloxidase activities, but activities peaking early in the decomposition process are more likely to correspond to tannin release during the initial stage of leaf leaching rather than to lignin-olytic activity. The latter activity generally occurs later in the process, when more recalcitrant compounds such as lignin remain on leaves [50].

The potential activities of β-glucosidase, which are an indicator of carbon acquisition from cellulose, differed between circumneutral and acidic sites, with higher cumulative potential activities being measured with time. High exocellulase activities were previously reported in acidic streams [24, 48] and were found to be negatively related to leaf litter decomposition, as evidenced in our study by the highest β-glucosidase turnover activities in impacted sites. In the present study, β-glucosidase activities decreased with time in circumneutral sites, but remained at high levels in acidic sites throughout the experiment. These patterns suggest that enzyme production or activity may be regulated by substrate availability (i.e., remaining cellulose) since leaf litter decomposition is slower in acidic sites.

Similar patterns were observed for the potential activities of β-*N*-acetylglucosaminidase, which are involved in chitin decomposition for C and N acquisition. Previous studies have shown that nutrient addition enhanced leaf decomposition [16, 26, 54] and that N and P together could co-limit this process [23]. Our results showed that N acquisition was unaltered, N content of leaves increasing in all streams through time. In a recent study, Ely et al. [17] showed an increase in N uptake by leaf biofilms in acidified streams. However, atmospheric N deposition in the Vosges Mountains has led to high levels of nitrates in headwater streams. This could explain why N was probably non-limiting in our study, and this makes both studies difficult to compare. Yet, in line with their results, we found the highest enzyme turnover activities in acidified streams and we therefore hypothesize that an important loss of N from leaf litter could have occurred via increased production of N-rich exoenzymes, leading to higher N demand by microbial decomposers in impacted streams than in non-impacted ones.

On the other hand, leaf P content only increased at less impacted sites and showed a strong negative relationship with total Al concentrations during the same period. Generally, both Al and pH explained most of variables due to their high covariance. However, site N3, which was circumneutral but had fairly high Al concentrations displayed symptoms similar to more acidic streams, suggesting that Al in particular may be an important factor. The potential P limitation of leaf litter microbial decomposers was confirmed by the particularly high turnover activity for phosphatase measured in impacted sites, corroborating previous observations of increasing phosphatase activities in streams along an acidity gradient [48]. Ecoenzymatic stoichiometry shows generally balanced ratios between nutrient acquisition activities [52], and could be used to highlight nutrient constraints. Ratios between β-glucosidase and phosphatase activities were far lower in impacted sites than in non-impacted sites, that certainly indicates that P could be a limiting factor in acidified sites. The soluble reactive P

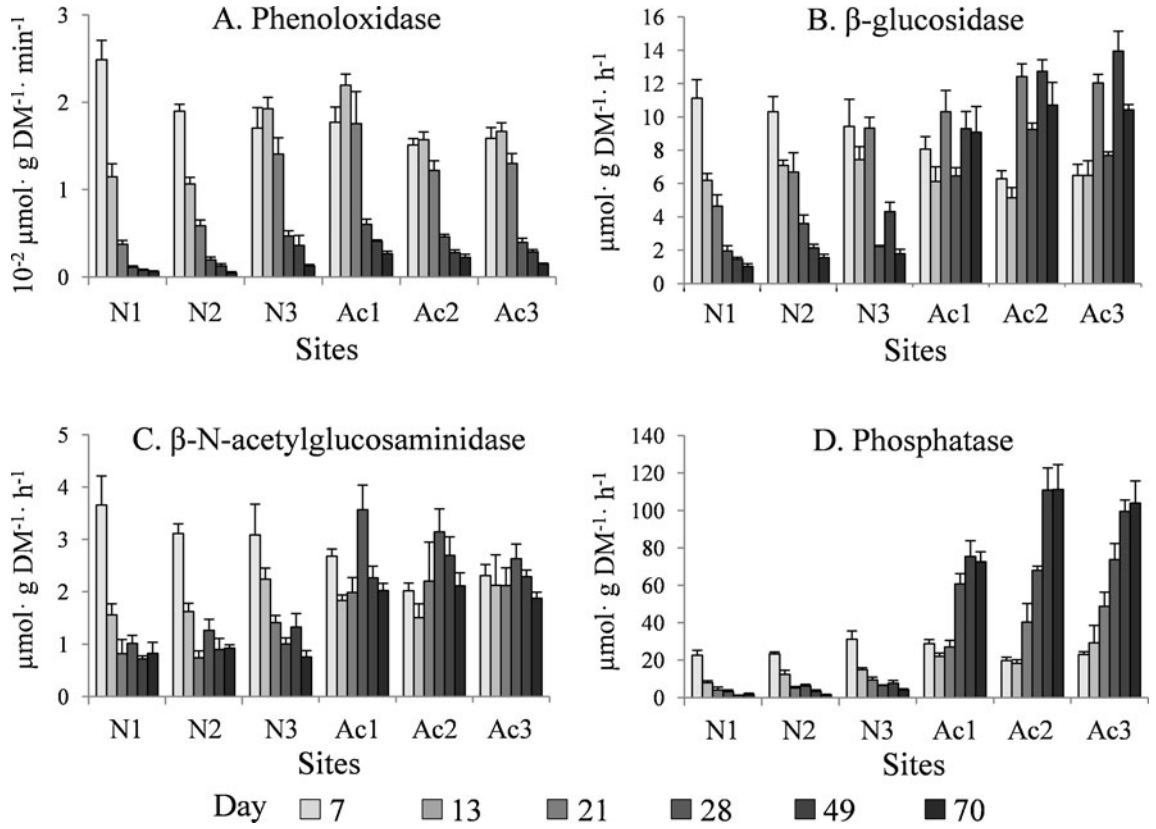

Figure 3 Potential enzyme activities associated with decaying leaves on the six sites over the 70-day study period. Values are the mean of potential enzyme activities ± SE (n=4)

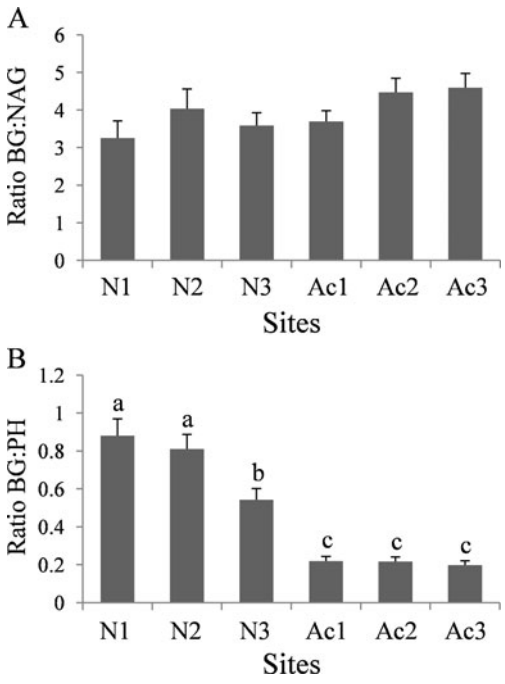

Figure 4 Ecoenzymatic ratios of C/N (**a**) and C/P (**b**) acquisition activities. Values are the mean of the ratios ± SE (n=24). Different letters represent statistical differences (ANOVA, p<0.05 followed by Tukey's test). *BG* β-glucosidase, *NAG* β-*N*-acetylglucosaminidase, *PH* phosphatase

concentrations at our study sites were generally very low (<5 µg l^{-1}), and it has been shown that mobilization of elevated Al concentrations could affect P availability. P inputs from the catchments may be reduced by efficient fixation with Al in soils [30]. Bittl et al. [5] demonstrated also that ionic Al could act as a competitive inhibitor of extracellular phosphatases in acidified lakes. The latter reported that phosphatase activity could be negatively affected in response to Al concentrations between 300 and 1,000 µg l^{-1} and pH values between 4.5 and 4.8. These

Table 4 Enzyme turnover activities (T_a) for the six sites

Site	PO	BG	NAG	PH
N1	9.3	7.1	2.6	9.5
N2	11.2	11	3.2	16.4
N3	27.8	17.5	5.1	33.3
Ac1	53.9	55.6	16.7	423.7
Ac2	54.3	100.6	24.4	823.7
Ac3	58	107.8	22.7	845.3

T_a is expressed in mmol g^{-1} for BG, NAG and PH and in µmol g^{-1} for PO

PO phenoloxidase, *BG* β-glucosidase, *NAG* β-*N*-acetylglucosaminidase, *PH* phosphatase

environmental conditions correspond well to those found in our strongly acidified sites and suggest that similar potential effects are likely to affect P acquisition in these streams. Moreover, in-lake P availability can be reduced at higher pH by binding of P with Al hydroxides in the surface sediment [33], revealing that other forms of Al can decrease P availability in the water column. All of these negative effects on P availability and phosphatase efficiency could lead to higher phosphatase production by microorganisms to overcome their P limitation. According to the strong correlation observed between leaf P content and leaf decomposition, we suggest that reduction of leaf litter processing at the impacted sites investigated in our study might primarily be due to P limitation.

It has been demonstrated that nutrients stimulate conidial production by aquatic hyphomycetes [26, 54]. In addition to the negative effects of Al on sporulation reported by Chamier and Tipping [9], reduced P acquisition could have a cascading effect on conidial production and contribute to both changes in aquatic hyphomycete assemblages and depletion of diversity observed by conidial identification under elevated Al concentrations [4]. In our study, fungal and bacterial assemblages were markedly different between circumneutral and acidic sites, confirming that microbial communities on leaves are primarily controlled by environmental factors [27]. However, in contrast to the results obtained by traditional methods (i.e., conidial identification; e.g., [4]), fungal diversity investigated using molecular approaches did not differ strongly among sites, as previously observed by Simon et al. [48]. Surprisingly, bacterial phylotype richness was higher in impacted sites. Pascoal and Cassio [43] showed that the relative contribution of bacteria to leaf litter decomposition increased in polluted rivers, while fungal activities were reduced. Unfortunately, in their study, bacterial diversity was not concurrently investigated. Nevertheless, the bacterial contribution to enzyme activities of decomposition is considered to be lower than that of fungi, despite complex antagonistic and synergistic interactions [47]. In addition, fungi are known to largely dominate microbial communities associated with decaying leaves in terms of biomass [18, 29]. We demonstrated that fungal biomass on leaves was not depressed by acidification. Similar findings were previously observed on beech leaves incubated in acidified streams in the Vosges Mountains [4, 10] and on chestnut oak leaves in some [48], but not all [17], acidified streams in the Appalachian Mountains (USA).

Toxic forms of Al can directly influence microorganisms, specifically their ion homeostasis, membrane transport, and enzymatic, metabolic and energy-requiring processes [45]. Nevertheless, it appears that Al could also have indirect effects on microorganisms by interacting with the P cycle, inducing a P limitation that could reduce their decomposing activity. Despite the clear gradient used in our experiment, evaluating the effects of acidification is not facilitated by the presence of covarying parameters in low pH waters. Base cation concentrations and pH often fluctuate together with Al in streams, and these parameters are well known to influence enzyme activities and leaf breakdown, particularly pectin lyase activity, which is involved in leaf maceration and reduced under acidic pH and low Ca concentrations [8, 31]. Further experiments in the laboratory are clearly needed to elucidate the respective effects of each factor. While reduced leaf litter breakdown would probably result from the combined effects of several parameters, this study demonstrates that P acquisition could represent a major factor limiting leaf litter processing by microorganisms in acidified streams. Due to the nutritional value of detritus being reduced in acidified streams through impacts on microbial leaf colonizers and the P cycle, acidification could lead to negative impacts on higher trophic levels and overall stream functioning.

Acknowledgements The present study was financed by the French ANR program (ANR Biodiversité, RECOVER Project). We thank the Conseil Général des Vosges, the Office National des Forêts and the Zone Atelier Moselle for their support. We are grateful to Virginie Baldy for ergosterol analyses. We also thank three anonymous reviewers for their constructive evaluation of the manuscript.

References

1. Arsuffi TL, Suberkropp K (1988) Effects of fungal mycelia and enzymatically degraded leaves on feeding and performance of Caddisfly (Trichoptera) larvae. J N Am Bentholl Soc 7:205–211
2. Bärlocher F (2005) Leaf mass loss estimated by litter bag technique. In: Graça M, Bärlocher F, Gessner MO (eds) Methods to study litter decomposition. Springer, Dordrecht, pp 37–42
3. Bärlocher F, Kendrick B (1981) The role of aquatic hyphomycetes in the trophic structure of streams. In: Wicklow DT, Carroll GC (eds) The fungal community. Marcel Dekker, New York, pp 743–760
4. Baudoin JM, Guérold F, Felten V, Chauvet E, Wagner P, Rousselle P (2008) Elevated Aluminium concentration in acidified headwater streams lowers aquatic hyphomycete diversity and impairs leaf-litter breakdown. Microb Ecol 56:260–269
5. Bittl T, Vrba J, Nedoma J, Kopácek J (2001) Impact of ionic aluminium on extracellular phosphatases in acidified lakes. Environ Microbiol 3:578–587
6. Bray JR, Curtis JT (1957) An ordination of the upland forest communities of Southern Wisconsin. Ecol Monogr 27:325–349
7. Chamier AC (1987) Effect of pH on microbial degradation of leaf litter in seven streams of the English Lake District. Oecologia 71:491–500
8. Chamier AC, Dixon PA (1982) Pectinases in leaf degradation by aquatic hyphomycetes: the enzymes and leaf maceration. J Gen Microbiol 128:2469–2483
9. Chamier AC, Tipping E (1997) Effects of aluminium in acid streams on growth and sporulation of aquatic hyphomycetes. Environ Pollut 96:289–298
10. Dangles O, Chauvet E (2003) Effects of stream acidification on fungal biomass in decaying beech leaves and leaf palatability. Water Res 37:533–538

11. Dangles O, Guérold F (1998) Influence of shredders in mediating breakdown rates of beech leaves in circumneutral and acidic forest streams. Arch Hydrobiol 151:649–666
12. Dangles O, Gessner MO, Guérold F, Chauvet E (2004) Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. J Appl Ecol 41:365–378
13. Dangles O, Guérold F (2001) Linking shredders and leaf litter processing: insights from an acidic stream study. Int Rev Hydrobiol 86:395–406
14. Duarte S, Cássio F, Pascoal C (2012) Denaturing Gradient Gel Electrophoresis (DGGE) in microbial ecology — Insights from freshwaters. In: Magdeldin S (ed) Gel electrophoresis — principles and basics. InTech, Rijeka, Croatia, pp 173–195
15. 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. Freshw Biol 53:91–101
16. Elwood JW, Newbold JD, Trimble AF, Stark RW (1981) The limiting role of phosphorus in a woodland stream ecosystem: effects of P enrichment on leaf decomposition and primary producers. Ecology 62:146–158
17. Ely DT, Schiller DV, Valett HM (2010) Stream acidification increases nitrogen uptake by leaf biofilms: implications at the ecosystem scale. Freshw Biol 55:1337–1348
18. Findlay SEG, Arsuffi TL (1989) Microbial growth and detritus transformations during decomposition of leaf litter in a stream. Freshw Biol 21:261–269
19. Gessner MO, Chauvet E (2002) A case for using litter breakdown to assess functional stream integrity. Ecol Appl 12:498–510
20. Gessner MO, Chauvet E (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. Appl Environ Microbiol 59:502–507
21. Gessner MO, Chauvet E (1994) Importance of stream microfungi in controlling breakdown rates of leaf litter. Ecology 75:1807
22. Gessner MO, Schmitt A (1996) Use of solid-phase extraction to determine ergosterol concentrations in plant tissue colonized by fungi. Appl Environ Microbiol 62:415–419
23. Grattan RM, Suberkropp K (2001) Effects of nutrient enrichment on Yellow Poplar leaf decomposition and fungal activity in streams. J N Am Bentholl Soc 20:33–43
24. Griffith MB, Perry SA, Perry WB (1995) Leaf litter processing and exoenzyme production on leaves in streams of different pH. Oecologia 102:460–466
25. Guérold F, Boudot J-P, Jacquemin G, Vein D, Merlet D, Rouiller J (2000) Macroinvertebrate community loss as a result of headwater stream acidification in the Vosges Mountains (N-E France). Biodivers Conserv 9:767–783
26. 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
27. Harrop BL, Marks JC, Watwood ME (2009) Early bacterial and fungal colonization of leaf litter in Fossil Creek, Arizona. J N Am Bentholl Soc 28:383–396
28. Heard RM, Sharpe WE, Carline RF, Kimmel WG (1997) Episodic acidification and changes in fish diversity in Pennsylvania headwater streams. Trans Am Fish Soc 126:977–984
29. Hieber M, Gessner MO (2002) Contribution of stream detrivores, fungi, and bacteria to leaf breakdown based on biomass estimates. Ecology 83:1026–1038
30. Jansson M, Persson G, Broberg O (1986) Phosphorus in acidified lakes: the example of Lake Gardsjon, Sweden. Hydrobiologia 139:81–96
31. Jenkins CC, Suberkropp K (1995) The influence of water chemistry on the enzymatic degradation of leaves in streams. Freshw Biol 33:245–253
32. Kellner H, Luis P, Buscot F (2007) Diversity of laccase-like multicopper oxidase genes in Morchellaceae: identification of genes potentially involved in extracellular activities related to plant litter decay. FEMS Microbiol Ecol 61:153–163
33. Kopácek J, Hejzlar J, Borovec J, Porcal P, Kotorova I (2000) Phosphorus inactivation by aluminum in the water column and sediments: lowering of in-lake phosphorus availability in an acidified watershed–lake ecosystem. Limnol Oceanogr 45:212–225
34. Legendre P, Legendre L (1998) Numerical ecology. Developments in environmental modelling. Elsevier, Amsterdam
35. Meegan SK, Perry SA, Perry WB (1996) Detrital processing in streams exposed to acidic precipitation in the Central Appalachian Mountains. Hydrobiologia 339:101–110
36. Mulholland PJ, Driscoll CT, Elwood JW, Osgood MP, Palumbo AV, Rosemond AD, Smith ME, Schofield C (1992) Relationships between stream acidity and bacteria, macroinvertebrates, and fish: a comparison of north temperate and south temperate mountain streams, USA. Hydrobiologia 239:7–24
37. Mulholland PJ, Palumbo AV, Elwood JW, Rosemond AD (1987) Effects of acidification on leaf decomposition in streams. J N Am Benthol Soc 6:147–158
38. Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schäfer H, Wawer C (1998) Denaturing Gradient Gel Electrophoresis (DGGE) in microbial ecology. In: Akkermans ADL, van Elsas JD, de Bruijn FJ (eds) Molecular microbial ecology manual, 3rd edn. Kluwer Academic Publishers, Dordrecht, pp 1–27, 3.4.4
39. Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700
40. Niyogi DK, Lewis WM, McKnight DM (2001) Litter breakdown in mountain streams affected by mine drainage: biotic mediation of abiotic controls. Ecol Appl 11:506–516
41. Ormaza-González FI, Statham PJ (1996) A comparison of methods for the determination of dissolved and particulate phosphorus in natural waters. Water Res 30:2739–2747
42. Oros-Sichler M, Gomes NCM, Neuber G, Smalla K (2006) A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. J Microbiol Methods 65:63–75
43. Pascoal C, Cássio F (2004) Contribution of fungi and bacteria to leaf litter decomposition in a polluted river. Appl Environ Microbiol 70:5266–5273
44. Petersen RC, Cummins KW (1974) Leaf processing in a woodland stream. Freshw Biol 4:343–368
45. Piña RG, Cervantes C (1996) Microbial interactions with aluminium. BioMetals 9:311–316
46. R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org
47. Romani A, Fischer H, Mille-Lindblom C, Tranvik L (2006) Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. Ecology 87:2559–2569
48. Simon KS, Simon MA, Benfield EF (2009) Variation in ecosystem function in Appalachian streams along an acidity gradient. Ecol Appl 19:1147–1160
49. Simpson KW, Bode RW, Colquhoun JR (1985) The macroinvertebrate fauna of an acid-stressed headwater stream system in the Adirondack Mountains, New York. Freshw Biol 15:671–681
50. Sinsabaugh RL, Carreiro MM, Alvarez S (2002) Enzyme and microbial dynamics of litter decomposition. In: Burns RG, Dick RP (eds) Enzymes in the environment: Activity, ecology and applications. Marcel Dekker, New York, pp 249–265
51. Sinsabaugh RL, Carreiro MM, Repert DA (2002) Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. Biogeochemistry 60:1–24

52. Sinsabaugh RL, Hill BH, Shah JJF (2009) Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature 462:795–798
53. Sinsabaugh RL, Horn DJ, Follstad Shah JJ, Findlay S (2010) Ecoenzymatic stoichiometry in relation to productivity for freshwater biofilm and plankton communities. Microb Ecol 60:885–893
54. Sridhar KR, Bärlocher F (2000) Initial colonization, nutrient supply, and fungal activity on leaves decaying in streams. Appl Environ Microbiol 66:1114–1119
55. Thompson PL, Bärlocher F (1989) Effect of pH on leaf breakdown in streams and in the laboratory. J N Am Bentholl Soc 8:203–210
56. Zar JH (1999) Biostatistical analysis. Prentice Hall, Englewood Cliffs

IV.3. Approche en microcosmes pour l'étude des effets de l'Aluminium et de la limitation en Phosphore sur les activités fongiques associées à la décomposition des litières.

Résumé

Les résultats obtenus dans l'étude précédente ont montré que les concentrations élevées en Al dans les cours d'eau acidifiés pourraient avoir des effets sur les micro-organismes décomposeurs, de part sa toxicité mais aussi en interférant avec l'acquisition du P, induisant potentiellement une limitation pour ces micro-organismes et leurs activités impliquées dans la décomposition des litières de feuilles.

Cependant, dans les cours d'eau impactés par l'acidification, l'augmentation des concentrations en Al est souvent corrélée à une diminution du pH et des concentrations en cations basiques (Ca et Mg). Ces autres facteurs sont également susceptibles d'influencer les activités microbiennes, rendant ainsi compliqué la compréhension des effets de l'acidification sur la dégradation des litières.

Pour évaluer précisément les effets de l'Al et d'une limitation en P dans les cours d'eau acidifiés, une approche in-vitro a ainsi été mise en place afin d'isoler les effets respectifs et interactifs de ces deux facteurs. Une expérience de trois semaines en microcosmes a donc été réalisée en évaluant les effets croisés en conditions acides de deux concentrations en Al (200 et 1000 $\mu g.L^{-1}$) et d'un témoin sans Al et de trois concentrations en P (20, 100 et 1000 $\mu g.L^{-1}$) sur la décomposition de litières pré-colonisées par un assemblage de 7 hyphomycètes aquatiques.

Les résultats ont montré que les taux de décomposition des litières et les biomasses fongiques associées étaient significativement réduits par l'Al aux deux concentrations les plus faibles en P, ces effets négatifs étant levés par la plus forte concentration en P. Aucun effet de l'Al n'a été observé sur les contenus en P des litières, mais aux deux plus faibles concentrations en P, l'Al avait un effet significatif sur les activités potentielles des phosphatases, ces dernières étant 5 à 7 fois plus importantes pour les deux traitements en Al par rapport aux traitements témoins. Les concentrations en Al ne semblaient pas modifier la structure des assemblages fongiques analysée par PCR-DGGE, alors que les communautés des plus fortes concentrations en P différaient légèrement des autres. En particulier, le développement de *Tetrachaetum elegans* a été favorisé par les forts traitements en P. Les analyses de spéciation de l'Al ont révélé que les fortes concentrations en P pouvaient

diminuer la proportion des formes les plus toxiques de l'Al. Cependant, le P n'a eu aucun effet sur l'accumulation d'Al dans les litières, de grandes quantités d'Al étant retrouvées dans celles-ci aux plus fortes concentrations en Al. Le contenu en Al dans les litières pouvait même correspondre jusqu'à la moitié des quantités totales d'Al introduites dans les microcosmes.

Cette étude montre ainsi que l'Al peut altérer aussi bien la décomposition des litières par les hyphomycètes aquatiques que leur développement mycélien, mais aussi que l'Al peut induire une limitation en P, tous ces effets ne pouvant être levés que par de fortes concentrations en P. De plus, nos résultats suggèrent que les effets négatifs de l'Al sur le conditionnement des litières et sur leurs contenus en Al pourraient avoir des répercussions sur les niveaux trophiques supérieurs, en raison de l'ingestion potentielle par les macro-invertébrés de matériel foliaire riche en Al, impactant ainsi toute la chaine trophique des écosystèmes aquatiques hétérotrophes.

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

Hugues Clivot[1,2], Faustine Charmasson[1,2], Vincent Felten[1,2], Christophe Pagnout[1,2], Jean-Pierre Boudot[3,4], François Guérold[1,2] and Michael Danger[1,2].

[1]Université de Lorraine; Laboratoire des interactions Ecotoxicologie Biodiversité Ecosystèmes (LIEBE); UMR 7146; Campus Bridoux rue du Général Delestraint, 57070 Metz, France

[2] CNRS; LIEBE; UMR 7146; 57070 Metz, France

[3] Université de Lorraine ; LIMOS ; UMR 7137 ; Faculté des Sciences, 54506 Vandoeuvre-lès-Nancy, France

[4]CNRS ; LIMOS ; UMR 7137 ; 54506 Vandoeuvre-lès-Nancy, France

Abstract

Decline in pH, elevated aluminum (Al) concentrations and base cation depletion are parameters often covarying in acidified streams and are supposed to impact microbial activities involved in leaf litter processing. In addition to the direct Al toxicity, Al is known to interact with phosphorus (P) cycle and is suspected to increase P limitation in acidified stream that could strongly constrain fungal decomposer activities. A 3-week microcosm experiment was carried out in acid conditions to assess the effects of Al (three levels: 0, 200 and 1000 $\mu g.L^{-1}$) at three different levels of P (20, 100 and 1000 $\mu g.L^{-1}$) on alder leaf litter processing by a consortium of 7 aquatic hyphomycete species. Results showed that Al significantly decreased leaf litter decomposition rates and leaf-associated fungal biomass at the two lowest P levels, these negative effects being alleviated at the highest P concentration. No effects of Al were observed on leaf litter P content, but Al exerted a significant effect on potential phosphatase activities, which were 5 to 7 times higher in both highest Al treatments than in control ones at the two lowest P levels. Concentration of Al did not modify the structure of fungal assemblages growing on leaf litter, while communities present in the highest P treatment slightly differed from the others. In particular, the growth of *Tetrachaetum elegans* was favored by high P concentrations. Al speciation analyses showed that the highest P concentration decreased the proportion of the most toxic monomeric forms of Al, but P concentration had no effect on leaf litter Al content. At the highest Al concentration, high amount of Al were found on leaf litter corresponding to about half of the total load of Al during the experiment. Our results showed that Al, as a sole factor, could alter not only fungal growth but also leaf litter processing and could accentuate P limitation, these effects being only alleviated by a high P supply. In addition, our results suggest that the impact of elevated Al concentrations on both conditioning and Al content of leaf litter in acidified streams could also have repercussions on higher trophic levels through alterations of detritus consumption and ingestion of Al by invertebrates and therefore on all the detritus-based food web.

Keywords: *leaf litter decomposition, microcosms, Aluminium, Phosphorus, aquatic hyphomycetes.*

Introduction

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

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

Nevertheless, high Al concentrations and base cation depletion are generally characteristics associated to pH decline in impacted streams. Thus, disentangling the influence of each factor in field-experiments is not facilitated by these covarying parameters, which could lead to confounding effects. By isolating one or a limited number of these parameters, laboratory experiments could therefore allow us to accurately evaluate their respective effects on microbial decomposers.

In this context, the objective of the current study was to assess *in vitro* the individual and combined effects of Al and P in acidic condition, since both parameters appear to be tied and could control microbial leaf litter processing in acidified streams. In a microcosm experiment, leaf disks pre-colonized by an assemblage of 7 aquatic hyphomycetes were exposed in artificial acidic water to 9 different combinations of 3 levels of both Al and P. After 3 weeks, we assessed leaf litter mass loss, fungal biomass and community structures, Al and nutrient contents on leaves and phosphatase activities. We expected that i) under acid stress and low P concentrations, high Al concentrations would reduce leaf litter processing by impacting microorganisms and notably by increasing their P limitation and that, ii) a high level of P could alleviate the negative effects of Al.

Material and methods

Leaf litter collection and conditioning

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

Mineral medium and experimental conditions

The basic mineral medium of our experiment was adapted from those used in Kilham et al. (1998) and Gessner & Chauvet (1993), but it was more diluted to correspond to physicochemical characteristics of headwater streams. This medium consisted of 7.4 mg of $CaCl_2$, 7.4 mg of $MgSO_4$-$7H_2O$, 8.5 mg of $NaNO_3$, 10.1 mg of KNO_3, 0.2 mg of $MnSO_4$-H_2O, 0.2 mg of $ZnSO_4$-H_2O, 0.02 mg of Na_2MoO_4-$2H_2O$, 0.02 mg of KI, 0.008mg of $CoCl_2$-$6H_2O$ and 0.005 mg of $NiCl_2$-$6H_2O$ in 1 liter of ultrapure Milli-Q water (Millipore, Molsheim, France). This basic medium was supplemented with NaH_2PO_4 to obtain 3 levels of P (20, 100 and 1000 µg $P.L^{-1}$, called P20, P100 and P1000) and with or without $AlCl_3$ to obtain 3 different levels of Al (0, 200 and 1000 µg $Al.L^{-1}$, called Al0, Al200 and Al1000). Acidic pH of solutions was adjusted to 4.5 with HCl (0.1 M). Real P concentrations were (mean ± S.D) 25.2 ± 5.9 µg L^{-1}, 98.8 ± 1.7 µg L^{-1} and 983.9 ± 29.4 µg L^{-1} in P20, P100, and P1000, respectively, whereas real Al concentrations were 207 ± 13 µg L^{-1} and 975 ± 57 µg L^{-1} in Al200 and Al1000, respectively.

Fungal inoculum

Seven common aquatic hyphomycete species constituted the initial inoculum of leaf litter (*Alatospora acuminata* (ALAC), *Anguillospora crassa* (ANCR), *Clavariopsis aquatic* (CLAQ), *Clavatospora longibrachiata* (CLLO), *Heliscus lugdunensis* (HULU), *Tetrachaetum elegans* (THEL) and *Tricladium chaetocladium* (TRCH)). Species were selected as non-comigrating in PCR-DGGE analyses (for conditions, see fungal biomass and assemblages part below), allowing us to follow distinctly each species contribution to fungal assemblage on leaf litter. Before starting the experiment, fungal strains were maintained on 2% Malt Agar and were grown for the experiment in P20 solution supplemented with 5 g/L of glucose as sole carbon source. An equivalent biomass of each hyphomycete mycelium (~ 5 mg of mycelial dry mass) was then rinsed twice with P20 solution to eliminate carbon traces. The seven strains were then grinded together in 30 ml of P20 solution and the homogenate was used to inoculate leaf litter.

Microcosms

Sets of 525 leaf disks were each inoculated with 1 ml of fungal homogenate in flasks containing 250 ml of P20 solution. Flasks were then incubated at 12°C on a rotary shaker during five days to allow fungal colonization of leaf litter. After this step, disks were placed in microcosms or were used to determine initial leaf parameters.

Aerated microcosms simulating stream conditions (Suberkropp, 1991) were used to perform the experiment. Sets of 40 leaf disks were randomly distributed in 36 microcosms containing 50 ml of solution from one of the 9 different combinations of Al and P (4 replicates each). Microcosms were incubated at 12°C for 3 weeks, solutions being renewed 3 times a week.

Water analysis and Al concentrations

Waters of each tested treatment were collected in initial solution and at the end of microcosm experiment. Al concentrations and speciation were performed and obtained as described in Boudot *et al*. (Boudot et al., 1994, 2000)

Leaf litter mass loss

Twenty-five disks from each microcosm were used to assess the remaining mass of leaf litter. Leaf disks were dried at 60°C for 48 h to constant mass and weighed to the nearest 0.1 mg (dry mass, DM).

Leaf litter nutrient contents

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

To determine N and P contents of leaf litter, ten dry leaf disks were finely ground. The N content was then assessed using a CHN elemental analyzer and the P content was assessed using a colorimetric method (Ormaza-González & Statham, 1996).

Al and P contents were expressed as µg. g^{-1} of DM and N content was expressed as mg.g^{-1} of DM.

Fungal biomass and assemblages

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

A two-step PCR-DGGE protocol (Oros-Sichler et al., 2006) was performed to compare fungal assemblages between treatments. For each microcosm, five leaf disks were finely ground and total DNA from leaf litter was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Fungal 18S rRNA gene fragments were amplified using using NS1 (5'-GTAGTCATATGCTTGTCTC-3') and EF3 (5'-TCCTCTAAATGACCAAGTTTG-3') primers. Diluted amplicons (1:500) obtained were used as the template for the second amplification with primers NS1 and FR1-GC (5'-CCCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGCCGAICCATTCAATCGGTAIT-3'). PCR mixture (100 µl) contained 6 U of Taq DNA Polymerase (5 PRIME, Hamburg, Germany), 1x Taq buffer (5 PRIME) formulated to automatically adjust the Mg^{2+} concentration, 200 µM of each dNTP, 0.5 µM of each primer and 20 ng of extracted DNA as template. The first PCR amplification protocol consisted of 5 min at 94°C, followed by 25 cycles of 30 s at 94°C, 45 s at 47°C, 3 min at 72°C, and a final extension of 10 min at 72°C. The second step was identical except that the number of cycles was reduced to 20 and the

annealing temperature was 48°C. The amplification products were separated with the DCODE Mutation Detection System (Bio-Rad, Hercules, CA). Electrophoresis were performed on 6% polyacrylamide gels with a denaturing gradient from 25 to 40%, 100% denaturant corresponding to 40% (v/v) formamide and 7 M urea. DGGE were run at 180 V and 58°C for 16 h. The gels were stained with SYBR Green I and imaged with a STARION FLA-9000 scanner (Fujifilm Life Sciences FSVT, Courbevoie, France).

Potential phosphatase activity

Potential phosphatase activity (EC 3.1.3.2) was measured on microcosm solution at the end of the experiment using 5 mM pNP-phosphate in 50 mM sodium acetate buffer (pH 5.0) (Sinsabaugh et al., 2002). For kinetic analyses, 150 µL of microcosm solution and 150 µL of substrate were homogenized and incubated at 22°C for 2 h. Three blanks consisted of 150 µL of microcosm solution with 150 µL of sodium acetate buffer, 150 µL of substrate with 150 µL of sodium acetate buffer and 300 µL of sodium acetate buffer. Reactions were stopped by adding 500 µL of NaOH (0.1 M), before reading absorbance at 405 nm. Potential phosphatase activity was expressed as µmol of *p*-nitrophenol formed mg^{-1} of fungal biomass day^{-1}.

Data analysis

Leaf litter decomposition was evaluated by subtracting the final leaf disks mass without fungal biomass to the initial mass of control disks. Decomposition rates (k) were then obtained by linear regression and were expressed as day^{-1}.

Two-way ANOVAs were carried out to test the effects of Al and P on decomposition rates, fungal biomass, Al and nutrient contents and phosphatase activities. To meet the assumptions of ANOVA, data were log transformed when necessary (Zar, 1999).

GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was used to align DGGE profiles. To identify species within assemblages, markers with the DNA of the seven aquatic hyphomycetes were loaded on each gel. For each sample, band relative intensities from DGGE profile were used to estimate the relative contribution of each species within the fungal assemblage. Data obtained were associated with those of fungal biomass to compare assemblages on leaf litter between treatments. Bray-Curtis distance matrices were then generated (Bray & Curtis, 1957) and the data were illustrated with non-metric multidimensional scaling (NMDS) (Minchin, 1987). Analyses of similarity (ANOSIM) were performed on these data to characterize differences between treatments (Clarke, 1993). An *R*-

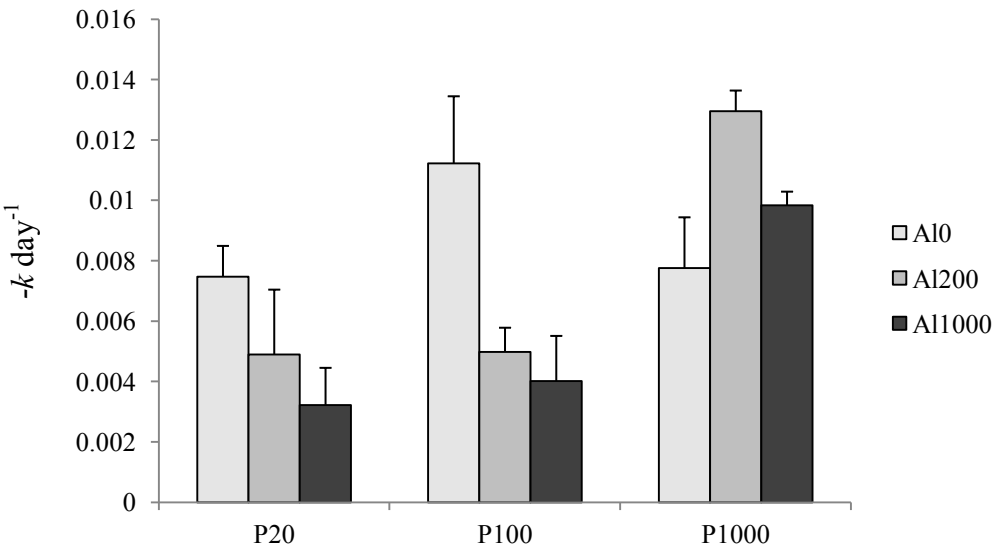

Figure 1. Decomposition rates (-*k*) of alder leaf disks exposed in microcosms to 9 combinations of 3 levels of both Al and P. Values are the mean of decomposition rates ± SE (n=4).

Table 1. Results of two-way ANOVA analyses performed on leaf litter decomposition rates, fungal biomass, N, P and Al content on leaves and phosphatase activity.

	F values - Two-way ANOVAs		
	Al (*df*=2)	P (*df*=2)	Al × P (*df*=4)
Decomposition rates	**3.6***	**9.4*****	**4.7****
Fungal biomass	**6.7****	**18.1*****	**3.1***
N content	**3.9***	**5.7****	**5.1****
P content	2.0	**43.0*****	1.1
Al content	**1250.0*****	0.1	2.1
Phosphatase activity	**5.7****	**23.8*****	1.4

Significant effects are indicated in bold.
Levels of significance are *p<0.05, **p<0.01, and ***p<0.001.

value of 0 indicates no difference whereas an *R*-value of 1 indicates completely dissimilar groups. All statistical analyses were performed using R Statistics (R Development Core Team, 2008). The level of significance was set at $p = 0.05$ for this study.

Results

Leaf litter decomposition rates ranged from 0.003 day^{-1} in P20Al1000 to 0.013 day^{-1} in P1000Al200 (Figure 1). Two-way ANOVA revealed that Al, P and the interaction between both factors had a significant effect on leaf litter decomposition rates (Table 1), showing a negative impact of Al and a positive effect of P on leaf litter decomposition. Specifically, for P20 and P100 treatments, decomposition rates were 2-3 times lower in Al1000 than in control treatments. In contrast, no effect was observed in P1000 (Figure 1).

Similarly, significant effects of Al, P and the interaction between both factors were observed on fungal biomass and N content (Two-way ANOVAs, Table 1). For P20 and P100 treatments, fungal biomass and N content were significantly lower in Al200 and Al1000 treatments than in controls (Figure 2A and 2B). No effect of Al was observed on fungal biomass and N content in P1000 treatments, but fungal biomass was significantly higher in P1000 than in P20 and P100 treatments.

A significant effect of P was observed on P leaf litter content (Two-way ANOVA, Table 1), which was significantly higher in P1000 than in P20 and P100 treatments (similar, Figure 2C). No effect of Al was observed on P leaf litter content.

Conversely, a significant effect of Al was observed on Al leaf litter content (Two-way ANOVA, Table 1), whereas P concentration had no effect on this leaf parameter. Compared to controls, Al contents of leaf litter were 2-3 and 20-25 times higher in Al200 and Al1000 treatments, respectively (Figure 2D).

Al and P exerted a significant effect on potential phosphatase activities, whereas no effect was observed for the interaction between both factors (Two-way ANOVA, Table 1). For P20 and P100 treatments, potential phosphatase activities were 5 to 7 times higher in Al200 and Al1000 than in control treatments, while very low levels of potential activities were measured in all P1000 treatments (Figure 3).

NMDS ordination of fungal communities on leaf litter showed that assemblages from P1000 slightly differed from those of P20 (ANOSIM, R=0.48 and p<0.001) and P100 (ANOSIM, R=0.27 and p=0.002), growth of most species being governed by a high P supply

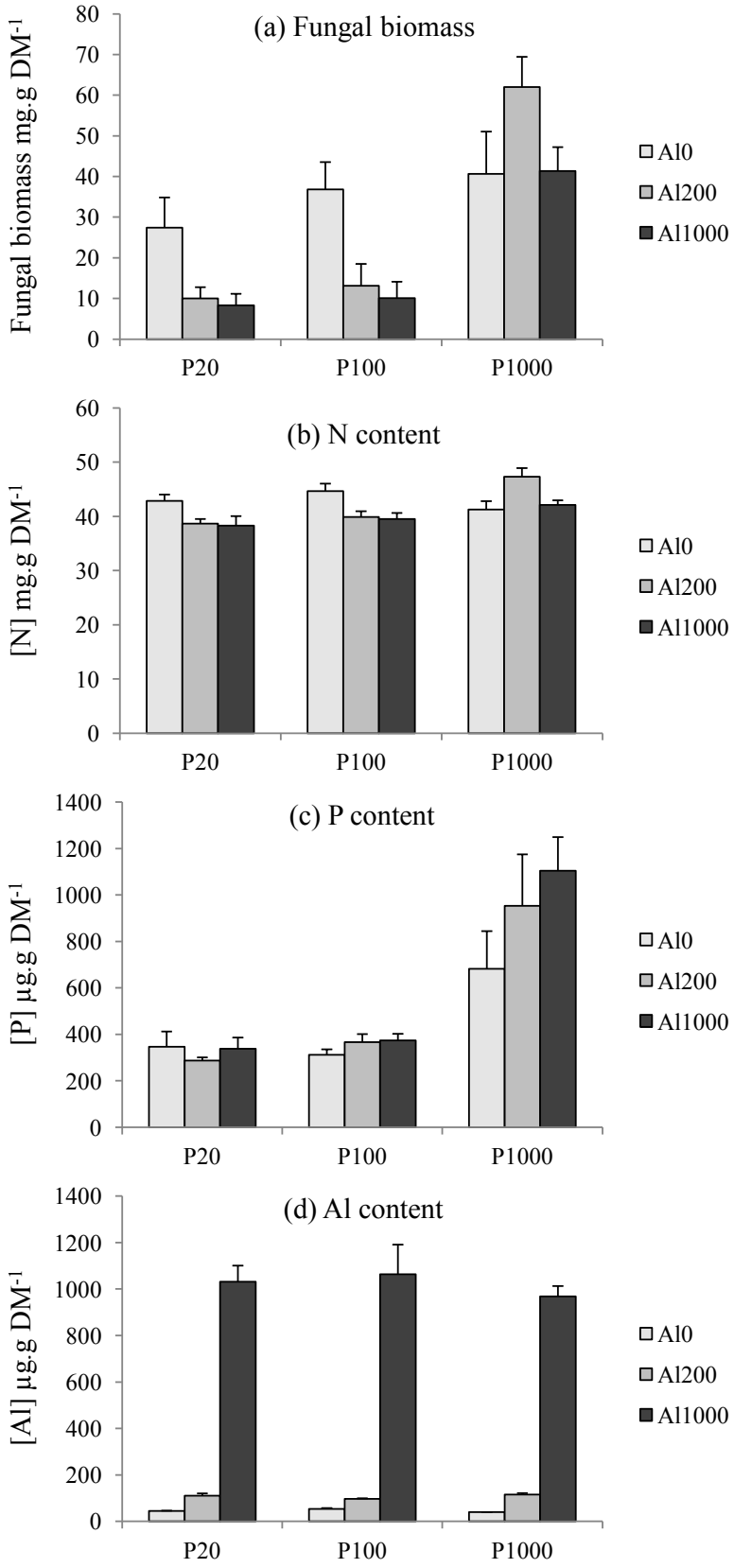

Figure 2. Fungal biomass (a), N (b) and P (c) contents and Al concentrations (d) on leaf litter after 3 weeks of exposure to 9 combinations of 3 levels of both Al and P in microcosms. Values are the mean of fungal biomass, Al or nutrient contents ± SE (n=4).

(Figure 4). Notably, we noticed that growth of THEL was particularly favored in P1000Al1000 treatment. Whatever the P treatment, no clear effect of Al was reported on fungal richness and structure of fungal assemblages. It should be also noted that the occurrence of CLLO was never recorded.

Total Al concentrations were lower in solutions collected at medium replacement date, after staying 3 days in microcosms, than in initial solutions (Figure 5). As expected, speciation analysis showed that the monomeric toxic form Al^{3+} was the prevalent Al species in initial P20 and P100 solutions. In P1000 initial solutions, high P concentrations decreased Al^{3+} and AlOH proportions leading to significant levels of $AlPO_4$, a less toxic form of Al, and to higher proportions of non-monomeric forms of Al. In solutions collected in microcosms, non-monomeric forms of Al were largely predominant at the end of the experiment.

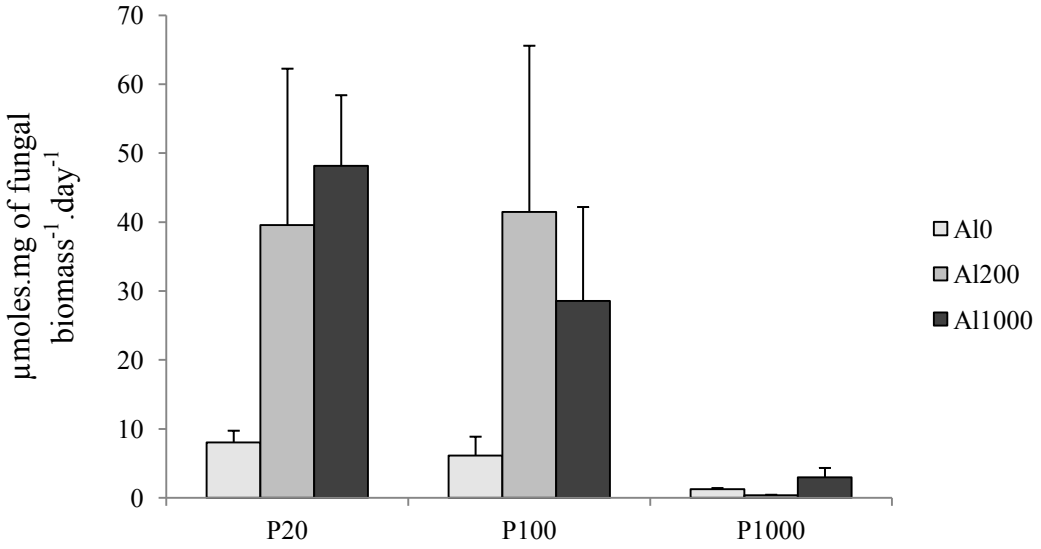

Figure 3. Potential phosphatase activities assessed on microcosm solutions after 3 weeks of leaf litter exposure to 9 combinations of 3 levels of both Al and P in microcosms. Values are the mean of potential activity ± SE (n=4).

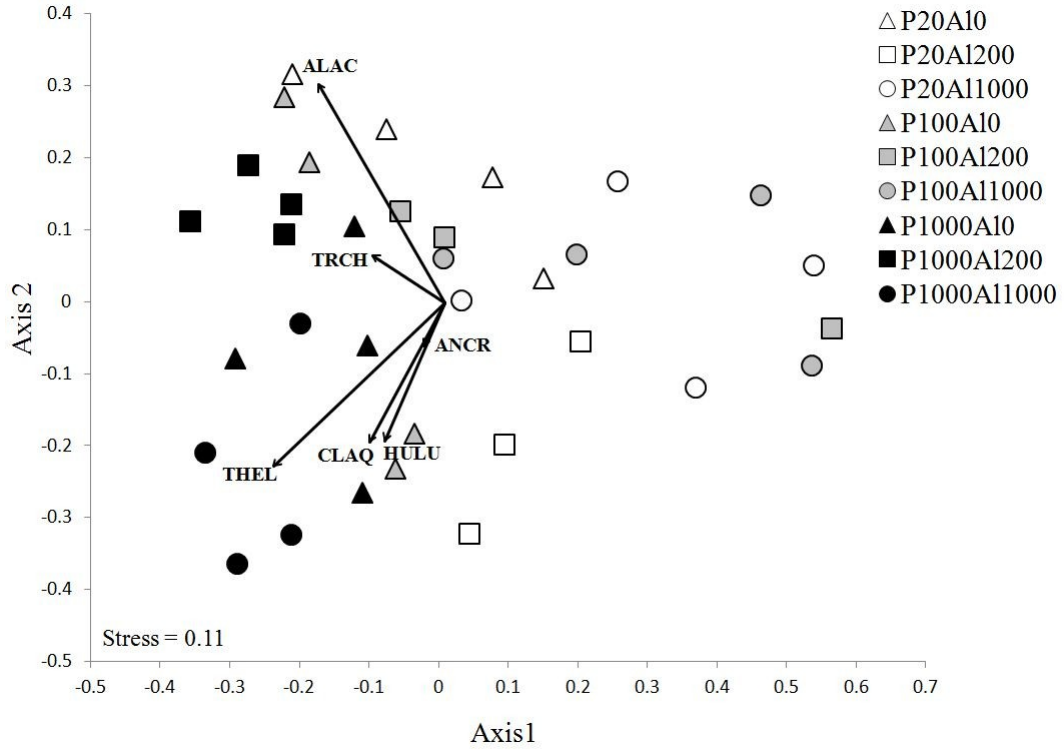

Figure 4. NMDS ordination of fungal assemblages on leaf litter after 3 weeks of exposure to 9 combinations of 3 levels of both Al and P in microcosms. Arrows indicate increasing abundance of the different aquatic hyphomycete species.

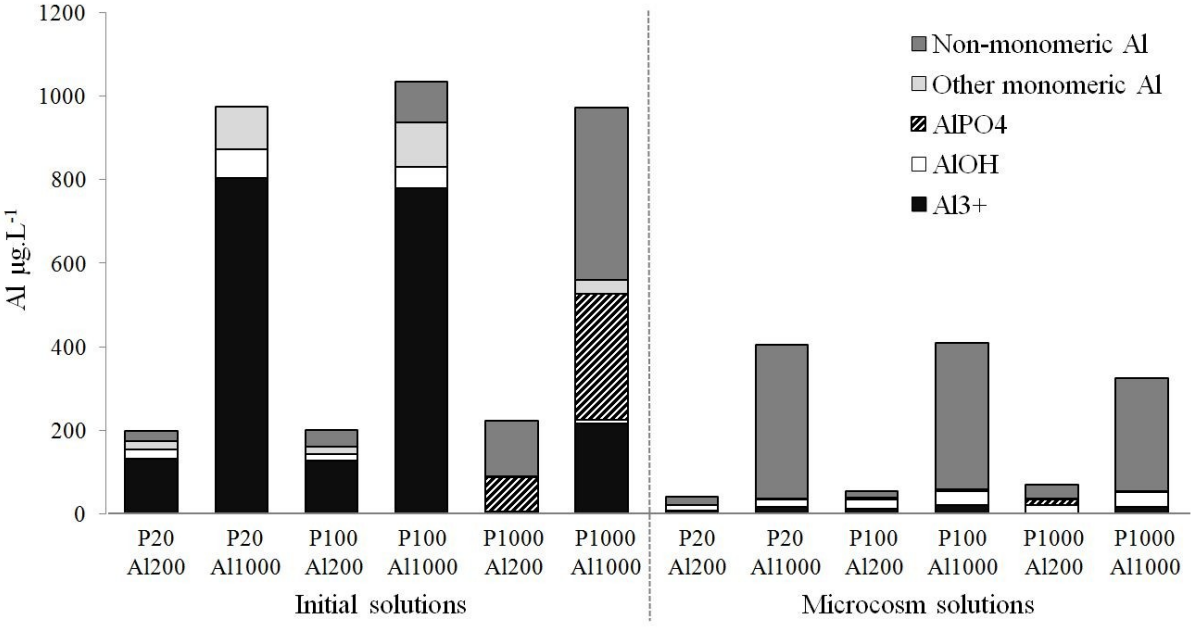

Figure 5. Al speciation in initial solutions and in solutions collected at the end of the experiment, after staying 3 days in microcosms.

Discussion

Al impact on aquatic hyphomycetes and leaf litter processing

In this study under controlled conditions, we showed that at acidic pH and at P concentrations comprised between 20 and 100 $\mu g.L^{-1}$, Al could be a factor reducing leaf litter processing by aquatic hyphomycetes. At these P levels, fungal growth on leaves was negatively affected by both levels of Al, contrasting with some *in situ*-results reported in acidified headwater streams (Dangles & Chauvet, 2003; Baudoin et al., 2008; Simon et al., 2009; Clivot et al., in press; Cornut et al., in press), despite that the lowest Al concentrations used in our study (i.e. 200 $\mu g.L^{-1}$) was more than 3 times lower than those measured in most of these field-experiments. This could result from the high sensitivity of aquatic hyphomycetes to anthropogenic stress. Indeed, other groups of fungi may greatly contribute to natural fungal assemblages growing on leaves in streams (Nikolcheva & Bärlocher, 2004; Seena et al., 2008). By extension, these latter could contribute significantly to fungal biomass measured on leaves in field-experiments and potentially hide some negative effects of acidification on aquatic hyphomycetes. Nevertheless, acidification effects on these other groups of fungi could have less repercussion on stream ecosystem functioning, these latter being comparatively considered as less efficient leaf litter decomposers (Barlocher & Kendrick, 1974). Another hypothesis could be that the difference observed in fungal biomass at the end of this experiment with a single date measurement could be related to the slight delay in colonization time reported in acidified streams (Clivot et al., in press). On the other hand, the negative effects observed on aquatic hyphomycetes are in line with previous results reported by Chamier and Tipping (1997), which showed that their growth rates were inhibited by Al. Thus, the negative impact of Al on aquatic hyphomycetes and on leaf litter processing confirmed that both could be useful indicators of anthropogenic disturbances (Gessner & Chauvet, 2002; Solé et al., 2008).

Al and P interactions

In accordance with our hypotheses, the negative effects of Al on leaf decomposition rates appeared to be alleviated by a high P supply. Similar effects were observed on fungal growth, which was enhanced by the highest P concentration whatever the Al level. Beyond the fact that a high P supply could directly allow fungi to face high Al concentrations through reductions of nutrient limitations, results showed that high P concentrations could also potentially decrease Al toxicity. Indeed, Al speciation revealed that the strong affinity

between Al species and PO_4 anions has led to significant levels of $AlPO_4$ in P1000 treatments, the proportions of the most toxic forms of Al (i.e. Al^{3+} and AlOH) being much lower than in the two lowest P levels treatments.

Potential phosphatase activities showed that a high P supply could meet microbial P need but also that Al could induce a P limitation at lowest P levels. As previously reported in acidified streams and under elevated Al concentrations (Simon et al., 2009; Clivot et al., in press), higher potential phosphatase activities were found with increasing Al concentrations. Surprisingly, no effects of Al were observed on leaf litter P content. However, total leaf litter P content was quantified and it is not excluded that a part of P could be abiotically bound to leaf litter and thus not directly accessible for microorganisms. Another hypothesis could be that aquatic hyphomycetes would have to produce a higher enzyme amount to obtain similar levels of P when subject to high Al concentrations.

Concerning fungal assemblages on leaf litter, the main difference observed was that THEL growth was favored in high P treatments. Interestingly, Gulis and Suberkropp (2003) demonstrated that the relative abundance of conidia produced by this species not only in water but also on rhododendron and maple leaves increased in a reach of a nutrient-enriched (N and P) stream. In addition, the contribution of this species to total conidial production from alder leaves has been shown to decrease across a gradient of increasing acidification and Al concentrations in headwater streams (Cornut et al., in press). Owing to the potential P limitation of microbial leaf decomposers under elevated Al concentrations (Clivot et al., in press), we could therefore hypothesize that THEL would not be favored in conditions of low P availability that could prevail in anthropogenically acidified headwater streams.

Leaf litter quality: implications for stream ecosystem functioning

Leaf litter conditioning by microorganisms for shredding invertebrates is a crucial step at the base of the headwater stream food webs. This study showed that Al could severely affect the quality of leaf litter through its impact on microbial decomposers and on leaf litter nutrient content.

The most worrying effect of Al on leaf characteristics was certainly the particularly high amount of Al found on leaf litter, even at the highest level of P. At the end of the experiment, Al contents on leaf litter corresponded to about 50 and 25% of the total loads of Al in microcosms for Al1000 and Al200 treatments, respectively. These results could explain the important decrease of Al concentrations in solutions collected in microcosms compared to

concentrations initially introduced. Leaf litter fragmentation can increase leaf surface area, providing more sites available for complexation and biosorption of contaminants with organic matter and fungi (Schaller et al., 2011), which can also accumulate high amount of metals (Purchase et al., 2009). Chamier et al. (1989) showed that Al on leaf litter can reach levels 10 times higher than those found in our study, when leaves were exposed during 12 weeks in acidified streams. Furthermore, the latter authors suggested that Al on leaves could be an additional factor of toxicity for efficient shredders such as Gammarids through the ingestion of contaminated leaf litter, affecting ecosystem functioning at higher levels.

At the two lowest P levels, fungal growth and leaf litter conditioning were impaired by Al, suggesting that leaf litter in acidified streams could be a poor quality resource, since these parameters are known to influence leaf litter consumption rates by invertebrate shredders (Bärlocher, 1985; Suberkropp, 1992; Graça, 2001). Moreover, N leaf litter content, which was the lowest under high Al concentrations and low P levels, can also influence invertebrate-mediated leaf litter breakdown (Hladyz et al., 2009). More importantly, P contents in leaf litter were strongly enhanced with the highest P level. In aquatic ecosystems, phosphorus content in resources is known to impact consumer consumption rates (Stelzer & Lamberti, 2002; Fink & Von Elert, 2006) and can be related to consumer growth rates (Gergs & Rothhaupt, 2008; Danger et al., 2012). This parameter could thus lead to increased leaf litter consumption rates through compensatory feeding on low quality resources, and modify secondary production in headwater streams.

Conclusion

In this microcosm-based experiment, we isolated for the first time Al from its covarying parameters in acidified streams, notably fluctuating pH and base cation concentrations, which are also supposed to control some microbial activities associated with leaf litter breakdown. We demonstrated that Al could be able to alter microbial growth and leaf litter processing, and could potentially induce a P limitation that could be alleviated only by a high P supply. Moreover, Al impact on leaf litter conditioning and nutrient content could play a key role by reducing its nutritive quality for invertebrate shredders and by concentrating the toxic metal on their main food resource. These evidences showed that Al, as a sole factor, could be involved in the deleterious effects of acidification on the stream ecosystem functioning and on the associated biota.

Acknowledgements

We are grateful to Philippe Rousselle for technical assistance. The present study was financed by the French ANR program (ANR-07-BDIV-007-01 Recover project). We thank the Conseil Général des Vosges, the Office National des Forêts and the Zone Atelier Moselle for their support.

References

Arsuffi, T.L., Suberkropp, K., 1988. Effects of Fungal Mycelia and Enzymatically Degraded Leaves on Feeding and Performance of Caddisfly (Trichoptera) Larvae. Journal of the North American Benthological Society 7, 205-211.

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., 1981. The role of aquatic hyphomycetes in the trophic structure of streams. In:The fungal community (eds D.T. Wicklow, G.C. Carroll), Marcel Dekker, New York, p. 743-760.

Bärlocher, F., Kendrick, B., 1974. Dynamics of the Fungal Population on Leaves in a Stream. Journal of Ecology 62, 761-791.

Baudoin, J., Guérold, F., Felten, V., Chauvet, E., Wagner, P.., Rousselle, P., 2008. Elevated Aluminium Concentration in Acidified Headwater Streams Lowers Aquatic Hyphomycete Diversity and Impairs Leaf-Litter Breakdown. Microbial Ecology 56, 260-269.

Boudot, J.P., Maitat, O., Merlet, D., Rouiller, J., 2000. Soil solutions and surface water analysis in two contrasted watersheds impacted by acid deposition, Vosges mountains, N.E. France: interpretation in terms of Al impact and nutrient imbalance. Chemosphere 41, 1419-1429.

Boudot, J.P., Merlet, D., Rouiller, J., Maitat, O., 1994. Validation of an operational procedure for aluminium speciation in soil solutions and surface waters. Science of The Total Environment 158, 237-252.

Bray, J.R., Curtis, J.T., 1957. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecological Monographs 27, 325-349.

Chamier, A.C., 1987. Effect of pH on microbial degradation of leaf litter in seven streams of the English Lake District. Oecologia 71, 491-500.

Chamier, A.C., Dixon, P.A., 1982. Pectinases in Leaf Degradation by Aquatic Hyphomycetes: the Enzymes and Leaf Maceration. Journal of General Microbiology 128, 2469-2483.

Chamier, A.C., Sutcliffe, D.W., Lishman, J.P., 1989. Changes in Na, K, Ca, Mg and Al content of submersed leaf litter, related to ingestion by the amphipod Gammarus pulex (L.). Freshwater Biology 21, 181–189.

Chamier, A.C., Tipping, E., 1997. Effects of aluminium in acid streams on growth and sporulation of aquatic hyphomycetes. Environmental Pollution 96, 289-298.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18, 117–143.

Clivot, H., Danger, M., Pagnout, C., Wagner, P., Rousselle, P., Poupin, P., Guérold, F., 2012. Impaired Leaf Litter Processing in Acidified Streams. Microbial Ecology.

Cornut, J., Clivot, H., Chauvet, Eric., Elger, A., Pagnout, C., Guérold, F., 2012. Effect of acidification on leaf litter decomposition in benthic and hyporheic zones of woodland streams. Water Research.

Danger, M., Cornut, J., Elger, A., Chauvet, E., 2012. Effects of burial on leaf litter quality, microbial conditioning and palatability to three shredder taxa. Freshwater Biology 57, 1017–1030.

Dangles, O., Chauvet, E., 2003. Effects of stream acidification on fungal biomass in decaying beech leaves and leaf palatability. Water Research 37, 533-538.

Dangles, O.., Guérold, F., 1998. Influence of shredders in mediating breakdown rates of beech leaves in circumneutral and acidic forest streams. Archiv für Hydrobiologie 151, 649-666.

Dangles, O., Gessner, M.O., Guérold, F., Chauvet, E., 2004. Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. Journal of Applied Ecology 41, 365-378.

Dangles, O., Guérold, F., 2001. Linking Shredders and Leaf Litter Processing: Insights from an Acidic Stream Study. International Review of Hydrobiology 86, 395-406.
Fink, P., Von Elert, E., 2006. Physiological responses to stoichiometric constraints: nutrient limitation and compensatory feeding in a freshwater snail. Oikos 115, 484–494.
Fisher, S.G., Likens, G.E., 1972. Stream Ecosystem: Organic Energy Budget. BioScience 22, 33-35.
Gensemer, R.W., Playle, R.C., 1999. The Bioavailability and Toxicity of Aluminum in Aquatic Environments. Critical Reviews in Environmental Science and Technology 29, 315-450.
Gergs, R., Rothhaupt, K.O., 2008. Feeding rates, assimilation efficiencies and growth of two amphipod species on biodeposited material from zebra mussels. Freshwater Biology 53, 2494–2503.
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., 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., Schmitt, A., 1996. Use of Solid-Phase Extraction To Determine Ergosterol Concentrations in Plant Tissue Colonized by Fungi. Applied and Environmental Microbiology 62, 415-419.
Graça, M.A.S., 2001. The Role of Invertebrates on Leaf Litter Decomposition in Streams – a Review. International Review of Hydrobiology 86, 383–393.
Gulis, V., Suberkropp, K., 2003. Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. Freshwater Biology 48, 123–134.
Hladyz, S., Gessner, M.O., Giller, P.S., Pozo, J., Woodward, G., 2009. Resource quality and stoichiometric constraints on stream ecosystem functioning. Freshwater Biology 54, 957–970.
Jenkins, C.C., Suberkropp, K., 1995. The influence of water chemistry on the enzymatic degradation of leaves in streams. Freshwater Biology 33, 245-253.
Kilham, S., Kreeger, D., Lynn, S., Goulden, C., Herrera, L., 1998. COMBO: a defined freshwater culture medium for algae and zooplankton. Hydrobiologia 377, 147-159.
Meegan, S.K., Perry, S.A., Perry, W.B., 1996. Detrital processing in streams exposed to acidic precipitation in the Central Appalachian Mountains. Hydrobiologia 339, 101-110.
Minchin, P.R., 1987. An evaluation of the relative robustness of techniques for ecological ordination. Vegetatio 69, 89-107.
Mulholland, P.J., Palumbo, A.V., Elwood, J.W., Rosemond, A.D., 1987. Effects of Acidification on Leaf Decomposition in Streams. Journal of the North American Benthological Society 6, 147-158.
Nikolcheva, L., Bärlocher, F., 2004. Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. Mycological Progress 3, 41-49.
Ormaza-González, F.I., Statham, P.J., 1996. A comparison of methods for the determination of dissolved and particulate phosphorus in natural waters. Water Research 30, 2739-2747.
Oros-Sichler, M., Gomes, N.C.M., Neuber, G., Smalla, K., 2006. A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. Journal of Microbiological Methods 65, 63-75.
Piña, R.G., Cervantes, C., 1996. Microbial interactions with aluminium. BioMetals 9, 311-316.
Purchase, D., Scholes, L., Revitt, D., Shutes, R., 2009. Effects of temperature on metal tolerance and the accumulation of Zn and Pb by metal-tolerant fungi isolated from urban runoff treatment wetlands. Journal of Applied Microbiology 106, 1163–1174.

R Development Core Team., 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.
Schaller, J., Brackhage, C., Mkandawire, M., Dudel, E.G., 2011. Metal/metalloid accumulation/remobilization during aquatic litter decomposition in freshwater: A review. Science of The Total Environment 409, 4891-4898.
Seena, S., Wynberg, N., Baerlocher, F., 2008. Fungal diversity during leaf decomposition in a stream assessed through clone libraries. Fungal Diversity 30, 1-14.
Simon, K.S., Simon, M.A., Benfield, E.F., 2009. Variation in ecosystem function in Appalachian streams along an acidity gradient. Ecological Applications 19, 1147–1160.
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.
Solé, M., Fetzer, I., Wennrich, R., Sridhar, K.R., Harms, H., Krauss, G., 2008. Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. Science of The Total Environment 389, 557-565.
Stelzer, R.S., Lamberti, G.A., 2002. Ecological stoichiometry in running waters: periphyton chemical composition and snail growth. Ecology 83, 1039-1051.
Suberkropp, K., 1992. Interactions with invertebrates. In:The ecology of aquatic hyphomycetes. (ed F. Bärlocher), Springer-Verlag, Berlin, p. 118-134.
Suberkropp, K., 1991. Relationships between growth and sporulation of aquatic hyphomycetes on decomposing leaf litter. Mycological Research 95, 843-850.
Vannote, R., Minshall, G., Cummins, K., Sedell, J., Cushing, C., 1980. River Continuum Concept. Canadian Journal of Fisheries and Aquatic Sciences 37, 130-137.
Webster, J.R., Meyer, J.L., 1997. Organic Matter Budgets for Streams: A Synthesis. Journal of the North American Benthological Society 16, 141-161.
Zar, J.H., 1999. Biostatistical Analysis. Prentice Hall.

Conclusion générale et perspectives

Un des objectifs affichés par le Grenelle de l'environnement est d'atteindre une part de 23% d'énergies renouvelables pour 2020 en France. Le développement de la production de bois à des fins énergétiques et la construction de centrales à biomasse sont des projets intégrant ce programme. Les besoins de production vont ainsi entrainer la diminution des périodes rotations mais aussi le prélèvement des rémanents. La mise en place de ces mesures risque d'appauvrir d'autant plus les sols les plus sensibles. Dans certaines régions, le recours à des épandages d'amendements calco-magnésiens pourrait donc se généraliser face à l'exploitation forestière intensive. Ainsi, différents projets d'amendements sont à l'étude compte tenu des carences minérales avérées dans les sols et les symptômes de dépérissement observés dans certains secteurs. Ainsi, 800 ha ont été amendés en 2008 puis près de 10 000 ha dans la région de la Vôge en 2011. Face au recours aux amendements qui semble s'opérer actuellement, il apparait nécessaire d'approfondir nos connaissances sur les effets engendrés par ces opérations de restauration, ces effets pouvant se traduire par des modifications marquées de la biodiversité et des processus associés dans les écosystèmes forestiers et *in fine* des services rendus par ces derniers.

Les travaux réalisés dans cette thèse participaient ainsi à l'étude des effets d'amendements calco-magnésiens et portaient plus particulièrement sur les communautés microbiennes de sols se trouvant sur des bassins versants forestiers acidifiés. L'objectif était d'évaluer si ces opérations de restauration pouvaient avoir un effet durable sur la reminéralisation des sols et si des indicateurs microbiens pouvaient être associés à une amélioration de la qualité des sols.

Les études réalisées ont montré que, quatre à cinq ans après les amendements, les sols traités présentaient un pH et des concentrations en cations basiques (Ca et Mg) supérieurs à ceux des sols de bassins versants témoins. Ces observations ont suggéré que les amendements pouvaient avoir un effet durable sur les caractéristiques physico-chimiques des sols se développant sur deux types de roches mères différentes (*i.e.* gréseuse et granitique). En parallèle, l'étude des communautés bactériennes a notamment permis de mettre en évidence un ratio plus important du nombre de *Proteobacteria* par rapport à celui d'*Acidobacteria* dans les sols amendés comparativement à leurs témoins. L'augmentation de ce ratio est supposée révéler l'évolution d'un sol oligotrophe vers un sol plus riche en nutriments (Smit et al., 2001) et l'utilisation de cet indicateur a été démontré comme étant un potentiel bioindicateur dans le cadre de restaurations (Hartman et al., 2008). Ces résultats montrent que l'amélioration à moyen terme des caractéristiques du sol a été accompagnée d'une évolution des communautés

microbiennes, ces dernières indiquant elles aussi une amélioration de la qualité des sols amendés.

Conjointement à ces analyses, d'autres études ont été entreprises sur ces sites dans le cadre de notre programme ANR et montrent également des effets des amendements sur la faune des sols (Auclerc, 2012; Auclerc et al., 2012) et sur la dynamique de la matière organique dans les sols (Rizvi, 2012; Rizvi et al., 2012). Les résultats obtenus ont rapporté que les amendements n'avaient aucun effet sur la richesse taxonomique des macro-invertébrés vivant dans les sols, mais que leur abondance était comparativement inférieure dans les sols amendés par rapport aux témoins. Cependant, quelques espèces détritivores et ingénieures du sol telles que des Curculionidae ou le vers de terre *Lumbricus castaneus* semblent avoir été favorisées par les amendements. Ainsi, une augmentation de l'abondance de ces organismes suggèrerait un meilleur fonctionnement du recyclage de la matière et des nutriments dans les sols amendés (Auclerc et al., 2012). Ces changements associés à ceux observées sur les communautés microbiennes pourraient être également en relation avec les évolutions de la morphologie et des caractéristiques des humus observées dans les sols amendés (Rizvi et al., 2012).

L'ensemble de ces résultats met en évidence que les processus de recyclage de la matière et des nutriments ont pu évoluer dans les sols amendés et qu'il serait intéressant d'étudier comparativement le processus de décomposition de la matière organique dans les sols traités et les sols témoins. Dans ce but, des expérimentations de décomposition de litières de feuilles ont été mises en place sur ces sites, cependant, pour des raisons techniques et de temps ce projet n'a pas pu être mené à son terme lors de cette thèse.

Quelques années après les amendements, les forêts traitées semblent donc répondre favorablement aux effets de l'amendement. Des études doivent être envisagées pour déterminer si ces effets seront durables et pour observer d'éventuels changements engendrés à plus long terme sur la biodiversité et les services rendus par ces écosystèmes.

L'amélioration de la qualité de la ressource en eau est également au centre des préoccupations lors de ces opérations de restauration. En effet, l'acidification prononcée des cours d'eau et ses effets délétères sur les organismes aquatiques et sur leur fonctionnement restent aujourd'hui une problématique majeure. Il a ainsi été rapporté que, suite à l'amendement, l'amélioration de la qualité de l'eau dans le cours d'eau drainant le bassin versant granitique avait entrainé une augmentation des taux de décomposition des litières et de la diversité d'espèces sporulantes de champignons associées. Les travaux présentés dans la deuxième partie de cette thèse s'inscrivaient donc dans la continuité de ces observations et

avaient pour objectifs d'apporter, à l'échelle microbienne, une meilleure compréhension des facteurs limitant le processus de décomposition des litières dans les cours d'eau acidifiés.

La première étude présentait l'originalité d'étudier en parallèle la décomposition de litières de feuilles et les communautés de décomposeurs associées dans les compartiments benthiques et hyporhéiques de cinq cours d'eau présentant différents degrés d'acidification. Les résultats ont montré que l'acidification pouvait altérer le processus de décomposition des litières et les communautés associées dans les deux compartiments des cours d'eau, mais que la subsistance des activités microbiennes permettait un maintien minimum des flux de carbone et de nutriments dans les zones hyporhéiques et dans les écosystèmes impactés, assurant ainsi à ces derniers un potentiel de résilience. En effet, en dépit du fait que leurs activités peuvent être modifiées par certaines perturbations, les micro-organismes présentent généralement de meilleures capacités d'adaptation et de résistance aux pressions environnementales que les organismes supérieurs.

L'étude des communautés fongiques a montré que l'acidification pouvait entrainer conjointement une sévère diminution de la diversité d'espèces sporulantes d'hyphomycètes aquatiques mais également de la contribution de ces derniers aux assemblages fongiques étudiés par méthode moléculaire. Bien qu'ils soient considérés comme étant moins impliqués dans le processus de décomposition des litières, une importante contribution d'autres groupes de champignons a été observée par analyse moléculaire et particulièrement dans le cours d'eau acidifié. Ainsi, contrairement à l'étude microscopique, les méthodes moléculaires n'ont pas révélé d'effets négatifs de l'acidification sur la richesse spécifique de champignons. Peu d'approches moléculaires se sont précédemment intéressées aux communautés fongiques associées aux litières. Des analyses plus poussées doivent maintenant être réalisées afin de comprendre quels facteurs structurent ces communautés et quelles peuvent être les interactions entre les différents champignons présents sur les feuilles en décomposition. La caractérisation, chez différentes espèces, de gènes fonctionnels codant pour des enzymes extracellulaires impliquées dans la décomposition des litières pourrait également permettre de connaitre leur rôle respectif dans ce processus.

Si des études ont pu mettre en évidence des effets de l'acidification sur la structure des communautés de macro-invertébrés (Meegan et al., 1996; Dangles & Guérold, 2001) ou de champignons associées aux litières (Chamier, 1987; Baudoin et al., 2008), en revanche, ses effets sur les activités microbiennes impliquées dans la décomposition des litières étaient très peu compris.

Des études antérieures s'accordaient à dire que l'altération du processus de décomposition des litières de feuilles dans les cours d'eau acidifiés était probablement due à une diminution des activités enzymatiques ciblant la dégradation des pectines (Chamier & Dixon, 1982; Jenkins & Suberkropp, 1995). En effet, ces enzymes sont impliquées dans le processus de macération des feuilles et sont reconnues pour nécessiter un pH optimal de fonctionnement alcalin (pH ~ 8) et pour être dépendantes des concentrations en Ca. De façon surprenante, peu d'études se sont intéressées aux effets de l'Al sur les communautés microbiennes associées à la décomposition des feuilles en dépit de la toxicité de ce métal pour les micro-organismes en général (Piña & Cervantes, 1996) et en particulier pour les hyphomycètes aquatiques (Chamier & Tipping, 1997; Baudoin et al., 2008). Les travaux de cette thèse ont ainsi permis de mettre en évidence que l'Al pouvait être en partie responsable de la réduction de la décomposition des litières dans les cours d'eau acidifiés en induisant notamment une limitation en P pour les micro-organismes associés à ce processus.

Les résultats obtenus ont révélé que la mobilisation de l'Al dans les cours d'eau acidifiés pouvait engendrer un ralentissement du conditionnement des feuilles par les micro-organismes et une diminution de la qualité nutritive des litières, ces dernières pouvant être moins riches en P. De plus, les importantes concentrations en Al dans les litières pourraient aussi avoir des effets délétères sur les macro-invertébrés, en raison de l'ingestion potentielle de matériel foliaire riche en Al. Cette diminution de la qualité de la ressource et l'accumulation d'Al dans les litières pourraient donc se répercuter sur les niveaux trophiques supérieurs en induisant des carences et une toxicité métallique accrue et venir ainsi aggraver les effets directs des stress acides et aluminiques sur les organismes.

Bien que de nombreux ruisseaux ne montrent aucune amélioration significative de la qualité de l'eau suite à la réduction des émissions et des dépôts atmosphériques (Probst et al., 1999; Angéli et al., 2009), l'entreprise de restauration forcée du bassin versant granitique a montré qu'il existait néanmoins un bon espoir de résilience pour ces écosystèmes. En effet, la subsistance des activités microbiennes dans les ruisseaux impactés et la proximité de zones non altérées par l'acidification permettent ainsi d'envisager une recolonisation par les espèces acido-sensibles et une reprise rapide du fonctionnement des cours d'eau acidifiés lorsque les conditions redeviendront plus favorables.

Une question importante est de savoir quel sera le temps nécessaire pour observer un retour vers un fonctionnement plus originel des ces écosystèmes. La restauration forcée par amendements calco-magnésiens peut permettre une amélioration à plus ou moins long terme

des écosystèmes les plus fragilisés par l'acidification. Cependant, à l'heure actuelle, les opérations de restauration des écosystèmes forestiers semblent être en partie gouvernées par le besoin d'améliorer leur productivité. La surexploitation pouvant être un frein au rétablissement des écosystèmes les plus sensibles, il convient donc de savoir si ces derniers doivent être préservés ou si une exploitation raisonnée doit être également envisagée afin d'observer un retour durable de leur fonctionnement originel.

Références bibliographiques

Adams, M.B., Burger, J., Jenkins, A., Zelazny, L., 2000. Impact of harvesting and atmospheric pollution on nutrient depletion of eastern US hardwood forests. Forest Ecology and Management 138, 301-319.

Adams, M.B., 1999. Acidic deposition and sustainable forest management in the central Appalachians, USA. Forest Ecology and Management 122, 17-28.

Alewell, C., Manderscheid, B., Meesenburg, H., Bittersohl, J., 2000. Environmental chemistry: Is acidification still an ecological threat? Nature 407, 856-857.

Anderson, J.M., 1988. Spatiotemporal effects of invertebrates on soil processes. Biology and Fertility of Soils 6, 216-227.

Angéli, N., Dambrine, E., Boudot, J.P., Nedeltcheva, T., Guérold, F., Tixier, G., Probst, A., Party, J.P., Pollier, B., Bourrié, G., 2009. Evaluation of streamwater composition changes in the Vosges Mountains (NE France): 1955-2005. Science of The Total Environment 407, 4378-4386.

Angéli, N., 2006. Evolution de la composition chimique des ruisseaux vosgiens: Analyse rétrospective et effet d'un amendement calco-magnésien. Thèse de doctorat, Université Henri Poincaré.

Arsuffi, T.L., Suberkropp, K., 1988. Effects of Fungal Mycelia and Enzymatically Degraded Leaves on Feeding and Performance of Caddisfly (Trichoptera) Larvae. Journal of the North American Benthological Society 7, 205-211.

Auclerc, A., 2012. Effets d'amendements calco-magnesiens sur la diversité des macroinvertébrés de sols forestiers et sur certains processus fonctionnels associés. Thèse Université de Lorraine, Metz.

Auclerc, A., Nahmani, J., Aran, D., Baldy, V., Callot, H., Gers, C., Iorio, E., Lapied, E., Lassauce, A., Pasquet, A., Spelda, J., Rossi, J.P., Guérold, F., 2012. Changes in soil macroinvertebrate communities following liming of acidified forested catchments in the Vosges Mountains (North-eastern France). Ecological Engineering 42, 260-269.

Bååth, E., Berg, B., Lohm, U., Lundgren, B., Lundvist, H., Rosswall, T., Oederstroem, B., Wiren, A., 1980. Effects of experimental acidification and liming on soil organisms and decomposition in a Scots pine forest. Pedobiologia 20:2, 85-100.

Bååth, E., Lundgren, B., Söderström, B., 1979. Effects of artificial acid rain on microbial activity and biomass. Bulletin of Environmental Contamination and Toxicology 23, 737-740.

Badalucco, L., Grego, S., Dell'Orco, S., Nannipieri, P., 1992. Effect of liming on some chemical, biochemical, and microbiological properties of acid soils under spruce (Picea abies L.). Biology and Fertility of Soils 14, 76-83.

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, Virginie., Gessner, M.O., 1997. Towards a budget of leaf litter decomposition in a first-order woodland stream. Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie 320, 747-758.

Bärlocher, F., 1982. The contribution of fungal enzymes to the digestion of leaves by Gammarus fossarum Koch (Amphipoda). Oecologia 52, 1-4.

Bärlocher, F., 1985. The role of fungi in the nutrition of stream invertebrates. Botanical Journal of the Linnean Society 91, 83-94.

Bärlocher, Félix., 1992. The Ecology of aquatic hyphomycetes. Springer-Verlag, Berlin.

Bärlocher, Felix., Kendrick, B., 1976. Hyphomycetes as intermediaries of energy flow in streams. In:Recent Advances in Aquatic Mycology, (eds E.B.G. Jones), Elek, London, 435-446.

Baudoin, J.M., Guérold, F., Felten, V., Chauvet, E., Wagner, P., Rousselle, P., 2008. Elevated Aluminium Concentration in Acidified Headwater Streams Lowers Aquatic Hyphomycete Diversity and Impairs Leaf-Litter Breakdown. Microbial Ecology 56, 260-269.

Baudoin, J.M., 2007. Biodiversité et fonctionnement de cours d'eau forestiers de tête de bassin: Effet de l'acidification anthropique et d'une restauration. Thèse Université Paul Verlaine Metz.

Beckett, K.P., Freer-Smith, P.H., Taylor, G., 1998. Urban woodlands: their role in reducing the effects of particulate pollution. Environmental Pollution 99, 347-360.

Bonneau, M., Fricker, C., 1985. Le Dépérissement des forêts dans le massif vosgien: relations possibles avec la pollution atmosphérique. Revue forestière française XXXVI, 105-126.

Bonneau, M., Landmann, G., Adrian, M., 1992. La fertilisation comme remède au dépérissement des forêts en sol acide: essais dans les Vosges. Revue forestière française 3, 207-223.

Bonneau, M., Nys, C., 1997. Effets des amendements calci-magnésiques en forêt. Compte rendu de l'Académie d'Agriculture Française 83, 161-169.

Boudot, J.P., Becquer, T., Merlet, D., Rouiller, J., 1994. Aluminium toxicity in declining forests: a general overview with a seasonal assessment in a silver fir forest in the Vosges mountains (France). Annales des Sciences Forestières 51, 27-51.

Boudot, J.P., Maitat, O., Merlet, Denis., Rouiller, J., 2000. Soil solutions and surface water analysis in two contrasted watersheds impacted by acid deposition, Vosges mountains, N.E. France: interpretation in terms of Al impact and nutrient imbalance. Chemosphere 41, 1419-1429.

Bourrié, G., 1978. Acquisition de la composition chimique des eaux en climat tempéré. Application aux granites des Vosges et de la Margeride. Thèse Université Louis Pasteur Strasbourg.

Buckton, S.T., Brewin, P.A., Lewis, A., Stevens, J., Ormerod, S.J., 1998. The distribution of dippers, Cinclus cinclus (L.), in the acid-sensitive region of Wales 1984-95. Freshwater Biology 39, 387-396.

Campbell, P.G.C., Bisson, M., Bougie, R., Tessier, A., Villeneuve, J.P., 1983. Speciation of aluminum in acidic freshwaters. Analytical Chemistry 55, 2246-2252.

Chamier, A.C., 1987. Effect of pH on microbial degradation of leaf litter in seven streams of the English Lake District. Oecologia 71, 491-500.

Chamier, A.C., Dixon, P.A., 1982. Pectinases in Leaf Degradation by Aquatic Hyphomycetes: the Enzymes and Leaf Maceration. Journal of General Microbiology 128, 2469-2483.

Chamier, A.C., Tipping, E., 1997. Effects of aluminium in acid streams on growth and sporulation of aquatic hyphomycetes. Environmental Pollution 96, 289-298.

Clair, T.A., Hindar, A., 2005. Liming for the mitigation of acid rain effects in freshwaters: A review of recent results. Environmental Reviews 13, 91-128.

Costanza, R., Arge, R., De Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., Van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. Nature 387, 253-260.

Cronan, C.S., Schofield, C.L., 1979. Aluminum Leaching Response to Acid Precipitation: Effects on High-Elevation Watersheds in the Northeast. Science 204, 304 -306.

Cummins, K.W., Wilzbach, M.A., Gates, D.M., Perry, J.B., Taliaferro, W.B., 1989. Shredders and Riparian Vegetation. BioScience 39, 24-30.

Dambrine, E., Party, J.P., Pollier, B., Nicolaï, M., Probst, A., Rozin, C., Duc, M., 1999. Acidification des eaux de source et saturnisme dans le Massif vosgien. Revue Forestière Française 2, 173-183.

Dambrine, E., Ulrich, E., Cénac, N., Durand, P., Gauquelin, T., Mirabel, P., Nys, C., Probst, A. Ranger, J., Zéphoris, M., 1995. Atmospheric deposition in France and possible relation with forest decline. In: Forest decline and atmospheric deposition effects in the French mountains (eds G. Landmann, M. Bonneau), Springer, Berlin, Heildeberg, New York, 177-199.

Dangles, O., Chauvet, E., 2003. Effects of stream acidification on fungal biomass in decaying beech leaves and leaf palatability. Water Research 37, 533-538.

Dangles, O., Guérold, F., 1998. Influence of shredders in mediating breakdown rates of beech leaves in circumneutral and acidic forest streams. Archiv für Hydrobiologie 151, 649-666.

Dangles, O., Guérold, F., 2000. Structural and functional responses of benthic macroinvertebrates to acid precipitation in two forested headwater streams (Vosges Mountains, northeastern France). Hydrobiologia 418, 25-31.

Dangles, O., Gessner, M.O., Guérold, F., Chauvet, E., 2004. Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. Journal of Applied Ecology 41, 365-378.

Dangles, O., Guérold, F., 2001. Linking Shredders and Leaf Litter Processing: Insights from an Acidic Stream Study. International Review of Hydrobiology 86, 395-406.

Davidson, E.A., Hart, S.C., Firestone, M.K., 1992. Internal Cycling of Nitrate in Soils of a Mature Coniferous Forest. Ecology 73, 1148-1156.

Driscoll, C.T., Baker, J.P., Bisogni, J.J., Schofield, C.L., 1980. Effect of aluminium speciation on fish in dilute acidified waters. Nature 284, 161-164.

Driscoll, C.T., Lawrence, G.B., Bulger, A.J., Butler, T.J., Cronan, C.S., Eagar, C., Lambert, K.F., Likens, G.E., Stoddard, J.L., Weathers, K.C., 2001. Acidic Deposition in the Northeastern United States: Sources and Inputs, Ecosystem Effects, and Management Strategies. BioScience 51, 180-198.

Ducros, M., 1845. Observation d'une pluie acide. Journal de Pharmacie et de Chimie 3, 273-277.

Dupouey, J.L., Thimonnnier, A., Lebourgeois, F., Becker, M., Picard, J.F., Timbal, J., 1999. Changes in the vegetation of the forests of northeastern France between 1970 and 1990. Revue Forestiere Francaise 2, 219-230.

Edwards, P.J., Kochenderfer, J.N., Coble, D.W., Adams, M.B., 2002. Soil Leachate Responses During 10 Years of Induced Whole-Watershed Acidification. Water, Air, & Soil Pollution 140, 99-118.

Ericsson, T., Göransson, A., Oene, H.V., Gobran, G., 1995. Interactions between Aluminium, Calcium and Magnesium: Impacts on Nutrition and Growth of Forest Trees. Ecological Bulletins 44, 191-196.

Falkengren-Grerup, U., 1989. Soil Acidification and Its Impact on Ground Vegetation. Ambio 18, 179-183.

Farmer, A.M., 1990. The effects of lake acidification on aquatic macrophytes—A review. Environmental Pollution 65, 219-240.

Feeney, D., Crawford, J., Daniell, T., Hallett, P., Nunan, N., Ritz, K., Rivers, M., Young, I., 2006. Three-dimensional Microorganization of the Soil-Root-Microbe System. Microbial Ecology 52, 151-158.

Fernandez, I.J., Rustad, L.E., Norton, S.A., Kahl, J.S., Cosby, B.J., 2003. Experimental Acidification Causes Soil Base-Cation Depletion at the Bear Brook Watershed in Maine. Soil Science Society of America Journal 67, 1909-1919.

Fierer, N., Strickland, M.S., Liptzin, D., Bradford, M.A., Cleveland, C.C., 2009. Global patterns in belowground communities. Ecology Letters 12, 1238-1249.

Findlay, S., Tank, J., Dye, S., Valett, H.M., Mulholland, P.J., McDowell, W.H., Johnson, S.L., Hamilton, S.K., Edmonds, J., Dodds, W.K., Bowden, W.B., 2002. A cross-system comparison of bacterial and fungal biomass in detritus pools of headwater streams. Microbial Ecology 43, 55-66.

Fisher, B., Turner, R.K., Morling, P., 2009. Defining and classifying ecosystem services for decision making. Ecological Economics 68, 643-653.

Fjellheim, A., Raddum, G.G., 1992. Recovery of acid-sensitive species of ephemeroptera, plecoptera and trichoptera in river audna after liming. Environmental Pollution 78, 173-178.

Francis, A., 1982. Effects of acidic precipitation and acidity on soil microbial processes. Water, Air, & Soil Pollution 18, 375-394.

Francis, A.J., 1986. Acid rain effects on soil and aquatic microbial processes. Experientia 42, 455-465.

Frostegård, Å., Bååth, E., Tunlid, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology and Biochemistry 25, 723-730.

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., Robinson, C.T., Ward, J.V., 1998. Leaf Breakdown in Streams of an Alpine Glacial Floodplain: Dynamics of Fungi and Nutrients. Journal of the North American Benthological Society 17, 403-419.

Gobran, G.R., Bosatta, E., 1988. Cation depletion rate as a measure of soil sensitivity to acidic decomposition: Theory. Ecological Modelling 40, 25-36.

Goodale, C.L., Apps, M.J., Birdsey, R.A., Field, C.B., Heath, L.S., Houghton, R.A., Jenkins, J.C., Kohlmaier, G.H., Kurz, W., Liu, S., Nabuurs, G.J., Nilsson, S., Shvidenko, A.Z., 2002. Forest carbon sinks in the northern hemisphere. Ecological Applications 12, 891-899.

Gorham, E., 1998. Acid deposition and its ecological effects: a brief history of research. Environmental Science & Policy 1, 153-166.

Graça, M.A.S., 2001. The Role of Invertebrates on Leaf Litter Decomposition in Streams - a Review. International Review of Hydrobiology 86, 383-393.

Grattan, R.M., Suberkropp, K., 2001. Effects of Nutrient Enrichment on Yellow Poplar Leaf Decomposition and Fungal Activity in Streams. Journal of the North American Benthological Society 20, 33-43.

Griffith, M., Perry, S., 1994. Fungal biomass and leaf litter processing in streams of different water chemistry. Hydrobiologia 294, 51-61.

Griffith, M., Perry, S.A., Perry, W.B., 1995. Leaf litter processing and exoenzyme production on leaves in streams of different pH. Oecologia 102, 460-466.

Groom, A.P., Hildrew, A.G., 1989. Food Quality for Detritivores in Streams of Contrasting pH. Journal of Animal Ecology 58, 863-881.

Guérold, F., Boudot, J.P., Jacquemin, G., Vein, D., Merlet, D., Rouiller, J., 2000. Macroinvertebrate community loss as a result of headwater stream acidification in the Vosges Mountains (N-E France). Biodiversity and Conservation 9, 767-783.

Guérold, F., Boudot, J.P., Merlet, D., Rouiller, J., Vein, D., Jacquemin, G., 1997. Evaluation de l'état d'acidfication des cours d'eau du département des Vosges. Conseil Général des Vosges, Rapport de convention N° 14/95C.

Gyaneshwar, P., Naresh Kumar, G., Parekh, L.J., Poole, P.S., 2002. Role of soil microorganisms in improving P nutrition of plants. Plant and Soil 245, 83-93.

Halder, A.K., Mishra, A.K., Bhattacharyya, P., Chakrabartty, P.K., 1990. Solubilization of rock phosphate by Rhizobium and Bradyrhizobium. The Journal of General and Applied Microbiology 36, 81-92.

Hames, R.S., Rosenberg, K.V., Lowe, J.D., Barker, S.E., Dhondt, A.A., 2002. Adverse effects of acid rain on the distribution of the Wood Thrush Hylocichla mustelina in North America. Proceedings of the National Academy of Sciences 99, 11235-11240.

Hansen, J., Johnson, D., Lacis, A., Lebedeff, S., Lee, P., Rind, D., Russell, G., 1981. Climate impact of increasing atmospheric carbon dioxide. Science 213, 957-966.

Harris, J.A., 2003. Measurements of the soil microbial community for estimating the success of restoration. European Journal of Soil Science 54, 801-808.

Hart, S.C., Nason, G.E., Myrold, D.D., Perry, D.A., 1994. Dynamics of Gross Nitrogen Transformations in an Old-Growth Forest: The Carbon Connection. Ecology 75, 880-891.

Harte, J., Hoffman, E., Vertucci, F.A., Corn, S., 1994. Acidification and Salamander Recruitment. BioScience 44, 125-126.

Hartman, W.H., Richardson, C.J., Vilgalys, R., Bruland, G.L., 2008. Environmental and anthropogenic controls over bacterial communities in wetland soils. Proceedings of the National Academy of Sciences 105, 17842-17847.

Hättenschwiler, S., Tiunov, A.V., Scheu, S., 2005. Biodiversity and litter decomposition in terrestrial ecosystems. Annual review of ecology, evolution, and systematics 36, 191-218.

Heard, R.M., Sharpe, W.E., Carline, R.F., Kimmel, W.G., 1997. Episodic Acidification and Changes in Fish Diversity in Pennsylvania Headwater Streams. Transactions of the American Fisheries Society 126, 977-984.

Hedin, L.O., Granat, L., Likens, G.E., Adri Buishand, T., Galloway, J.N., Butler, T.J., Rodhe, H., 1994. Steep declines in atmospheric base cations in regions of Europe and North America. Nature 367, 351-354.

Henrikson, L., Hindar, A., Thörnelöf, E., 1995. Freshwater liming. Water, Air, & Soil Pollution 85, 131-142.

Hesthagen, T., Larsen, B.M., 2003. Recovery and re-establishment of Atlantic salmon, Salmo salar, in limed Norwegian rivers. Fisheries Management and Ecology 10, 87-95.

Hieber, M., Gessner, M.O., 2002. Contribution of Stream Detrivores, Fungi, and Bacteria to Leaf Breakdown Based on Biomass Estimates. Ecology 83, 1026-1038.

Hindar, A., Wright, R.F., Nilsen, P., Larssen, T., Høgberget, R., 2003. Effects on stream water chemistry and forest vitality after whole-catchment application of dolomite to a forest ecosystem in southern Norway. Forest Ecology and Management 180, 509-525.

Hooper, D.U., Chapin, F.S., Ewel, J.J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J.H., Lodge, D.M., Loreau, M., Naeem, S., Schmid, B., Setälä, H., Symstad, A.J., Vandermeer, J., Wardle, D.A., 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs 75, 3-35.

Huettl, R.F., Zoettl, H.W., 1993. Liming as a mitigation tool in Germany's declining forests-reviewing results from former and recent trials. Forest Ecology and Management 61, 325-338.

Illmer, P., Schinner, F., 1991. Effects of lime and nutrient salts on the microbiological activities of forest soils. Biology and Fertility of Soils 11, 261-266.

Ingerslev, M., 1997. Effects of liming and fertilization on growth, soil chemistry and soil water chemistry in a Norway spruce plantation on a nutrient-poor soil in Denmark. Forest Ecology and Management 92, 55-66.

Ingold, C.T., 1942. Aquatic hyphomycetes of decaying alder leaves. Transactions of the British Mycological Society 25, 339-417.

Irwin, J., 1989. Acid-Rain - Emissions and Deposition. Archives of Environmental Contamination and Toxicology 18, 95-107.

Jenkins, C.C., Suberkropp, K., 1995. The influence of water chemistry on the enzymatic degradation of leaves in streams. Freshwater Biology 33, 245-253.

Johnson, A.H., Siccama, T.G., 1983. Acid deposition and forest decline. Environmental Science and Technology 17, 294A-305A.

Johnson, D.W., Taylor, G.E., 1989. Role of air pollution in forest decline in Eastern North America. Water, Air, & Soil Pollution 48, 21-43.

Jones, D.L., 1998. Organic acids in the rhizosphere - a critical review. Plant and Soil 205, 25-44.

Joslin, J.D., Wolfe, M.H., 1988. Responses of red spruce seedlings to changes in soil aluminum in six amended forest soil horizons. Canadian Journal of Forest Research 18, 1614-1623.

Kawakami, T., Honoki, H., Yasuda, H., 2001. Acidification of a Small Stream on Kureha Hill Caused by Nitrate Leached from a Forested Watershed. Water, Air, & Soil Pollution 130, 1097-1102.

Kinraide, T., 1991. Identity of the rhizotoxic aluminium species. Plant and Soil 134, 167-178.

Kreiser, A., Rose, N.L., Probst, A., Massabuau, J.C., 1995. Relationship Between Lake-Water Acidification in the Vosges Mountains and SO2 - NOx Emissions in Western Europe. In: Forest decline and atmospheric deposition effects in the French mountains (eds G. Landmann, M. Bonneau) Springer, Berlin, Heildeberg, New York, 363-370.

Kreutzer, K., 1995. Effects of forest liming on soil processes. Plant and Soil 168-169, 447-470.

Landeweert, R., Hoffland, E., Finlay, R.D., Kuyper, T.W., Van Breemen, N., 2001. Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. Trends in Ecology & Evolution 16, 248-254.

Landmann, G., Bonneau, M., 1995. Forest decline and atmospheric deposition effects in the French mountains. Springer-Verlag, Berlin, 461 pp.

Larssen, T., Lydersen, E., Tang, D., He, Y., Gao, J., Liu, H., Duan, L., Seip, H.M., Vogt, R.D., Mulder, J., Shao, M., Wang, Y., Shang, H., Zhang, X., Solberg, S., Aas, W., Okland, T., Eilertsen, O., Angell, V., Li, Q., Zhao, D., Xiang, R., Xiao, J., Luo, J., 2006. Acid Rain in China. Environmental Science and Technology 40, 418-425.

Larssen, T., Seip, H.M., Semb, A. Mulder, J., Muniz, I.P., Vogt, R.D., Lydersen, E., Angell, V., Dagang, T., Eilertsen, O., 1999. Acid deposition and its effects in China: an overview. Environmental Science & Policy 2, 9-24.

Lavelle, P., Decaëns, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerie, P., Mora, P., Rossi, J.P., 2006. Soil invertebrates and ecosystem services. European Journal of Soil Biology 42, Supplement 1, S3-S15.

Lawrence, G.B., David, M.B., Lovett, G.M., Murdoch, P.S., Burns, D.A., Stoddard, J.L., Baldigo, B.P., Porter, J.H., Thompson, A.W., 1999. Soil Calcium Status And The Response Of Stream Chemistry To Changing Acidic Deposition Rates. Ecological Applications 9, 1059-1072.

Likens, G. E., Driscoll, C. T., Buso, D. C., 1996. Long-Term Effects of Acid Rain: Response and Recovery of a Forest Ecosystem. Science 272, 244-246.

Likens, G.E., Butler, T.J., Buso, D.C., 2001. Long- and short-term changes in sulfate deposition: Effects of the 1990 Clean Air Act Amendments. Biogeochemistry 52, 1-11.

Löfgren, S., Cory, N., Zetterberg, T., Larsson, P.E., Kronnäs, V., 2009. The long-term effects of catchment liming and reduced sulphur deposition on forest soils and runoff chemistry in southwest Sweden. Forest Ecology and Management 258, 567-578.

Lundström, U.S., Öhman, L.O., 1990. Dissolution of feldspars in the presence of natural, organic solutes. Journal of Soil Science 41, 359-369.

Lundström, U.S., Bain, D.C., Taylor, A.F.S., Van Hees, P. A.W., 2003. Effects of Acidification and its Mitigation with Lime and Wood Ash on Forest Soil Processes: A Review. Water, Air, & Soil Pollution: Focus 3, 5-28.

Lundström, U.S., Van Breemen, N., Bain, D.C., Van Hees, P. A.W., Giesler, R., Gustafsson, J.P., Ilvesniemi, H., Karltun, E., Melkerud, P.A., Olsson, M., Riise, G., Wahlberg, O., Bergelin, A., Bishop, K., Finlay, R., Jongmans, A.G., Magnusson, T., Mannerkoski, H., Nordgren, A., Nyberg, L., Starr, M., Tau Strand, L., 2000. Advances in understanding the podzolization process resulting from a multidisciplinary study of three coniferous forest soils in the Nordic Countries. Geoderma 94, 335-353.

Mason, C.F., MacDonald, S.M., 1989. Acidification and other (*Lutra lutra*) distribution in Scotland. Water, Air, & Soil Pollution 43, 365-374.

Massabuau, J.C., Fritz, B., Burtin, B., 1987. Mise en évidence de ruisseaux acides (pH≤5) dans les Vosges. Comptes rendus de l'Académie des sciences. Série 3, Sciences de la vie 305, 121-124.

Mayer, R., 1998. Soil acidification and cycling of metal elements: cause-effect relationships with regard to forestry practices and climatic changes. Agriculture, Ecosystems & Environment 67, 145-152.

Meegan, S.K., Perry, S.A., 1996. Periphyton Communities in Headwater Streams of Different Water Chemistry in the Central Appalachian Mountains. Journal of Freshwater Ecology 11, 247-256.

Meegan, S.K., Perry, S.A., Perry, W.B., 1996. Detrital processing in streams exposed to acidic precipitation in the Central Appalachian Mountains. Hydrobiologia 339, 101-110.

Messant, D., Baudoin, J.M., Bock, J., Dambrine, E., Guérold, F., Nys, C., 2008. Restauration de deux bassins versants acidifiés des Vosges par amendement calco-magnésien. Rendez-vous Techniques 22, 3-13.

Mulholland, P.J., Driscoll, C.T., Elwood, J.W., Osgood, M.P., Palumbo, A.V., Rosemond, A.D., Smith, M.E., Schofield, C., 1992. Relationships between stream acidity and bacteria, macroinvertebrates, and fish: a comparison of north temperate and south temperate mountain streams, USA. Hydrobiologia 239, 7-24.

Mulholland, P.J., Palumbo, A.V., Elwood, J.W., Rosemond, A.D., 1987. Effects of Acidification on Leaf Decomposition in Streams. Journal of the North American Benthological Society 6, 147-158.

Neale, S.P., Shah, Z., Adams, W.A., 1997. Changes in microbial biomass and nitrogen turnover in acidic organic soils following liming. Soil Biology and Biochemistry 29, 1463-1474.

Nisbet, M., 1958. Aperçu chimique sur quelques ruisseaux des Vosges: le Rabodeau et ses affluents. Annales de la station d'hydrobiologie 7, 270-284.

Niyogi, D.K., Lewis, W.M., McKnight, D.M., 2001. Litter breakdown in mountain streams affected by mine drainage: biotic mediation of abiotic controls. Ecological Applications 11, 506-516.

Nys, C., Picart, J.F., Dambrine, E., 2004. Restauration de la qualité des sols et des eaux de deux bassins versants situés dans les Vosges gréseuses et les Vosges cristallines. Caractérisation initiale des sites ateliers d'amendement calco magnésien. Bassins versants de Cornimont et Senones. INRA Nancy ONF, Nancy.

Nys, C., 1981. Réponse d'un peuplement adulte d'Epicéa commun à la fertilisation dans le Limousin. Revue Forestière Française XXXIII(3), 217-227.

Odén, S., 1968. The acidification of air and precipitation and its consequences in the natural environment. Ecology Committee Bulletin Stockholm, Sweden. National Science Research Council. Translation Consultants TR-1172, 1, 1-86.

Olsson, B.A., 1999. Effects of biomass removal in thinnings and compensatory fertilization on exchangeable base cation pools in acid forest soils. Forest Ecology and Management 122, 29-39.

Olsson, M., Rosén, K., Melkerud, P.A., 1993. Regional modelling of base cation losses from Swedish forest soils due to whole-tree harvesting. Applied Geochemistry 8, Supplement 2, 189-194.

Party, J.P., 1999. Acidification des sols et des eaux de surface des écosystèmes forestiers français: facteurs, mécanismes et tendances. Taux d'altération sur petits bassins-versants silicates. Application au calcul des charges critiques d'acidité. Université de Strasbourg 1: Strasbourg, France.

Pennanen, T., Fritze, H., Vanhala, P., Kiikkilä, O., Neuvonen, S., Bååth, E., 1998. Structure of a Microbial Community in Soil after Prolonged Addition of Low Levels of Simulated Acid Rain. Applied and Environmental Microbiology 64, 2173-2180.

Pimm, S.L., Russell, G.J., Gittleman, J.L., Brooks, T.M., 1995. The future of biodiversity. Science 269, 347-350.

Piña, R.G., Cervantes, C., 1996. Microbial interactions with aluminium. BioMetals 9, 311-316.

Postel, S.L., Thompson, B.H., 2005. Watershed protection: Capturing the benefits of nature's water supply services. Natural Resources Forum 29, 98-108.

Prechtel, A., Alewell, C., Armbruster, M., Bittersohl, J., Cullen, J.M., Evans, C.D., Helliwell, R., Kopácek, J., Marchetto, A., Matzner, E., Meesenburg, H., Moldan, F., Moritz, K., Veselý, J., Wright, R. F., 2001. Response of sulphur dynamics in European catchments to decreasing sulphate deposition. Hydrology and Earth System Sciences Discussions 5, 311-326.

Probst, A., Party, J.P., Fevrier, C., Dambrine, E., Thomas, A.L., Stussi, J.M., 1999. Evidence of Springwater Acidification in the Vosges Mountains (North-East of France): Influence of Bedrock Buffering Capacity. Water, Air, and Soil Pollution 114, 395-411.

Probst, A., Fritz, B., Ambroise, B., Viville, D., 1987. Forest influence on the surface water chemistry of granitic basins receiving acid precipitation in the Vosges massif, In: Forest hydrology and watershed management, August 1987, Vancouver.

Probst, A., Massabuau, J.C., Probst, J.L., Fritz, B., 1990. Acidification des eaux de surface sous l'influence des précipitations acides: rôle de la végétation et du substratum, conséquences pour les populations de truites. Le cas des ruisseaux des Vosges. Comptes-rendus de l'Académie des Sciences de Paris - Série II, Mécanique, physique, chimie, astronomie 311, 405-411.

Probst, A., Probst, J.L., Massabuau, J.C., Fritz, B., 1995. 5.2 Surface Water Acidification in the Vosges Mountains: Relation to Bedrock and Vegetation Cover. In: Forest decline and atmospheric deposition effects in the French mountains (eds G. Landmann, M. Bonneau), Springer, Berlin, Heildeberg, New York, 371-386.

Qin, G., Huang, M., 2001. A Study on Rain Acidification Processes in Ten Cities of China. Water, Air, & Soil Pollution 130, 163-174.

Reuss, J.O., Cosby, B. J., Wright, R. F., 1987. Chemical processes governing soil and water acidification. Nature 329, 27-32.

Rillig, M.C., Mummey, D.L., 2006. Mycorrhizas and soil structure. New Phytologist 171, 41-53.

Rineau, F., Garbaye, J., 2009. Effects of liming on ectomycorrhizal community structure in relation to soil horizons and tree hosts. Fungal Ecology 2, 103-109.

Rineau, F., Maurice, J.P., Nys, C., Voiry, H., Garbaye, J., 2010. Forest liming durably impact the communities of ectomycorrhizas and fungal epigeous fruiting bodies. Annals of Forest Science 67, 110.1-110.12.

Rizvi, S.H., Gauquelin, T., Gers, C., Guérold, F., Pagnout, C., Baldy, V., 2012. Calcium–magnesium liming of acidified forested catchments: Effects on humus morphology and functioning. Applied Soil Ecology 62, 81-87.

Rizvi, S.H., 2012. Amendements calco-magnésiens de bassins versants forestiers acidifiés: effets sur la dynamique de la matière organique et l'activité biologique du sol. Thèse Université Aix-Marseille, Marseille.

Romani, A., Fischer, H., Mille-Lindblom, C., Tranvik, L., 2006. Interactions of bacteria and fungi on decomposing litter: Differential extracellular enzyme activities. Ecology 87, 2559-2569.

Rosemond, A.D., Reice, S.R., Elwood, J.W., Mulholland, P.J., 1992. The effects of stream acidity on benthic invertebrate communities in the south-eastern United States. Freshwater Biology 27, 193-209.

Rusek, J., Marshall, V.G., 2000. Impacts of airborne pollutants on soil fauna. Annual Review of Ecology and Systematics 31, 395-423.

Schindler, D.W., 1997. Liming to Restore Acidified Lakes and Streams: A Typical Approach to Restoring Damaged Ecosystems? Restoration Ecology 5, 1-6.

Schloter, M., Dilly, O., Munch, J., 2003. Indicators for evaluating soil quality. Agriculture, Ecosystems & Environment 98, 255-262.

Schulze, E.D., 1989. Air Pollution and Forest Decline in a Spruce (Picea abies) Forest. Science 244, 776 -783.

Shah, Z., Adamst, W.A., Haven, C.D.V., 1990. Composition and activity of the microbial population in an acidic upland soil and effects of liming. Soil Biology and Biochemistry 22, 257-263.

Sinsabaugh, R.L., Benfield, E.F., Linkins, A.E., 1981. Cellulase Activity Associated with the Decomposition of Leaf Litter in a Woodland Stream. Oikos 36, 184-190.

Sinsabaugh, R.L., Carreiro, M.M., Alvarez, S., Burns, R.G., Dick, R.P., 2002. In: Enzymes in the environment: Activity, ecology and applications (eds R.G. Burns, R.P. Dick) Marcel Dekker, New York, 249-265.

Skjelkvåle, B., Tørseth, K., Aas, W., Andersen, T., 2001. Decrease in Acid Deposition - Recovery in Norwegian Waters. Water, Air, & Soil Pollution 130, 1433-1438.

Smit, E., Leeflang, P., Gommans, S., Van den Broek, J., Van Mil, S., Wernars, K., 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Applied and Environmental Microbiology 67, 2284-2291.

Smith, R.A., 1872. Air and rain: The beginnings of a chemical climatology. Longmans, Green, and co., London.

Smolander, A., Mälkönen, E., 1994. Microbial biomass C and N in limed soil of norway spruce stands. Soil Biology and Biochemistry 26, 503-509.

Solé, M., Fetzer, I., Wennrich, R., Sridhar, K.R., Harms, H., Krauss, G., 2008. Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. Science of The Total Environment 389, 557-565.

Stoddard, J. L., Jeffries, D.S., Lükewille, A., Clair, T. A., Dillon, P.J., Driscoll, C. T., Forsius, M., Johannessen, M., Kahl, J. S., Kellogg, J.H., Kemp, A., Mannio, J., Monteith, D.T., Murdoch, P. S., Patrick, S., Rebsdorf, A., Skjelkvåle, B.L., Stainton, M.P., Traaen, T., Van Dam, H., Webster, K.E., Wieting, J., Wilander, A., 1999. Regional trends in aquatic recovery from acidification in North America and Europe. Nature 401, 575-578.

Streets, D., Tsai, N., Akimoto, H., Oka, K., 2001. Trends in Emissions of Acidifying Species in Asia, 1985-1997. Water, Air, & Soil Pollution 130, 187-192.

Swift, M.J., Heal, O.W., Anderson, J.M., 1979. Decomposition in Terrestrial Ecosystems. University of California Press.

Tang, D., Lydersen, E., Seip, H.M., Angell, V., Eilertsen, O., Larssen, T., Liu, X., Kong, G., Mulder, J., Semb, A., Solberg, S., Torseth, K., Vogt, R.D., Xiao, J., Zhao, D., 2001.

Integrated Monitoring Program on Acidification of Chinese Terrestrial Systems (Impacts) — A Chinese-Norwegian Cooperation Project. Water, Air, & Soil Pollution 130, 1073-1078.

Thiebaut, G., Vanderpoorten, A., Guérold, F., Boudot, J.P., Muller, S., 1998. Bryological patterns and streamwater acidification in the Vosges Mountains (N.E. France): An analysis tool for the survey of acidification processes. Chemosphere 36, 1275-1289.

Tisdall, J., 1994. Possible role of soil microorganisms in aggregation in soils. Plant and Soil 159, 115-121.

Turner, B.L., 2010. Variation in pH Optima of Hydrolytic Enzyme Activities in Tropical Rain Forest Soils. Applied and Environmental Microbiology 76, 6485-6493.

Van Der Heijden, M.G.A., Bardgett, R.D., Van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11, 296-310.

Van Hees, P.A. W., Jones, D.L., Godbold, D.L., 2002. Biodegradation of low molecular weight organic acids in coniferous forest podzolic soils. Soil Biology and Biochemistry 34, 1261-1272.

Vannote, R., Minshall, G., Cummins, K., Sedell, J., Cushing, C., 1980. River Continuum Concept. Canadian Journal of Fisheries and Aquatic Sciences 37, 130-137.

Vitousek, P.M., Mooney, H.A., Lubchenco, J., Melillo, J.M., 1997. Human Domination of Earth's Ecosystems. Science 277, 494 -499.

Wall, D.H., Moore, J.C., 1999. Interactions Underground: Soil Biodiversity, Mutualism, And Ecosystem Processes. Bioscience 49, 109-117.

Webster, J.R., Meyer, J.L., 1997. Organic Matter Budgets for Streams: A Synthesis. Journal of the North American Benthological Society 16, 141-161.

Webster, J.R., Newbold, J.D., Thomas, S.A., Valett, H.M., Mulholland, P. J., 2009. Nutrient Uptake and Mineralization during Leaf Decay in Streams - a Model Simulation. International Review of Hydrobiology 94, 372-390.

Wright, R.F., Larssen, T., Camarero, L., Cosby, B.J., Ferrier, R.C., Helliwell, R., Forsius, M., Jenkins, A., Kopáček, J., Majer, V., Moldan, F., Posch, M., Rogora, M., Schöpp, W., 2005. Recovery of Acidified European Surface Waters. Environmental Science and Technology 39, 64A-72A.

Résumé : De nombreux écosystèmes forestiers subissent les effets de l'acidification d'origine anthropique, à travers ses effets délétères sur la biodiversité et le fonctionnement de ces écosystèmes. Pour contrer ces effets, des amendements calco-magnésiens peuvent être utilisés pour améliorer les caractéristiques physico-chimiques des sols et des cours d'eau afin de restaurer l'état sanitaire des forêts et le fonctionnement des cours d'eau. En particulier, il a été démontré que les amendements, pouvaient permettre la reprise de la décomposition des litières, qui est un processus clé dans le fonctionnement des cours d'eau forestiers. Dans ce contexte, le premier objectif de cette étude était d'étudier si les amendements calco-magnésiens, à travers leurs effets sur les caractéristiques des sols, pouvaient induire des changements au sein des communautés microbiennes des sols. Le deuxième objectif était d'identifier quels facteurs pouvaient être responsables, à l'échelle microbienne, du ralentissement de la décomposition des litières dans les cours d'eau acidifiés. Les résultats ont montré que des amendements raisonnés et à grande échelle pouvaient avoir un effet durable sur les communautés bactériennes des sols. Les principaux changements taxonomiques ont notamment révélé que le ratio entre *Proteobacteria* et *Acidobacteria* était supérieur dans les sols amendés par rapport à leurs témoins, confirmant que ce ratio pouvait être un indicateur de l'amélioration de la qualité des sols. Les résultats obtenus dans la seconde partie de ce travail ont révélé que la diversité d'espèces sporulantes d'hyphomycètes aquatiques était fortement altérée dans les cours d'eau acidifiés, alors que la diversité fongique, analysée par méthodes moléculaires n'était pas affectée. Ces dernières ont révélé une plus faible proportion d'hyphomycètes aquatiques et une plus importante proportion de champignons d'origine terrestre sur les feuilles exposées dans un cours d'eau impacté. L'analyse des activités microbiennes a permis de mettre en évidence que l'aluminium était un facteur pouvant entrainer la diminution de la décomposition des feuilles, ce métal induisant notamment une limitation en phosphore pour les micro-organismes décomposeurs. Ces effets pourraient en retour avoir des répercussions sur les niveaux trophiques supérieurs et sur tout le fonctionnement de l'écosystème.

Mots-clés : *acidification, amendements calco-magnésiens, micro-organismes, décomposition des litières, cours d'eau forestiers, sols.*

Abstract : Many terrestrial and freshwater forested ecosystems are affected by anthropogenic acidification, which can led to deleterious effects on biodiversity and ecosystem functioning. To counteract acidification, liming can be used to improve soil and water physicochemical characteristics in order to restore tree health and headwater stream functioning. In particular, liming has been shown to enhance leaf litter breakdown, which is a key ecosystem process in headwater streams. In this context, the aims of this study were, first to investigate if liming, through its effects on soil chemical characteristics, could induce changes on soil microbial communities, and second to identify what factors could be responsible, at the microbial level, of reduced leaf litter breakdown in acidified headwater streams. Results showed that moderate large-scale liming can induce sustainable changes in soil bacterial communities. Major taxonomic changes revealed notably that the ratio between *Proteobacteria* and *Acidobacteria* was higher in limed soils compared to their control counterparts, confirming that this ratio could be a microbial indicator of soil quality improvement. Results obtained in the second part of this work showed that sporulating aquatic hyphomycete diversity on leaves was strongly impaired in acidified streams, whereas fungal diversity investigated by molecular analyses was not depressed. The latter showed a lower proportion of aquatic hyphomycetes and a higher proportion of terrestrial fungi on leaves when exposed in an acidified stream compared to a circumneutral one. Microbial activity analyses bring out that Al may be an important factor that could reduce microbial leaf litter processing, this metal inducing notably a P limitation for microbial decomposers. These effects may in turn have repercussions on higher trophic levels and whole ecosystem functioning.

Keywords : *acidification, liming, microorganisms, litter decomposition, forested headwater streams, soils.*